UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

IMPLICACIONES DEL ÓXIDO NÍTRICO EN LA DIFERENCIACIÓN Y APOPTOSIS DE NEURONAS DOPAMINÉRGICAS : PAPEL DE LA GLÍA

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Para Alicia, por su apoyo,
paciencia y comprensión...

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12-HETE:	Acido 12(S)-hidroxieicosatetraenoico
12-HPETE:	Acido 12(S)-hidroperoxieicosatetraenoico
12-LOX:	Lipoxigenasa 12
AA:	Acido araquidónico
BSO:	L-butionina-(S,R)-sulfoximina
CAT:	Catalasa
DA:	Dopaminérgico/a
DEA/NO:	Sodio-(Z)-1-(N,N-dietilamina) diazen-1-ium-1,2-diolato
EP:	Enfermedad de Parkinson
ERK-1/2:	Proteína kinasa regulada por señal extracelular 1 y 2 (*extracellular signal-regulated protein kinase 1 and 2*)
GAPDH:	Gliceraldehido 3-fosfato deshidrogenasa
GC:	Guanilato ciclasa
GPx:	Glutation peroxidasa
GRx:	Glutation reductasa
GSH:	Glutation reducido
GSNO:	S-nitrosoglutation
GSSG:	Glutation Oxidado
L-NAC:	N-acetil-L-cisteína
MAPK:	Proteína kinasa activada por mitógeno (*mitogen-activated protein kinase*)
MPTP:	1-metil-4-fenil-1,2,3,6-tetrahidropiridina
NMDA:	N-metil-D-aspartato
NO:	Oxido nítrico
$NO^{\bullet}$:	Radical óxido nítrico
NOSe:	Oxido nítrico sintasa endotelial
NOSi:	Oxido nítrico sintasa inducible
NOSn:	Oxido nítrico sintasa neuronal
$O_2^{\bullet -}$:	Radical superóxido
$OH^{\bullet}$:	Radical hidroxilo
$ONOO^-$:	Peroxinitrito
PARP:	Poli(ADP-ribosa) polimerasa
PKC:	Proteína kinasa C
PLA_2:	Fosfolipasa A_2
SN:	*Substantia nigra*
SNAP:	S-nitroso-N-acetilpenicilamina
SOD:	Superóxido dismutasa
TH:	Tirosina hidroxilasa

INTRODUCCIÓN GENERAL

En 1980 el Dr. R.F. Furchgott del Departamento de Farmacología de la Universidad de Nueva York, descubrió un factor soluble liberado por el endotelio vascular, responsable de la relajación del musculo liso vascular en la aorta de conejo, en respuesta a acetilcolina (Furchgott and Zawadzki, 1980). Este factor, al que Furchgott denominó EDRF (*endotelial derived relaxing factor*), resultó ser de gran importancia en la regulación del flujo y la presión sanguíneos. Siete años después, los grupos dirigidos por S. Moncada y L.J. Ignarro identificaron el EDRF producido y liberado por arterias y venas como óxido nítrico (NO) (Palmer et al., 1987; Ignarro et al., 1987). En 1988 se reconoce por primera vez el papel del NO en el sistema nervioso, cuando se demostró que el neurotransmisor excitatorio glutamato, actuando sobre los receptores de N-metil-D-aspartato (NMDA), estimulaba la producción de NO en cultivos de células granulares de cerebelo (Garthwaite et al., 1988). Pese a que con anterioridad se había estudiado el papel del NO en la estimulación de la guanilato ciclasa (GC) (Murad et al., 1978), la identificación del NO como EDRF y su relevancia en el sistema nervioso, intensificó la actividad investigadora en el campo. Desde entonces hasta la fecha, la participación del NO en numerosos procesos fisiológicos y patológicos ha sido ampliamente estudiada. Reflejo de ello son las más de 50.000 entradas relativas al NO que se pueden encontrar en Medline y que crecen, en la actualidad, a razón de 6.000 nuevos trabajos al año.

El estudio del NO en el sistema nigro-estriatal, y concretamente su toxicidad para las neuronas dopaminérgicas (DA), adquiere gran interés con los numerosos trabajos que indican la participación de este radical libre en la enfermedad de Parkinson (EP). Los estudios bioquímicos e histológicos en los cerebros *post-mortem* de estos pacientes, han revelado un incremento en la concentración de nitritos en el líquido cefaloraquídeo (Qureshi et al., 1995) y la presencia de radicales NO en la *substantia nigra* (SN) (Shergill et al., 1996). Las inclusiones intracelulares denominadas cuerpos de Lewy, característica histopatológica de la enfermedad, se tiñen positivamente con anticuerpos anti-3-nitrotirosina (Good et al., 1998) y recientemente se ha descrito que uno de los componentes mayoritarios de estas inclusiones, la proteína α-sinucleina, se encuentra nitrosilada en la EP (Giasson et al., 2000). Finalmente, se ha demostrado un marcado incremento en el número de células gliales que expresan un enzima de síntesis del NO, la NO sintasa inducible (NOSi) en la SN de los enfermos de EP (Hunot et al., 2001). Todos estos datos indican una sobreproducción de NO, cuya fuente parece ser las células gliales. Por otra parte, la información que se obtiene en modelos animales de la enfermedad, refuerza la

participación del NO en el proceso degenerativo. En ratones tratados con 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), se detecta un incremento en 3-nitrotirosina (Schulz et al., 1995; Pennathur et al., 1999) y un aumento en la expresión de NOSi en la microglía (Liberatore et al., 1999; Dehmer et al., 2000). Además, tanto la delección del gen de la NOSi como la inhibición farmacológica de este enzima, protege a las neuronas DA de la toxicidad del MPTP (Liberatore et al., 1999; Dehmer et al., 2000).

Un exceso de producción de NO puede producirse por un incremento en la síntesis del enzima NOSi, o por un aumento de calcio intracelular que activa los enzimas constitutivos NOS neuronal (NOSn) o NOS endotelial (NOSe). La concentración local de NO es una función de la relación entre su producción y su desaparición, bien por reacción o por difusión (Murphy, 1999). Cuando la concentración de NO se eleva, puede causar daño celular por diversos mecanismos (Fig. 1): (1) formando S-nitrosotioles, (2) reaccionando con el radical superóxido ($O_2^{\bullet -}$) para formar peroxinitrito ($ONOO^-$), una molécula mucho más reactiva y citotóxica que su precursora, (3) desregulando enzimas con grupo hemo y (4) liberando hierro de la proteína intracelular ferritina.

Una de las dianas principales donde el NO (y el $ONOO^-$) ejercen sus efectos deletéreos, es la mitocondria. El NO puede inhibir reversible o irreversiblemente la respiración mitocondrial, inhibir la creatina kinasa e inducir la permeabilidad mitocondrial transitoria (PT), con la liberación de pequeñas proteínas de la matriz (Revisado en Murphy 1999). El NO puede afectar tanto la cantidad como el reclutamiento en la membrana mitocondrial externa, de proteínas pro-apoptóticas (Bax, Bcl-X_S, Bak) y anti-apoptóticas (Bcl-2, Bcl-X_L) (Levine, 1997; Hsu et al., 1997). Por otro lado, la S-nitrosilación del enzima gliceraldehido 3-fosfato deshidrogenasa (GAPDH) produce su inactivación y ADP-ribosilación no enzimática (Molina y Vedia et al., 1992), inhibiendo la glicolisis. Otro punto importante de la toxicidad del NO es la inducción de daño sobre el ADN y la consiguiente activación de la poli(ADP-ribosa) polimerasa (PARP) para asistir su reparación. Este enzima transfiere más de 100 grupos ADP-ribosa del NAD^+ a proteínas nucleares (Szabo et al., 1996). La resíntesis de NAD^+ que sigue a este ciclo, consume gran cantidad de ATP, a la vez que la menor disponibilidad de NAD^+ compromete la síntesis de ATP. Por tanto, la activación de PARP produce la depleción de ATP y una muerte celular que se previene con inhibidores de este enzima (Eliasson et al., 1997). Todos estos resultados indican, en conjunto, que la causa principal de la toxicidad del NO es la

inducción de un déficit energético, bien sea inhibiendo la GAPDH, alterando la mitocondria o activando PARP. Pero además, la acumulación de hierro libre intracelular inducida por el NO, participa en la reacción de Fenton produciendo radicales hidroxilo ($OH^{\cdot}$), con gran capacidad oxidativa y citotóxica.

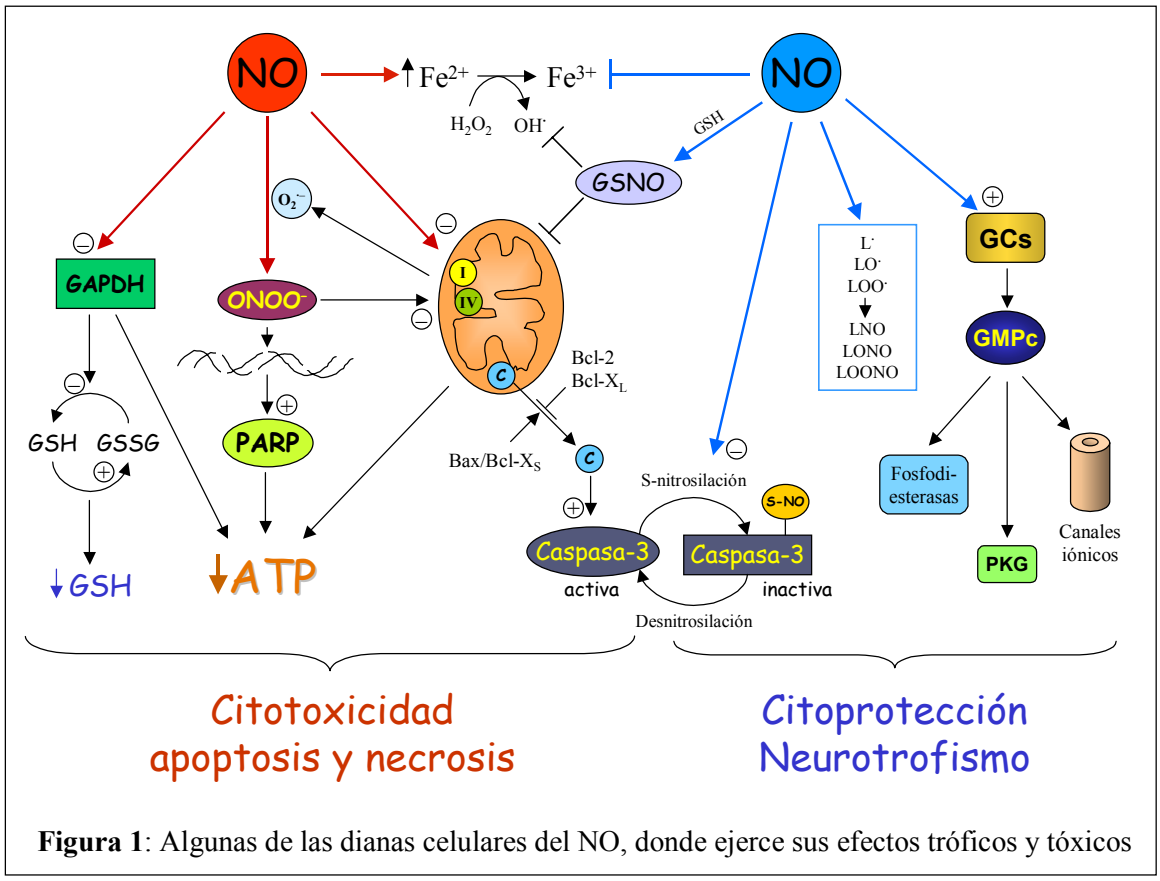

Figura 1: Algunas de las dianas celulares del NO, donde ejerce sus efectos tróficos y tóxicos

Una observación interesante en los cerebros de pacientes con EP, enfermedad de Alzheimer y tras una isquemia, es que las neuronas que expresan NOSn aparecen relativamente preservadas del daño (Bockelmann et al., 1994; Mufson and Brandabur, 1994). Una posible explicación a este hecho, es que las neuronas que expresan NOSn tienen defensas antioxidantes más efectivas que otras neuronas (Bolaños et al., 1997). Pero además, en modelos *in vivo* de EP se ha demostrado que el NO y el nitrosoglutation (GSNO), producto de la reacción entre el NO y el glutation (GSH), protegen a las neuronas DA del daño oxidativo causado por radicales $OH^{\cdot}$. (Rauhala et al., 1996; Mohanakumar et al., 1998; Rauhala et al., 1998). Los mecanismos por los que el NO y el GSNO pueden actuar como neuroprotectores (Fig. 1) incluyen: (1) inhibición de la producción de $OH^{\cdot}$ estimulada por hierro en la rección de Fenton, (2) finalización de la reacción en cadena de peroxidación lipídica, (3) aumento de la potencia antioxidante del GSH (el GSNO es 100

veces más potente que el GSH atrapando $OH^{\cdot}$ y peróxidos lipídicos), y (4) inhibición por S-nitrosilación de cisteinil proteasas como la caspasa-3 y la proteasa HIV-1 (Revisado en Chiueh and Rauhala, 1999). Incluso se ha descrito que el efecto neuroprotector del BDNF (*Brain-Derived Neurotrophic Factor*) es mediado por NO, a través de la producción de GMPc (Thippeswamy and Morris, 1997; Estévez et al., 1998). Todos estos resultados experimentales ponen de manifiesto el problema, intensamente discutido, del NO como molécula "buena" neuroprotectóra y neurotrófica, o como molécula "mala", inductora de muerte neuronal. Como se verá más adelante, la conclusión más importante de este debate ha sido que el entorno celular en cada momento, pude condicionar la acción final de este radical libre.

En lo que se refiere a la EP, la coexistencia en el sistema nigro-estriatal de otras alteraciones relacionadas con el estrés oxidativo, podría condicionar el efecto final del NO en esta enfermedad. Por ejemplo, se ha descrito una severa disminución en los niveles de GSH en la SN de pacientes con EP (Perry et al., 1982; Riederer et al., 1989; Sian et al., 1994), así como una acumulación de hierro intracelular (Riederer et al., 1989; Dexter et al., 1989; Sofic et al., 1991). La depleción de GSH es la primera alteración bioquímica encontrada, hasta la fecha, en cerebros con EP. Aparece antes de la degeneración neuronal en la enfermedad de los cuerpos de Lewy, considerada como una manifestación presintomática de la EP (Riederer et al., 1989), por lo que se puede descartar que sea una consecuencia del proceso degenerativo. Esto sugiere una posible relación entre ambos hechos, aunque debe ser establecido si la depleción de GSH contribuye a la degeneración.

El tripéptido GSH, γ-L-glutamil-L-cisteinilglicina, es el compuesto tiólico presente en mayor concentración en las células de cualquier órgano (1-10 mM). Tiene importantes funciones como antioxidante, en la detoxificación de xenobióticos, es cofactor en diversas reacciones y constituye el almacen y transporte de cisteina (Revisado en Dringen, 2000). La homeostasis del GSH (Fig. 2) es muy importante para la defensa celular frente al estrés oxidativo. Puede reaccionar directamente con radicales de forma no enzimática y también es un donador de electrones en la reducción de peróxidos catalizada por la GSH peroxidasa. A pesar de su importancia, la depleción de GSH en proporciones similares e incluso superiores a las que ocurren en la EP (50-70% de disminución), no induce degeneración del sistema DA en animales de experimentación (Wullner et al., 1996; Toffa et al., 1997). Es más, las neuronas DA en cultivo son más resistentes a la depleción de

GSH que otras poblaciones celulares del mesencéfalo (Nakamura et al., 1997; Nakamura et al., 2000; Nakamura et al., 2001). No obstante, la disminución de GSH puede aumentar la sensibilidad de las neuronas DA frente a algunas toxinas como el MPTP y la 6-hidroxi dopamina (Pileblad et al., 1989; Wullner et al., 1996; Nakamura et al., 1997) y así, promover el proceso degenerativo.

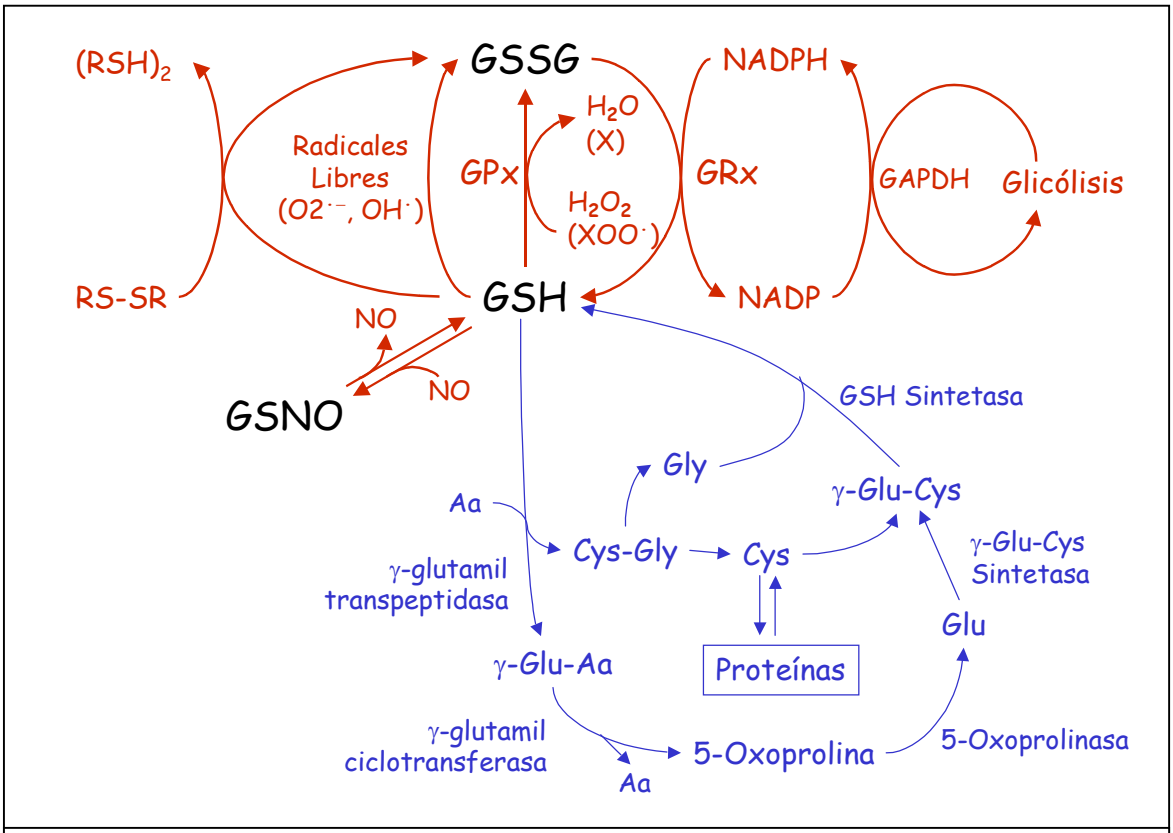

Figura 2: Metabolismo del glutation. En rojo se indican las reacciones correspondientes a la función antioxidante del glutation y en azul el ciclo γ-glutamilo, en el que se produce su síntesis. El enzima limitante en la síntesis de glutation es la γ-Glu-Cys sintetasa y es la diana farmacológica del L-butionina-(S,R)-sulfoximina (BSO).

GSH, glutation reducido; GSSG, glutation oxidado; GPx, GSH peroxidasa; GRx, GSSG reductasa; GAPDH, gliceraldehido 3-fosfato deshidrogenasa; Aa, aminoácido.

Aunque no sabemos si las numerosas alteraciones bioquímicas e histológicas descritas en estudios *postmortem* de pacientes con enfermedades neurológicas, son causa, parte o consecuencia del proceso degenerativo, la coexistencia espacio-temporal de algunas de ellas podría ser relevante en el proceso de pérdida neuronal.

OBJETIVOS

Objetivo 1: Estudiar el efecto del óxido nítrico en la diferenciación, funcionalidad y supervivencia de las neuronas dopaminérgicas, teniendo en cuenta las posibles interacciones neurona-glía en el resultado final.

Objetivo 2: Investigar la influencia de la concentración intracelular y extracelular de glutation, en el efecto final del óxido nítrico sobre las neuronas dopaminérgicas.

Objetivo 3: Buscar estrategias neuroprotectoras frente a la toxicidad producida por un incremento agudo y elevado, en la concentración de NO, así como estudiar los mecanismos asociados a dicha protección.

Objetivo 4: Estudiar la resistencia de las neuronas dopaminérgicas a la disminución de la concentración intracelular de glutation y el efecto del óxido nítrico en dichas condiciones.

Objetivo 5: Investigar las rutas de señalización intracelular que median los efectos del óxido nítrico y del glutation, en cultivos primarios de mesencéfalo.

RESULTADOS

Trabajo 1

Neurotrophic and neurotoxic effects of nitric oxide on fetal midbrain cultures

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Neurotrophic and neurotoxic effects of nitric oxide on fetal midbrain cultures

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Abstract

There is evidence suggesting that nitric oxide (NO) may play an important role in dopamine (DA) cell death. Thus, the aim of this study was to investigate the effects of NO on apoptosis and functionality of DA neurones and glial cells. The experiments were carried out in neuronal-enriched midbrain cultures treated with the NO donor diethylamine–nitric oxide complexed sodium (DEA–NO). DEA–NO, at doses of 25 and 50 μM, exerted neurotrophic effects on dopamine cells, increasing the number of tyrosine hydroxylase positive (TH^+) cells, TH^+ neurite processes, DA levels and [^{3}H]DA uptake. A dose of 25 μM DEA–NO protected DA cells from apoptosis. In addition, it induced *de novo* TH synthesis and increased intracellular reduced glutathione (GSH) levels, indicating a possible neuroprotective role for GSH. However, in doses ranging from 200 to 400 μM, DEA–NO decreased TH^+ cells, DA levels, [^{3}H]DA uptake and the number of mature oligodendrocytes ($O1^+$ cells). No changes in either the amount or morphology of astrocytes and glial progenitors were detected. A dose- and time-dependent increase in apoptotic cells in the DEA–NO-treated culture was also observed, with a concomitant increase in the proapoptotic Bax protein levels and a reduction in the ratio between Bcl-xL and Bcl-xS proteins. In addition, DEA–NO induced a dose- and time-dependent increase in necrotic cells. 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ, 0.5 μM), a selective guanylate cyclase inhibitor, did not revert the NO-induced effect on [^{3}H]DA uptake. Glia-conditioned medium, obtained from fetal midbrain astrocyte cultures, totally protected neuronal-enriched midbrain cultures from NO-induced apoptosis and rescued [^{3}H]DA uptake and TH^+ cell number. In conclusion, our results show that low NO concentrations have neurotrophic effects on DA cells via a cGMP-independent mechanism that may implicate up-regulation of GSH. On the other hand, higher levels of NO induce cell death in both dopamine neurones and mature oligodendrocytes that is totally reverted by soluble factors released from glia.

Keywords: apoptosis, Bcl-2 proteins, dopamine neurones, glia, glutathione, nitric oxide.

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There is great controversy, to date, regarding the role of nitric oxide (NO) on dopamine cell death. Several studies suggest that NO is a toxic factor mediating dopamine cell death (Dawson *et al*. 1992; Smith *et al*. 1994; Przedborski *et al*. 1996; LaVoie and Hastings 1999; Liberatore *et al*. 1999) whereas others have demonstrated that it protects against oxidative stress (Lipton *et al*. 1993; Wink *et al*. 1996; Rauhala *et al*. 1998). NO, synthesized by neuronal NO synthase (nNOS), is thought to modulate MPTP-induced neurotoxicity (Schulz *et al*. 1995; Przedborski *et al*. 1996; Liberatore *et al*. 1999). Conversely, NOS-containing neurones are relatively spared in parkinsonian brains (Bockelmann *et al*. 1994; Mufson and Brandabur 1994).

Astrocytes, on the other hand, can synthesize NO either constitutively or by induction of the enzyme NOS. The function of astrocyte-derived NO is not yet clear. However, it is well established that astrocytes comprise an effective antioxidant system (Sagara *et al*. 1993; Makar *et al*. 1994; Mena *et al*. 1997, 1998a, 1999) and are highly resistant to elevated concentrations of the deleterious NO-derived oxidant, peroxynitrite (Bolaños *et al*. 1995). However, these cells are not immune to MPTP toxicity (Di Monte *et al*. 1992; Tsai and Lee 1994).

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Abbreviations used: DA, dopamine; DEA–NO, diethylamine–nitric oxide complex sodium; GC, guanylate cyclase; GCM, glia-conditioned medium; GSH, reduced glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; ODQ, 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one, NO, nitric oxide; NOS, nitric oxide synthase; PD, Parkinson's disease; TH, tyrosine hydroxylase.

A redox-based mechanism for the neuroprotective and neurodestructive effects of NO and related nitroso compounds has been postulated (Lipton *et al*. 1993). Glutathione is an endogenous thiol that reacts with NO to form *S*-nitrosoglutathione (GSNO) and which protects dopamine neurones from oxidative stress (Rauhala *et al*. 1998; Chiueh and Rauhala 1999).

In view of the above, the present study was undertaken to examine whether NO can modulate dopamine function and to determine the role of glial cells on NO-induced DA cell death and survival.

Materials and methods

Materials

Culture media

Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L), Ham's F12 nutrient mixture, Eagle's minimal essential medium (EMEM) with Earl's salts and Leibovitz's L-15 medium, all of which were supplemented with L-glutamine, fetal calf serum (FCS), sodium pyruvate and L-glutamine were purchased from Gibco BRL (Paisley, Scotland, UK). Glucose 45%, insulin, putrescine, progesterone and sodium selenite were from Sigma (Madrid, Spain) and human transferrin, 30% iron-saturated, from Boehringer-Mannheim (Barcelona, Spain).

Antibodies

Mouse monoclonal anti-β-actin antibody and antirabbit IgG conjugated with tetramethylrhodamine (TRITC) were purchased from Sigma (Madrid, Spain); mouse monoclonal antityrosine hydroxylase (TH) antibody and antimouse Ig fluorescein were from Boehringer-Manheim (Barcelona, Spain); O1 and A2B5 were obtained from hybridoma supernatants (Raff *et al*. 1979, 1983); polyclonal anti-GFAP antibody, raised in rabbits, was from Dako (Glostrup, Denmark); antirabbit polyclonal Bcl-2, Bax and Bcl-$x_{S/l}$ antibodies were from Sta. Cruz Biotechnology, Inc. (CA, USA).

Chemicals

Trypan blue, bovine serum albumin, poly-D-lysine, *p*-phenylenediamine, bis-benzimide, pargyline, *N*-(1-napthyl)-ethylenediamine, sulfanilamide, 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB), reduced and oxidized forms of glutathione and 2-vinylpyridine were from Sigma (Madrid, Spain); NADPH, lactate dehydrogenase standard (LDH), the cytotoxicity detection kit (LDH) and glutathione reductase (GR) were from Boehringer-Manheim (Barcelona, Spain); ascorbic acid was from Merck (Darmstadt, Germany), 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ) from Biomol Research Lab., Inc. (PA, USA), and diethylamine/nitric oxide complex sodium (DEA–NO) from RBI (Natick, MA, USA). The radiochemical [^{3}H]DA (70 Ci/mmol) was obtained from Dupont NEN (Boston, MA, USA) and ^{3}H-guanosine (6.3 Ci/mmol) from Moravek Biochemicals (CA, USA). The apoptosis TUNEL detection kit was obtained from Promega (Madison, WI, USA). The BCA protein assay kit was from Pierce (Rockford, Illinois, USA). All other reagents were of the highest purity commercially available from Merck or Sigma.

Neuronal and glial cultures

Neuronal-enriched cultures from embryonic Sprague–Dawley rat midbrain E-14 (crown–rump length 10–12 mm) were obtained and prepared as previously described (Mena *et al*. 1993; Pardo *et al*. 1997). The cells were seeded in DMEM with 15% fetal calf serum (DMEM-FCS) at a density of 10^5 cells/cm^2 in multiwells or glass cover slides previously coated with poly-D-lysine, 4.5 μg/cm^2, in 0.1 M borate buffer, pH 8.4. The cultures were kept in a humidified chamber at 37°C in a 5% CO_2 atmosphere. Twenty-four hours after plating, the cells were changed to serum-free defined medium (EF12) as previously reported (Mena *et al*. 1993; Pardo *et al*. 1997). EF12 consisted of a 1 : 1 (v/v) EMEM and nutrient mixture of Ham's F-12, supplemented with D-glucose (6 mg/mL), insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), progesterone (20 nM) and sodium selenite (30 nM).

Glia-enriched mesencephalic cultures were obtained from sibling cells kept in culture for 10–15 days in DMEM-FCS (Levison and McCarthy 1991). The DMEM-FCS medium was then discarded, the cells were washed out three times with Leibovitz's L-15 and subsequently cultured in serum-free defined medium. After 24 h of culture in such conditions, the medium was collected and stored frozen. This medium was considered glia-conditioned medium (GCM). Positive staining with anti-GFAP antibody identified the astrocytes in these cultures. After 10–15 days in culture, the number of astrocytes constituted around 80–90% of total cells.

Experimental treatments

After 5 days in culture, the cells, randomly allocated to the different experimental groups, received two main treatments with no change of culture media: a dose–response curve of DEA–NO (0, 5, 10, 25, 50, 100, 200 and 400 μM), dissolved in defined medium, for 24 h and a time-response curve of 0, 4 and 8 h with 400 μM DEA–NO in defined medium.

The effect of low doses of DEA–NO on cell survival and phenotype expression was investigated in sister cultures plated at similar cell density and treated with solvent or DEA–NO. TH^+ cell count was carried out after 1, 5 and 6 days in culture. After 5 days in culture, some wells were treated with 25 μM DEA–NO, the culture maintained for an additional 24 h and the number of TH^+ cells counted.

Finally, the effect of GCM on DEA–NO-induced toxicity was evaluated in a set of cultures that were changed to GCM or fresh DM and treated with 200 μM DEA–NO or solvent (defined medium) for 24 h.

Measurement of cGMP formation

For cGMP determination, the culture medium was removed from the wells and the cells were incubated for 24 h with 2 μCi/ml [^{3}H]guanosine in 1 mL of serum-free defined medium. After radiolabelling, cells were washed once with Krebs–Henseleit–Hepes buffer (KHH) (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 20 mM Hepes, 25 mM $NaHCO_3$, pH 7.4 and 0.5 mM IBMX). After washing, the cells were incubated at 37°C in KHH for 30 min prior to addition of DEA–NO, at different concentrations, for 5 min. The reaction was stopped by aspiration of the buffer and addition of 1 mL of cold methyl alcohol (MeOH) and 0.12 M HCl (1 : 1). Plates were then

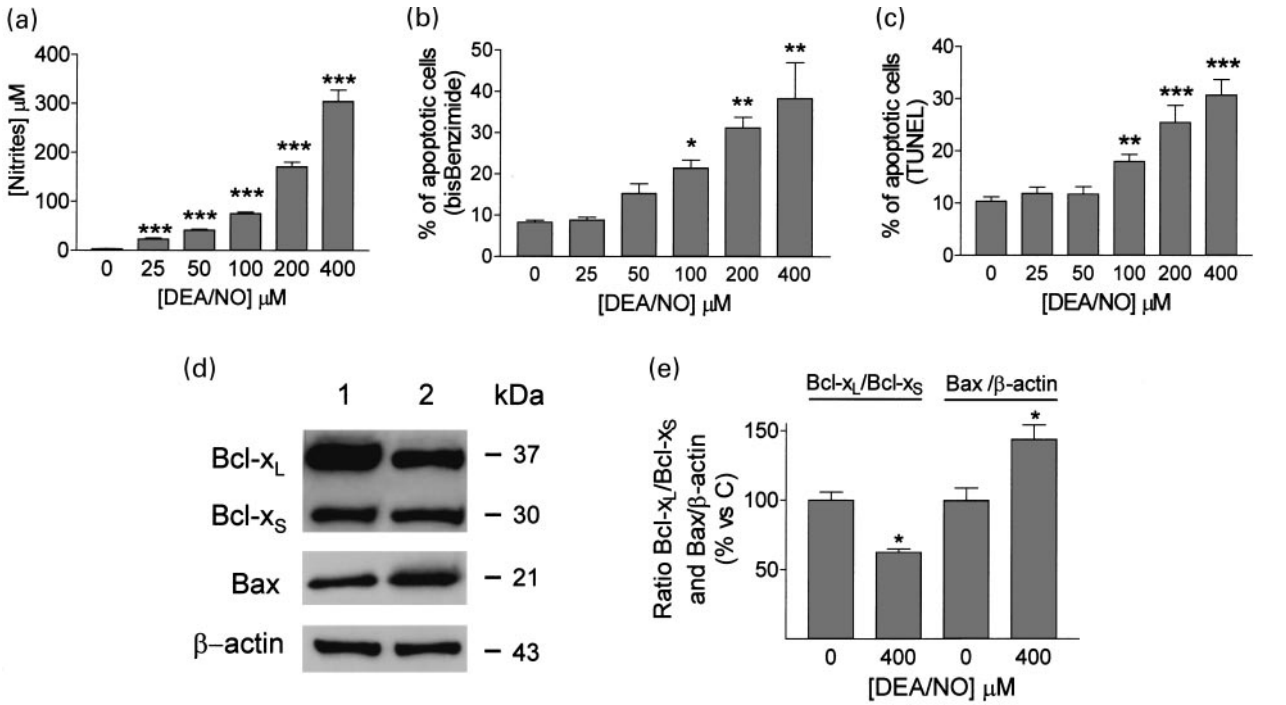

Fig. 1 Dose-dependent effects of DEA–NO on fetal midbrain cultures. Cells were cultured for 5 days and subsequently treated with DEA–NO, in concentrations ranging from 25 to 400 μM, for 24 h. (a) Nitrite concentration in the culture medium induced by DEA–NO. (b) Apoptotic cells induced by DEA–NO, counted with bis-benzimide and expressed as a percentage of chromatin condensed nuclei with respect to the total number of cells. (c) Apoptotic cells counted by the TUNEL assay, expressed as a percentage of positive nuclei with respect to the total number of cells. (d) Western blot analysis of proteins from the Bcl-2 family in control cultures (1) and cultures treated with 400 μM DEA–NO for 24 h (2). Charge control with β-actin. (e) Data represent the ratio Bax/β-actin and the ratio Bcl-xL/S in control cultures and in cultures treated with 400 μM DEA–NO for 24 h. Values are expressed as the mean ± SEM (for at least three independent experiments $n = 3 - 9$) Statistical analysis was performed by ANOVA followed by the Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. controls.

kept in the freezer at − 20°C for 1 h and aliquots (900 μL) of the MeOH–HCl extract were used to determine cGMP production as previously described (Hernández 1995).

Immunocytochemistry

DA neurones were characterized by immunostaining with a mouse monoclonal anti-TH antibody (1 : 100), astrocytes with a rabbit policlonal anti-GFAP antibody (1 : 500); glial progenitors with monoclonal anti-A2B5 antibody (1 : 10) (Raff *et al*. 1979, 1983) and oligodendrocytes with monoclonal anti-O1 (1 : 10) (Sommer and Schachner 1981). In brief, cultures were fixed with 4% paraformaldehyde, washed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, permeabilized with ethanol–acetic acid (19 : 1) and incubated at 4°C for 24 h with primary antibodies diluted in PBS containing 10% fetal calf serum. Fluorescein- and rhodamine-conjugated secondary antibodies were employed to visualize positive cells under fluorescent microscopy. The number of immunoreactive cells was counted in 1/7 of the total area of the cover slides. The cells were counted in predefined parallel strips using a counting reticule inserted in the ocular.

Cell death measurements

Cells growing on cover slides were treated with DEA–NO at the concentrations and incubation times indicated in the figure legends. Apoptosis was measured by DNA staining and the TUNEL assay. Cultures were fixed in 4% paraformaldehyde, nuclei were stained with bis-benzimide 33342 Hoechst added in the antifading solution (3×10^{-6} M final concentration) (Hilwig and Gropp 1975; Pardo *et al*. 1997) and counted in 1/14 of the cover slide area; apoptotic cells were identified by chromatin condensation.

The apoptosis TUNEL detection system measures the fragmented DNA of apoptotic cells by incorporating fluorescein-12-dUTP* at the 3′-OH ends of the DNA using the enzyme terminal deoxynucleotidyl transferase (TdT) (Kerr *et al*. 1972; Gavrieli *et al*. 1992). For this assay, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The fluorescein-12-dUTP-labelled DNA of apoptotic cells was visualized by fluorescence microscopy (positive cells with green fluorescence). The number of apoptotic cells was counted in 1/14 of the cover slide area. Cells were counted in predefined parallel strips using a counting reticule in the ocular. Cells incubated with buffer in the absence of TdT enzyme were used as negative controls.

For necrotic cell death determination, Trypan blue dye exclusion assay was performed (Pardo *et al*. 1997) and LDH activity was measured in the culture medium by using a cytotoxicity detection kit (Decker and Lohmann-Matthes 1988).

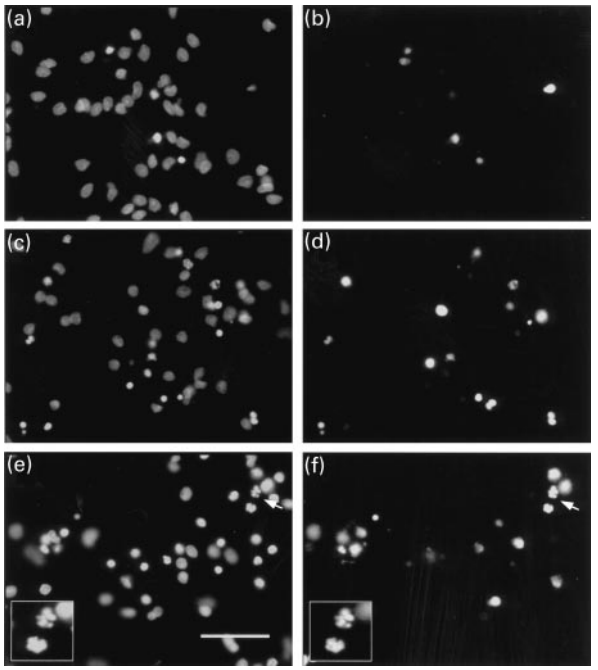

Fig. 2 Effect of DEA–NO (200 and 400 μM) for 24 h on apoptotic cell death in fetal midbrain cultures. Photomicrograph of total nuclei stained with bis-benzimide (a c and e) and of apoptotic cells obtained by the TUNEL assay (b, d and f), corresponding to the same field. (a) and (b): Control cells, (c) and (d): Cultures treated with 200 μM DEA–NO, (e) and (f): cultures treated with 400 μM DEA–NO. Scale bar = 50 μm. Inset shows that both methods mark the same apoptotic cells (arrows).

Nitrite measurement

NO production was routinely quantified by measuring nitrite, a stable oxidation end product of NO (Green *et al*. 1982). Briefly, 400 μL of culture medium were mixed with 800 μL of Griess reagent (1.5% sulfanilamide in 1 M HCl plus 0.15% *N*-(1-naphtyl)ethylenediamine dihydrochloride in distilled water, v:v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined, using sodium nitrite as a standard.

Uptake studies

[^{3}H]DA uptake was measured after incubation of the cells with 10^{-8} M [^{3}H]DA (70 Ci/mmol), in the presence of pargyline 10^{-5} M, and ascorbic acid (AA) 10^{-3} M, at 37°C for 30 min. Non-specific uptake/binding was calculated in the presence of 10^{-5} M mazindol (Beart and McDonald 1980). Proteins were measured according to the Bradford assay (1976).

Western blot analysis

Primary midbrain cultures were homogenized with a sonicator in buffer containing 20 mM TrisHCl, 10 mM AcK, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 0.25% NP-40, pH 7.4 (Sigma), and then centrifuged at 12 000 *g* for 30 min at 4°C.

The supernatant was used for protein determination by the BCA protein assay kit and for electrophoretical separation. Samples (30 μg) were added to SDS sample loading buffer, electrophoresed in Bio-Rad SDS-polyacrilamide gels (4–20%) and then electroblotted to 0.45 μm nitrocellulose membranes. For immunolabelling, the blots were blocked with TTBS (20 mM Tris-HCl pH 7.6, 137 mM NaCl plus 0.1% (v/v) Tween-20 and 5% dry skimmed milk) for 1 h at room temperature. After blocking non-specific binding, the membranes were incubated with mouse anti-TH (1 : 2000), rabbit anti-Bax (1 : 1000), mouse anti-Bcl-2 (1 : 250), rabbit anti Bcl-$x_{S/l}$ (1 : 500) and mouse anti-β-actin (1 : 5000) in blocking solution overnight at 4°C. The blots were developed by chemiluminiscence detection using a commercial kit (Amersham)

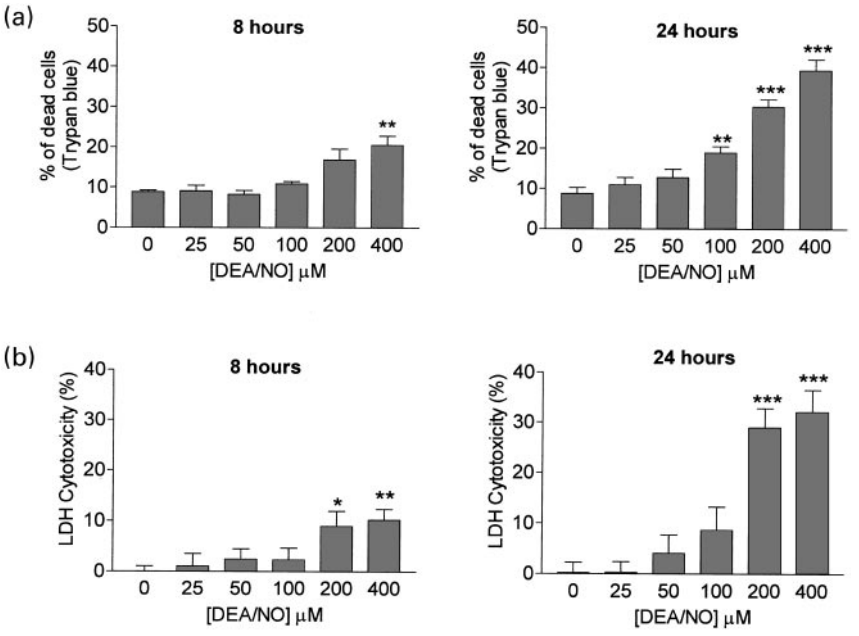

Fig. 3 Dose- and time-dependent effects of DEA–NO on necrotic cell death in fetal midbrain cultures. (a) Trypan blue dye exclusion assay at 8 and 24 h and (b) LDH activity in the culture medium at 8 and 24 h after DEA–NO (25–400 μM) treatment. Values are expressed as the mean (SEM from $n = 4$–6. Statistical analysis was performed by ANOVA followed by the Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. controls.

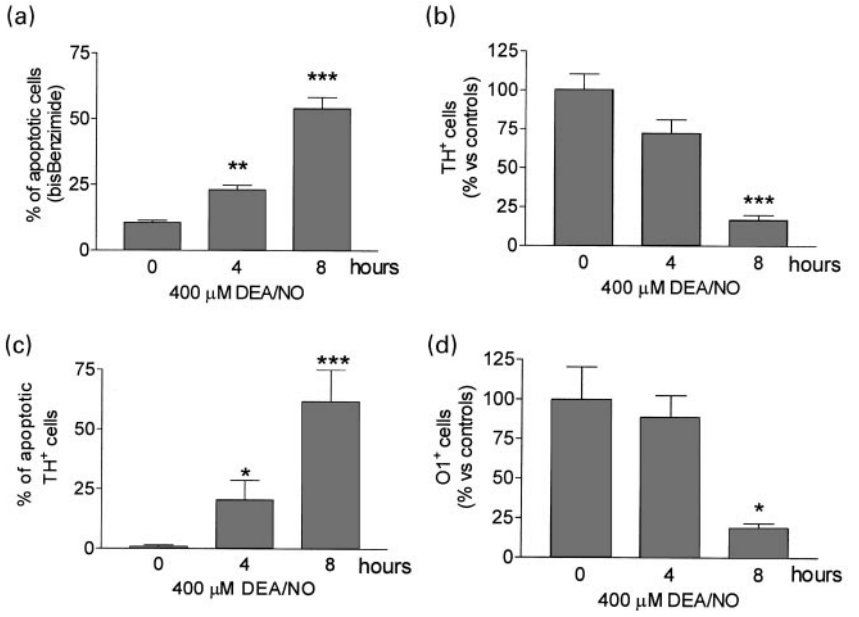

Fig. 4 Time-dependent effects and cell selectivity of DEA–NO-induced neurotoxicity. Cells were cultured for 5 days and then treated with 400 μM DEA–NO for 4 h or 8 h. (a) Cellular nuclei were stained with bis-benzimide, the chromatin-condensed nuclei were counted and expressed as a percentage of apoptotic cells with respect to the total number of cells. (b) Number of DA neurones expressed as a percentage vs. control. (c) Apoptotic DA neurones expressed as the percentage of TH^+-chromatin condensed cells vs. total number of TH^+ cells. (d) Number of oligodendrocytes expressed as a percentage vs. control. Values are the mean ± SEM from $n = 4 - 6$. Control values (per well): 2171 ± 239 TH^+ cells; 6884 ± 1315 $O1^+$ cells. Statistical analysis was performed by ANOVA followed by the Student's *t*-test. $^*p < 0.05$, $^{***}p < 0.001$ vs. controls.

and quantified by computer-assisted videodensitometry. β-Actin was employed as a control of charge.

Catecholamine levels

CA levels were measured by HPLC with an ESA coulochem detector, according to Mena *et al*. (1989, 1995a), as follows: The cells were detached from the wells and centrifuged. The resultant supernatant was discarded and the pellets were sonicated in 0.4 M perchloric acid (PCA) with 0.5 mM $Na_2S_2O_5$ and 2% EDTA and then centrifuged for 5 min. CA levels were determined in 20 μL of the latter supernatant, and the pellet was used for protein determination.

Glutathione measurements

Total glutathione levels were measured by the method of Tietze (1969). Briefly, 4×10^5 cells were washed with PBS, lysed in 100 μL of 3% perchloric acid (PCA) for 30 min at 4°C, centrifuged, and the supernatants were neutralized with four volumes of 0.1 M NaH_2PO_4, 5 mM EDTA, pH 7.5. Glutathione content was measured in a P96 automatic reader by the addition of DTNB (0.6 mM), NADPH (0.2 mM) and glutathione reductase (1 U) and the reaction monitored at 412 nm during 6 min. Oxidized glutathione (GSSG) was measured in the cells by the method of Griffith (1980). Briefly, after PCA extraction and pH neutralization, reduced glutathione (GSH) was derivatized with 2-vinylpyridine at

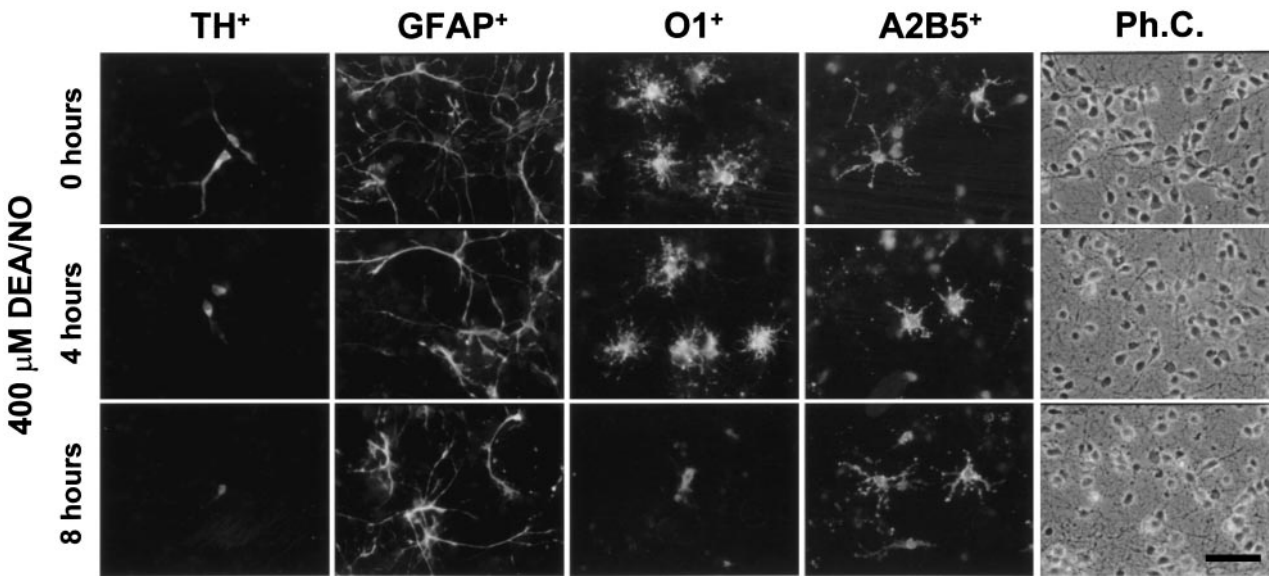

Fig. 5 Time-selectivity of the DEA–NO-induced neurotoxic effect for the different cell types in fetal midbrain cultures. Cells were cultured for 5 days and then treated with 400 μM DEA–NO for 4 h or 8 h. Photo-micrographs show DA neurones (TH^+), astrocytes ($GFAP^+$), mature oligodendrocytes ($O1^+$), glial progenitors ($A2B5^+$) and their corresponding phase-contrast images. Scale bar = 50 μm.

Table 1 Neurotrophic and neurotoxic effects of DEA–NO on dopamine neurones, intracellular glutathione concentration and nitrite levels in the culture medium

[DEA–NO] (μM)	Number of TH^+ cells	[^{3}H]DA uptake (cpm x 10^3/well)	Endogenous DA (pg/well)	[Glutathione] (ng/μg of protein)	[Nitrites] (μM)
0	3036 ± 188	15.6 ± 1.6	575 ± 66	20.2 ± 1.2	3.3 ± 0.1
5	3061 ± 220	15.7 ± 1.7	499 ± 26	24.1 ± 3.1	8.8 ± 0.9**
10	3696 ± 333	13.6 ± 1.3	558 ± 83	21.8 ± 1.6	12.7 ± 1.6**
25	4454 ± 255**	27.1 ± 4.5*	1006 ± 164*	35.0 ± 4.3**	23.3 ± 1.8**
50	3461 ± 172	22.0 ± 2.0*	821 ± 111	30.3 ± 2.3**	41.3 ± 2.0***
100	2459 ± 142	19.6 ± 2.8	628 ± 111	20.2 ± 1.1	75.2 ± 3.2***
200	1943 ± 177**	13.2 ± 1.0	507 ± 97	17.1 ± 1.0	171.1 ± 9.4***
400	486 ± 145***	6.3 ± 0.9**	266 ± 17**	15.5 ± 0.8*	304.5 ± 23.3***

Cells were cultured for 5 days and subsequently treated with DEA–NO in doses ranging from 5 to 400 μM for 24 h. Values are expressed as the mean ± SEM from three independent experiments with $n = 4 - 6$ replicates in each experiment. Statistical analysis was performed by ANOVA followed by the Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p <$ Temp 0.001 vs. controls.

room temperature for 1 h and the reaction carried out as above. GSH was obtained by substracting GSSG levels from total glutathione levels. Extracellular glutathione measurements were made in culture media treated with PCA (3% final concentration) and monitored as above.

Statistical analysis

The results were statistically evaluated for significance using one way analysis of variance followed by the Student's *t*-test as a *post hoc* evaluation. Differences were considered statistically significant when $p < 0.05$.

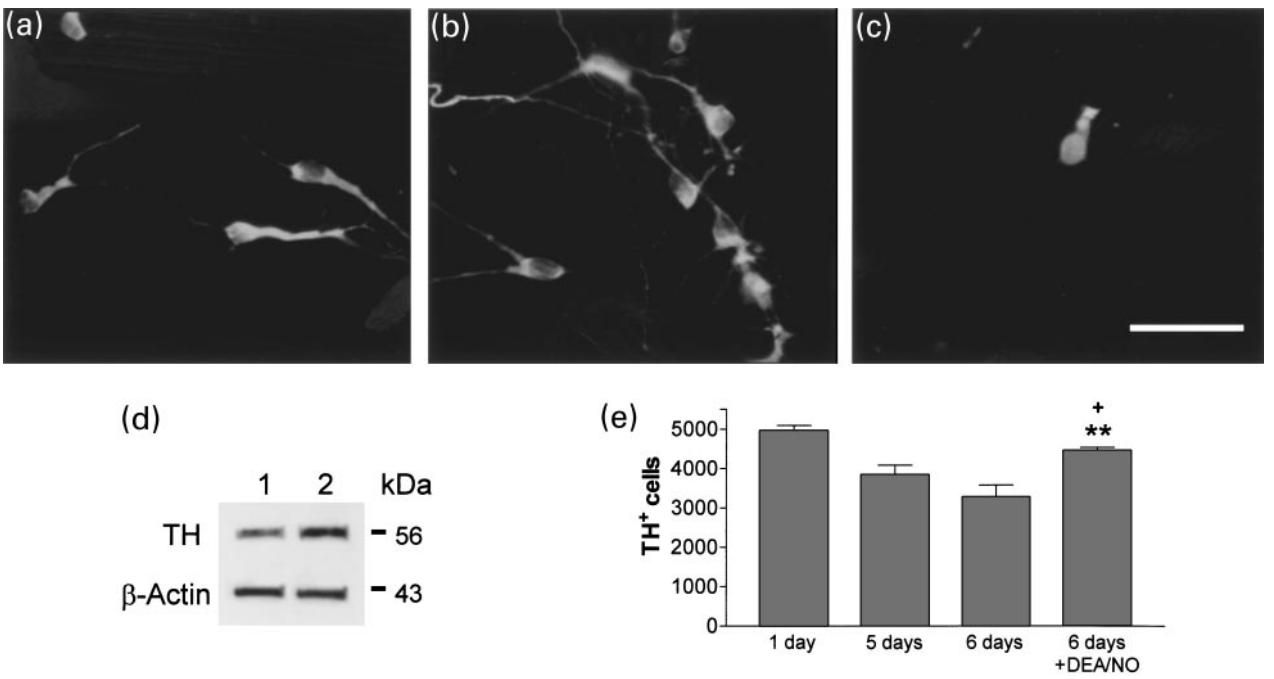

Fig. 6 Neurotrophic and neurotoxic effects of DEA–NO on DA neurones. TH immunocytochemistry of six day-postplating fetal midbrain cultures, treated for 24 h with: vehicle (a), 25 μM DEA–NO (b), or 400 μM DEA–NO (c). Scale bar = 50 μm. (d) Western blot analysis of TH protein in control cultures (1) and cultures treated with 25 μM DEA–NO for 24 h (2). Charge control with β-actin. (e) Effect of 25 μM DEA–NO on DA cell survival. Values are the mean (SEM from $n = 4 - 6$. Statistical analysis was performed by ANOVA followed by the Student's *t*-test. **$p < 0.01$, 6-day cultures treated with 25 μM DEA–NO, from day 5–6, vs. their respective controls; †$p < 0.05$, 6-day cultures treated with 25 μM DEA–NO vs. control cultures of 5 days.

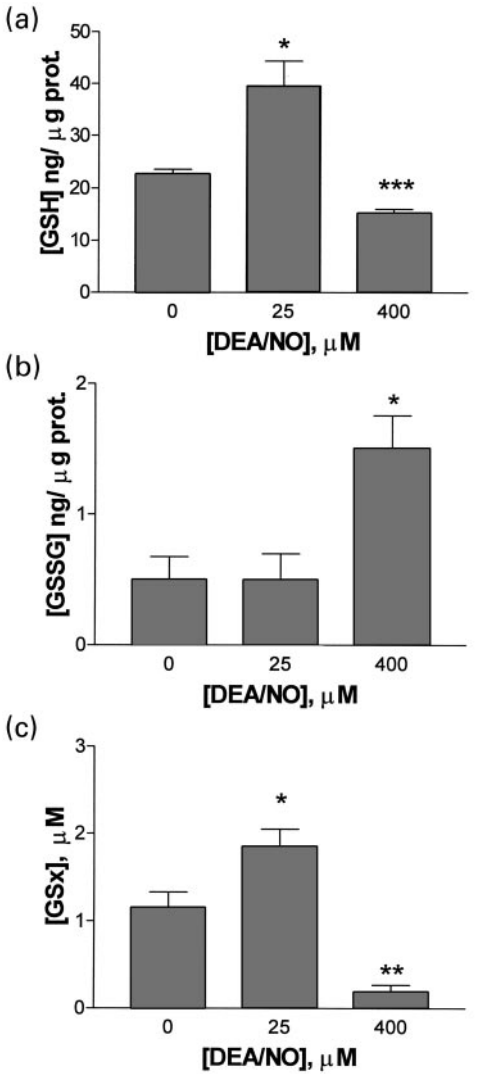

Fig. 7 Effect of 25 and 400 μM DEA–NO on glutathione levels. Intracellular reduced form (GSH) (a), intracellular oxidized form (GSSG) (b), and extracellular reduced + oxidized (GSx) forms (c). Values are the mean (SEM from $n = 6$. Statistical analysis was performed by ANOVA followed by the Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. controls.

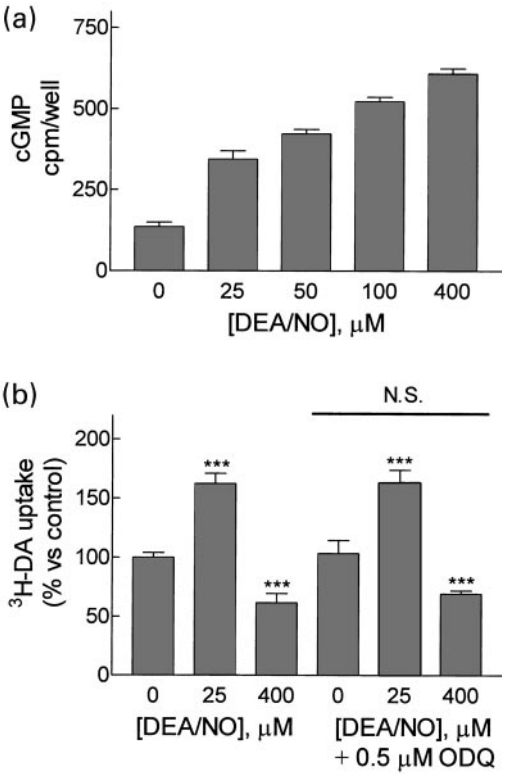

Fig. 8 (a) Dose-dependent increase in cGMP production induced by DEA–NO in fetal midbrain cultures. Results are expressed as the mean ± SEM ($n = 3$). (b) Effect of ODQ (0.5 μM) on [^{3}H]DA uptake in DEA–NO (25 and 400 μM)-treated cell cultures. Results are expressed as a percentage of the control group and are the mean ± SEM ($n = 3$–6). Basal levels: 16 045 ± 581 cpm/well. Statistical analysis was performed by ANOVA followed by the Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group. No significance differences (N.S.) were detected with ODQ-treatment when compared to their respective controls.

Results

Effects of NO on cell death

Dose-dependent effect

Treatment of fetal midbrain cultures with increasing concentrations of DEA–NO, ranging from 25 to 400 μM, for 24 h produced a dose-dependent increase in nitrite concentration in the culture medium (Fig. 1a) as a consequence of NO released from the donor (Keefer *et al.* 1996; Ferrero *et al.* 1999). In addition, a dose-dependent increase in apoptotic cell number was detected in the culture by chromatin condensation with bis-benzimide nuclear staining (Fig. 1b and Fig. 2c,e) and by DNA fragmentation with the TUNEL assay (Fig. 1c, 2d,f). The apoptotic nature of cell death in our system was corroborated by Western blot analysis of Bcl-2 family protein levels. A 24 h treatment with 400 μM DEA–NO increased Bax protein levels and reduced the ratio between Bcl-x_L/Bcl-x_S proteins (144 ± 10.3% and 62.6 ± 2.4%, respectively) (Fig. 1e), indicating a role for these proteins in the apoptotic process triggered by NO. No changes in Bcl-2 protein expression were detected in the same blots (data not shown). DEA–NO induced a dose- and time-dependent increase in necrotic cell number, as determined by Trypan blue dye exclusion (Fig. 3), concomitant with an increase in LDH activity, which was measured in the culture media (Fig. 3).

Time-dependent effects and cell selectivity

The apoptotic effect was observed at 4 h after treatment with 400 μM DEA–NO (23 ± 2% of apoptotic cells vs.

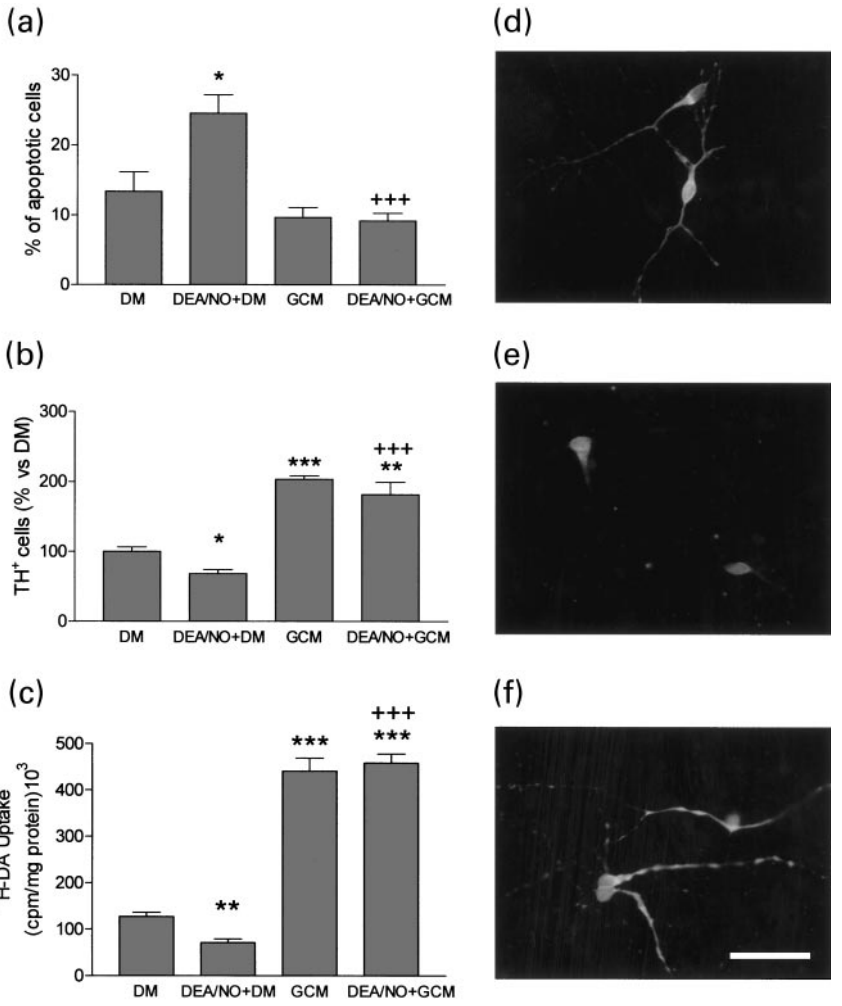

Fig. 9 Protective effects of glia-conditioned medium (GCM) on DEA–NO-induced toxicity in fetal midbrain cultures. After 5 days in culture, cells were changed to GCM or freshly defined medium (DM) and then treated with vehicle or 200 μM DEA–NO for 24 h. (a) Cellular nuclei were stained with bis-benzimide, chromatin-condensed nuclei were counted and expressed as a percentage of apoptotic cells with respect to the total cell number. (b) Number of DA neurones expressed as a percentage vs. DM. (c) High-affinity [^{3}H]DA uptake normalized by protein content. Right panels show TH immunocytochemistry of cultures treated in DM with vehicle (d) or 200 μM DEA–NO (e), and cultures treated in GCM with 200 μM DEA–NO (f). Scale bar = 50 μm. Values are expressed as the mean ± SEM for $n = 4$–6. Basal level for the number of TH$^+$ cells per well was 1638 ± 101. Statistical analysis was performed by ANOVA followed by the Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. DM. $\dagger\dagger\dagger p < 0.001$ vs. DEA–NO + DM.

11 ± 1% in controls) and increased to 54 ± 4% at 8 h (Fig. 4a). Immunocytochemical characterization of cell death in these time-related treatments (Figs 4 and 5) revealed that dopamine neurones (TH$^+$ cells, Fig. 4b) and mature oligodendrocytes (O1$^+$ cells, Fig. 4d) were the cell types most affected by 400 μM DEA–NO at 4 h (72 ± 9% and 85 ± 12% vs. control values, respectively) and 8 h (17 ± 3% and 18 ± 3%, respectively). Astrocytes (GFAP$^+$ cells, Fig. 5) and glial progenitors (A2B5$^+$ cells, Fig. 5) were unaffected, both in number and morphology, by this treatment at any of the time periods studied. Using double staining with bis-benzimide and TH$^+$ immunoreactivity, we proved that dopamine cell death occurs by an apoptotic mechanism (Fig. 4c).

Neurotrophic and neurotoxic effects of NO on dopamine cells

We have found that treatment of fetal midbrain cultures with DEA–NO, ranging from 5 to 400 μM, exerts neurotrophic and neurotoxic effects on functional dopamine parameters. Doses of 25–50 μM DEA–NO enhanced dopamine function (Table 1 and Fig. 6), increasing the number of TH$^+$ cells by 147 ± 10%, the TH$^+$ neurite processes (Fig. 6b), high affinity [^{3}H]DA uptake (173 ± 25%), intracellular DA levels (175 ± 29%) and TH protein levels (Fig. 6d).

To determine if these changes were due to *de novo* synthesis of TH$^+$ cells in the culture, we counted these neurones at 24 h, 5 and 6 days *in vitro*, in cultures treated with 25 μM DEA–NO from days 5–6. This dose of DEA–NO not only protected TH$^+$ cells from apoptosis, but also induced *de novo* synthesis of these cells; the number of TH$^+$ cells at 5 days *in vitro* was 3841 ± 243, increasing to 4473 ± 66 at 6 days (Fig. 6e).

Higher doses of DEA–NO (200 and 400 μM), however, were neurotoxic since they produced a dose-dependent decrease in the number of TH$^+$ cells (64 ± 6.8% and 16 ± 2.7%, respectively) and in the TH$^+$ neurite processes (Fig. 6c and Table 1). High affinity [^{3}H]DA uptake and endogenous DA levels were also decreased by 400 μM DEA–NO (40 ± 6% and 46 ± 3%, respectively) (Table 1).

Effect of DEA–NO on glutathione homeostasis

When primary midbrain cultures were treated with DEA–NO in doses ranging from 5 to 400 μM, an increase in glutathione levels was observed at 25–50 μM and a decrease at 400 μM, coincident with an increase and decrease, respectively, in DA function (Table 1). Extracellular glutathione levels were altered in a similar fashion as the intracellular GSH levels (Fig. 7a,c). Glutathione disulfide (GSSG) was increased only at 400 μM DEA–NO (Fig. 7b). Altogether, these data suggest that low doses of NO enhance the free radical neutralizing capacity of cells whereas high doses produce an increase in oxidative stress.

Effect of DEA–NO on cGMP levels

cGMP levels, as well as nitrite levels, in fetal midbrain cultures were affected by DEA–NO in a dose-dependent manner (Fig. 8a). To determine if cGMP is involved in the NO-induced neurotoxic/neurotrophic effects in fetal midbrain cultures, the effect of DEA–NO was examined in the presence of the specific soluble guanylate cyclase inhibitor, ODQ. The DEA–NO-induced increase of cGMP levels in the cell cultures was inhibited by ODQ (0.001–10 μM) in a concentration-dependent manner ($IC_{50} = 0.072$ μM, data not shown). Subsequent experiments were therefore conducted at 0.5 μM ODQ. The cells were treated with ODQ 30 min before addition of DEA–NO. Control groups were treated with ODQ solvent (3.75×10^{-4} % DMSO). ODQ (0.5 μM) did not affect the cell viability (data not shown), nor did it revert the DEA–NO-induced effect on [^{3}H]DA uptake (Fig. 8b).

Glia-conditioned medium protects fetal midbrain cultures from NO-induced toxicity

Cultures that were treated with the neurotoxic dose of 200 μM DEA–NO, in the presence of GCM, did not show any signs of toxicity. No neurite degradation was detected, as shown by phase contrast and TH immunostaining, nor was a decrease in TH^+ cell number or [^{3}H]DA uptake observed. In addition, we detected no increase in apoptotic cell death (Fig. 9). GCM protects from DEA–NO-induced neurotoxic effects when given simultaneously.

Discussion

NO donors exert their effects through the spontaneous release of NO. Most often, NO is toxic for nerve cells and induces apoptosis and necrosis. In some cases, however, NO reduces neuronal death resulting from excitotoxicity or serum deprivation (Lei *et al*. 1992; Kim *et al*. 1999). The differential effects of NO on cell death are due, in part, to the type of NO donor and cells used (Keefer *et al*. 1996; Ferrero *et al*. 1999; Yamamoto *et al*. 2000). In this regard, the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), which has a half-life of 37.2 ± 13.8 min (in Krebs buffer, at 24°C), induces differentiation of NB69 neuroblastoma cells (Rodríguez-Martín *et al*. 2000). In this study, we have investigated the effects of DEA–NO, a nitric oxide donor, on neuronal-enriched fetal rat midbrain cultures. We found that the effects of NO depend on the dose, time and cell type. At low concentrations, NO has anti-apoptotic effects on DA cells whereas at high concentrations, it increases apoptosis in neurones and oligodendrocytes. Cell death, induced by DEA–NO, takes places by apoptosis and necrosis. Early neurotoxicity, consisting in apoptosis, occurs in DA neurones at high DEA–NO concentrations (400 μM). GCM, obtained from fetal midbrain astrocyte cultures, totally protects neuronal-enriched midbrain cultures from NO-induced apoptosis and rescues [^{3}H]DA uptake and TH^+ cell number.

The family of Bcl-2-related proteins, that includes Bcl-2, Bcl-x, Bad, Bak and Bax, constitutes a class of apoptosis regulatory gene products that act at the effector stage of apoptosis. It has been shown recently that NO-induced cell death alters the expression of Bcl-2 and Bax (Tamatani *et al*. 1998; Matsuzaki *et al*. 1999). We found that treatment with 400 μM DEA–NO increases Bax levels but reduces the ratios of Bcl-xL/Bcl-xS and Bcl-2/Bax.

Our results suggest that NO may be neurotrophic or neurotoxic for fetal DA cells. Neurotrophism, as shown by an increase in the number of TH^+ cells, the number and branching of DA processes, TH-protein content, [^{3}H]DA uptake and DA levels, takes place at low doses of DEA–NO (25 and 50 μM); neurotoxicity, as shown by a reduction of all these parameters, occurs at higher DEA–NO concentrations. The neuroprotective and neurotoxic roles of brain NO may derive from the local intracellular oxidation–reduction potential, since a reductive environment favours the production of NO-free radicals (Lipton *et al*. 1993; Smith *et al*. 1994). NO reacts with the superoxide anion and produces peroxynitrite. This reactive species damages proteins by sulfydryl oxidation and nitration, mechanisms that inactivate TH (Ara *et al*. 1998; Kuhn *et al*. 1999).

Regulatory effects of NO on TH activity may be due to a transcriptional mechanism, since we found an increase of TH protein levels, as assessed by Western blotting. Other mechanisms underlying TH activation by NO, including post-transcriptional phosphorylation of the protein, cannot be excluded (Ohki *et al*. 1995; Kumer and Vrana 1996; Bhat *et al*. 1998; Rodríguez-Pascual *et al*. 1999). At low concentrations, NO may act as a second messenger that activates the TH protein producing the opposite effects at high doses, probably related to a nitrosylation or a sulfydryl oxidation of the TH enzyme. Rauhala *et al*. (1998) reported that *S*-nitrosylation of GSH by NO and oxygen may be part of the antioxidative cellular defense system as well as the inactivation of caspases by GSNO via *S*-nitrosylation of cysteine residues. In this context, Wink *et al*. (1993) have proposed that NO protects against cellular damage and cytotoxicity from reactive oxygen species. Mena *et al*.

(1998b) suggested that mild pro-oxidant treatments could protect different types of neurones from death by upregulating GSH. In the present study, we observed a significant increase in intracellular GSH levels at low doses of DEA–NO (25 and 50 μM), and a significant and progressive decrease from 200 to 400 μM, indicating a neuroprotective role for GSH. The ratio of GSSG/GSH has been used as an index of oxidative stress in the brain (Slivka *et al*. 1987) and GSH/total glutathione as an antioxidant index (Weber 1999). There is relevant *in vivo* evidence suggesting that nanomolar concentrations of GSNO can protect brain DA neurones from iron-induced oxidative stress and degeneration. GSNO may be formed in NO-generating cells such as astrocytes, which also contain millimolar concentrations of GSH (Murphy *et al*. 1993; Bolaños *et al*. 1995). The cellular redox status is an important factor during neuronal apoptosis. Under conditions of increased reactive oxygen species, production of high intracellular glutathione content could protect neurones from apoptotic injury. Likewise, drugs inhibiting GSH depletion could prevent neurones from oxidative damage (Ahlemeyer and Krieglstein 2000). The intracellular redox status determines whether NO is toxic or protective for rat oligodendrocytes in culture (Rosenberg *et al*. 1999). Generation of NO and subsequent formation of $ONOO^-$ or nitrite may contribute to the selective vulnerability of DA neurones through the oxidation of DA and protein modification. GSH is the most potent endogenous antioxidant known so far and blocks the binding of the DA quinone to the protein induced by $ONOO^-$ (LaVoie and Hastings 1999).

We observed a dose-dependent increase of nitrite levels in the supernatant of DEA–NO-treated cells that appeared less than 10 min after addition of the NO donor. The nitrite levels were identical in the absence of cells. It is unlikely that the effect of DEA–NO on DA levels is related to a competition between NOS and TH for BH_4, the cofactor of both enzymes (Hwang *et al*. 1998), since NO production from DEA–NO does not require NOS activity.

DEA–NO produces a dose-dependent increase of cGMP levels in midbrain cells. We used ODQ, a guanylate cyclase inhibitor, to investigate the role of the cGMP pathway in cell function (Moro *et al*. 1998; Kim *et al*. 1999) and DEA–NO-induced neurotrophic and neurotoxic effects. ODQ (0.5 μM) did not revert the DEA–NO-induced effect on [^{3}H]DA uptake. These data suggest that the mechanism through which NO modulates DA function is independent of cGMP. GSNO and NO have antioxidant properties independent of cGMP in other systems (Chiueh and Rauhala 1999), as well as in DA differentiation in human neuroblastoma cells (Rodríguez-Martín *et al*. 2000).

Altogether, our findings may have important clinical implications. Low GSH levels are present in the brains of patients with Parkinson's disease (PD) (Jenner and Olanow 1998; Weber 1999). GSH is enriched in the mitochondria (Meister 1988), where the thiolic groups play an essential role in the activity of complex I (Martínez-Banaclocha 2000). In addition, GSH depletion in the substantia nigra is an early indicator of oxidative stress in PD (Jha and Andersen 1999). Peroxynitrite may also play a role in this disease; an increase of nitrite concentration in the cerebrospinal fluid of patients with PD has been reported (Qureshi *et al*. 1995) and NO radicals have been detected in PD substantia nigra (Shergill *et al*. 1996). Finally, the core of Lewy bodies in PD are immunoreactive for nitrotyrosine (Good *et al*. 1998).

GCM protects from DEA–NO-induced neurotoxic effects when given simultaneously. It inhibits apoptosis but is also neurotrophic for DA neurones (Mena *et al*. 1996, 1997, 1999). GCM is very rich in antioxidants (mainly GSH and ascorbic acid), molecules that are neuroprotective during acute exposure to DEA–NO. In addition, glia produces neurotrophic and neurite-promoting agents that enhance development, survival and neurite extension of DA neurones and confer resistance to neurotoxins (Engele *et al*. 1991; Nagata *et al*. 1993; Hoffer *et al*. 1994; Takeshima *et al*. 1994a, 1994b; Muller *et al*. 1995). Several gliotrophic factors, such as fibroblast growth factor (Gall *et al*. 1994; Mena *et al*. 1995b; Hou *et al*. 1997) and glial-derived neurotrophic factor (GDNF) (Lin *et al*. 1994), share with the antioxidants the neuroprotective effects of GCM. We have shown that the neurotrophic effect of GCM on DA neurones is greater than what could be attributed to GDNF (Mena *et al*. 1997). Other neurotrophic factors (Mena *et al*. 1995b; Engele *et al*. 1996; Grove *et al*. 1997; Mena *et al*. 1998a) could also participate in the neurotrophic effect of GCM. Thus, GCM seems a promising source of neurotrophic agents for PD.

These studies provide new insights into the complex regulatory activity of NO on DA neurones *in vitro*. The role of NO in the regulation of these neurones *in vivo*, in health and disease, and the putative pharmacological manipulation of the NO pathway in neurodegenerative diseases, must be further investigated.

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Trabajo 2

Nitric oxide induces differentiation in the NB69 human catecholamine-rich cell line

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Nitric oxide induces differentiation in the NB69 human catecholamine-rich cell line

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Abstract

The nitric oxide (NO) donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), induced differentiation of human neuroblastoma NB69 cells to a dopamine phenotype, as shown by phase-contrast microscopy and tyrosine hydroxylase (TH) immunocytochemistry. NB69 cells were treated with 50 to 750 μM SNAP in serum-free-defined medium for 24 h. SNAP treatment did not increase the number of necrotic or apoptotic cells. However, a decrease in the number of viable cells was observed at 750 μM SNAP. In addition, a decrease in ^{3}H-thymidine uptake was detected at the highest dose of SNAP. An increase in the antiapoptotic Bcl-2 and Bcl-xL protein levels and a decrease in the proapoptotic Bax and Bcl-xS protein levels were also detected by Western blot analysis after SNAP treatment. At low doses (50–125 μM), SNAP induced an increase in catecholamine levels, ^{3}H-dopamine uptake, TH activity and monoamine metabolism, while a decrease in all these parameters was observed at high doses (250–750 μM). The TH protein content, analyzed by Western blot, remained unchanged in SNAP-treated cells throughout the range of doses studied, when compared with the control group. SNAP produced a dose-dependent decrease in the glutathione (GSH) content of the culture medium, without altering intracellular GSH. In addition, cGMP levels and nitrite concentration, measured in the supernatant of SNAP-treated cells, increased in a dose-dependent manner, as compared to control levels. The guanylate cyclase inhibitor lH-[1,2,4]oxadiazolo[4,3a]quinoxaline-l-one (ODQ) did not revert the SNAP-induced effect on ^{3}H-dopamine uptake to control values. These results suggest that NO, released from SNAP, induces differentiation of NB69 cells and regulates TH protein at the post-transcriptional level through a cGMP-independent mechanism.

Keywords: Nitric oxide; Dopamine; Tyrosine hydroxylase; Differentiation; Bcl-2 family proteins; Glutathione; cGMP; Neuroblastoma cells

1. Introduction

Nitric oxide (NO) is synthesized by various isoforms of nitric oxide synthase (NOS) that catalyse the conversion of L-arginine to L-citrulline and NO in the presence of oxygen and NADPH (Bredt and Snyder, 1990; Knowles and Moncada, 1994). NO plays an important role in cell-to-cell modulation (Garthwaite and Boulton, 1995) and vasodilation via activation of NO-sensitive guanylyl cyclase and the generation of cGMP (Miki et al., 1977; Moncada et al., 1991). NO has been shown to exert a dual action: it ameliorates glutamate neurotoxicity by reducing N-methyl-D-aspartate (NMDA) currents when converted to its oxidized form (NO+) but has a neurotoxic action when converted to its reduced form ($NO^{\bullet}$) (Lipton et al., 1993). NO formation may be neuroprotective or neurotoxic (Bolaños et al., 1997). It has been reported that primary cortical neurons exposed to NO donors such as S-nitroso-N-acetyl-D,L-penicillamine (SNAP), sodium nitroprusside (SNP), or 3-morpholinosydnonimine (SIN-l) exhibit concentration-dependent cytotoxicity, indicating that increasing concentrations of NO can be neurotoxic (Dawson et al. 1991, 1993). Several authors have found neuroprotection with NOS inhibitors (Izumi et al., 1992; Schulz et al., 1995), while others report no neuroprotection (MacKenzie et al., 1995) or exacerbation of NMDA-induced toxicity (Connop et al., 1995). On the other hand, Sawada et al. (1996) have shown that the mechanism which protects from NO neurotoxicity in dopamine neurons is based on an inhibition of the conversion of NO to the peroxynitr-

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ite anion, independent of the NO redox state, and is possibly due to a suppression of superoxide anion production. Recently, it has been described that the protective actions of NO could be mediated by cGMP-dependent mechanisms (Hindley et al., 1997; Kim et al., 1999).

In the present study, we examined the effects of the NO donor SNAP on human neuroblastoma NB69 cells, a catecholamine-rich cell line whose biochemical and pharmacological profile has been described previously (Mena et al. 1989, 1992; Pardo et al., 1995). For this purpose, endogenous catecholamine levels, TH activity, TH protein levels, monoamine metabolism, ^{3}H-dopamine uptake, glutathione (GSH) levels, cGMP formation, nitrite levels, viability and necrotic and apoptotic cell death were determined in NB69 cells. In order to evaluate the differentiation of this catecholamine cell line, microscopy and immunocytochemical studies were carried out. In addition, to determine if NB69 cell differentiation is mediated through a cGMP-dependent mechanism, 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ), a guanylate cyclase inhibitor, was used.

2. Materials and methods

2.1. Material

The culture reagents Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L), fetal calf serum, penicillin-streptomycin, pyruvate, glutamine, Nutrient mixture F-12 (Ham) with L-glutamine and minimum essential medium (MEM) with Earle's salts and L-glutamine, were obtained from GIBCO-Life Technologies (Scotland). Trypan blue, bovine serum albumin, mouse monoclonal β-actin antibody, pargyline, 3-hydroxibenzyl hydrazine (NSD 1015), N-(1-napthyl)-ethylenediamine and sulfanilamide were purchased from Sigma (Madrid, Spain); ascorbic acid was from Merck (Darmstadt, Germany), lH-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ) from Biomol Research Lab., Inc. (PA, USA), S-Nitroso-N-acetylpenicillamine (SNAP) from Tocris (UK), mouse monoclonal anti-tyrosine hydroxylase (TH) antibody and anti-mouse Ig fluorescein from Boehringer-Manheim (Barcelona, Spain). Anti-rabbit polyclonal Bax and Bcl-xS/L antibodies were from Sta. Cruz Biotechnology, Inc. (CA, USA) and mouse monoclonal Bcl-2 antibody was from Dako (Denmark). ^{3}H-dopamine (53.7 Ci/mmol) (^{3}H-DA) and ^{3}H-methyl-thymidine (20 Ci/mmol) were from American Radiolabeled Chemical, Inc. (St. Louis, MO, USA) and ^{3}H-guanosine (6.3 Ci/mmol) from Moravek Biochemicals (CA, USA). The apoptosis TUNEL detection kit was obtained from Promega (Madison WI, USA). All other agents were of the highest purity commercially available from Merck or Sigma. 3,4-dihydroxy-4′-methyl-S-nitrobenzenophenone (RO-40-7592) was kindly donated by Hoffman LaRoche (Basel, Switzerland).

2.2. Cell culture

Human neuroblastoma NB69 cells were grown and maintained as described previously (Mena et al. 1989, 1992). In brief, the cells were grown in DMEM supplemented with 15% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/ml), pyruvate (1 mM) and glutamine (4 mM) (DMEM-FCS). Cells were obtained from 4–6 subcultures after thawing. Five days after plating at a density of 1×10^5 cells/mL on 35 mm-diameter multiwells, the culture medium was changed to a serum-free defined medium (EF12) and the cells were treated with SNAP or vehicle, with slight modifications of the method described by O'Malley et al. (1991). The cells were incubated with SNAP, dissolved in EF12, at five different concentrations (50, 125, 250, 500, 750 μM) for 24 h.

The number of viable cells was estimated by trypan blue dye exclusion in a Neubauer hemocytometer. The cells were collected in the culture medium and an aliquot of these cells was used for cell viability determination. Protein content was determined by the method of Bradford (1976), using bovine serum albumin as the standard. Estimation of deoxyribonucleic acid (DNA) was performed according to Burton (1956). For the biochemical assays, cultured cells were harvested by scraping in Ca^{2+}-and Mg^{2+}- free phosphate-buffered saline (PBS), centrifuged at $500\times\boldsymbol{g}$ for 7 min, and resuspended in PBS. Differentiation of the NB69 cells was evaluated according to morphological changes detected by microscopy, by TH immunostaining, and by determination of catecholamine (CA) levels and ^{3}H-DA uptake. Cell proliferation was detected by ^{3}H-thymidine uptake (Mena et al., 1995).

2.3. Catecholamine levels, TH immunostaining and ^{3}H-DA uptake

CA levels were measured by HPLC with an ESA coulochem detector, according to Mena et al. (1989, 1995), as follows: The cells were detached from the wells and centrifuged. The resultant supernatant was discarded and the pellets were sonicated in 300 μL of 0.4 N perchloric acid (PCA) with 0.5 mM $Na_2S_2O_5$ and 2% EDTA and then centrifuged for 5 min. CA levels and TH activity were determined in 20 μL of the resulting supernatant, and the pellet was used for protein and DNA determination. CA neurons were characterized by immunostaining with a mouse monoclonal anti-TH antibody (Mena et al., 1995). Nuclei were stained with bisbenzimide (Hoechst 33342) (Hilwig and Gropp, 1975). High affinity ^{3}H-DA uptake was evaluated according to the method of Beart and McDonald (1980).

TH activity was evaluated by measuring the accumulation of DOPA during the 2 h following inhibition of its decarboxylation by NSD 1015 (150 mg/L) (Carlsson et al., 1973). Monoamine metabolism was measured after inhibition of monoamine oxidase (MAO) and catechol O-methyl transferase (COMT), for 4 h, using pargyline (10^{-5} M) and RO-40-7592 (30 mg/L), respectively.

2.4. ^{3}H-thymidine uptake

The rate of cell division was evaluated by the uptake and incorporation of ^{3}H-(methyl)thymidine in NB69 cells cultures, according to the following experimental procedure: After 5 days in culture, ^{3}H-thymidine (specific radiactivity 20 Ci/mmol; 0.008 μCi/ml of culture medium) was added at the same time as SNAP treatment. Incubation was continued for 24 h at 37°C. The medium was then removed, the cells were washed twice with PBS, dislodged from the wells and suspended in 200 μL, of 5% perchloric acid. The cell suspension was then vortexed and incubated at 70°C for 15 min. After this incubation, 100 μL aliquots of the supernatants were suspended in 3 mL of scintillation liquid (Optiphase) and counted in a scintillation counter.

2.5. Measurement of glutathione levels

Glutathione (GSH) levels were measured according to Hyland and Bottiglieri (1992) by HPLC using a spectrofluorimeter detector, with minor modifications as described previously (Mena et al., 1998a). Extracellular GSH levels were measured in the culture medium of NB69 cells previously deproteinized with 0.4 N PCA. Intracellular GSH levels were determined in 20 μL of the second supernatant, as explained above.

The chromatographic system consisted of two Beckman-112 pumps, a 231 XL-Gilson autosampler, and a 5 μm Beckman ODS (15 cm×4.6 mm I.D.) reversed-phase column. The OPA-derivatized GSH was achieved using a Perkin Elmer LS4 spectrofluorimeter. Excitation and emission wavelengths were set at 365 and 455 nm, respectively.

Mobile phase A was 0.05 M sodium acetate (pH 6.8), and phase B was acetonitrile. The flow rate was 1.3 ml/min and a linear gradient from 8% to 25% B in 5 min was performed. A volume of 20 μl of sample (culture media plus PCA to a final concentration of 0.4 N or intracellular supernatant) was injected every 10 min.

In order to evaluate the content of oxidized (GSSG) and reduced (GSH) glutathione, GSSG levels were determined by the method of Griffith (1980) and GSH levels by the method of Tietze (1969).

2.6. Immunoblot analysis

Analysis of TH and Bcl-2 family proteins was performed according to the method described by Labatut et al. (1988). The cells were detached from the wells in analysis buffer (20 mM Tris HCl, 10 mM AcK, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 0.25% NP-40, pH 7.4), homogenized and then centrifuged at 12.000***g*** for 30 min at 4°C. The supernatant was used for electrophoretical assay and protein determination. Samples (30 μg) were added to SDS sample loading buffer, electrophoresed in Bio-Rad SDS-polyacrilamide gels (4–20%) and then electroblotted to 0.45 μm nitrocellulose membranes. For immunolabeling, the blots were blocked with TTBS (20 mM Tris-HCI pH 7.6, 137 mM NaCl plus 0.1% (v/v) Tween-20 and 5% dry skimmed milk) for 1 h at room temperature. After blocking non-specific binding, the membranes were incubated with mouse anti-TH (1:2000), rabbit anti-Bax (1:1000), mouse anti-Bcl-2 (1:250), rabbit anti Bcl-xS/L (1:500) and mouse anti-β-actin (1:5000) in blocking solution for 16 h at 4°C. The blots were appropriately washed and further incubated for 1 h at room temperature with anti-mouse Ig- or anti-rabbit Ig-antibodies linked to horseradish peroxidase (HRP) (1:1000) in blocking solution. After washing, the immunoreactive bands were visualized by chemiluminescence detection using a commercial kit (Amersham). Autoradiograms were quantitated by computer-assisted videodensitometry. Neither TH nor Bcl-2, Bax, Bcl-xS/L or β-actin immunoreactivity was observed when primary antibodies were removed from the assay (data not shown). The same test was carried out for HRP-conjugated secondary antibody and no immunoreactivity was detected at that time. In addition, membranes were immunolabeled for control of charge using β-actin or 0.1% fast green diluted in 25% methanol and 10% acetic acid.

2.7. Measurement of cGMP formation

For cGMP determination, the culture medium was removed from the wells and the cells were incubated for 24 h with 2 μCi/mL [^{3}H]guanosine in 1 mL of serum-free defined medium. After radiolabelling, cells were washed once with Krebs-Henseleit-Hepes buffer (KHH) (118 mM NaCl, 4.7 mM KCI, 2.5 mM $CaCl_2$, 1.2 mM $MgS0_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 20 mM Hepes, 25 mM $NaHC0_3$, pH 7.4 and 0.5 mM IBMX). After washing, the cells were incubated at 37°C in KHH for 30 min prior to addition of SNAP, at different concentrations, for 5 min. The reaction was stopped by aspiration of the buffer and addition of 1 ml of cold methyl alcohol (MeOH) and 0.12 N HCl (1:1). Plates were then kept in the freezer at -20°C for 1 h and aliquots (900 μL) of the MeOH-HCl extract were used to determine cGMP production as described previously (Hernández, 1995).

2.8. Apoptosis detection

The apoptosis TUNEL detection system measures the fragmented DNA of apoptotic cells by incorporating fluorescein-12-dUTP* at the 3′-OH ends of the DNA using the enzyme Terminal deoxynucleotidyl Transferase (TdT), which forms a polymeric tail using the principle of the TUNEL (TdT-mediated dTP Nick-End Labeling) assay (Kerr et al., 1972; Gavrieli et al., 1992). The assay was performed on glass cover slides at a density of 15 000 cells per cover slide. The cells were incubated with SNAP at different concentrations for 24 h, fixed in 4% p-formaldehide and permeabilized with 0.2% Triton X-100. The number of apoptotic cells was counted in 21 fields of the coverslip, at random. Cells were counted in pre-defined parallel strips using a counting reticule in the ocular. Cell nuclei, stained by *bis*-Benzimide, were counted in 21 fields of the coverslip, at random.

2.9. Nitrite measurement

NO production was quantified by measuring nitrite, a stable oxidation end product of NO (Green et al., 1982). Briefly, 400 μL of culture medium were mixed with 800 μL of Griess reagent (1.5% sulfanilamide in 1N HCl plus 0.15% N-(1-naphtyl)-ethylenediamine dihydrochloride in distilled water, v:v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined. Sodium nitrite was used as a standard.

2.10. Data analysis

All data presented in this study are expressed as the mean ±S.E.M. The results were statistically evaluated for significance by using one-way analysis of variance followed by the Student's *t* test. Means among groups were considered significantly different when the *p* value was less than 0.05.

3. Results

3.1. Effect of SNAP on cell death

SNAP (50–750 μM) induced differentiation of NB69 cells, as shown by phase-contrast microscopy and by tyrosine hydroxylase immunocytochemistry (Figs. 1A and B).

The treatment of NB69 cells with SNAP did not induce any significant change in the number of dead cells at any of the doses studied. We did find, however, a decrease in the number of viable cells at 750 μM of SNAP (Table 1), as measured by trypan blue dye exclusion. The cells were collected in the culture medium and an aliquot of these cells was used for cell viability determination. In this regard, a decrease was also shown at 750 μM of SNAP by the ^{3}H-thymidine uptake assay (Table 1) when compared to controls. These results are in agreement with the cell differentiation shown previously (Figs. 1A and B). The treatment with SNAP did not increase the number of apoptotic cells (Table 1). Protein and DNA levels were reduced to 90% and 79% of control values, respectively, in cultures treated with 750 μM of SNAP (Table 1). In addition, an increase in the antiapoptotic Bcl-2 and Bcl-xL protein levels and a decrease in the proapoptotic Bax protein levels were detected by western blot analysis (Fig. 2).

3.2. Effect of SNAP on catecholamine levels and TH activity

In NB69 cells, SNAP induced an increase in CA, noradrenaline (NA) and DA levels at 50 and 125 μM, and a decrease in these levels at doses from 250 to 750 μM of SNAP, when compared to controls (Table 2). In parallel, a similar effect of SNAP on high affinity ^{3}H-DA uptake was observed in NB69 cells, with an increase from 50 to 250 μM of SNAP and a decrease from 500 to 750 μM when compared to control levels (Table 2). In both cases, the maximal effect was detected at 50 μM SNAP. In addition, TH activity and monoamine metabolism assays showed a similar pattern: 50 μM SNAP increased both parameters whereas 500–750 μM SNAP induced a decrease in DOPA as well as in NA and DA levels, respectively.

3.3. Effect of SNAP on glutathione, nitrite and cGMP levels

SNAP, at doses ranging from 250 to 750 μM, induced a dose-dependent reduction in the level of GSH in the culture medium of NB69 cells, without affecting the intracellular levels (Table 3). Moreover, no GSSG was detectable in either control or in SNAP-treated cells (data not shown).

Cells that were not treated with SNAP released very little nitrite into the supernatant. After addition of SNAP, nitrite levels in the supernatant were increased in a dose-dependent manner (213.29 μM±1.54 vs 3.31 μM±0.15, SNAP 750 μM vs control cells). In addition, we detected an identical nitrite concentration in the absence or presence of cells at the different doses of SNAP (Fig. 3A). cGMP levels in NB69 cells were also affected by SNAP in a dose-dependent manner (Fig. 3B). To determine if cGMP is the mechanism involved in the NO-induced NB69 cell differentiation, the effect of SNAP was examined in the presence of the specific inhibitor of soluble guanylate cyclase, ODQ. The SNAP-induced increase in cGMP levels in the NB69 cells was inhibited by ODQ (0.001–10 μM) in a concentration-dependent manner (IC_{50}=0.072 μM, data not shown). The experiments were

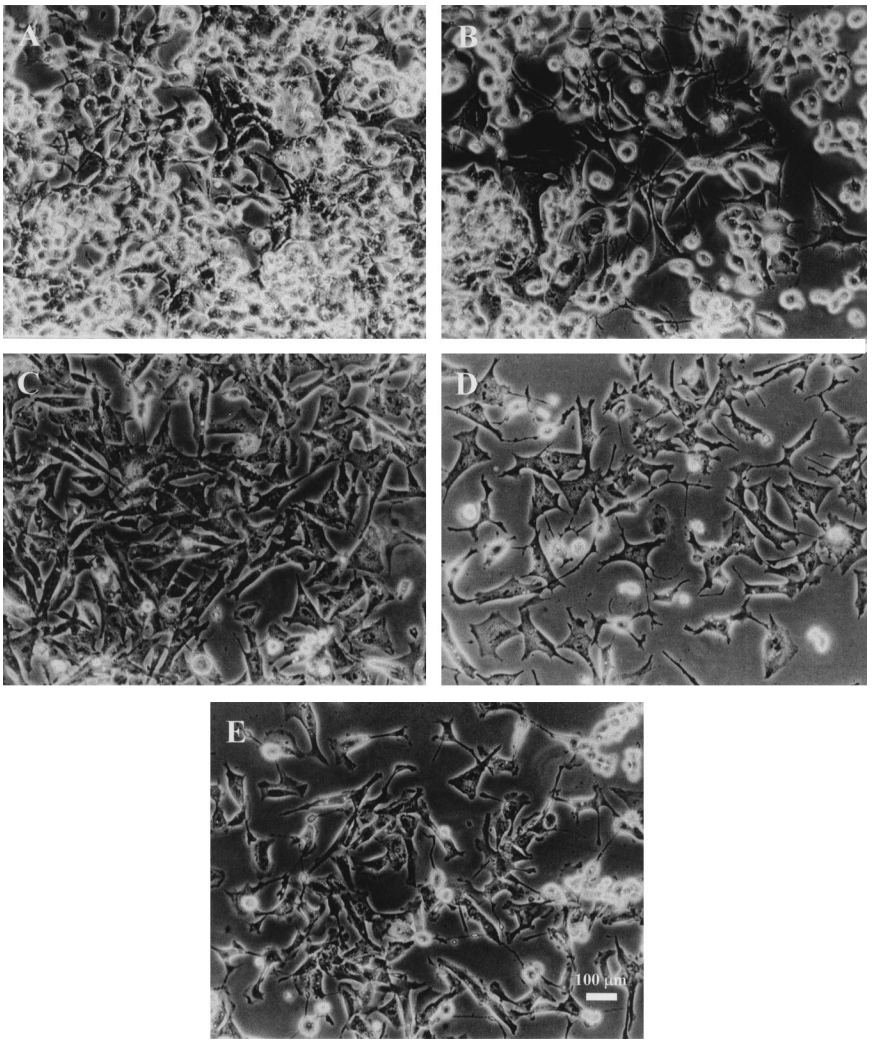

Fig. 1. (A) Phase-contrast microscopy and (B) Tyrosine hydroxylase immunocytochemistry of NB69 human neuroblastoma cells after six days in culture. On the 5th day, the cultures were treated with SNAP or vehicle (serum-free defined medium) for 24 h. (a) Control cells treated with vehicle. (b) Cells treated with 50 μM SNAP. (c) Cells treated with 125 μM SNAP. (d) Cells treated with 250 μM SNAP. (e). Cells treated with 500 μM SNAP. Scale bar=100 μm.

therefore conducted at 1 μM ODQ. The cells were treated with ODQ 30 min before addition of SNAP. ODQ (1 μM) did not affect the cell viability (data not shown) and did not revert the SNAP-induced effect on ^{3}H-DA uptake (Fig. 4).

3.4. Effect of SNAP on TH protein

To evaluate whether the changes in TH activity in NB69 cells were due to post-transcriptional activation or de novo protein synthesis, we measured TH protein levels by Western blot analysis (Fig. 5). TH protein levels were not altered by any of the SNAP doses studied (50–750 μM). We also tested β-tubulin levels by immunoblot and detected no changes in the amount of protein (data not shown).

4. Discussion

NO donors produce their effects through the spontaneous release of NO. We have investigated the effect of SNAP, a nitric oxide donor, on human neuroblastoma NB69 cells, a catecholamine-rich cell line. Our studies reveal that SNAP induces differentiation of NB69 cells to a mature DA phenotype, as shown by phase contrast microscopy and by tyrosine hydroxylase immunocytochemistry. Arrest of cell division is a prerequisite for cells to enter a program of terminal differentiation. In this regard, the decrease in the number of viable cells with no change in the number of dead cells, and the reduction in the rate of division, expressed by the ^{3}H-thymidine uptake, observed at 750 μM SNAP, also support the NO-induced-differentiation of NB69 cells. These results are

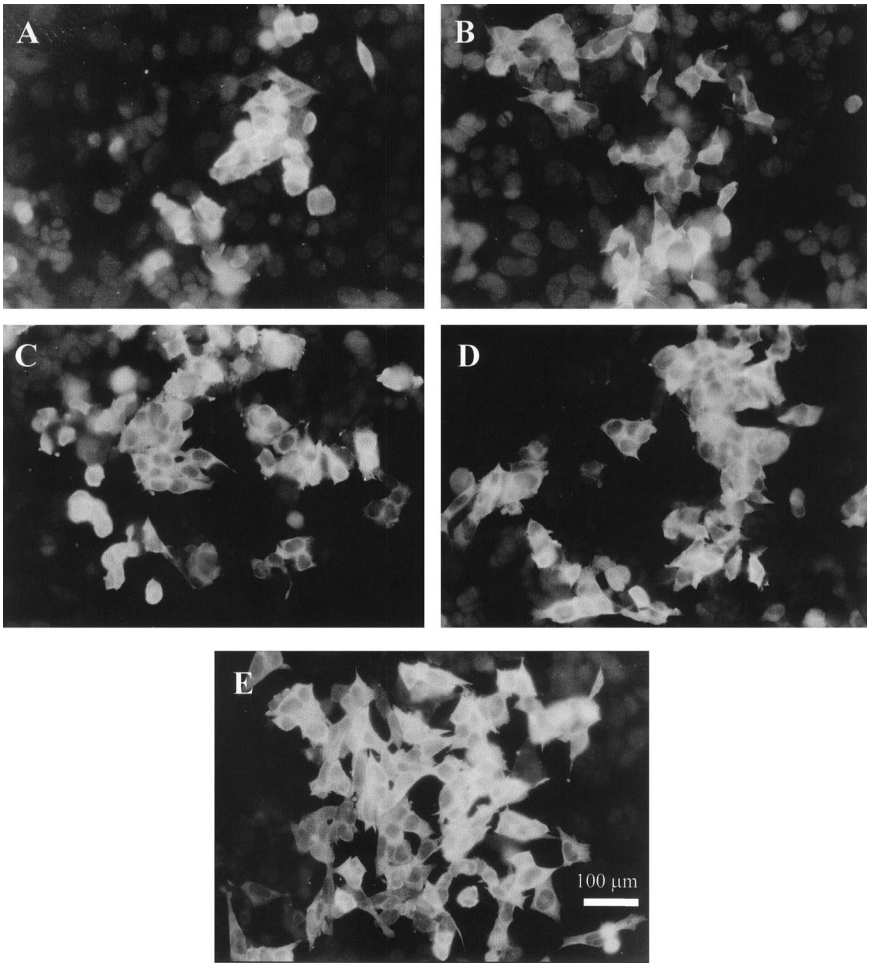

Fig. 1. (*continued*).

Table 1
Effect of SNAP on NB69 cell viability[a]

SNAP (μM)	Viable cells ($\times 10^6$/ml)	Dead cells ($\times 10^6$/ml)	% apoptotic cells	Proteins (μg/ml)	DNA (μg/ml)	^{3}H-thymidine uptake cpm$\times 10^3$/ml
Control (0 μM)	2.25±0.17 100%	0.91±0.06 100%	0.50±0.03	1470±49 100%	101.84±1.55 100%	78.51±3.80 100%
SNAP 50 μM	2.48±0.17 110%	0.85±0.16 93%	0.46±0.05	1595±39 109%	107.35±3.92 105%	82.83±4.00 106%
SNAP 125 μM	2.59±0.12 115%	0.77±0.05 85%	0.47±0.02	1570±49 107%	109.77±3.16 108%	84.01±4.07 107%
SNAP 250 μM	2.34±0.08 104%	0.84±0.02 92%	0.46±0.05	1570±30 107%	108.43±3.03 106%	96.57±4.67 123%
SNAP 500 μM	2.42±0.07 108%	0.82±0.09 90%	0.48±0.02	1523±100 104%	108.13±1.56 106%	76.16±3.69 97%
SNAP 750 μM	1.21±0.28* 54%	0.90±0.11 99%	0.42±0.01	1320±106* 90%	80.83±3.00*** 79%	58.89±2.85*** 75%

[a] Cells were cultured for 5 days in complete medium. On the 5th day, the cultures were treated with SNAP or vehicle (serum-free defined medium) for 24 h. Results are representative of two experiments and are expressed as the mean±S.E.M. (n=10). Statistical analysis was performed by one-way analysis of variance followed by the Student's "t" test. *$p<0.05$, ***$p<0.001$.

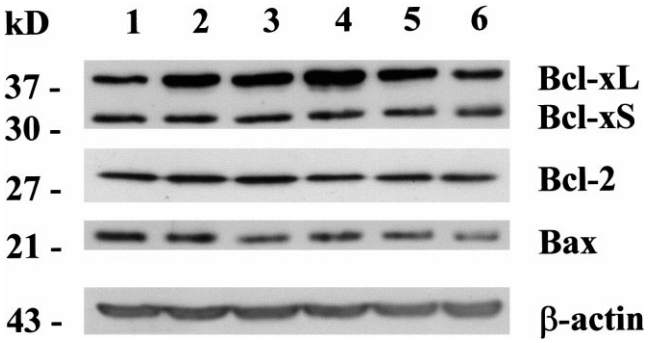

Fig. 2. Top panel: Representative Western blot of Bcl-2, Bax, Bcl-$x_{S/L}$ proteins from control (lane 1) and SNAP-treated cells (50, 125, 250, 500 and 750 μM SNAP, lanes 2 to 6). The protein levels were determined by computer-assisted videodensitometry from Western blot films. Each lane contains 30 μg of total protein. Control of charge with *β*-actin was carried out and no changes between lanes were detected. Bottom pannel: Data represent the ratio Bcl-2 family proteins/*β*-actin at the different SNAP doses used. Values are expressed as the mean+S.E.M. (*n*=3). Statistical analysis was performed by one-way analysis of variance followed by the Student's "*t*" test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Bottom panel.

SNAP (μM)	Bcl-xL/β-actin	Bcl-xS/β-actin	Bcl-2/β-actin	Bax/β-actin
Control (0μM)	0.534±0.09	0.4510±0.04	0.6950±0.05	0.7960±0.02
SNAP 50μM	1.200±0.14*	0.4560±0.04	0.8360±0.07	0.6910±0.02*
SNAP 125 μM	1.200±0.10**	0.4300±0.03	0.8120±0.06	0.4920±0.05**
SNAP 250 μM	1.622±0.19**	0.3880±0.05	0.6050±0.08	0.6210±0.01**
SNAP 500 μM	1.042±0.13*	0.4240±0.02	0.7020±0.05	0.5440±0.01***
SNAP 750 μM	0.593±0.02	0.4030±0.02	0.6530±0.02	0.4090±0.01***

Table 3
Effect of SNAP on glutathione (GSH) content[a]

SNAP (μM)	GSH in the culture medium (μM)	Intracellular GSH (nmoles/mg of protein)
Control (0 μM)	1.08±0.04 100%	9.17±1.02 100%
SNAP 50 μM	1.08±0.03 100%	8.29±0.40 90%
SNAP 125 μM	0.93±0.05 86%	9.28±1.01 101%
SNAP 250 μM	0.70±0.06*** 65%	8.01±0.43 87%
SNAP 500 μM	0.60±0.05*** 56%	9.63±1.27 105%
SNAP 750 μM	0.62±0.07*** 57%	8.48±0.71 92%

[a] Cells were cultured for 5 days in complete medium. On the 5th day, the cultures were treated with SNAP or vehicle (serum-free defined medium) for 24 h. Results are expressed as the mean±S.E.M. (*n*=6). Statistical analysis was performed by one-way analysis of variance followed by the Student's "*t*" test. ***$p<0.001$.

in agreement with other studies reporting a relationship between NO and cell differentiation. In this context, it has been shown that NO may play a role in the differentiation of neuronal and glial cells (Tanaka et al., 1994; Peunova and Enikolopov, 1995; Viani et al., 1997).

Although NO donors can be toxic and cause changes in cellular morphology such as apoptosis and necrosis, NO can also block neuronal death resulting from various cytotoxic stimuli (Lei et al., 1992; Kim et al., 1999). The effects of NO on cell death are due, in part, to the type of NO donor used. Nisoli et al. (1998) have reported that 300 μM SNAP decreases cell proliferation and is

Table 2
Effect of SNAP on the catecholaminergic system in NB69 cells[a]

SNAP (μM)	TH activity Δ DOPA (ng/well)	MAO+COMT inhibition		Endogenous levels		^{3}H-DA uptake cpm/well
		NA (ng/well)	DA (ng/well)	NA ng/mg protein	DA ng/mg protein	
Control (0 μM)	1.94±0.12 100%	4.22±0.11 100%	2.52±0.11 100%	2.70±0.03 100%	1.20±0.07 100%	6988±216 100%
SNAP 50 μM	2.46±0.1** 127%	5.01±0.36 119%	4.39±0.27*** 174%	3.81±0.14*** 141%	2.43±0.08*** 202%	26813±935*** 383%
SNAP 125 μM				2.65±0.15 98%	2.02±0.06*** 168%	23130±1665** 352%
SNAP 250 μM				1.37±0.06*** 51%	0.9±0.07* 75%	13170±1170*** 188%
SNAP 500 μM	1.19±0.07*** 61%	0.77±0.05*** 21%	0.75±0.02*** 30%	0.59±0.06*** 22%	0.27±0.02*** 22%	3285±148*** 47%
SNAP 750 μM	1.08±0.003*** 56%	0.28±0.01*** 7%	0.37±0.03*** 15%	0.39±0.03*** 14%	0.15±0.01*** 12%	1233±110*** 17%

[a] Cells were cultured for 5 days in complete medium. On the 5th day, the cultures were treated with SNAP or vehicle (serum-free defined medium) for 24 h. For TH activity experiments, cells were treated with NSD 1015 (150 mg/l) for 2 h. For metabolism experiments, MAO and COMT enzymes were inhibited by pargyline (10^{-5} M) or RO-40-7592 (30 mg/l), respectively, for 4 h. Results are expressed as the mean±S.E.M. (*n*=8). Statistical analysis was performed by one-way analysis of variance followed by the Student's "*t*" test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

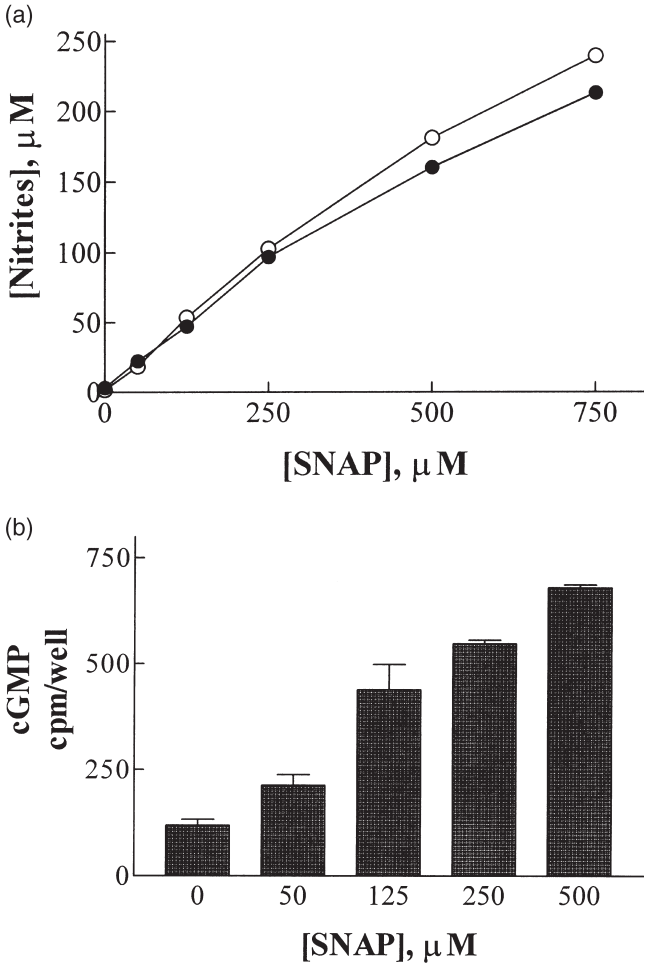

Fig. 3. (A) Dose-dependent increase of nitrite concentration in the culture medium induced by SNAP, in the presence (●) or absence of (○) cells (n=10), and (B) dose-dependent increase in cGMP production induced by SNAP in NB69 cells (n=10). For sake of clarity, the S.E.M. are not represented in the graph since they were always below 5% of the mean values. Statistical analysis was performed by one-way analysis of variance followed by the Student's "t" test. ***$p<0.001$.

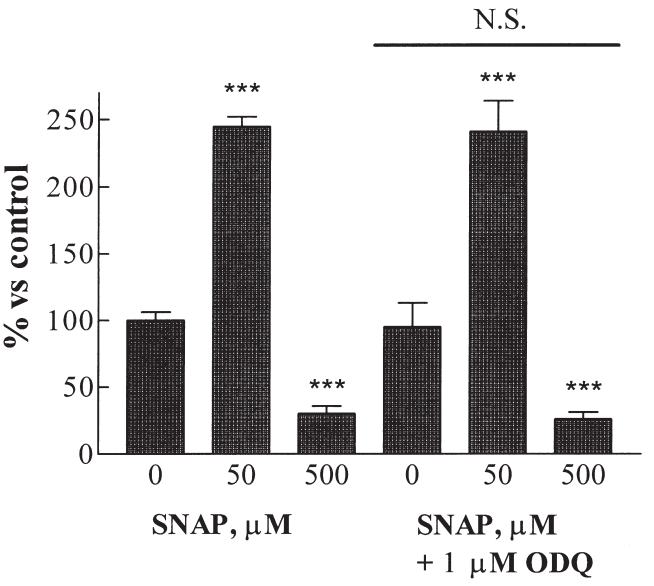

Fig. 4. Effect of ODQ (1μM) on ^{3}H-DA uptake in SNAP (50 and 500 μM)-treated cells. Results are expressed as a percentage of the control group (245, 30, 95, 241, 26%). Basal levels: 39365±2350 cpm/mf protein (n=12). Statistical analysis was performed by one-way analysis of variance followed by the Student's "t" test. ***$p<0.001$ vs control group. No significance differences (N.S.) were detected with ODQ-treatment when compared to their respective controls.

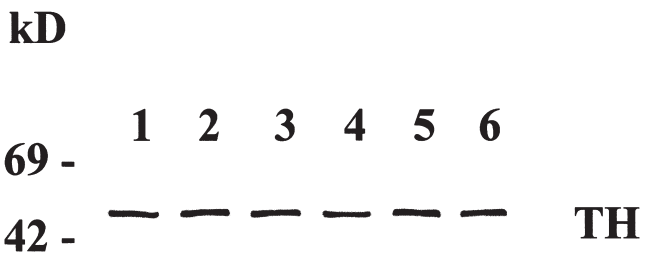

Fig. 5. Representative Western blot of total TH protein from control (lane 1) and SNAP-treated cells (50, 125, 250, 500 and 750 μM SNAP, lanes 2 to 6) (n=3). Values are expressed as a percentage of the control blot (111, 104, 89, 110, 106%). The protein level was determined by computer-assisted videodensitometry from Western blot films. Each lane contains 30 μg of total protein. Control of charge with fast green was carried out and no changes between lanes were detected.

accompanied by the expression of two genes which are upregulated during differentiation.

The family of Bcl-2-related proteins, that includes Bcl-2, Bcl-x, Bad, Bak and Bax, constitutes a class of apoptosis regulatory gene products that act at the effector stage of apoptosis. In this context, Bax, a proapoptotic protein, forms a dimer with Bcl-2 and prevents the death repressor activity of the antiapoptotic protein Bcl-2 (Sedlak et al., 1995). The ratio of endogenous Bcl-2 to Bax is suggested to determine the sensitivity of cells to apoptosis response to specific stimuli (Oltvai et al., 1993). It has been demonstrated recently that changes in Bcl-2 and Bax expression are involved in NO-induced cell death (Tamatani et al., 1998). Our results show that Bax protein content was decreased after SNAP treatment, whereas Bcl-2 and Bcl-xL protein levels were increased, that is, NO induces a decrease in pro-apoptotic protein expression and an increase in anti-apoptotic protein expression. In addition, no changes in apoptotic or necrotic cells were detected in SNAP-treated cells. Taken together, these results suggest that NO did not induce apoptosis, at the doses studied, in the human NB69 catecholamine-rich cell line.

We have found that NO exerts a biphasic effect on NB69 cells, increasing TH activity, monoamine metabolism, ^{3}H-DA uptake and catecholamine levels at low doses and exerting the opposite effect at high doses. The neuroprotective and neurotoxic roles of brain NO may derive from the local intracellular oxidation-reduction potential with a reductive environment favoring the production of NO free radical (Lipton et al., 1993; Smith et al., 1994). TH activity was increased by SNAP prob-

ably due to a post-translational mechanism because no changes in the amount of TH protein, as assessed by Western blotting, were observed in the present study. It has been reported that TH can be phosphorylated at different amino acid residues, mainly at Ser-40 (Kumer and Vrana, 1996). This mechanism is related to an activation of the preexisting protein and could explain the results observed at low doses of SNAP. There is, however, no information available regarding the effects of low concentrations of NO on protein phosphorylation, although it has been reported that NO interacts with cAMP-dependent protein kinases (Ohki et al., 1995; Bhat et al., 1998).

On the other hand, it has been reported recently that NO can react with the superoxide anion to produce peroxynitrite. This reactive species damages proteins by sulfhydryl oxidation and nitration, mechanisms that inactivate TH (Ara et al., 1998; Kuhn et al., 1999). Therefore, NO, at low doses, may act as a second messenger that activates TH protein and produces the opposite effects at high doses, probably related to a nitrosylation or a sulfhydryl oxidation of the TH enzyme. Further studies are needed to elucidate the mechanism that leads to NO-induced TH activation.

SNAP produces spontaneous release of NO, but it may also be catalyzed at the cell membrane (Uehara et al., 1999). We observed a dose-dependent increase in nitrite levels in the supernatant of SNAP-treated cells. In addition, we detected an identical nitrite concentration in the absence or presence of cells at the different doses of SNAP. The hypothetic mechanism of action of SNAP in our system would not include a competition of these cells for the NOS cofactor BH_4, required for TH and NOS activity (Hwang et al., 1998), because we found that NOS activity is not necessary for NO production from SNAP.

We have shown that cGMP levels in NB69 cells are affected by SNAP in a dose-dependent manner. NO mediates signal transduction in the brain via glutamate receptor stimulated NO formation, guanylate cyclase activation and, subsequently, increasing cGMP levels (Moncada et al., 1991). ODQ, a guanylate cyclase inhibitor, is commonly used to determine if the cGMP pathway is implicated in the regulation of cellular function (Moro et al., 1998; Kim et al., 1999). We used ODQ to test the possible role of cGMP in cell differentiation detected after SNAP treatment. ODQ (1 μM) did not revert the SNAP-induced effect on ^{3}H-DA uptake and catecholamine levels (data not shown) in NB69 cells. These data suggest that the mechanism through which NO may act to cause cell differentiation is independent of cGMP.

Rauhala et al. (1998) have described that S-nitrosylation of GSH by NO and oxygen may be part of the antioxidative cellular defense system. In this context, Wink et al. (1993) have proposed that NO protects against cellular damage and cytotoxicity from reactive oxygen species. In a recent study, Mena et al. (1998b) suggest that mild pro-oxidant treatments could protect a range of cells from death by up-regulating GSH. In our study, we observed a significant and progressive decrease in the level of GSH in the culture medium from 250–750 μM SNAP-treated cells, in comparison with control levels. In contrast, no changes in intracellular GSH levels were detected. Thus, NB69 cells may use GSH from the medium as a mechanism to protect themselves from high concentrations of free radicals.

In summary, the NO donor SNAP induces a differentiation of NB69 cells. These results suggest that NO generated from SNAP has no neurotoxic effects in the conditions studied, and that it regulates TH activity in a human catecholamine-rich cell line through a cGMP-independent mechanism.

These studies provide new insights, into the complex regulatory activity of NO on dopamine neurons in vitro. The role of NO in the regulation of these neurons in vivo, in health and disease, and the putative pharmacological manipulation of the NO pathway in neurodegenerative diseases, should be investigated.

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Trabajo 3

Thiolic antioxidants protect from nitric oxide-induced toxicity in fetal midbrain cultures

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Thiolic antioxidants protect from nitric oxide-induced toxicity in fetal midbrain cultures

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Abstract

Nitric oxide (NO) may act as a neuroprotector or neurotoxic agent in dopamine neurons, depending on cell redox status. We have investigated the effect of several thiolic antioxidants, glutathione (GSH), its cell permeable analog GSH ethyl ester (GSHEE), and the GSH synthesis precursor L-*N*-acetyl cysteine (L-NAC), as well as non-thiolic antioxidants like ascorbic acid (AA) and uric acid, on NO-induced toxicity in fetal midbrain cultures. The cultures were treated for 8–24 h with neurotoxic doses of the NO donor diethylamine/nitric oxide complex sodium DEA/NO (200–400 μM) and/or antioxidants. Thiolic antioxidants, at equimolar concentrations, added at the same time or previous to DEA/NO, protected from cell death, from tyrosine hydroxylase (TH) positive cell number decrease and from intracellular GSH depletion, induced by DEA/NO, without increasing intracellular GSH content. In these conditions, *S*-nitrosothiol compound formation was detected in the culture media. Protection disappeared when antioxidants were supplied 30 min after NO treatment. Nevertheless, non-thiolic antioxidants, AA and uric acid, with similar peroxynitrite scavenging activity to thiolic antioxidants, and free radical-scavenging enzymes as catalase and Cu/Zn-superoxide dismutase, which prevent extracellular peroxynitrite ion formation, and 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), which prevents intracellular peroxynitrite ion formation, did not rescue cell cultures from neurotoxicity induced by NO. In addition, AA exacerbated DEA/NO-induced toxicity in a dose-dependent manner from 200 μM AA. The present results suggest that only antioxidants with thiol group exert neuroprotection from NO-induced toxicity in fetal midbrain cultures, probably by direct interaction of NO and thiol groups, resulting in NO blocking. On the other hand, some classical antioxidants, like AA, exacerbate neurotoxicity due to NO.

Keywords: Nitric oxide; Dopamine neurons; Glutathione; Ascorbic acid; Cu/Zn-superoxide dismutase; Catalase; Superoxide scavengers; Free radicals

1. Introduction

Oxidative stress has been implicated in the development of several neurodegenerative diseases including Parkinson's disease (PD). Consistent with this, a decreased activity of several antioxidant enzymes, as well as reduction of glutathione (GSH) content, have been reported in substantia nigra of parkinsonian brain (Di Monte et al., 1992; Perry et al., 1982). Loss of dopaminergic neurons in PD substantia nigra may be due to the effects of nitric oxide (NO). NO, a modulator of cell activity may act as a neuroprotector or neurotoxic agent depending on cell redox status. Our previous results have shown that the NO donor diethylamine/NO complex sodium (DEA/NO), at doses of 25 and 50 μM, exerts neurotrophic effects on dopamine (DA) cells, by increasing the number of tyrosine hydroxylase positive (TH+) cells, TH+ neurite processes, DA levels, 3H-DA uptake and by elevating intracellular and extracellular GSH concentration. However, doses ranging from 200 to 400 μM had neurotoxic effects in midbrain cultures, decreasing TH+ cells and GSH levels and increasing both necrosis and apoptosis (Canals et al., 2001b). Furthermore, GSH depletion switches NO neurotrophic effects to cell death

Abbreviations: NO, nitric oxide; DEA/NO, diethylamine/nitric oxide complex sodium; TH, tyrosine hydroxylase; DA, dopamine; GSH, reduced glutathione; GSSG, oxidized glutathione; GSHEE, glutathione ethyl ester; GSNO, *S*-nitrosoglutathione; AA, ascorbic acid; PD, Parkinson's disease; Cu/Zn-SOD, Cu/Zn-superoxide dismutase; L-NAC, L-*N*-acetyl cysteine

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in DA neurons (Canals et al., 2001a). We have also reported that glia-conditioned medium (GCM), obtained from fetal midbrain astrocyte cultures, totally protects DA neurons from NO-induced apoptotic and necrotic cell death and rescues 3H-DA uptake and TH+ cell number (Canals et al., 2001b). GCM is rich in small antioxidants and peptidic growth factors, that increase free radical scavengers enzymatic activities (Mena et al., 1997a, 1998a, 2002).

NO may react with superoxide anion to produce peroxynitrite, a powerful oxidant (Beckman et al., 1990; Bolaños et al., 1995; Lizasoain et al., 1996). There is evidence to indicate that peroxynitrite-mediated damage occurs in the PD brain (Good et al., 1998; Giasson et al., 2000). Furthermore, it has been shown that peroxynitrite inactivates TH and consequently L-DOPA synthesis in PC12 (Ischiropoulos et al., 1995). Tyrosine nitration, as well as sulfhydryl oxidation, have been proposed as the mechanisms for TH inactivation (Ara et al., 1998; Kuhn et al., 1999). NO also exerts its effects by reacting with intracellular GSH to form a nitrosylated adduct, *S*-nitrosoglutathione (GSNO), which may regulate cellular functions and protect DA neurons from oxidative stress and damage caused by reactive oxygen species (Rauhala et al., 1996, 1998; Clancy et al., 1994).

The aim of this study was to investigate whether thiolic and non-thiolic antioxidants, as well as free-radical scavenging enzymes, can modulate NO-induced toxicity for midbrain cultures and DA neurons. The mechanisms of neuroprotection were also addressed.

2. Materials and methods

2.1. Materials

The culture reagents Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l), Ham's F12 nutrient mixture, Eagle's minimal essential medium (EMEM) with Earl's salts and Leibovitz's L-15 medium, all of which were supplemented with L-glutamine, fetal calf serum (FCS), sodium pyruvate and L-glutamine, were purchased from Gibco BRL (Paisley, Scotland, UK). Glucose 45%, insulin, putrescine, progesterone and sodium selenite were from Sigma (Madrid, Spain) and human transferrin, 30% iron-saturated, from Boehringer-Mannheim (Barcelona, Spain). Trypan blue, bovine serum albumin, poly-D-lysine, *p*-phenylenediamine, bisBenzimide, pargyline, *N*-(1-naphthyl) ethylenediamine, sulfanilamide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), reduced and oxidized forms of glutathione, glutathione ethyl ester (GSHEE), catalase, Cu/Zn-superoxide dismutase (Cu/Zn-SOD), 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron) and diethylamine/nitric oxide complex sodium (DEA/NO) were from Sigma (Madrid, Spain); L-*N*-acetyl cysteine (L-NAC) from Calbiochem (CA, USA); NADPH, the cytotoxicity detection kit (LDH), cell proliferation kit I (MTT) and GSH reductase (GR) were from Boehringer-Mannheim (Barcelona, Spain) and ascorbic acid (AA) was from Merck (Darmstadt, Germany). The apoptosis TUNEL detection kit was obtained from Promega (Madison, WI, USA). The BCA protein assay kit was from Pierce (Rockford, IL, USA), mouse monoclonal anti-tyrosine hydroxylase (TH) antibody from Chemicon International, Inc. (CA, USA). All other reagents were of the highest purity commercially available from Merck or Sigma.

2.2. Cell culture

Neuronal-enriched cultures from embryonic Sprague–Dawley rat midbrain E-14 (crown-rump length 10–12 mm) were obtained and prepared as described previously (Pardo et al., 1997; Mena et al., 1993). The cells were seeded in DMEM with 15% fetal calf serum (DMEM-FCS) at a density of 10^5 cells/cm^2 in multiwells or glass cover slides previously coated with poly-D lysine, 4.5 μg/cm^2, in 0.1 M borate buffer, pH 8.4. The cultures were kept in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. Twenty-four hours after plating, the cells were changed to serum-free defined medium (EF12) as reported previously (Pardo et al., 1997; Mena et al., 1993), supplemented with D-glucose (6 mg/ml), insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM) and sodium selenite (30 nM).

After 7 days in culture, the cells were mostly β-tubulin III$^+$ cells (neurons), non-neuronal cells were also present in low numbers: GFAP+(astrocytes) were around 2% of the total cells. Slightly more abundant were glial progenitors and oligodendrocytes (A2B5+ and O-1+, respectively), nestin-positive cells, which stain pluripotential neural progenitors, as well as radial glial cells, were present in the culture. Microglia accounted for less than 0.5%. TH+ cells ranged between 2.1 and 5.4% of the total, and the rest of neurons were mainly GABAergic (Pardo et al., 1997; Mena et al., 1998a, 1999).

2.3. Experimental treatments

After 5 days in culture, the cells, randomly allocated to the different experimental groups, were treated with thiolic and non-thiolic antioxidants, L-NAC, GSH, GSHEE or AA, uric acid, and free-radical scavenging enzymes, catalase and Cu/Zn-SOD, Tiron and/or neurotoxic doses of DEA/NO (200–400 μM) for 8 or 24 h, dissolved in distilled water, with no change of culture media. Antioxidants were added 30 min or 24 h before, or 30 min, 1 or 2 h after DEA/NO treatment, catalase, Cu/ZN-SOD and Tiron, a cell-permeable superoxide

scavenger, were added 30 min before treatment with DEA/NO.

2.4. Immunocytochemistry

DA neurons were characterised by immunostaining with a mouse monoclonal anti-TH antibody (1:100) (Mena et al., 1993). In brief, cultures were fixed with 4% paraformaldehyde, washed in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), permeabilized with ethanol-acetic acid (19:1) and incubated at 4 °C for 24 h with primary antibodies diluted in PBS containing 10% FCS. A fluorescein-conjugated secondary antibody was employed to visualize positive cells under fluorescent microscopy. The number of immunoreactive cells was counted in 20 fields that represent 1/7 of the total area of the cover slides. The cells were counted in predefined parallel strips using a counting reticule inserted in the ocular.

2.5. Cell death measurements

Apoptosis was measured by light microscopy morphological features, DNA staining with bisBenzimide (Hoechst 33342), and the TUNEL assay. Briefly, cultures were fixed in 4% paraformaldehyde, nuclei were stained with bisBenzimide added in the anti-fading solution (3×10^{-6}M final concentration) (Hilwig and Gropp, 1975; Pardo et al., 1997) and counted in 10 predefined fields that represent 1/14 of the cover slide area; apoptotic cells were identified by condensation and fragmentation of chromatin.

The apoptosis TUNEL detection system measures the fragmented DNA of apoptotic cells by incorporating fluorescein-12-dUTP∗ at the 3′-OH ends of the DNA using the enzyme Terminal deoxynucleotidyl Transferase (TdT) (Kerr et al., 1972; Gavrieli et al., 1992). For this assay, the cells were fixed in 4% *p*-formaldehyde and permeabilized with 0.2% Triton X-100. The fluorescein-12- dUTP-labeled DNA of apoptotic cells was visualized by fluorescence microscopy (positive cells with green fluorescence). The number of apoptotic cells was counted in 1/14 fields of the coverslide area. Cells were counted in predefined parallel strips using a counting reticule in the ocular. Cells incubated with buffer in the absence of TdT enzyme were used as negative controls (Rodríguez-Martín et al., 2000).

For necrotic cell death determination, trypan blue dye exclusion assay and lactate dehydrogenase activity were performed (Pardo et al., 1997).

Mitochondrial activity was measured with the MTT assay. The MTT assay determines the ability of cells to metabolize 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). At the end of the cell treatment period, 300 μl of culture medium were removed from total 500 μl of each well and 20 μl of MTT solution (5 mg/ml) were added and incubated for 1 h. At this time, 200 μl of solubilization solution (10% SDS in HCl 0.01 M) were then added to the wells and after 24 h of incubation at 37 °C, 100 μl were transferred into 96-well microtitre plates, and the absorption value at 540 nm was measured in an automatic microtitre reader (Spectra Fluor, Tecan). The thiolic antioxidants used in this study, with exception for L-NAC; interfered with the MTT assay and all of them interfered with the LDH assay.

2.6. Glutathione measurements

Total glutathione levels were measured by the method of Tietze (1969). Briefly, 4×10^5 cells were washed with PBS, lysed in 100 μl of 3% perchloric acid (PCA) for 30 min at 4 °C, centrifuged, and the supernatants were neutralized with 4 vol of 0.1 M NaH_2PO_4, 5 mM EDTA, pH 7.5. Glutathione content was measured in a P96 automatic reader by the addition of DTNB (0.6 mM), NADPH (0.2 mM) and glutathione reductase (1 U) and the reaction monitored at 412 nm during 6 min. Oxidized glutathione (GSSG) was measured in the cells by the method of Griffith (1980). Briefly, after PCA extraction and pH neutralization, reduced glutathione (GSH) was derivatized with 2-vinylpyridine at room temperature for 1 h and the reaction carried out as above. GSH was obtained by subtracting GSSG levels from total glutathione levels. Extracellular glutathione measurements were made in culture media treated with PCA (3% final concentration) and monitored as above. Formation of GSNO in fetal midbrain culture media was measured by incubation with/without 100 μM $CuSO_4$, which breaks thiol bonds, for 20 min at 37 °C (Clancy et al., 1994). After incubation, extracellular GSH levels were determined as described (Cook et al., 1996).

2.7. Uptake studies

High affinity ^{3}H-DA uptake was measured after incubation of the cells with 10^{-8} M [3H]DA (70 Ci/mmol), in the presence of pargyline 10^{-5} M and AA 10^{-3} M, at 37 °C for 20 min. Non-specific uptake/binding was calculated in the presence of 10^{-5} M mazindol (Beart and McDonald, 1980). Proteins were measured according to the BCA assay.

2.8. Nitrite measurement

NO production was quantified by measuring nitrite, a stable oxidation end product of NO (Green et al., 1982). Briefly, 400 μl of culture medium were mixed with 800 μl of Griess reagent (1.5% sulfanilamide in 1 N HCl plus 0.15% *N*-(1-naphtyl)-ethylenediamine dihydrochloride in distilled water, v:v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined. Sodium nitrite was used as a standard.

2.9. Statistical analysis

The results were statistically evaluated for significance using one way analysis of variance followed by the Newman–Keuls test as a post hoc evaluation. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Cell viability and GSH depletion time course after DEA/NO treatment

Cell death induced by 400 μM DEA/NO has features of both necrosis and apoptosis. DNA condensation of NO-treated cells, determined by bisBenzimide staining, correlates with the TUNEL assay results, as showed in Fig. 1(A). Necrosis was measured by Trypan blue and LDH activity assays. The percentage of dead cells measured by Trypan blue after 24 h of 400 μM DEA/NO treatment (39.4 ± 2.8%) was concomitant with an increase in LDH activity, which was measured in the culture media (32.16 ± 4.34%) (Fig. 1(B)).

A time course of injury along with the measurement of intracellular GSH is indicated in Fig. 1(C) and (D). As shown, a time-dependent decrease in cell viability, analyzed by the MTT assay, was detected at 4 h after treatment with 400 μM DEA/NO, decreasing at 8 and 24 h after NO addition. However, GSH decrease was not observed until 24 h after DEA/NO treatment. A decrease in intracellular GSH levels does not precede cell death and as such this cannot be the mechanism by which NO mediates cell death in this model.

Immunocytochemical characterization of cell death revealed that DA neurons TH+ cells and mature oligodendrocytes O1+ cells were the cell types most affected by 400 μM DEA/NO at 4 and 8 h (Canals et al., 2001b).

3.2. Thiolic antioxidants protect from DEA/NO-induced neurotoxicity

We have previously described that neurotrophic doses (25–50 μM) of the NO donor DEA/NO increase GSH levels, whereas high doses (200–400 μM) exert the opposite effect on fetal midbrain cultures (Canals et al., 2001b). Pretreatment for 24 h with 600 μM GSH before treatment with 400 μM DEA/NO for additional 8 h, protected from DEA/NO-induced decrease of TH+ cell number (Fig. 2(A) and (C)) and increase of apoptosis (Fig. 2(B) and (D)). In addition, GSH rescued TH+ cells and midbrain neurons from the loss of their neurite processes

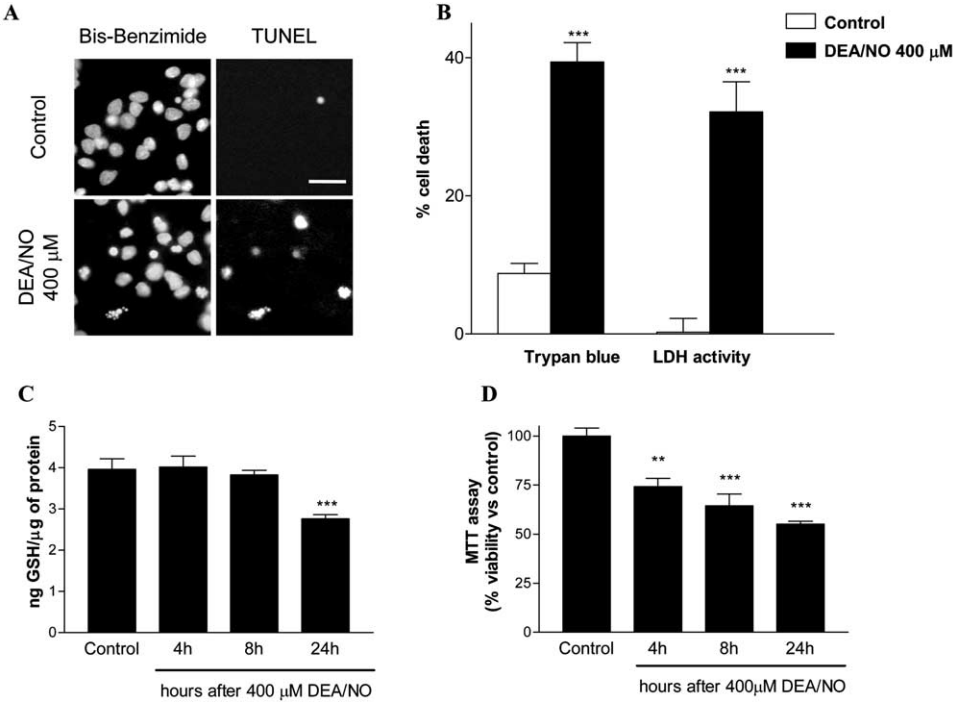

Fig. 1. Effects of 400 μM DEA/NO treatment on cell death and GSH depletion in fetal midbrain cultures. (A) Photomicrographs of cellular nuclei stained with bisBenzimide (left panels) and of apoptotic cells stained by the TUNEL assay (right panels), corresponding to the same field. Cells were treated with 400 μM DEA/NO or vehicle for 24 h. Scale bar = 25μm. (B) Trypan blue dye exclusion assay and LDH activity in the culture medium at 24 h after 400 μM DEA/NO treatment. (C) Time-course of glutathione intracellular levels after DEA/NO addition. (D) Time-dependent effects of DEA/NO treatment on cell viability measured by MTT assay and presented as a percentage vs controls. Results are expressed as the mean ± SEM ($n = 4-6$). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $**p < 0.01$, $***p < 0.001$ vs controls.

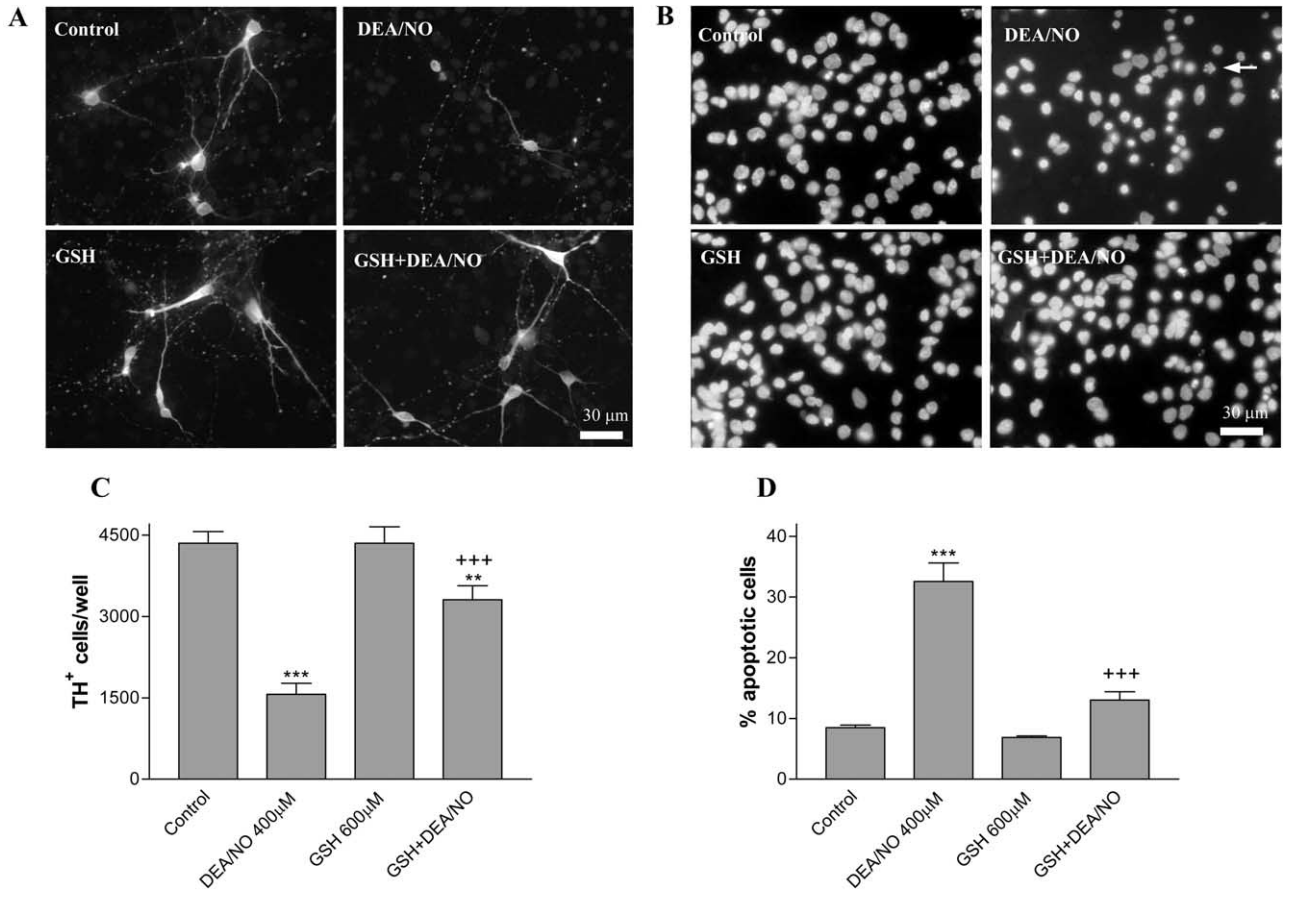

Fig. 2. Protective effects of GSH pretreatment for 24 h on DEA/NO-induced cell death in fetal midbrain cultures. After 4 days in vitro, the cultures were treated with GSH 600 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 400 μM for additional 8 h. (A) TH immunocytochemistry and (B) cellular nuclei stained with bisBenzimide (Hoechst 33342). (C) Number of DA neurons expressed as TH+ cells/well. (D) Chromatin-condensed nuclei were counted and expressed as a percentage of apoptotic cells with respect to the total cell number. Arrow indicates a representative apoptotic cell. Values represent the mean ± SEM from ($n = 4-6$). Control basal levels were 4350 ± 214 TH+ cells/well and 8.47 ± 0.45% of apoptotic cells. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $**p < 0.01$, $***p < 0.001$ vs controls; $^{+++}p < 0.001$ GSH + DEA/NO vs DEA/NO.

(Fig. 2(A)). The pretreatment with the GSH synthesis precursor L-NAC also showed a neuroprotector dose-dependent effect on DEA/NO-treated cells, rescuing DA neurons (Fig. 3(A) and (C)) and midbrain cultures from apoptosis (Fig. 3(B), (D) and (E)). A total protection was detected when 600 μM L-NAC was used previous to the addition of 400 μM DEA/NO for 24 h (Fig. 3(E)).

DA neurons treated with thiolic antioxidants were protected from DEA/NO toxicity more effectively than other cells in the culture. As shown, DA neurons TH+ cells are recovered to 100% when NO is added in the presence of 300 μM L-NAC (Fig. 3(C)). However, the rest of the cells, mainly neurons, are partially recovered by L-NAC treatment (Fig. 3(D)). In addition, L-NAC and GSH protected from DEA/NO-induced decrease of 3H-DA uptake (Fig. 4(B)).

Both antioxidants, GSH and L-NAC, prevent from intracellular GSH depletion without increasing its intracellular levels (Fig. 4(C) and (D)). With regard to this, neither GSHEE, a cell permeable compound, nor cysteine or cystine addition to the cell culture increased the GSH intracellular levels (data not shown), these data support that the GSH synthesis enzymatic system is saturated in our experimental model. Experiments carried out with astrocyte cultures and NB69 cells-treated with GSH synthesis precursors (L-NAC, cysteine and cystine from 300 to 600 μM), and PC12 treated with L-NAC 300–600 μM (Mena et al., 1998b) increased intracellular GSH levels (data not shown). Although thiolic antioxidants did not increase intracellular GSH content, they protected midbrain cultures from DEA/NO-induced GSH decrease (Fig. 4(A)–(D)).

We have investigated the effect of several thiolic, GSH, GSHEE, L-NAC, and non-thiolic antioxidants, AA and uric acid, on NO-induced cell death in fetal midbrain cultures. Cell treatment with thiolic antioxidants protected from cell death induced by DEA/NO, whereas non-thiolic antioxidants did not exert neuroprotection (Fig. 4(A)). The same results were observed for permeable and non-permeable GSH compounds and for

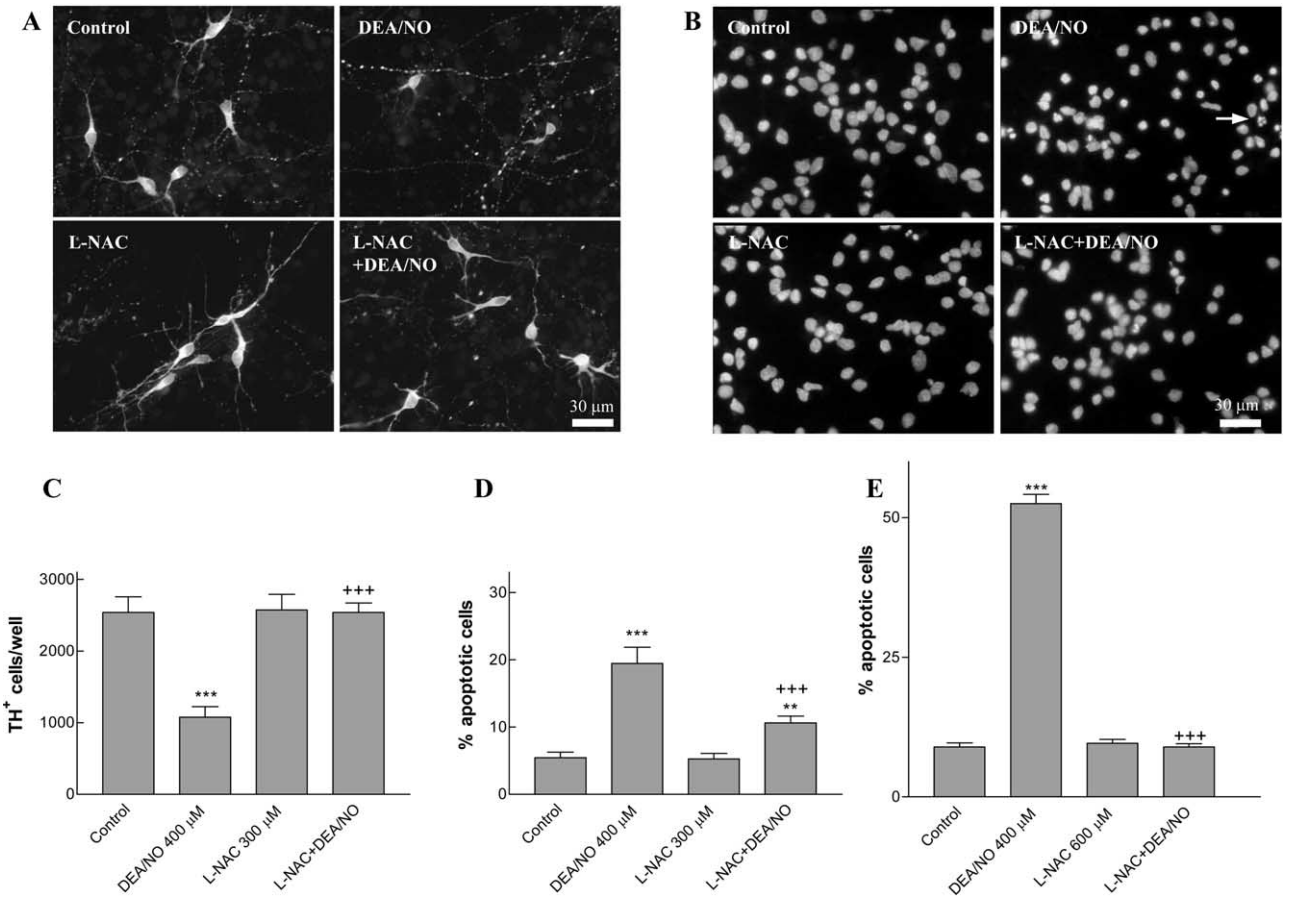

Fig. 3. Protective effects of 24 h L-NAC pretreatment on DEA/NO-induced cell death in fetal midbrain cultures. After 4 days in vitro, the cultures were treated with L-NAC 300–600 µM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 400 µM for additional 8 or 24 h. (A) TH immunocytochemistry and (B) cellular nuclei stained with bisBenzimide (Hoechst 33342). (C) Number of DA neurons expressed as TH+ cells/well. (D, E) Chromatin-condensed nuclei were counted and expressed as a percentage of apoptotic cells with respect to the total cell number. In (E), 24 h of DEA/NO treatment instead 8 h (A–D) was used. Arrow indicates a representative apoptotic cell. Values represent the mean ± SEM from ($n = 4-6$). Control levels were 2540 ± 132 TH+ cells/well and 5.4 ± 0.8% of apoptotic cells. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $**p < 0.01$, $***p < 0.001$ vs controls; $^{+++}p < 0.001$ L-NAC+DEA/NO vs DEA/NO.

L-NAC, suggesting that antioxidant mechanism of protection occur extracellularly. In addition, the neuroprotection exerted by L-NAC showed that antioxidant had to be present in the culture medium previous to DEA/NO, because 30 min, 1 and 2 h after it did not protect (Fig. 5(A) and (B)).

Antioxidants, at the doses used, did not modify by themselves the percentage of cell death, measured by trypan blue dye exclusion (data not shown). Treatment with antioxidants did not change the amount of nitrites measured in the culture media. DEA/NO addition increased nitrite concentration in an equimolar range, but this production was not diminished by pretreatment with antioxidants (data not shown).

These results suggest that NO interacts with thiol groups at the extracellular level. To confirm this hypothesis, GSNO formation in the culture media was determined by measurement of extracellular GSH levels after incubation with/without 100 µM $CuSO_4$, a compound that breaks thiol bonds. Extracellular GSH levels were decreased to 59.16 ± 1.16% in GSH + DEA/NO-treated cells when compared to GSH-treated group levels (Fig. 6). After breaking the S–N bond with $CuSO_4$ the GSH amount was restored to 92.17 ± 4.77% in the GSH + DEA/NO-treated cells vs GSH-treated group. GSNO formation in the culture media is involved in the decrease of GSH levels and after GSNO breakdown, GSH amount was recovered because of GSH release.

Our data show that in the neuroprotective effects of thiolic antioxidants from NO-induced toxicity, extracellular mechanisms play an important role. However, we cannot exclude that an intracellular mechanism may also be involved.

3.3. Non-thiolic antioxidant effects on DEA/NO-induced cell death. Exacerbation of toxicity by AA

The non-thiolic antioxidants AA and uric acid, with similar peroxynitrite scavenging activities to thiolic anti-

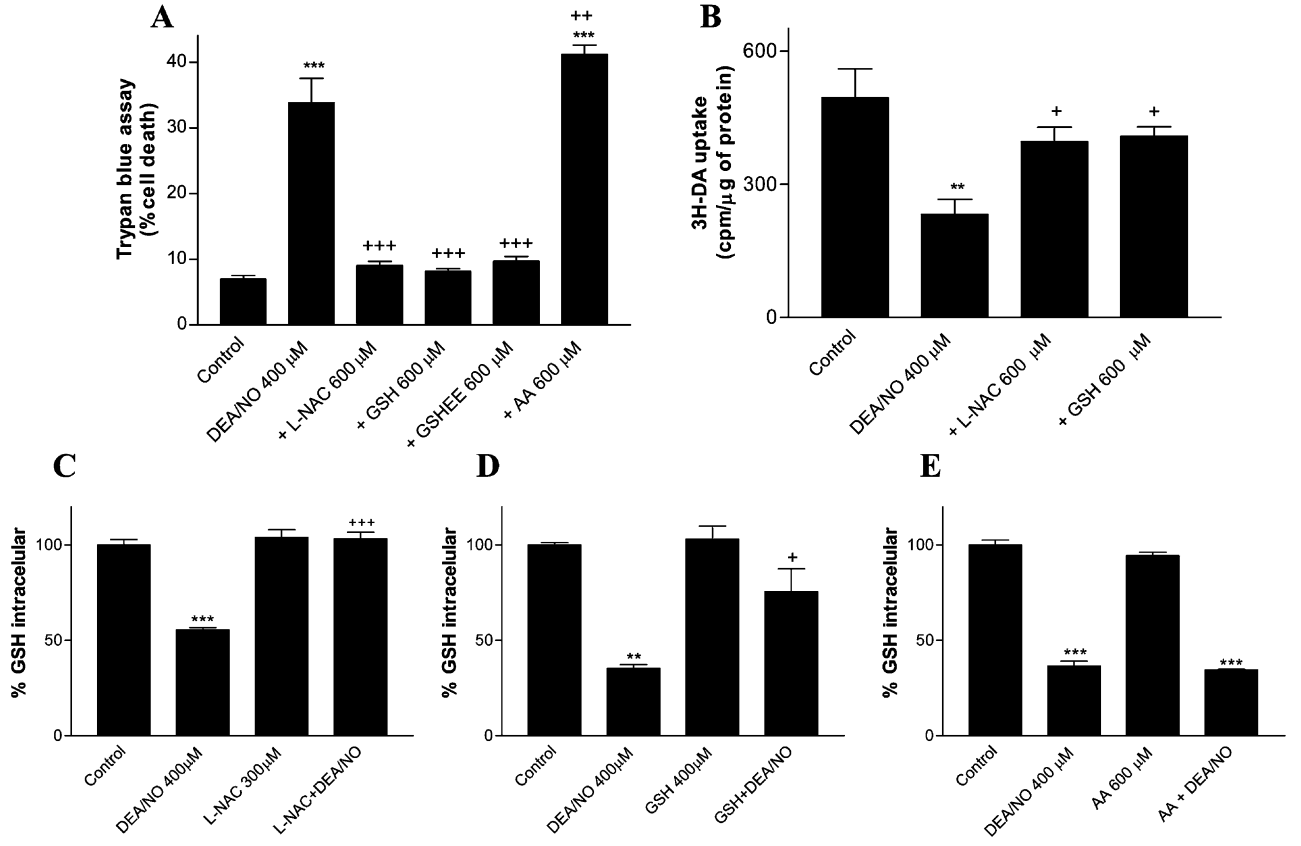

Fig. 4. Comparison of pretreatment with several antioxidants on DEA/NO-induced cell death in fetal midbrain cultures. After 5 days in vitro, the cells were treated with antioxidants (L-NAC, GSH, GSHEE and AA) 600 μM or vehicle 30 min previous to DEA/NO 400 μM for additional 24 h. (A) Cell viability measured by trypan blue dye exclusion is presented as a percentage of cell death. (B) High-affinity [^{3}H]-dopamine uptake expressed as cpm/μg of protein. After 5 days in vitro, the cultures were treated with 600 μM L-NAC, GSH or vehicle 30 min previous to 400 μM DEA/NO addition for 24 h. (C–E) Intracellular GSH levels measured after DEA/NO and/or antioxidants (L-NAC, GSH and AA, respectively) treatment. Values are presented as percentage vs control levels. GSH intracellular basal levels are 17.23 ± 0.48ng/μg of protein. Results are expressed as the mean ± SEM from $n = 4-6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $**p < 0.01$, $***p < 0.001$ vs controls; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, antioxidant + DEA/NO vs DEA/NO.

oxidants, were also used to investigate mechanisms of neuroprotection from NO toxicity. Neither AA nor uric acid rescued cell cultures from cell death induced by neurotoxic doses of DEA/NO (Figs. 4(A), 7 and 8(A)). In addition, AA did not protect from GSH decrease induced by DEA/NO (Fig. 4(E)) and potentiated neurotoxicity in a dose-dependent manner, increasing the percentage of cell death in the culture (Fig. 7(A)) and exacerbated the decrease of 3H-DA uptake in DEA/NO-treated cells (Fig. 7(B)).

3.4. Free radical scavengers

The effects of DEA/NO and catalase plus Cu/Zn-SOD enzymes on cell death, measured as mitochondrial activity by the MTT assay, is summarized in Fig. 8(B). Fifty and 100 U/ml of catalase and Cu/Zn-SOD, enzymes that prevent extracellular peroxynitrite formation, did not recover the increase of cell death induced by 200 μM DEA/NO treatment for 24 h to basal levels. Tiron 1–2 mM, a cell-permeable superoxide scavenger used 30 min before NO treatment, did not protect from 400 μM DEA/NO-induced toxicity at 24 h of treatment (Fig. 8(C)).

All together, these data support that mechanism of neuroprotection by antioxidants is not mediated through peroxynitrite scavenging. The presence of a thiol group in the antioxidant to exert neuroprotection from DEA/NO-induced toxicity is also important, probably by a direct interaction of NO and thiolic group through formation of *S*-nitrosothiol compounds.

4. Discussion

NO has been implicated to play an important role in a number of physiological processes in the CNS, such as pain perception, synaptic plasticity and learning (Moncada et al., 1991; Bredt and Snyder, 1992; Garthwaite and Boulton, 1995). However, NO, by itself or by reaction with superoxide anion giving peroxynitrite ($ONOO^-$), has also been involved in the pathogenesis

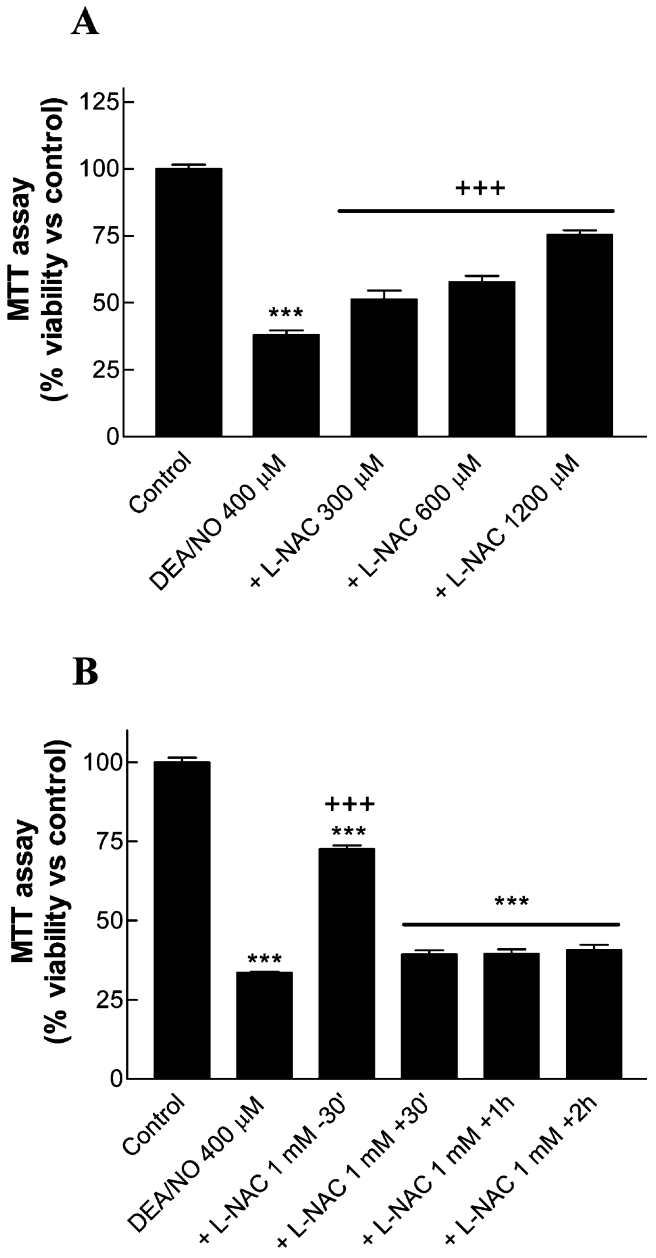

Fig. 5. (A) Dose- and (B) time-response-curves of L-NAC effects on DEA/NO-induced mitochondrial activity in fetal rat midbrain cultures. Cells were treated with L-NAC at different doses and times, and/or DEA/NO 400 μM, for 24 h. Cell viability was measured by MTT assay and is presented as a percentage vs controls. Values are the mean ± SEM from $n = 4$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $***p < 0.001$ vs control; $^{+++}p < 0.001$ L − NAC + DEA/NO vs DEA/NO.

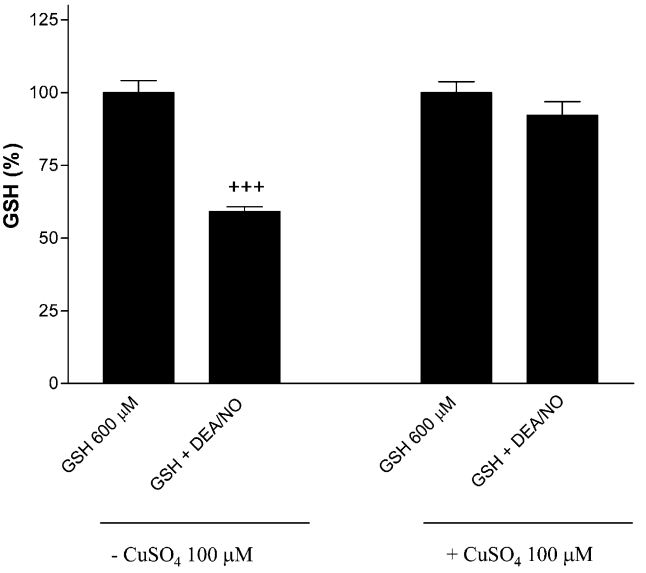

Fig. 6. GSNO formation in the media of fetal midbrain cultures treated with DEA/NO and a thiolic antioxidant. After 5 days in vitro, the cultures were pretreated with 600 μM GSH or vehicle 30 min previous to 400 μM DEA/NO addition for 8 h. GSNO production was determined by measurement of GSH levels after incubation with/without 100 μM $CuSO_4$, a compound that breaks thiolic bonds. Basal levels of extracellular GSH: 1.30 ± 0.14μM. Values are the mean ± SEM from $n = 6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{+++}p < 0.001$ GSH + DEA/NO vs GSH.

of neurodegenerative disorders as PD (Dawson, 1995; Bolaños et al., 1997; Good et al., 1998; Heales et al., 1999). We have previously reported that the biphasic effects of NO depend on the dose, time of exposure and cell type, as well as cell redox status. At low concentrations (25–50 μM), DEA/NO has neurotrophic effects on fetal midbrain cultures, and increases intracellular GSH levels. Neurotoxicity occurs at high DEA/NO doses (200–400 μM), and is accompanied by a decrease in GSH levels. Cell death, induced by DEA/NO, takes place by apoptosis and necrosis, and is detected at 4 h of NO treatment, while GSH depletion occurs after 24 h of treatment.

It has also been proven that antioxidant therapies are a powerful instrument against oxidative stress which is closely related to neurodegenerative diseases (Ho et al., 1997; Iwata-Ichikawa et al., 1999; Gilgun-Sherki et al., 2001; Lee et al., 2001a). In this regard the role of thiolic and non-thiolic antioxidants, as well as free radical scavenger enzymes, was implicated on DEA/NO-induced cell death in neuronal-enriched fetal midbrain cultures. We have found that thiolic antioxidants, as GSH, GSHEE or L-NAC, exert neuroprotection on DA neurons and midbrain cultures when they are present previously or at the same time as NO in the culture media; their effects were dose- and time-dependent and did not implicate up-regulation of the GSH synthesis. Formation of *S*-nitrosothiol compounds from NO and GSH was detected in the culture media. However, neither non-thiolic antioxidants such as AA and uric acid, nor extracellular free-radical scavenging enzymes like catalase and Cu/Zn-SOD or the intracellular superoxide scavenger Tiron, protected midbrain cultures from cell death induced by DEA/NO, suggesting that the mechanism of

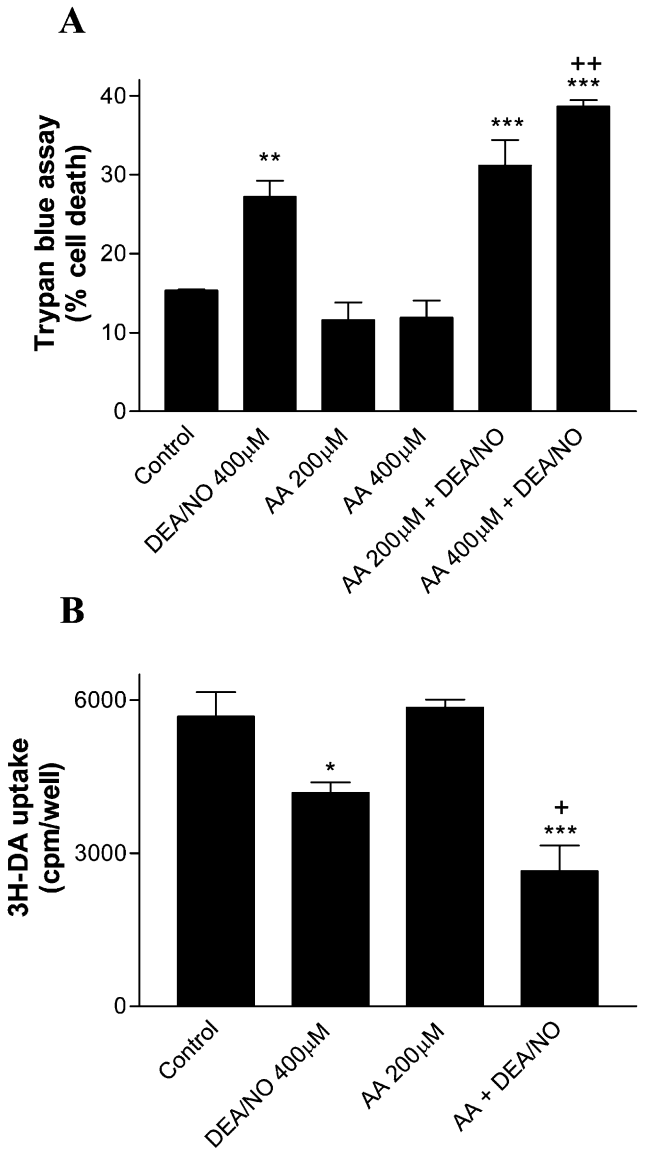

Fig. 7. A Effect of AA on DEA/NO-induced neurotoxicity. Cell viability in fetal rat midbrain cultures pretreated with AA (200–400 μM) for 30 min previous and/or 400 μM DEA/NO for additional 24 h. Cell viability was determined by trypan blue dye exclusion and is presented as a percentage of cell death. (B) High-affinity [^{3}H]-dopamine uptake expressed as cpm/well. After 5 days in vitro, the cultures were treated with AA 200 μM or vehicle 30 min previous to 400 μM DEA/NO addition for 24 h. Basal levels: 5678 ± 476cpm/well. Values represent the mean ± SEM from ($n = 4$). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs controls; $^{+}p < 0.05$, $^{+\,+}p < 0.01$ AA + DEA/NO vs DEA/NO.

protection by antioxidants is not mediated through peroxynitrite scavenging.

DEA/NO-induced cell death is totally abolished by astroglia-conditioned medium treatment (Canals et al., 2001b). It is known that GCM is rich in small antioxidants, and peptidic growth factors, that increase free radicals scavengers enzymatic activities (Takeshima et al., 1994; Muller et al., 1995; Mena et al., 1997a,b, 1998a,

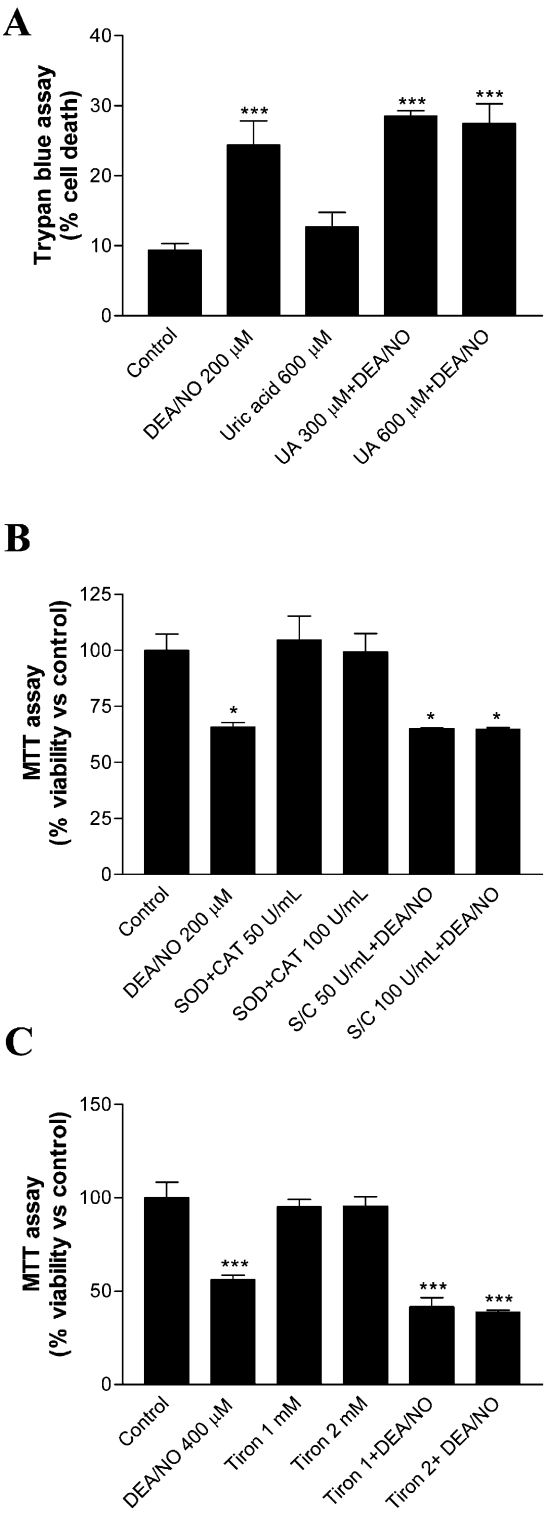

Fig. 8. Effect of peroxynitrite scavengers, 600 μM uric acid, 50 and 100 U/ml catalase and Cu/Zn-SOD, as well as the cell-permeable superoxide scavenger Tiron 1–2 mM, on 200–400 μM DEA/NO-induced cell death in fetal midbrain cultures treated for 24 h. Peroxynitrite and superoxide scavengers were added 30 min before DEA/NO treatment. (A) Cell viability was determined by trypan blue dye exclusion and is presented as a percentage of cell death. (B, C) Mitochondrial activity measured by MTT assay is presented as viability percentage vs controls. Values represent the mean ± SEM from ($n = 4-6$). Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $*p < 0.05$, $***p < 0.001$ vs controls.

2002), and enhance development, survival, neurite extension and resistance to neurotoxins of DA neurons. Glial cells protect neurons from oxidative stress by transcriptional up-regulation of oxidative stress-related genes, such as the γ-glutamyl-cysteine synthetase gene (Iwata-Ichikawa et al., 1999). Furthermore, we have recently described that GSH depletion switches NO neurotrophic effects to cell death in DA neurons (Canals et al., 2001a).

The present study shows that DA neurons are rescued from NO-induced cell death by thiolic antioxidants more effectively than other cells of the culture. Buettner and Jurkiewicz (1993) have described that DA cells are more resistant to GSH depletion; the preferential resistance of DAergic neurons to the toxicity induced by GSH depletion has also been reported by Nakamura et al. (2000), which explains that less susceptibility to cell death is independent of cellular GSH peroxidase and is mediated from the antioxidant capacity of tetrahydrobiopterin (BH_4). In addition, they have shown that BH_4 decreases superoxide in vitro through a direct scavenging mechanism independent of its role as a cofactor for DA or NO synthesis. Superoxide levels are inversely related to BH_4 levels in mesencephalic neurons (Nakamura et al., 2001).

We have found that AA exacerbates DEA/NO-induced cell death in a dose-dependent manner. AA is a well-known reducing agent and is involved in several types of protective mechanisms (Rice, 2000). However, AA can also exert a pro-oxidant activity related to ascorbyl radicals produced through a non-enzymatic degradation of AA. This latter action is stimulated under a higher oxidation potential environment (Choi et al., 2000). Dehydroascorbate, the total oxidized form of AA, is carried into cells and reduction of the cytosolic dehydroascorbate to AA causes oxidative stress (Song et al., 1999), which decreases cellular reducing agents as GSH and other thiols (Winkler et al., 1994). The balance between anti- and pro-oxidant effect of AA is dependent on vitamin E, GSH or other reducing agents (Wefers and Sies, 1988). A recent report has proven that vitamin C lacks efficacy as a cancer chemoprevention agent by generating bifunctional electrophiles and enhancement of hydroperoxide-dependent lipid peroxidation (Lee et al., 2001b). Taken altogether, our data may be explained by formation of ascorbyl radicals from AA in an oxidising environment induced by high doses of DEA/NO.

In the cascade of NO-induced neurotoxicity, three types of oxygen radicals, NO, superoxide, and peroxynitrite anions play an important role. NO is easily converted into peroxynitrite anion in the presence of superoxide anion (Beckman et al., 1990; Radi et al., 1991). This anion is highly reactive and can cause subsequent cytotoxic radical chain reactions (Dawson et al., 1993). Therefore, a possibility to explain NO-induced toxicity in fetal midbrain cultures could be the NO conversion to its metabolite peroxynitrite. In this study, uric acid and AA, with similar peroxynitrite scavenging activities to thiolic antioxidants, (Keller et al., 1998; Ciriolo et al., 2000), failed to protect from cell death induced by DEA/NO. Furthermore, catalase plus Cu/Zn-SOD, detoxifying enzymes that avoid extracellular peroxynitrite formation, and the cell-permeable superoxide scavenger Tiron, also failed to protect cell culture from DEA/NO-induced neurotoxicity. We may assess that the scavenging of peroxynitrite ion is not involved in the mechanism of protection in our model of neurotoxicity.

We found GSNO in the culture media of GSH plus DEA/NO-treated cells. *S*-nitrosothiol compounds such as GSNO may function as stable intracellular intermediates of NO activity and, perhaps, protect against NO-induced neurotoxicity. Analysis of *S*-nitrosothiols may help to understand the biology of NO. Rauhala et al. (1998) have reported that *S*-nitrosylation of GSH by NO may be part of the antioxidative cellular defence system of brain DA neurons. Our results show that NO reacts extracellularly with GSH to form a nitrosylated adduct which may regulate cellular functions and exert neuroprotective effects. These data could help to elucidate the protective role of thiolic antioxidants from neurotoxicity induced by DEA/NO.

This study supports the involvement of oxidative stress in the DEA/NO-induced neurotoxicity on neuronal-enriched fetal midbrain cultures and the neuroprotective role of thiolic antioxidants rescuing DA neurons from NO-induced cell death. Our results may be useful for the development of new therapeutic strategies against neurodegenerative disorders such as PD.

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Trabajo 4

Glutathione depletion switches nitric oxide neurotrophic effects to cell death in midbrain cultures: implications for Parkinson's disease

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Glutathione depletion switches nitric oxide neurotrophic effects to cell death in midbrain cultures: implications for Parkinson's disease

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Abstract

Nitric oxide (NO) exerts neurotrophic and neurotoxic effects on dopamine (DA) function in primary midbrain cultures. We investigate herein the role of glutathione (GSH) homeostasis in the neurotrophic effects of NO. Fetal midbrain cultures were pretreated with GSH synthesis inhibitor, L-buthionine-(*S,R*)-sulfoximine (BSO), 24 h before the addition of NO donors (diethylamine/nitric oxide-complexed sodium and *S*-nitroso-*N*-acetylpenicillamine) at doses tested previously as neurotrophic. Under these conditions, the neurotrophic effects of NO disappeared and turned on highly toxic. Reduction of GSH levels to 50% of baseline induced cell death in response to neurotrophic doses of NO. Soluble guanylate cyclase (sGC) and cyclic GMP-dependent protein kinase (PKG) inhibitors protected from cell death for up to 10 h after NO addition; the antioxidant ascorbic acid also protected from cell death but its efficacy decreased when it was added after NO treatment (40% protection 2 h after NO addition). The pattern of cell death was characterized by an increase in chromatin condensed cells with no DNA fragmentation and with breakdown of plasmatic membrane. The inhibition of RNA and protein synthesis and of caspase activity also protected from cell death. This study shows that alterations in GSH levels change the neurotrophic effects of NO in midbrain cultures into neurotoxic. Under these conditions, NO triggers a programmed cell death with markers of both apoptosis and necrosis characterized by an early step of free radicals production followed by a late requirement for signalling on the sGC/cGMP/PKG pathway.

Keywords: ascorbic acid, cGMP-dependent protein kinase, dopamine neurones, glutathione, guanylate cyclase, nitric oxide.

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Glutathione (GSH) depletion occurs in several forms of cell death and is associated with Parkinson's disease (PD). GSH has been reported to be markedly reduced in PD, particularly in patients with advanced disease (Perry *et al*. 1982; Di Monte *et al*. 1992). Furthermore, the GSH decrease seems to appear before neurodegeneration in presymptomatic PD (Sian *et al*. 1994; Merad-Boudia *et al*. 1998) and is not a consequence thereof. This suggests that a link may exist between these two events although it remains to be established whether or not the loss of GSH can induce neurodegeneration. Nitric oxide (NO) has been also implicated in neurodegenerative diseases. Several authors have reported markers that suggest a NO overproduction in PD brains, i.e. NO radicals detected in PD substantia nigra (Shergill *et al*. 1996), as well as increased nitrosilated proteins such as α-synuclein (Giasson *et al*. 2000) and increased nitrite concentration in cerebrospinal fluid (Qureshi *et al*. 1995). Finally, the core of Lewy bodies in PD are immunoreactive for nitrotyrosine (Good *et al*. 1998).

Decreased GSH may predispose cells to the toxicity of other insults that are selective targets for dopaminergic neurones. GSH depletion synergistically increases the selective toxicity of MPP^+ in dopamine (DA) cell cultures (Nakamura *et al*. 1997), and the toxicity of 6-OHDA and MPTP *in vivo* (Pileblad *et al*. 1989; Wullner *et al*. 1996). GSH peroxidase (GPx)-knockout mice show increased

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Abbreviations used: AA, ascorbic acid; BSO, L-buthionine-(*S,R*)-sulfoximine; cGMP, cyclic GMP; DA, dopamine; DEA/NO, diethylamine/nitric oxide-complexed sodium; DMSO, dimethyl sulfoxide; GSH, glutathione; LDH, lactate dehydrogenase; NO, nitric oxide; PKG, cGMP-dependent protein kinase; PBS, phosphate-buffered saline; PD, Parkinson's disease; sGC, soluble guanylate cyclase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TH, tyrosine hydroxylase.

vulnerability to MPTP (Klivenyi *et al*. 2000). There is evidence that NO may play an important role in DA cell death and functionality (Przedborski *et al*. 1996; LaVoie and Hastings 1999; Liberatore *et al*. 1999; Rodríguez-Martín *et al*. 2000; Canals *et al*. 2001). A redox-based mechanism for the neuroprotective and neurodestructive effects of NO and related nitroso-compounds has been postulated (Lipton *et al*. 1993). In this regard, GSH is an endogenous thiol that reacts with NO to form *S*-nitrosoglutathione and which protects DA neurones from oxidative stress (Rauhala *et al*. 1998; Chiueh and Rauhala 1999).

We show that the NO donor diethylamine/nitric oxide complexed sodium (DEA/NO) at doses of 25 and 50 μM exert neurotrophic effects on DA cells, by increasing the number of tyrosine hydroxylase positive (TH^+) cells, TH^+ neurite processes, DA levels, [^{3}H]DA uptake and by elevating intracellular and extracelular GSH concentration (Canals *et al*. 2001). When we tried to block the NO neurotrophic effect by GSH synthesis inhibition with the γ-glutamylcysteine synthetase inhibitor L-buthionine-(*S*,*R*)-sulfoximine (BSO), NO effects switched from neurotrophic to induce cell death.

In this work, we study the combined effect of nontoxic GSH down-regulation and neurotrophic doses of NO for midbrain cultures and DA neurones. Cell viability in the culture was analysed, the nature of cell death, the cell type susceptibly, the time course of cell death were characterized, and the mechanism of cell death induction and neuroprotection were addressed.

Materials and methods

Materials

Culture media

Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L), Ham's F12 nutrient mixture, Eagle's minimal essential medium (EMEM) with Earl's salts and Leibovitz's L-15 medium, all of which were supplemented with L-glutamine, fetal calf serum (FCS), sodium pyruvate and L-glutamine, were purchased from Gibco BRL (Paisley, Scotland, UK). Glucose 45%, insulin, putrescine, progesterone and sodium selenite were from Sigma (Madrid, Spain) and human transferrin, 30% iron-saturated, from Boehringer-Mannheim (Barcelona, Spain).

Antibodies

Rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody was from Chemicon International, Inc. (CA, USA), anti-microtubule-associated protein 2a + 2b (MAP-2) antibody and anti-rabbit IgG conjugated with tetramethylrhodamine (TRITC) were purchased from Sigma (Madrid, Spain) and anti-mouse Ig fluorescein was from Jackson (West grove, PA, USA).

Chemicals

Trypan blue, bovine serum albumin, poly D-lysine, p-phenylenediamine, bis-benzimide, BSO, pargyline, *N*-(1-naphthyl)ethylenediamine, sulfanilamide, dimethyl sulfoxide (DMSO), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), reduced and oxidized forms of glutathione, and diethylamine/nitric oxide complexed sodium (DEA/NO) were from Sigma (Madrid, Spain), denitrosylated DEA/NO (– NO) was obtained by incubating a 5-mM stock solution of DEA/NO for 2 h at room temperature (22–24°C) under illumination. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was from Tocris (Bristol, UK); NADPH, lactate dehydrogenase standard (LDH), the cytotoxicity detection kit (LDH), cell proliferation kit I (MTT) and GSH reductase (GR) were from Boehringer-Mannheim (Barcelona, Spain); methylene blue and ascorbic acid were from Merck (Darmstadt, Germany); LY-83583 was from Biomol (Plymouth, PA, USA) and KT5823, caspase inhibitor III (Boc-D-FMK), cycloheximide and actinomycin D were from Calbiochem (Darmstadt, Germany). The radiochemicals [^{3}H]DA (70 Ci/mmol) and [^{3}H]GABA (90 Ci/mmol) were obtained from Dupont NEN (Boston, MA, USA). The apoptosis TUNEL detection kit was from Promega (Madison, WI, USA) and the Live/Dead Viability/Cytotoxicity kit from Molecular Probes (Eugene, OR, USA). The BCA protein assay kit was from Pierce (Rockford, IL, USA). All other reagents were of the highest purity commercially available from Merck or Sigma.

Neuronal culture

Neuronal-enriched cultures from embryonic Sprague–Dawley rat midbrain E-14 (crown-rump length 10–12 mm) were obtained and prepared as previously described (Mena *et al*. 1993; Pardo *et al*. 1997). The cells were seeded in DMEM with 15% fetal calf serum (DMEM–FCS) at a density of 10^5 cells/cm^2 in multiwells or glass cover slides previously coated with poly-D-lysine, 4.5 μg/cm^2, in 0.1 M borate buffer, pH 8.4. The cultures were kept in a humidified chamber at 37°C in a 5% CO_2 atmosphere. Twenty-four hours after plating, the cells were changed to serum-free defined medium (EF12) as reported elsewhere (Mena *et al*. 1993; Pardo *et al*. 1997). EF12 consisted of a 1 : 1 (v/v) EMEM and nutrient mixture of Ham's F-12, supplemented with D-glucose (6 mg/mL), insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), progesterone (20 nM) and sodium selenite (30 nM).

Immunocytochemistry

To study cell type susceptibility to BSO + DEA/NO treatment in the fetal midbrain cultures, we performed immunostaining techniques. Rabbit polyclonal anti-TH antibody (1 : 500) was employed to identify DA neurones and mouse monoclonal anti-MAP-2 antibody (1 : 250) to detect all neurones in the culture. In brief, cultures were fixed with 4% paraformaldehyde, washed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, permeabilized with ethanol-acetic acid (19 : 1) and incubated at 4°C for 24 h with primary antibodies diluted in PBS containing 10% FCS. Fluorescein- and rhodamine-conjugated secondary antibodies were employed to visualise positive cells under fluorescent microscopy. The number of immunoreactive cells was counted in 1/7 of the total area of the cover slides. The cells were counted in predefined parallel strips using a counting reticule inserted in the ocular.

Cell viability measurements

Mitochondrial activity was measured with the MTT assay. Cells were grown on 24-well culture plates with 500 μL defined medium and treated with various reagents according to the experimental design. The MTT assay measures the ability of cells to metabolize 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). At

the end of the treatment period, 300 μL of culture medium were removed from each well and 20 μL of MTT solution (5 mg/mL) were added and incubated for 1 h. At this time, 200 μL of solubilization solution (10% SDS in HCl 0.01 M) were then added to the wells and after 24 h of incubation at 37°C, 100 μL were transferred into 96-well microtitre plates, and the absorption value at 540 nm was measured in an automatic microtitre reader (Spectra Fluor; Tecan, Mannedorf, Switzerland).

In some experiments, calcein acetoxymethyl ester and ethidium homodimer staining (live/dead viability/cytotoxicity assay) was employed to check viability under fluorescence microscopy (Mena *et al*. 1997). Calcein acetoxymethyl ester is a membrane-permanent dye that labels cells with esterase activity and an intact membrane is required to retain the esterase products (viable cells). Ethidium homodimer is a membrane-impermeable DNA dye that identifies cells in which plasma membrane integrity has been disrupted (dead cells). Cultures were washed and then incubated with 0.5 μM calcein acetoxymethyl ester and 1 μM ethidium homodimer for 30 min at room temperature before examination.

Apoptosis was measured by light microscopy features, DNA staining and the TUNEL assay. Cells growing on cover slides were fixed in 4% paraformaldehyde, nuclei were stained with bis-benzimide (Hoechst 33342) added in the antifading solution (3×10^{-6} M final concentration) (Hilwig and Gropp 1975; Pardo *et al*. 1997) and counted in 1/14 of the cover slide area; apoptotic cells were identified by chromatin condensation. TUNEL detection system for apoptosis measures the fragmented DNA of cells by incorporating fluorescein-12-dUTP* at the 3′-OH ends of the DNA by using the enzyme terminal deoxynucleotidyl transferase (TdT) (Kerr *et al*. 1972; Gavrieli *et al*. 1992). For this assay, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The fluorescein-12-dUTP-labelled DNA of apoptotic cells was visualized by fluorescence microscopy (positive cells with green fluorescence). The number of $TUNEL^+$ cells was counted in 1/14 of the cover slide area. Cells were counted in predefined parallel strips by using a counting reticule in the ocular. Cells incubated with buffer in the absence of TdT enzyme were used as negative controls.

For necrotic cell death determination, trypan blue dye exclusion assay was performed (Pardo *et al*. 1997), and lactate dehydrogenase (LDH) activity was measured in the culture medium by using a cytotoxicity detection kit (Decker and Lohmann-Matthes 1988), and expressed as a percentage versus detergent-extracted controls (100% cytotoxicity).

Nitrite measurement

NO production was routinely quantified by measuring nitrite, a stable oxidation end product of NO (Green *et al*. 1982). Briefly, 400 μL of culture medium were mixed with 800 μL of Griess reagent [1.5% sulfanilamide in 1 N HCl plus 0.15% *N*-(1-naphthyl]ethylenediamine dihydrochloride in distilled water, v/v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined in an automatic microtitre reader, by means of sodium nitrite as standard.

Uptake studies

[^{3}H]DA uptake was measured after incubation of the cells with 10^{-8} M [^{3}H]DA (70 Ci/mmol), in the presence of pargyline 10^{-5} M, and ascorbic acid 10^{-3} M, at 37°C for 30 min. Non-specific uptake/binding was calculated in the presence of 10^{-5} M mazindol and represented ≤ 5% (Beart and McDonald 1980). [^{3}H]GABA uptake was performed in the presence of 10^{-5} M aminooxyacetic acid and 10^{-3} M ascorbic acid and incubated for 4 min with 10 nM [^{3}H]GABA (90 Ci/mmol). Non-specific uptake/binding was calculated by incubating cultures at 0°C and represented ≤ 7% of the total (Michel and Hefti 1990). Proteins were measured by the BCA protein assay kit.

Glutathione measurements

Total glutathione levels were measured by the method of Tietze (1969). Briefly, 1×10^5 cells were washed with PBS, lysed in 100 μL of 3% perchloric acid (PCA) for 30 min at 4°C, centrifuged, and the supernatants were neutralized with 4 volumes of 0.1 M NaH_2PO_4, 5 mM EDTA, pH 7.5. Fifty microlitres of resulting supernatants were mixed with DTNB (0.6 mM), NADPH (0.2 mM) and glutathione reductase (1 U) and the reaction monitored in a P96 automatic microtiter reader at 412 nm during 6 min. Oxidized glutathione (GSSG) was measured in the cells by the method of Griffith (1980). Briefly, after PCA extraction and pH neutralization, GSH was derivatized with 2-vinylpyridine at room temperature for 1 h and the reaction carried out as above. GSH was obtained by subtracting GSSG levels from total glutathione levels.

Statistical analysis

The results were statistically evaluated for significance with one-way analysis of variance followed by the Newman–Keuls multiple comparison test as a *post-hoc* evaluation. Differences were considered statistically significant when $p < 0.05$.

Results

GSH depletion switches NO-induced neurotrophic effect on DA function to neurotoxic

Low doses of NO released by the NO-donor DEA/NO (25–50 μM) induce a neurotrophic effect on fetal midbrain cultures, characterized by increased TH^+ cell number and arborization, DA levels, [^{3}H]DA uptake, TH protein by western blot and GSH levels (Canals *et al*. 2001). Figure 1 shows how 50 μM DEA/NO-induced up-regulation of [^{3}H]DA uptake in midbrain cultures disappeared with 24 h of 20 μM BSO pretreatment and turned on down-regulation of neurotransmitter uptake. Cultures treated with 20 μM BSO alone without DEA/NO addition, did not show changes in neurotransmitter uptake. NO-induced neurotrophic effect is DA function-specific since [^{3}H]GABA uptake was not up-regulated, but in combination with GSH depletion, NO caused greater decrease in [^{3}H]GABA uptake than in [^{3}H]DA uptake (Fig. 1).

Neurotrophic levels of NO cause a loss of viability when applied to GSH down-regulated fetal midbrain cultures

NO treatment on GSH-down-regulated cultures not only decreased neurotransmitter uptake but also resulted in a loss of cell viability. Several doses of the NO donors DEA/NO (Figs 2a and b) and SNAP (Figs 2c and d) were applied to

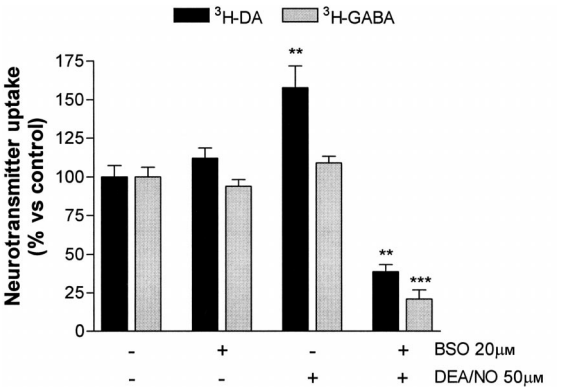

Fig. 1 GSH depletion switches NO-induced up-regulation of DA function to neurotoxic effect in midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for additional 24 h. The figure shows high-affinity [^{3}H]DA and [^{3}H]GABA uptakes expressed as a percentage versus controls. Values are the mean ± SEM from $n = 6$. Control values were 6.6×10^5 cpm/mg protein for [^{3}H]DA and 4.0×10^5 cpm/mg protein for [^{3}H]GABA. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{**}p < 0.01$; $^{***}p < 0.001$ versus their respective controls.

cultures pretreated for 24 h either with BSO 20 μM or vehicle. BSO 20 μM treatment without DEA/NO addition did not show any changes in cell viability. Neurotrophic doses of NO that caused no alterations in cell viability parameters (50 μM DEA/NO or 100 μM SNAP), turned on highly toxic in GSH-down-regulated cultures, increasing LDH release by 20–30% (Figs 2a and c) and decreasing MTT viability assay values by 75–85% (Figs 2b and d). A minimal amount of NO was required to switch on the cell death cascade, since 25 μM DEA/NO or 50 μM SNAP did not alter cell viability in the cultures. Both NO donors had similar effects on BSO-pretreated cultures and the addition of denitrosylated DEA/NO 50 μM (2 h after having been dissolved) exerted no effects on cell viability (Fig. 2, last bar to the right of graphs in (a) and (b), indicated as –NO), suggesting that NO is essential to initiate the cell death cascade.

GSH depletion after BSO treatment was dose- and time-dependent (Figs 3a and b). Treatment of cell cultures for 24 h with 1, 3, 10 and 20 μM BSO decreased intracellular GSH to 60%, 50%, 30% and 20% versus controls, respectively. GSH content in cultures treated with 20 μM BSO decreased from 20% at 24 h to 10% at 48 h of treatment, respectively. No signs of toxicity were seen with

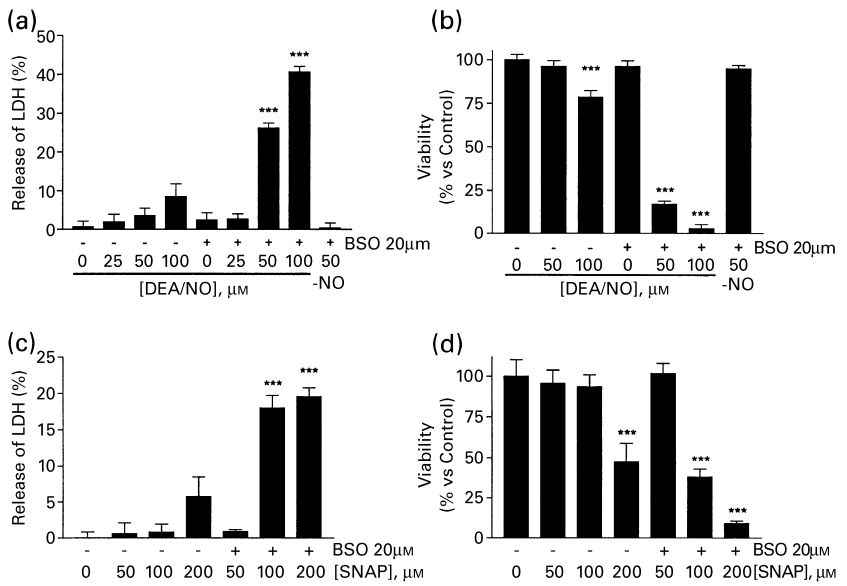

Fig. 2 Effects of NO donors and GSH depletion on cell viability. After 4 days *in vitro*, the cells were treated with BSO 20 μM or vehicle, and then on the fifth day, pre-established groups were treated with either the NO donors DEA/NO or SNAP in concentrations ranging from 25 to 200 μM, for 24 h. Cell viability was measured by MTT assay and is presented as a percentage versus controls. Cell cytotoxicity was measured by LDH activity in the culture medium and is expressed as a percentage versus detergent-extracted controls (100% cytotoxicity). (a) and (b) represent release of LDH and viability, respectively, in midbrain cultures treated with BSO 20 μM and DEA/NO in concentrations ranging from 25 to 100 μM. The last graph-bar on the right (– NO) corresponds to groups that were treated with denitrosylated DEA/NO 50 μM (added to the culture 2 h after DEA/NO reconstitution). (c) and (d) represent LDH cytotoxicity and MTT viability, respectively, in midbrain cultures treated with BSO 20 μM and SNAP in concentrations ranging from 50 to 200 μM. Values are the mean ± SEM from $n = 4$–6. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{***}p < 0.001$ versus controls.

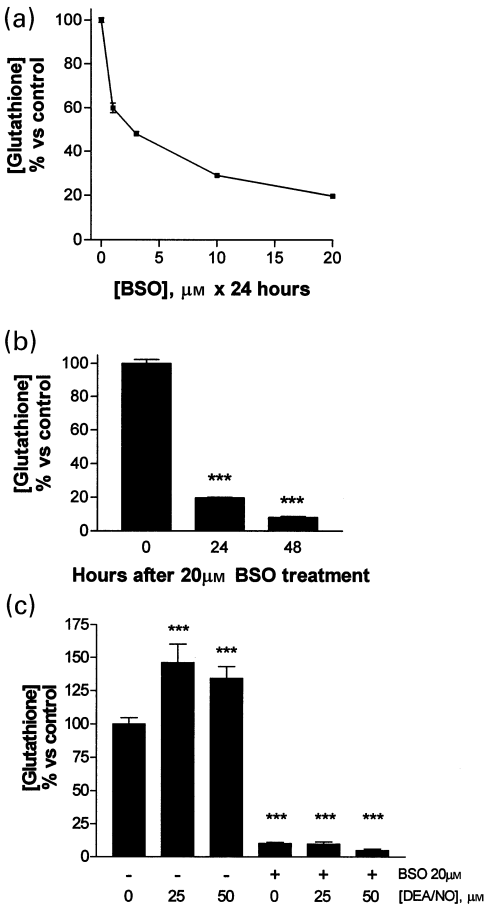

Fig. 3 Intracellular GSH levels in midbrain cultures treated with BSO and DEA/NO separately and in combination. GSH concentration is normalized by protein content and expressed as a percentage versus controls. (a) Dose–response curve of BSO (1, 3, 10 and 20 μM) treated for 24 h; (b) time-response curve of 20 μM BSO treatment; and (c) effect of pretreatment with 20 μM BSO for 24 h on DEA/NO-induced up-regulation of GSH synthesis. Basal levels of GSH were 17.2 ± 0.3 μg/mg protein in (a), 16.9 ± 0.4 μg/mg protein in (b), and 18.8 ± 0.9 μg/mg protein in (c). Values are the mean ± SEM from $n = 4$–8. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus control.

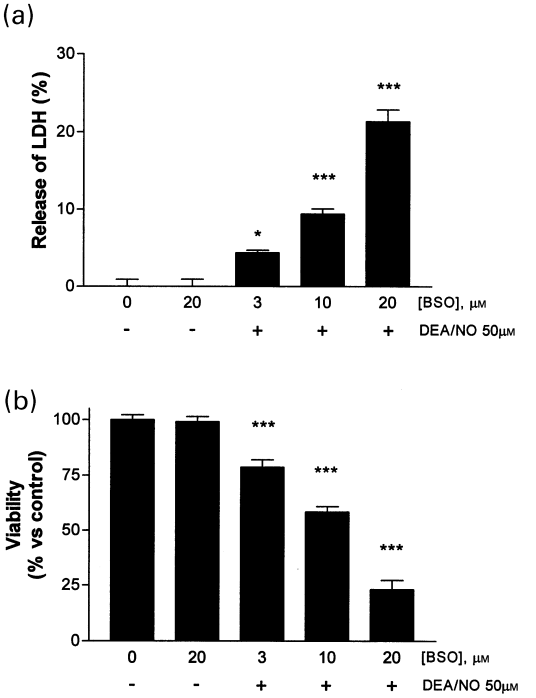

Fig. 4 Effect of pretreatment with different doses of BSO followed by DEA/NO 50 μM on cell viability. After 4 days *in vitro*, the cultures were treated with increasing concentrations of BSO (3, 10 and 20 μM) or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for an additional 24 h. (a) Cell cytotoxicity measured in the culture medium by LDH activity. (b) Cell viability measured by MTT assay and presented as a percentage versus controls. Values are the mean ± SEM from $n = 4$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. *$p < 0.05$; ***$p < 0.001$ versus controls.

BSO 20 μM at 48 h (BSO-pretreated DEA/NO-untreated groups) and at 72 h (data not shown) in midbrain cultures. When used in combination with 25 and 50 μM DEA/NO, 20 μM BSO prevented NO-induced GSH up-regulation in the cultures and depleted intracellular GSH to the same extent that when applied alone (Fig. 3c).

To investigate which level of GSH depletion is sufficient to trigger NO-induced cell death cascade, midbrain cultures were pretreated for 24 h with the same doses of BSO than in Fig. 3, and then 50 μM DEA/NO was added for additional 24 h. Results, as shown in Fig. 4, indicate that a neurotrophic dose of NO turns on neurotoxic from 3 μM BSO pretreatment, that is 50% depletion of intracelular GSH, and the toxicity increases in a dose-dependent manner in parallel with BSO concentration. We have used a pretreatment with 20 μM BSO for the cell death characterization and the cell death protection studies because such dose was not toxic by itself but it produced a high degree of toxicity when combined with 50 μM DEA/NO.

Soluble guanylate cyclase (sGC) and cyclic GMP-dependent protein kinase (PKG) are involved in the cell death cascade

GSH depletion has been shown to increase cyclic GMP (cGMP) synthesis in rat brain and primary culture neurones (Heales et al. 1996). Also, it is well known that NO is an endogenous activator for sGC (Bredt and Snyder 1989). However, we have previously shown that neurotrophism induced by 50 μM DEA/NO and cell death induced by 400 μM DEA/NO in fetal midbrain cultures are not mediated by cGMP (Canals et al. 2001). To study the implication of cGMP in the toxicity induced by low doses of NO in GSH down-regulated cultures, two structurally differentiated sGC inhibitors, LY83583 and methylene blue (MB), were used.

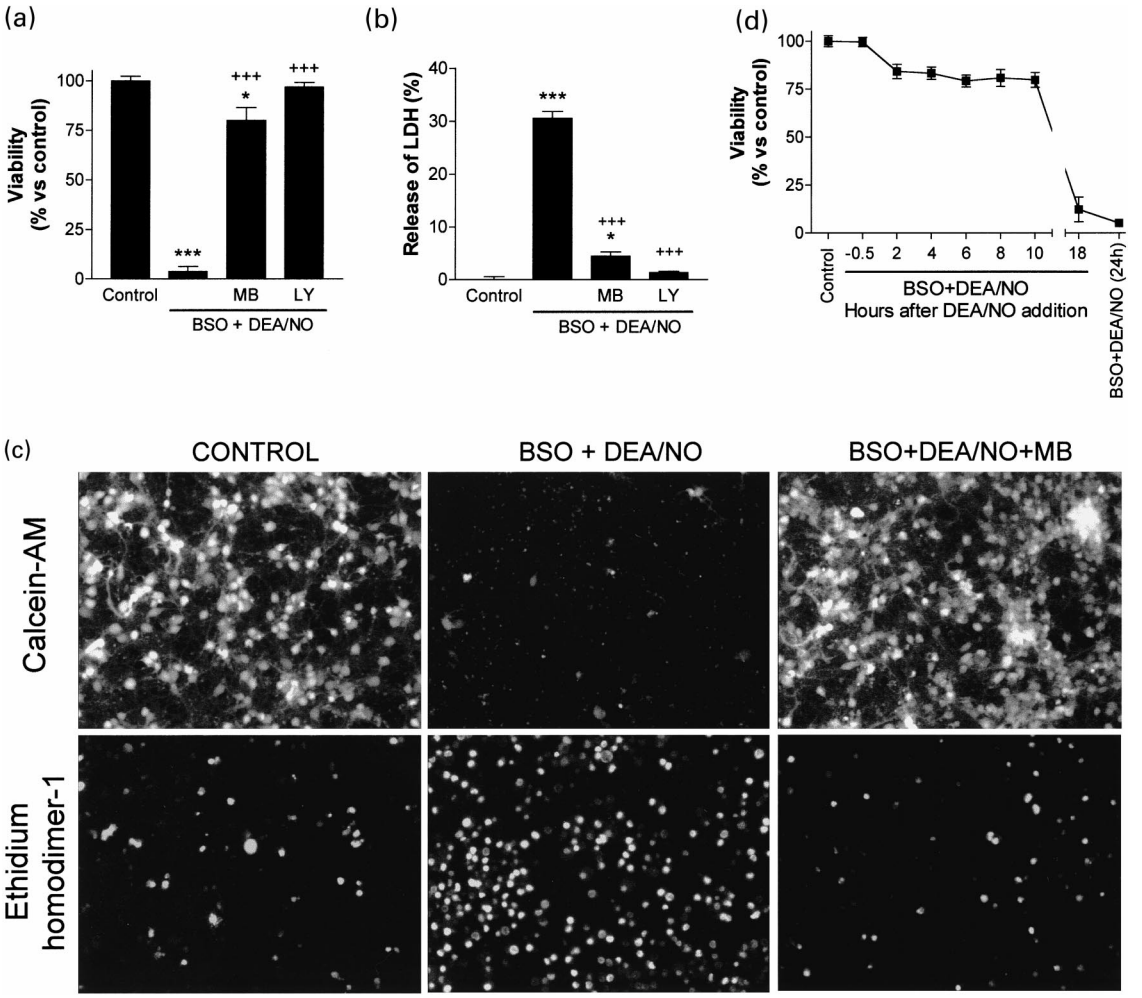

Fig. 5 Guanylate cyclase inhibitors protect from NO-induced toxicity in GSH-down-regulated midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM, then on the fifth day, pre-established groups were treated with methylene blue 0.1 μM (MB), 0.2 μM LY83583 (LY) or vehicle and 30 min later with DEA/NO 50 μM for 24 h. (a) Cell viability measured by MTT assay and presented as a percentage versus controls. (b) Cell cytotoxicity measured by LDH activity in the culture medium. (c) Photomicrographs of cells stained with calcein-AM and ethidium homodimer-1. (d) LY83583 was added to the cultures 30 min before DEA/NO addition or up to 10 h later, in 2 h-intervals. At 24 h of DEA/NO addition, cell viability was measured by MTT assay and is expressed as a percentage versus controls. Values are expressed as the mean ± SEM from $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{*}p < 0.05$, $^{***}p < 0.001$ versus controls; $^{+++}p < 0.001$ versus BSO 20 μM + DEA/NO 50 μM.

Addition of 0.2 μM LY83583 or 0.1 μM MB to the cultures 30 min before DEA/NO treatment (Figs 5a and b) or up to 10 h after (Fig. 5d), rescued the cultures from cell death as seen by MTT viability assay (Figs 5a and d), LDH activity assay (Figs 5b and 7), and calcein-AM/ethidium homodimer-1 staining (Fig. 5c). Protection was 100% when inhibitors were applied before NO treatment and by about 80% when applied 2–10 h after DEA/NO treatment (Fig. 5d). sGC inhibitors protected from cell death but not from GSH depletion (data not shown).

The PKG inhibitor KT5823 was used to investigate the way in which cGMP participates in the induced cell death. At concentrations of 0.5 and 1 μM, this inhibitor added to GSH-depleted cultures 30 min before DEA/NO treatment and up to 10 h after, strongly prevented the loss of viability as measured by MTT (Figs 6a and c) and LDH assays (Figs 6b and 7), indicating that cGMP-induced cell death occurs through a PKG-dependent mechanism. Since dimethyl sulfoxide (DMSO), used as solvent for KT5823, interfered with our model in a dose-dependent manner (Figs 6d and e), we reduced DMSO concentration as much as allowed by KT5823 solubility and treated the cultures with the usual dose of DEA/NO and a higher one. PKG inhibitor protected from cell death but not from GSH depletion (data not shown).

Free radicals are also involved in cell death

The antioxidant ascorbic acid (AA) was supplied to BSO-pretreated cultures 30 min before DEA/NO addition or up to 10 h after (Fig. 7). AA protected from cell death when was present in the culture before NO addition. The protection was progressively decreasing from 2 h (40% protection) to 10 h (no protection) after NO treatment, suggesting that free radical production occurs soon in the cell death cascade and that free radical levels correlate with cell death in the culture. When the ability of AA, sGC and PKG inhibitors to prevent cell death was compared in parallel, the protective effect of AA was lost sooner after NO treatment than the inhibitors protective effects (Fig. 7). This suggests that free radical production precedes sGC and PKG activation in the cell death cascade.

Involvement of free radicals in the BSO + DEA/NO-induced cell death is also suggested by GSH/GSSG ratios. Cultures treated with 50 μM DEA/NO alone doubled its GSH/GSSG ratio, 20 μM BSO treatment decreased it and BSO + DEA/NO combined treatment, although not statistically significant versus BSO alone, further decreased the GSH/GSSG ratio. Inhibition of PKG protected from cell death but did not restore GSH/GSSG ratio, indicating that free radical production precedes PKG activation (Table 1).

Cell type susceptibility to NO-induced toxicity in GSH-down-regulated cultures

Immunocytochemical characterization of cell death in cultures pretreated for 24 h with 20 μM BSO and treated for additional 24 h with 50 μM DEA/NO, reveals that all neurones in the cultures (TH^+ and MAP-2^+ cells) are strongly affected by the treatment (Fig. 8b), but TH^+ cells remain more preserved. Untreated groups showed that TH^+ cells represent a minor proportion of the total neurones in the cultures (Fig. 8a, top panels), but when treated with

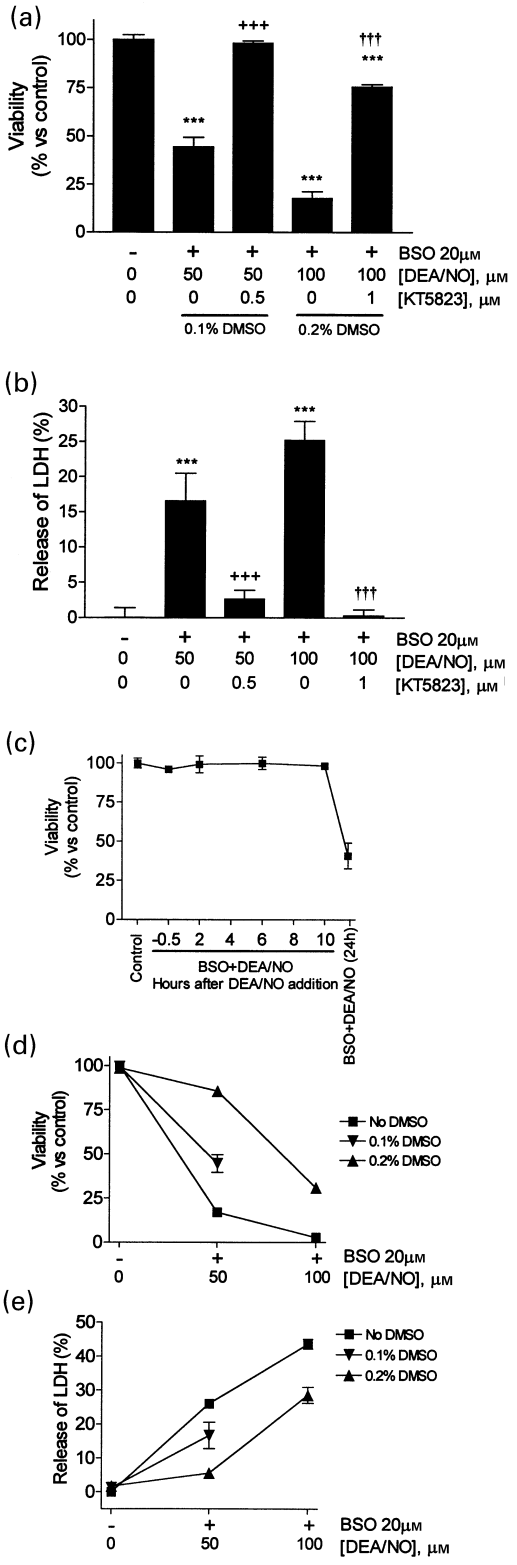

Fig. 6 The cGMP-dependent kinase inhibitor KT5823 protects from NO-induced toxicity in GSH-down-regulated midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM, then on the 5th day, preestablished groups were treated with DEA/NO 50–100 μM for 24 h. Cell viability was measured by MTT assay and is presented as a percentage versus controls. Cell cytotoxicity was measured by LDH activity in the culture medium. (a) and (b) represent viability and release of LDH, respectively, in cultures treated with 0.5 μM KT5823 in 0.1% DMSO or 1 μM KT5823 in 0.2% DMSO, 30 min before DEA/NO addition. Control and BSO + DEA/NO groups received the appropriate dose of DMSO under each condition. ***$p < 0.001$ versus controls; $^{+++}p < 0.001$ versus BSO 20 μM + DEA/NO 50 μM in 0.1% DMSO; †††$p < 0.001$ versus BSO 20 μM + DEA/NO 100 μM in 0.2% DMSO. (c) 0.5 μM KT5823 was added to the cultures 30 min before and 2, 6 and 10 h after 50 μM DEA/NO treatment. At 24 h of DEA/NO addition, cell viability was measured by MTT assay. Values are expressed as a percentage versus controls. (d) and (e) show viability and release of LDH, respectively, in BSO + DEA/NO-treated midbrain cultures in the absence or presence of 0.1% or 0.2% DMSO. ***$p < 0.001$ versus DMSO untreated group. Values are the mean ± SEM from $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test.

Table 1 Effects of treatments with BSO, DEA/NO and PKG inhibitor on GSH/GSSG ratios

Control	29.6 ± 4.9
DEA/NO 50 μM	60.4 ± 5.5***
BSO 20 μM	6.1 ± 1.2***
BSO + DEA/NO 50 μM	3.6 ± 0.6***
BSO + DEA/NO + KT5823 0.5 μM	3.4 ± 0.5***
KT5823 0.5 μM	28.3 ± 3.1

After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then, on the fifth day, pre-established groups were pretreated with 0.5 μM KT5823 or vehicle and 30 min later with DEA/NO 50 μM for additional 24 h. Control values for GSH and GSSG are 22.2 ± 1.5 and 0.8 ± 0.1 ng/μg of protein, respectively. Values are expressed as the mean ± SEM for $n = 4$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus control.

BSO + DEA/NO, TH^+ cells reached the main population of surviving neurones (Fig. 8a, middle panels). These results are in agreement with those shown in Fig. 1 referred to [^{3}H]DA and [^{3}H]GABA uptakes, and indicate that DA neurones, although very affected, are the most resistant neurones to NO-induced toxicity in GSH-down-regulated midbrain cultures.

The sGC inhibitor MB, added to the culture 30 min before DEA/NO treatment in GSH-down-regulated cultures, totally protected MAP-2^+ and TH^+ cells from NO-induced toxicity, showing intact neuronal processes (Fig. 8a, bottom panels and 8b).

NO induces on GSH-down-regulated cultures a form of programmed cell death with characteristics of apoptosis and necrosis

Cell death induced by DEA/NO 50 μM in cultures pretreated with BSO 20 μM for 24 h is characterized by shrinkage, rounded cells with chromatin condensation without DNA fragmentation. Chromatin-condensed cells in the cultures

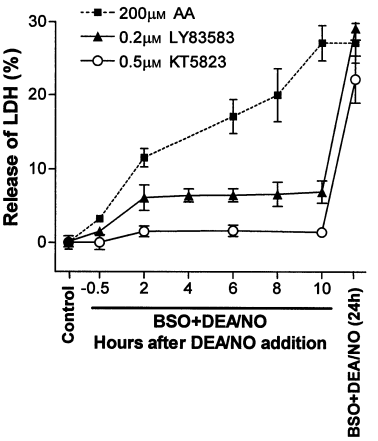

Fig. 7 Protection time pattern of the antioxidant ascorbic acid (AA), the sGC inhibitor LY83583 and the PKG inhibitor KT5823 on cell death induced by NO in GSH-down-regulated midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for an additional 24 h. At different time points after DEA/NO treatment, 200 μM AA, 0.2 μM LY83583 or 0.5 μM KT5823 were added to the culture. Cell death was measured at 24 h by LDH activity. Values are the mean ± SEM from $n = 4$.

increased above 70% with no increase in $TUNEL^+$ cells (Table 2). Chromatin condensed peripherally in the nucleus, as seen by bis-benzimide staining and phase contrast (inset in bis-benzimide photomicrograph and arrows in Fig. 9). TUNEL staining in the same fields showed that peripherally condensed chromatin did not comark as $TUNEL^+$ (insets in Fig. 9). Neither BSO 20 μM nor DEA/NO 50 μM alone caused changes in cell or nuclear morphology by phase contrast, or bis-benzimide or TUNEL staining. On the other hand, the combined treatment of BSO and DEA/NO gave rise to breakdown of plasmatic membrane, since LDH released to the culture medium increased to 30%, suggesting necrotic cell death. Furthermore, the membrane-impermeable DNA dye ethidium homodimer (Fig. 5c) and trypan

Table 2 Effects of BSO and DEA/NO treatment on chromatin condensation and DNA fragmentation

	TUNEL cells ($\times 10^3$)	Condensed nuclei ($\times 10^3$)	Condensed nuclei (% versus total nuclei)
Control	2.3 ± 0.3	2.6 ± 0.3	9.7 ± 0.9
DEA/NO 25 μM	2.3 ± 0.2	2.4 ± 0.1	9.0 ± 0.9
DEA/NO 50 μM	2.6 ± 0.2	2.9 ± 0.2	10.2 ± 1.1
BSO 20 μM	2.2 ± 0.2	2.5 ± 0.3	9.3 ± 0.6
BSO + DEA/NO 25 μM	2.2 ± 0.3	2.6 ± 0.3	9.8 ± 0.7
BSO + DEA/NO 50 μM	2.7 ± 0.2	19.3 ± 0.6***	72.6 ± 2.3***

After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then, on the fifth day, pre-established groups were treated with DEA/NO 25 or 50 μM for an additional 24 h. Values are expressed as the mean ± SEM for $n = 4$–6. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus control.

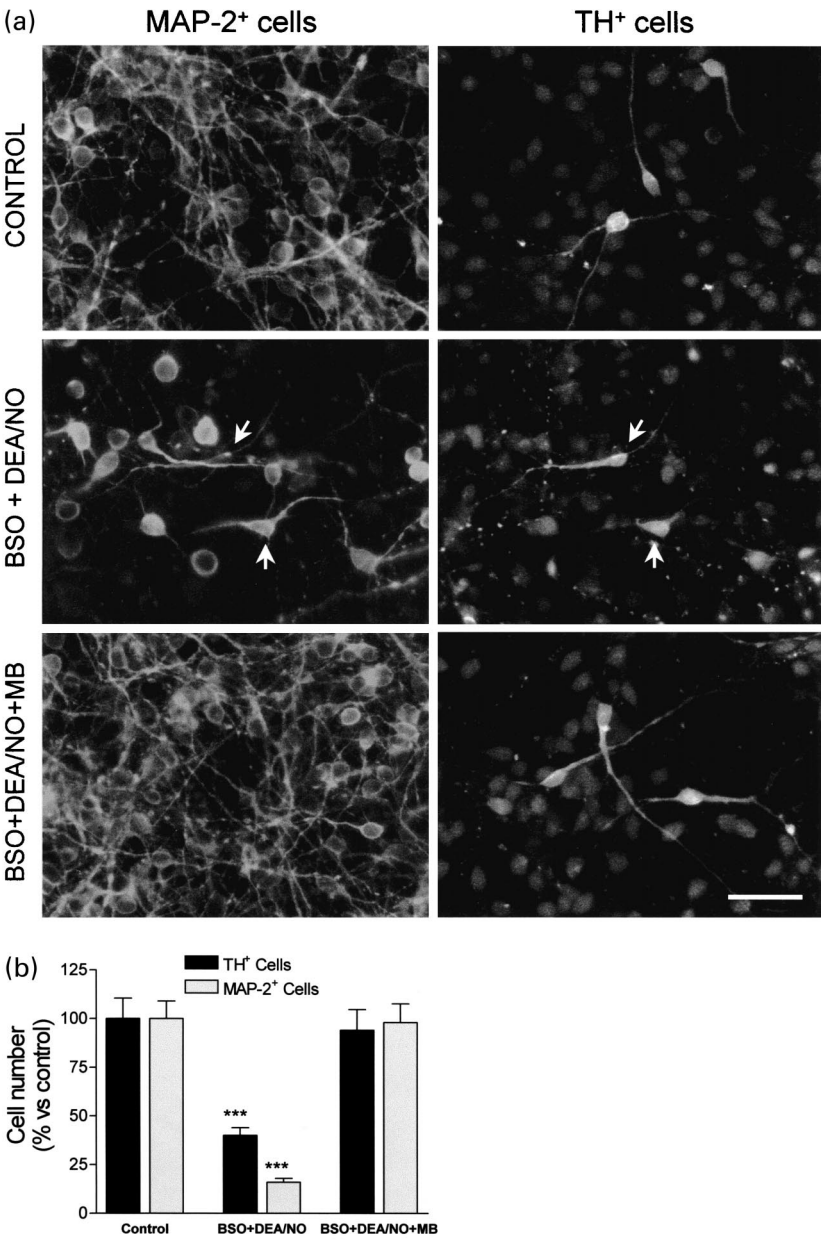

Fig. 8 Cell type selectivity of BSO + DEA/NO-induced neurotoxicity and cell protection by the sGC inhibitor methylene blue (MB). After 4 days *in vitro*, the cultures were treated with BSO 20 μM, then on the fifth day, pre-established groups were treated with MB 0.1 μM or vehicle and 30 min later with DEA/NO 50 μM for 24 h additional. (a) Photomicrographs show total neurones and DA neurones, corresponding to the same field, after cell treatments. Arrows indicate TH- and MAP-2-costained cells in the same field. Scale bar = 50 μm. (b) Number of total neurones (MAP-2$^+$) and DA neurones (TH$^+$) expressed as a percentage versus controls. Values are expressed as the mean ± SEM for $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. ***$p < 0.001$ versus their respective controls.

blue dye exclusion assay (data not shown), stained almost all the chromatin-condensed cells, indicating that both chromatin condensation and breakdown of plasmatic membrane occur simultaneously in the same cell. The definitive nature of programmed cell death is suggested because the inhibition of RNA and protein synthesis and of caspase activity did revert or attenuate cell death in the cultures (Fig. 10). The protein synthesis inhibitor, cycloheximide, at 0.01 μg/mL, added to the culture 30 min before DEA/NO 50 μM treatment, prevents the loss of viability as measured by MTT assay (Fig. 10a) and the increased LDH released to the culture medium (Fig. 10b). Similar results were obtained with the transcriptional inhibitor, actinomycin D, but the inhibitor by itself at 0.1 μg/mL caused more toxicity than did cycloheximide (Figs 10a and b), and lower concentrations failed to protect midbrain cultures from BSO + DEA/NO toxicity (data not shown). Furthermore, the broad spectrum inhibitor of caspases Boc-D-FMK also reverted the loss of viability induced by BSO- and DEA/NO-cotreatment (Fig. 10c).

Discussion

We have previously shown that the NO donor DEA/NO at low doses (25 and 50 μM) not only protects DA cells from apoptosis but also induces *de novo* TH synthesis and exerts

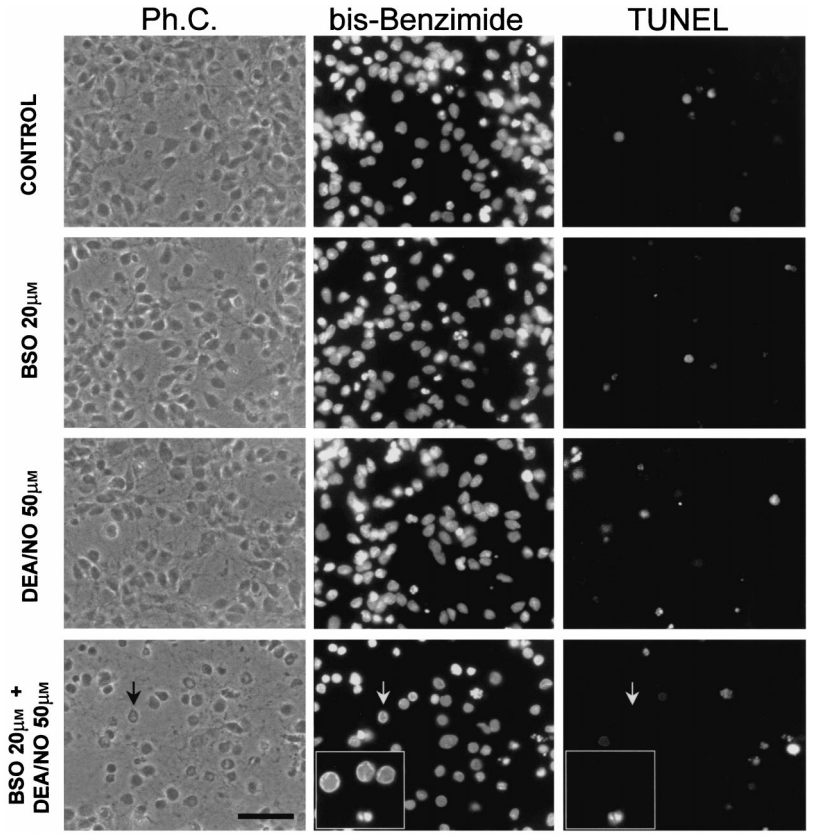

Fig. 9 Effect of GSH-down-regulation and NO treatment in apoptotic cell death of fetal midbrain cultures. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for additional 24 h. The figure shows the phase-contrast microscopy of midbrain cultures and of total nuclei stained with bis-benzimide and nuclear fragmented cells stained by the TUNEL assay, corresponding to the same field. Arrows mark a constant position in the photomicrographs of BSO + DEA/NO group. Inset shows that chromatin-condensed nuclei are not comarked by TUNEL assay. Scale bar = 50 μm.

neurotrophic effects on DA function. In addition, it increases intracellular and extracellular GSH (Canals *et al*. 2001). To investigate the importance of correct GSH homeostasis for the neurotrophic capacity of NO, we pretreated cell cultures with different doses of the γ-glutamylcysteine synthetase inhibitor BSO. With this model, we demonstrated that a GSH depletion of only 50% is sufficient to transform the neurotrophic effect on DA function exerted by low doses of NO into neurotoxic effects. Under these conditions, NO triggers a programmed cell death with markers of both apoptosis and necrosis that is characterized by an early step of free radicals production followed by a late requirement for signalling on the sGC/cGMP/PKG pathway.

Several authors have reported that experimental depletion of GSH potentiates the toxicity of 6OH-DA and MPTP *in vivo* (Pileblad *et al*. 1989; Wullner *et al*. 1996; Klivenyi *et al*. 2000), sulfite plus $ONOO^-$ in CSM14.1.4 cell line (Marshall *et al*. 1999) and $ONOO^-$ in astrocytes (Barker *et al*. 1996). Furthermore, cellular GPx-deficient mice show increased vulnerability to MPTP (Klivenyi *et al*. 2000). A very important aspect of our data is that a 50% depletion of GSH, similar to that observed in PD (Sian *et al*. 1994; Merad-Boudia *et al*. 1998), is enough to enable NO, at low doses, to trigger the cell death cascade. Although under these conditions, cell death induced by NO is less significant than that induced with higher GSH depletions, it may become more outstanding over longer time periods, like in neurodegenerative diseases with development over decades.

Soluble guanylate cyclase inhibitors, LY83583 and MB, protect from cell death induced by BSO 20 μM pretreatment and DEA/NO 50 μM treatment up to 10 h after NO addition, suggesting that sGC is directly linked to neuronal death. Also, it suggests that NO released from DEA/NO is not the major source of sGC activation in our model because DEA/NO totally decomposes in less than 15 min (half-life decomposition of 3.5 ± 0.2 min) and sGC activation occurs up to 10 h after NO donor addition. Only a 20% decrease in cell viability was seen when sGC was inhibited 2 h or more after NO treatment, which may be explained by direct activation of sGC by NO. In addition to NO, other compounds like hydroxyl radicals and lipid peroxides activate sGC (Weber 1999; Snider *et al*. 1984; Reiser 1990) and have been involved in this way, with cell death (Li *et al*. 1997a). These compounds might participate in the cell death cascade observed in our model. This hypothesis is further supported by the experiments showing AA protection from BSO + DEA/NO-induced cell death. Because AA protects at earlier stages of the cell death cascade than sGC and PKG inhibitors, free radical species may initiate the death signalling. Furthermore, GSH/GSSG ratios indicate an increase in intracellular oxidative stress after the treatments that is not reverted by PKG inhibition. These results corroborate the

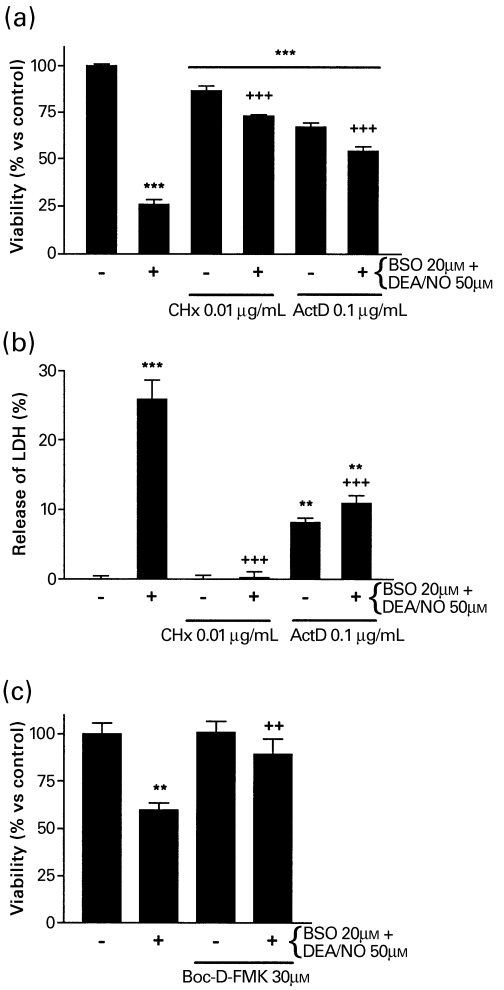

Fig. 10 Inhibition of macromolecular synthesis and caspase activity prevent cell death. After 4 days *in vitro*, the cultures were treated with BSO 20 μM or vehicle, then on the fifth day, pre-established groups were treated with DEA/NO 50 μM for an additional 24 h. The transcriptional inhibitor actinomycin D (ActD), the protein synthesis inhibitor cycloheximide (CHx), the broad spectrum caspase inhibitor Boc-D-FMK or its correspond solvents, were added 30 min before DEA/NO treatment. (a) and (c) show cell viability measured by MTT assay and presented as a percentage versus controls. (b) Cell cytotoxicity measured by LDH activity in the culture medium. Values are expressed as the mean ± SEM for $n = 4–6$. Statistical analysis was performed by ANOVA followed by the Newman–Keuls multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus untreated groups. $^{++}p < 0.01$, $^{+++}p < 0.001$ versus BSO 20 μM + DEA/NO 50 μM.

involvement of oxidative stress as the major mechanism in this process and the neuroprotective role of AA in PD.

The PKG inhibitor KT5823 totally protects midbrain cultures from BSO + DEA/NO-induced cell death for up to 10 h after NO addition. PKG has been identified in neurones of the basal ganglia (Walaas *et al*. 1989), where its major substrate, the protein phosphatase I inhibitor DARPP-32, is highly represented (Tsou *et al*. 1993; Wang and Robinson 1997). The NO/cGMP/PKG pathway has been implicated in protein phosphatase regulation, calcium signalling, cytoskeletal dynamics, neurotransmitter release (Wang *et al*. 1997), the regulation of catecholamine synthesis, and secretion in CNS (Rodríguez-Pascual *et al*. 1999), but no data is available about its participation in cell death processes in CNS. Since we noted that macromolecular synthesis inhibitors also protects from cell death induced by BSO + DEA/NO treatment, and PKG has been implicated in *fos* promoter activation (Gudi *et al*. 1996; Gudi *et al*. 1999), the sGC/cGMP/PKG pathway may activate genes implicated in the cell death induced by NO in GSH down-regulated midbrain cultures.

NO is essential to initiate the cell death cascade. This is supported because two structurally differentiated donors exerted the same results and denitrosylated DEA/NO did not cause loss of viability. Also, in our experimental model, BSO by itself at any of doses used and for up to 3 days in culture (data not shown), did not result in any signs of toxicity. This is in agreement with other data in midbrain cultures that show no toxicity after 3 days of 50 μM BSO treatment (Mytilineou *et al*. 1999) and in rat mesencephalic cell line CSM14.1.4, in which 100 μM BSO for up to 60 h does not compromise cell viability detected by trypan blue exclusion or MTT reduction (Marshall *et al*. 1999). However, in other models with embryonic cortical primary neurones and HT22 hippocampal nerve cell line, GSH depletion above 80%, induced with glutamate or BSO treatment, causes cell death by sGC activation and extracellular Ca^{2+} influx, without PKG participation (Li *et al*. 1997a,b).

Here we show that 50 μM DEA/NO induces a type of cell death in the GSH down-regulated cultures that integrates simultaneously in the same cell morphological characteristics of apoptosis and necrosis. The biochemical study indicates that this type of cell death constitutes an active process with cell participation because inhibitors of macromolecule synthesis, such as cycloheximide and actinomycin D, and caspase inhibitors attenuate cell death, like in many other cases of apoptosis (Oppenheim *et al*. 1990; Koh *et al*. 1995; Ahn *et al*. 2000). A type of cell death with characteristics of both apoptosis and necrosis has been previously reported by using a mouse hippocampal cell line (HT-22) treated with either 5 mM glutamate or 250 μM BSO (Tan *et al*. 1998). Intracellular ATP depletion switches the mode of cell death from apoptosis to necrosis (Leist *et al*. 1999). NO donors caused necrosis in the absence of glucose due to inhibition of respiration and subsequent ATP depletion, but in the presence of glucose, to maintain ATP level via glycolysis, NO donors caused apoptosis. GSH depletion inhibits mitochondrial complex I activity (Jha *et al*. 2000) leading to mitochondrial respiration failure and ATP depletion. Finally, ATP is essential for the morphological changes in the nuclei typical of apoptosis (Kass *et al*. 1996).

These experiments may contribute to understand the type of programmed cell death observed in GSH-depleted cultures.

We found that DA neurones are more resistant than GABA neurones to GSH depletion plus NO treatment. Nakamura *et al*. (2000) reported that the preferential resistance of DA neurones to the toxicity of GSH depletion was independent of cellular GPx and was mediated by tetrahydrobiopterin (BH4). The resistance of dopaminergic neurones to oxidative stress may be critical to their survival and disturbances in their capacity to produce BH4 or other antioxidants from genetic mutations or exposure to exogenous toxins could underlie their demise in PD. Such a multihit hypothesis for DA cell death is consistent with the current view that PD is a heterogeneous disease that can arise from combinations of genetic susceptibilities and environmental insults (Langston 1998).

If our results can be extended to *in vivo* situations, then intervention of the sGC/cGMP/PKG pathway could be beneficial to individuals suffering from PD or other pathologies associated with NO and GSH disregulation. Experiments to elucidate the molecular and cellular mechanisms, to identify of cell death genes and the role played by mitochondria, leading to cell death initiated by NO when GSH synthesis is compromised in neuronal models, could lead to novel preventive or therapeutic strategies for PD.

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Trabajo 5

Nitric oxide triggers the toxicity due to glutathione depletion in midbrain cultures through 12-lipoxygenase

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Nitric Oxide Triggers the Toxicity Due to Glutathione Depletion in Midbrain Cultures Through 12-Lipoxygenase*

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Glutathione (GSH) depletion is the earliest biochemical alteration shown to date in brains of Parkinson's disease (PD) patients. However, data from animal models show that GSH depletion by itself is not sufficient to induce nigral degeneration. We have previously shown that non-toxic inhibition of GSH synthesis with L-buthionine-(S,R)-sulfoximine (BSO) in primary midbrain cultures, transforms a nitric oxide (NO) neurotrophic effect, selective for DA neurons, into a toxic effect with participation of guanylate cyclase (GC) and cGMP-dependent protein kinase (PKG). Here we demonstrate that arachidonic acid (AA) metabolism through the 12-lipoxygenase (12-LOX) pathway is also central for this GSH-NO interaction. LOX inhibitors (NDGA and baicalein) but not cyclooxygenase (indomethacin) or epoxygenase (clotrimazole) ones, prevent cell death in the culture, even when added 10h after NO treatment. Furthermore, AA addition to GSH depleted cultures precipitates a cell death process that is indistinguishable from that initiated by NO, in its morphology, time course and 12-LOX, GC and PKG dependency. The first AA metabolite through 12-LOX enzyme, 12-HPETE, induces cell death in the culture and its toxicity is greatly enhanced by GSH depletion. In addition we show that if GSH synthesis inhibition persists for up to 4 days without any additional treatment, it will induce a cell death process that also depends on 12-LOX, GC and PKG activation. In this study, therefore we show that the signalling pathway AA/12-LOX/12-HPETE/GC/PKG may be important in several pathologies in which GSH decrease has been documented, like PD. The potentiating effect of NO over such signalling pathway, may be of relevance as part of the cascade of events leading to and sustaining nerve cell death.

In a number of neurological disorders including Parkinson's disease (PD)[1], several potentially toxic alterations related to oxidative stress may coexist simultaneously in the brain (1). Decreased levels of the thiolic antioxidant glutathione (GSH) have been reported in the *sustantia nigra* (SN) of PD patients (2-4), as well as iron accumulation (3, 5, 6) and decreased mitochondrial complex I (7, 8) and α-ketoglutarate dehydrogenase (9) activities. In addition, biochemical markers of lipid peroxidation (10, 11) and nitric oxide (NO) overproduction, i.e. increased nitrite concentration in cerebrospinal fluid (12), NO radicals detected in PD nigra (13), increased 3-nitrotyrosine immunostaining in Lewy bodies (14) and α-synuclein nitration (15) have been found in PD brains. Whether such alterations are a primary cause of the disease, part of the degenerative mechanism or secondary to the cell death process, remains to be resolved.

In cell cultures and animal models of several diseases, including PD, these alterations may contribute to neuronal degeneration. Furthermore, synergistic interactions between them have been demonstrated and greatly enhance the neurodegenerative process (1, 16, 17).

GSH depletion is the earliest biochemical alteration shown to date in PD brains. It seems to appear before neurodegeneration in incidental Lewy bodies disease, considered as the presymptomatic manifestation of PD (3). However, data from animal models show that GSH depletion by itself is not sufficient to induce nigral degeneration (18, 19). Furthermore, dopamine (DA) neurons in culture seem to be more resistant to GSH decrease than other midbrain cell populations (20-22). However, reduction of GSH levels may rather enhance the susceptibility of DA cells to the toxicity of other insults, promoting the neurodegenerative process. For example, GSH depletion induced by L-buthionine-(S,R)-sulfoximine (BSO) treatment, a selective GSH synthesis inhibitor (23), enhances the susceptibility of DA neurons to the toxicity of the mitochondrial complex I inhibitor MPTP/MPP^+ *in vivo* (18) and *in vitro* (20), potentiates the toxicity of 6-OHDA in rat striatum (24) and even changes the DA cell-specific trophic effect of NO in midbrain cultures into neurotoxic (25).

We have previously described a culture model in which one of these interactions was clearly stated. In midbrain cultures the short lived NO donor diethyl-amine/nitric oxide complexed sodium (DEA/NO) at doses of 25 and 50μM selectively increases the number of tyrosine hydroxylase positive (TH^+) cells, TH^+ neurite processes, DA syntheses and [3H]DA uptake (26). Interestingly, this DA cell-specific neurotrophism of NO disappears when GSH content is lowered to 50% by BSO pretreatment (25), a GSH depletion similar to that occurring in PD (2-4). In addition, under GSH-decreased conditions, neurotrophic doses of NO triggers a programmed cell death with dependence on guanylate cyclase (GC) and cyclic GMP-dependent protein kinase (PKG) activation. Also we have shown that the GC activation that mediates cell death is not produced by NO (25).

In the present work we go far inside the mechanism related to the neurotoxic interaction between decreased GSH levels and NO, by exploring the possible participation of arachidonic acid (AA) metabolism. AA is an important component of membrane lipids that can activate several signalling pathways directly by itself or by its metabolites through lipoxygenase (LOX), cyclooxygenase (COX), or epoxygenase pathways (27). In nervous tissue the major enzymatic route for AA metabolism is the 12-LOX pathway and the resulting metabolites play an important role in neuronal signalling and degeneration (28-30). Several observations suggest that 12-LOX may participate in cell death triggered by NO in GSH-decreased conditions. Fist, GSH depletion can induce the activation of 12-LOX (31) and such a mechanism is related to neuronal death in GSH-depleted cultures (32). Second, 12-HPETE, the initial AA metabolite from 12-LOX, is a potent activator of GC (33), and a NO-independent GC activation is necessary for cell

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[1]The abbreviations used are: NO, nitric oxide; DEA/NO, diethylamine/nitric oxide complexed sodium; TH, tyrosine hydroxylase; DA, dopamine; GSH, glutathione; LDH, lactate dehydrogenase; 12-LOX, 12-lipoxygenase; 12-HETE, (12s)-hydroxyeicosatetraenoic acid; 12-HPETE, (12s)-hydroperoxyeicosatetraenoic acid; AA, arachidonic acid; GC, guanylate cyclase; PKG, cGMP-dependent protein kinase; PD, Parkinson's disease.

death in our model (25). Finally, NO has been shown to potentiate AA metabolism by activating phospholipase (PLA) A2 (34, 35) or inhibiting AA re-esterification to membrane (36, 37). Here we show that NO and GSH synergically interact at the AA metabolic pathway to induce cell death in midbrain cultures.

EXPERIMENTAL PROCEDURES

Materials

Culture media: Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5g/l), Ham's F12 nutrient mixture, Eagle's minimal essential medium (EMEM) with Earl's salts and Leibovitz's L-15 medium, all of which were supplemented with L-glutamine, fetal calf serum (FCS), sodium pyruvate and L-glutamine, were purchased from Gibco BRL (Paisley, Scotland, UK). Glucose 45%, insulin, putrescine, progesterone and sodium selenite were from Sigma (Madrid, Spain) and human transferrin, 30% iron-saturated, from Boehringer-Mannheim (Barcelona, Spain).

Antibodies: rabbit polyclonal anti mouse leukocyte 12-lipoxygenase antibody was from Alexis (Carlsbad, CA, USA); mouse monoclonal anti-tyrosine hydroxylase (TH) antibody was from Chemicon (Temecula, CA, USA); O1 was obtained from hybridoma supernatants (38); polyclonal anti-GFAP antibody, raised in rabbits, was from DAKO (Glostrup, Denmark); anti-microtubule-associated protein 2a + 2b (MAP-2) antibody, mouse monoclonal anti-β-actin antibody and anti-rabbit IgG conjugated with tetramethylrhodamine (TRITC) were purchased from Sigma (Madrid, Spain); anti-mouse Ig fluorescein was from Jackson (PA, USA) and anti-mouse IgM Alexa Fluor® 488 was from Molecular Probes (Eugene, OR, USA).

Chemicals: poly-D-lysine, p-phenylenediamine, bis-Benzimide, L-buthionine-[S,R]-sulfoximine (BSO), dimethyl sulphoxide (DMSO), 5,5'-dithio-*bis*2-nitrobenzoic acid (DTNB), reduced and oxidised forms of glutathione and arachidonic acid (AA) were from Sigma (Madrid, Spain). Diethylamine/nitric oxide complexed sodium (DEA/NO) was from Alexis (Carlsbad, CA, USA). NADPH, the cytotoxicity detection kit (LDH), cell proliferation kit I (MTT) and GSH reductase (GR) were from Boehringer-Mannheim (Barcelona, Spain); 12-HPETE, 12-HETE and LY-83583 were from Biomol (Plymouth, PA, USA), KT5823, baicalein, nordihydroguaiaretic acid (NDGA), indomethacin and clotrimazole were from Calbiochem (Darmstadt, Germany). The BCA protein assay kit was from Pierce (Rockford, Ill, USA). All other reagents were of the highest purity commercially available from Merck or Sigma.

Neuronal culture—Neuronal-enriched cultures from embryonic Sprague-Dawley rat midbrain E-14 (crown-rump length 10-12 mm) were obtained and prepared as previously described (39, 40). The cells were seeded in DMEM with 15% fetal calf serum (DMEM-FCS) at a density of 10^5 cells/cm^2 in multiwells or glass cover slides previously coated with poly-D-lysine, 4.5 μg/cm^2, in 0.1 M borate buffer, pH 8.4. The cultures were kept in a humidified chamber at 37°C in a 5% CO_2 atmosphere. Twenty-four hours after plating, the cells were changed to a serum-free defined medium (EF12) as reported elsewhere (39, 40). EF12 consisted of a 1:1 (v/v) EMEM and nutrient mixture of Ham's F-12, supplemented with D-glucose (6 mg/ml), insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM) and sodium selenite (30 nM).

Experimental treatments—In the experiments designed to study interactions between NO and GSH depletion on cell viability, the cells, after 4 days in culture, received 20 μM BSO or vehicle, and then on the fifth day, pre-established groups were treated with the NO donor DEA/NO (50 or 100 μM) for additional 24 h. Enzymes inhibitors for the three pathways of AA metabolism, LOX pathway (NDGA and baicalein), COX pathway (indomethacin) and epoxygenase pathway (clotrimazole and proadifen) or its corresponding solvents, were routinely added 30 min before DEA/NO treatment or up to 10 h later. For experiments with AA and 12-LOX metabolites, cultures were treated with BSO as above and on the fifth day received several doses of AA, 12-HPETE, 12-HETE or solvent for additional 24h. Finally, experiments for time-course effects of 100 μM DEA/NO or 20 μM BSO alone or in combination, on 12-LOX expression were also depicted.

The long term effect of GSH synthesis inhibition on cell viability was also studied. The cells received 20 μM BSO as above and the treatment proceeded for up to 4 days (8th day *in vitro*). In this experimental design, enzymes inhibitors or vehicles were added to the culture on the fifth day *in vitro*. The participation of 12-LOX in neurotoxicity induced by higher doses of NO under normal GSH homeostasys was investigated by pretreating cultures with NDGA or baicalein 30 min before 200 and 400 μM DEA/NO addition.

Immunocytochemistry—DA neurons were characterized by immunostaining with a mouse monoclonal anti-TH antibody (1:100), astrocytes with a rabbit policlonal anti-GFAP antibody (1:500) and oligodendrocytes with monoclonal anti-O1 (1:10) (41). To detect all neurons in the culture, a mouse monoclonal anti-MAP-2 antibody (1:250) was used. For TH, GFAP and MAP-2 immunostaining, cultures were fixed with 4% paraformaldehyde, washed in 0.1 M Phosphate-buffered saline, pH 7.4 (PBS), permeabilized with ethanol-acetic acid (19:1) and incubated at 4ºC for 24h with primary antibodies diluted in PBS containing 10% fetal calf serum. Fluorescein- and rhodamine-conjugated secondary antibodies were employed to visualize positive cells under fluorescent microscopy. For oligodendrocytes detection, anti-O1 antibody was directly added (1:10) to living cells and incubated for 15 min at room temperature, washed in PBS and fixed with 4% paraformaldehyde previous to anti-mouse IgM Alexa Fluor® 488 development. The number of immunoreactive cells was counted in 1/7 of the total area of the cover slides. The cells were counted in pre-defined parallel strips using a counting reticule inserted in the ocular.

Cell viability measurements—Mitochondrial activity was measured with the MTT assay. Cells were grown on 24-well culture plates with 500 μL defined medium and treated with various reagents according to the experimental design. The MTT assay measures the ability of cells to metabolize 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). At the end of the treatment period, 300 μL of culture medium were removed from each well and 20 μL of MTT solution (5 mg/mL) were added and incubated for 1 h. At this time, 200 μL of solubilisation solution (10% SDS in HCl 0.01M) were then added to the wells and after 24 h of incubation at 37ºC, 100 μL were transferred onto 96-well microtiter plates, and the absorption value at 540 nm was measured in an automatic microtiter reader (Spectra Fluor, Tecan).

Chromatin condensation was assesed by DNA staining with bis-Benzimide (Hoechst 33342). Cells growing on cover slides were fixed in 4% paraformaldehyde, nuclei were stained with bisBenzimide added in the anti-fading solution ($3x10^{-6}$ M final concentration) (40, 42) and counted in 1/14 of the cover slide area.

For necrotic cell death determination, lactate dehydrogenase (LDH) activity was measured in the culture medium by using a cytotoxicity detection kit (43), and expressed as a percentage *vs.* detergent-extracted controls (100% cytotoxicity). In our system, LDH release to the culture medium correlates with cell death measured by trypan blue dye exclusion assay (26).

Western blot analysis—Primary midbrain cultures were homogenized with a sonicator in buffer containing 20 mM TrisHCl, 10 mM AcK, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, leupeptin, aprotinin, pepstatin 5μg/mL each, 0.25% NP-40, pH 7.4, and then centrifuged at 12.000 x g for 30 min at 4°C. The supernatant was used for protein determination by the BCA protein assay kit and for electrophoretical separation. Samples (30 μg) were added to SDS sample loading buffer, electrophoresed in 10% SDS-polyacrilamide gels and then electroblotted to 0.45 μm nitrocellulose membranes. For immunolabeling, the blots were blocked with TTBS (20 mM Tris-HCl pH 7.6, 137 mM NaCl plus 0.1% (v/v) Tween-20 and 5% dry skimmed milk) for 1 h at room temperature. After blocking non-specific binding, the membranes were incubated with rabbit anti-12-LOX (1:2000) and mouse anti-β-actin (1:10000) in blocking solution overnight at 4°C. The blots were developed by chemiluminiscence detection using a commercial kit (Amersham) and quantified by computer-assisted videodensitometry. β-actin was employed as a control of charge.

Glutathione measurements—Total glutathione levels were measured by the method of Tietze (1969)(44). Briefly, 1 x 10^5 cells were washed with PBS, lysed in 100 μL of 3% perchloric acid (PCA) for 30 min at 4ºC, centrifuged, and the supernatants were neutralised with 4 vol of 0.1 M NaH_2PO_4, 5 mM EDTA, pH 7.5. Fifty μL of resulting supernatants were mixed with DTNB (0.6 mM), NADPH (0.2 mM) and glutathione reductase (1U) and the reaction monitored in a P96 automatic microtiter reader at 412 nm during 6 minutes. Oxidised glutathione (GSSG) was measured in the cells by the method of Griffith (1980)(45). Briefly, after PCA extraction and pH neutralisation, GSH was derivatized with 2-vinylpyridine at room temperature for 1 hour and the reaction carried out as above. GSH was obtained by subtracting GSSG levels from total glutathione levels.

Statistical analysis—The results were statistically evaluated for significance with one-way analysis of variance followed by the Newman-Keuls multiple comparison test as a post-hoc evaluation. Differences were considered statistically significant when $p<0.05$.

RESULTS

Inhibitors of lipoxygenase block NO triggered cell death in GSH depleted cultures—Midbrain cultures treated for 24h with 20 μM BSO decrease its GSH content from 17.5 ± 0.4 μg/mg of protein to 3.7 ± 0.1 μg/mg of protein (≈20 % vs control). In these GSH depleted conditions, treatment of cultures with the NO donor DEA/NO at doses previously shown to be neurotrophic for DA neurons (50 μM), for additional 24h, results in extensive cell death in the culture (Fig. 1a-b). The death process is characterised by 75-85% loss of mitochondrial activity measured by MTT assay, 30-35% increase in LDH release to the culture medium and 70-80% of cells with chromatin condensation without DNA fragmentation (Fig. 1a-b and 2; and data not shown), as previously shown (25). However, this rate of GSH synthesis inhibition by itself did not induce cell death in the culture at 48 h (Fig. 2a and b) and at 72h (data not shown), in accordance with previously reported data for neuronal enriched midbrain cultures (25, 46). To investigate the possible role of lipoxygenase in cell death in the above conditions, we first used the general LOX inhibitor nordihydroguaiaretic acid (NDGA). Added 30 min before the NO treatment, NDGA preserves cell viability in the culture (75-80% of controls viability) and totally blocks LDH release and chromatin condensation induced by BSO + DEA/NO treatment (Fig. 1c and 2a-c). The concentration required for maximal protection was 0.5 μM, being toxic to the culture up to 2.5 μM (data not shown). Since 12-LOX is the predominant brain isoform of this enzyme (28), we tested baicalein, a selective 12-LOX inhibitor, for cell viability assays. As seen for NDGA, baicalein rescues all viability parameters measured in the culture, with maximal protection observed at 2.5 μM (Fig. 1d and 2a-d). Both inhibitors protect from cell death induced by 50 μM as well as 100 μM DEA/NO in BSO pretreated cultures (Fig. 2a-d), without interfering with BSO induced GSH depletion (data not shown).

In contrast, when indomethacin, a specific inhibitor of cyclooxygenses with an IC50 of 1 μM, was used at concentrations up to 50 μM, no protection from cell death was seen (Fig. 1e and 2a-b). In addition, indomethacin, which is not toxic for fetal midbrain cultures at doses used here, increases the toxicity of NO in GSH depleted midbrain cultures (Fig. 2a and b). Two inhibitors of epoxygenase, clotrimazole and proadifen, were also proved for cell death protection. Clotrimazole, with an IC50 of 0.4 μM, used at concentrations up to 5 μM, did not show any protective effect (Fig. 1f and 2a-b). In the same way as indomethacin, non toxic doses of clotrimazole further increased cell death in the culture (Fig. 2a and b). However, the other epoxygenase inhibitor, proadifen at 10 μM provide partial neuroprotection (Fig. 2a and b). Although widely used as an epoxygenase inhibitor, proadifen also inhibits LOX activity (47). In view of the above results, the proadifen protective effect is probably due to 12-LOX inhibition.

We have previously shown that NO induced cell death on GSH down-regulated midbrain cultures required GC activation. The protection afforded by GC inhibitors occurred up to 10h after NO addition (25). To determine the temporal correlation between GC inhibition and LOX inhibition on cell death prevention, we tested the efficacy of baicalein to protect cells, when it is added to the culture 2, 6 and 10 h after NO treatment. As shown in Fig. 2d, the 12-LOX inhibitor efficiently prevent cell death at any time used, similar to that occurring for GC inhibitors.

Effect of BSO and DEA/NO treatment on 12-LOX protein levels—Li et al. (32) have previously shown that GSH depletion induced by glutamate treatment in primary immature cortical cultures, induces a 2- to 3-fold increase in 12-LOX protein. To test this possibility in midbrain cultures depleted of GSH, we performed western blot analyses of 12-LOX protein. As shown in figure 3, GSH depletion for up to 48h, alone or in combination with NO treatment did not vary 12-LOX protein levels. Furthermore, NO alone (100 μM DEA/NO) neither changed 12-LOX levels at 1, 4 nor after 8h of treatment (Fig 3b).

12-LOX inhibition protects all cell types in the culture—Immunocytochemical characterization of cell death induced by BSO + DEA/NO treatment in fetal midbrain cultures shows that the most affected cell types were neurons ($MAP\text{-}2^+$ cells) and oligodendrocytes ($O1^+$ cells) (Fig. 4). Among neurons, TH^+ cells, although very affected, were more preserved (Fig. 4 and 5), in accordance with previously reported data (25). Astrocytes ($GFAP^+$ cells) was the most resistant cell type in the culture. When the ability of LOX inhibitors (NDGA and baicalein) to protect from cell death was studied for different cell populations, we observed that all cell types in the culture were protected from toxicity (Fig. 4 and 5).

Arachidonic acid and 12-HPETE but not 12-HETE, reproduce in GSH-depleted midbrain cultures, all features of cell death triggered by NO—Incubation of midbrain cultures for 24 h with increasing concentrations of AA resulted in no sign of toxicity in doses ranging from 0.3 to 6 μM and very slightly toxic for 12 μM. By contrast, after GSH depletion, AA induced cell death in the culture in a dose-dependent manner, reaching 100% toxicity from 3 μM (Fig. 6). Cell death is characterized by shrinkage, rounded cells with chromatin condensed peripherally in the nucleus without

DNA fragmentation (Fig. 6c and data not shown), loss of mitochondrial activity (measured by MTT assay; Fig. 6a) and breakdown of plasmatic membrane (measured as LDH released to culture medium; Fig. 6b), similar to that occurring with NO in GSH depleted cultures (Fig. 1 and 2). Also, the temporal course of the cell death process is similar to that initiated by NO since there was no change in cell viability for at least 16 h after AA treatment (data not shown). Furthermore, LOX inhibitors (NDGA and baicalein) but not cyclooxygense (indomethacin) or epoxygenase (clotrimazole) inhibitors protect from AA induced cell death (Fig. 6d). Finally, cell death was also blocked by the GC inhibitor LY83583 and the PKG inhibitor KT5823 (Fig. 6d) in the same way that NO triggered cell death.

These results demonstrate that GSH depletion by itself potentiates AA metabolism through the 12-LOX pathway and that AA is limiting LOX activity in our model since AA supplementation is sufficient to induce cell death in the culture. In addition, the results indicate that the cell death process initiated by AA under GSH-decreased conditions is indistinguishable from that induced by NO and suggest that a relationship may exist between these two events.

Next we tested the ability of 12-HPETE, the first AA metabolite through 12-LOX pathway, to induce cell death in solvent or BSO pre-treated cultures. Incubation of control cultures with increasing concentrations of 12-HPETE resulted in loss of viability from 3μM and BSO pretreatment greatly increased its toxicity (Fig. 7a and b). Unlike NO or AA in GSH decreased cultures, 12-HPETE did not require long exposure times for toxicity and cell death was visible as soon as 2h after treatment, by phase contrast microscopy (data not shown). This observation suggests that 12-HPETE participates in the execution phase of the cell death process. In addition, the main down-stream product of 12-HPETE in brain, 12-HETE, was unable to induce cell death in control nor GSH depleted cultures (Fig. 7b). Overall these data indicate that 12-HPETE is the metabolite responsible for cell death induction by NO and AA in our model.

GSH depletion rather than NO determines the LOX/GC/PKG dependency of cell death induced by BSO + DEA/NO treatment— GSH depletion induced by 20μM BSO results in no sign of toxicity in midbrain cultures after 48h of treatment (Fig. 2). If inhibition persists, loss of viability occurs on the fourth day after BSO addition, without NO requirement (Fig. 8). Cell death produced in these experimental conditions is prevented by 12-LOX, GC and PKG inhibition (Fig. 8). The toxicity of NO for DA neurons in midbrain cultures without pharmacological alteration of the GSH system, is observed from 200 to 400μM DEA/NO, but this toxic effect is not dependent on GC and LOX activation (26, and data not shown). Overall these data suggest that GSH depletion did not favour NO to be toxic due to loss of one of the most important antioxidant systems in the cell, but rather that NO precipitates the cell death process which takes place in GSH decreased conditions and that requires LOX, GC and PKG activation.

DISCUSSION

The effect of GSH depletion on neuronal viability has been widely studied in several experimental models of neurodegenerative diseases. PD models have received special attention since a severe GSH depletion in the SN of PD patients has been described (2-4). Experimental GSH depletion does not cause nigral degeneration by itself in the rat, but it renders DA neurons more susceptible to following insults (18, 19, 24). In cell culture models, different tolerance to GSH depletion has been reported depending on cell type and the brain region used for primary cultures. Cell death in primary immature cortical neurons and HT22 hippocampal nerve cell line is dose-dependently induced by 50-95% GSH depletion after 12h of BSO incubation and the death process depend on 12-LOX and GC activation, without PKG participation (32, 48). By contrast, neuronal enriched midbrain cultures and the CSM14.1.4 rat midbrain cell line are resistant to GSH depletions of about 95% for up to 3 days of BSO treatment (25, 46, 49). As we show in figure 8, if GSH synthesis inhibition in neuronal enriched midbrain cultures persists up to 4 days (by 20μM BSO treatment), it causes the death of the culture. Cell death in those GSH-depleted conditions is prevented by LOX, GC and PKG inhibitors. Despite the differences in susceptibility, cell death induced by GSH depletion seems to be tightly linked with 12-LOX and GC activation in different culture models.

We have previously shown that the tolerance to GSH depletion of neuronal enriched midbrain cultures and DA neurons is lost after non-toxic NO addition. Neurotrophic doses of NO for DA neurons, when added after 24h of 20μM BSO pretreatment, precipitate cell death in a GC and PKG dependent manner (25). Here we demostrate that 12-LOX is also central for this NO effect since NDGA and baicalein prevent cell death in the culture. Other pathways of AA metabolism in our model are excluded since cyclooxygenase and epoxygenase inhibitors did not confer any protection. Furthermore, they increase both NO and AA induced cell death. This potentiating effect may be related to a rise in AA availability for 12-LOX metabolism, as has been described for other systems (50). We conclude that GSH decrease and NO, interacting on AA metabolism through 12-LOX, cooperate to induce cell death in neuronal enriched midbrain cultures. Interestingly, midbrain cultures containing serum, with a great proportion of glial cells, become more sensitive to GSH depletion than neuronal enriched cultures and dies after 48h of BSO treatment (46, 51). In agreement with the role of NO in potentiating BSO toxicity through 12-LOX pathway, cell death in those glia-containing cultures is blocked by the NOS inhibitor L-NAME and by the LOX inhibitor NDGA (46).

The 12-LOX enzyme is clearly detected in primary midbrain cultures but its synthesis is not regulated by isolated or combined depletion of GSH and NO treatment. Nevertheless, addition of AA to BSO pretreated cultures precipitates neuronal cell death at doses in which AA is not toxic for midbrain cultures and the effect is prevented by NDGA and baicalein, indicating that GSH depletion by itself is sufficient to activate 12-LOX. These observations are in agreement with previously reported data showing that GSH directly inhibits LOX (31, 32, 52).

The exact mechanism by which NO potentiates BSO toxicity in our neuronal enriched midbrain cultures needs further investigation. Cell death produced by AA supplementation in GSH-depleted cultures is indistinguishable from that induced by NO, in its time course, morphology and molecular signalling pathway (12-LOX/GC/PKG), supporting the notion that increased AA metabolism is behind NO effects and linking 12-LOX metabolites of AA with GC/PKG activation and cell death. The fact that AA precipitates cell death in BSO-pretreated cultures indicates that AA is limiting LOX activity and therefore, one possibility for NO actions is by increasing non-esterified AA availability for 12-LOX metabolism. Several groups have implicated PLA2 activation and decreased rate of AA re-esterification to the membrane as mechanisms for NO induced increase in free AA (34-37). Since AA re-esterification is dependent on cellular ATP and GSH depletion potentiates NO inhibition of mitochondrial complex I (53) and thus decreases ATP levels, this mechanism of free AA up-regulation is possibly operating in our model.

Several sources of evidence support that 12-HPETE is the 12-LOX metabolite implicated in the cell death process. 12-HPETE induces cell death in midbrain cultures and its toxicity is greatly enhanced by GSH depletion. It has been shown that 12-HPETE activates GC (33) and a NO-independent GC activation is needed

for cell death induced by DEA/NO in GSH decreased midbrain cultures. The toxicity of AA under GSH depleted conditions as well as the toxicity of long term GSH synthesis inhibition, also require GC activation. There is a good temporal relation between GC inhibition and LOX inhibition on cell death prevention in our model. In addition, 12-HPETE plays a role in the execution phase of the cell death process since its toxic effect is observed as soon as 2h after treatment. Finally, 12-HETE, the main downstream metabolite of 12-HPETE and without GC activation capacity did not induce cell death in control nor BSO treated cultures. Although the reduction of 12-HPETE to 12-HETE is very rapid and this has long been known to be a major metabolite of AA in brain (28), 12-HPETE metabolism is not limited to its reduction and therefore, the participation of other derived compounds such as hepoxilins, in cell death, may not be formally discarded.

Overall, the results with AA and 12-HPETE suggest that GSH decrease fulfils at least two functions related to lipid peroxidation in our model: a) increased 12-LOX activity and b) increased 12-HPETE half life by decreasing its reduction rate. The last hypothesis is based on the fact that GSH depletion potentiates 12-HPETE toxicity and on data showing that the GSH system participates in the reduction of lipid hydroperoxydes into hydroxy acids (28). But another important function of GSH may be directly related to NO. GSH can compete with cellular targets of NO by conjugating with it to form nitrosoglutathione or by regenerating nitrosyl groups and thus, limiting NO actions (54, 55). When intracellular GSH is decreased, this NO-buffering effect, is expected to be lower.

Several observations in animal models of PD confer importance to our results; in the MPTP model, cell protection has been reported by neuronal and inducible (i) NOS gen ablation in mice (56, 57). Interestingly, PLA2 knock-out mice are protected from MPTP toxicity (58) and the PLA2 inhibitor, mepacrine, also prevents the toxin-induced DA depletion in rat *striatum* (59). All these data indicate that NO and AA are implicated in the degeneration of *nigro-striatal* system in rodents. Since GSH depletion potentiates MPTP toxicity in vivo, we propose that interactions between GSH, NO and AA metabolism may be important for DA cell toxicity in PD experimental models. In PD patients, along with GSH depletion a marked increase of glial cells expressing iNOS in the SN has been described (60). This indicates the possibility that, in PD nigra, NO concentration increases in the vicinity of DA neurons in a general environment of GSH depletion. In combination, NO overproduction and GSH depletion may interact, reaching a toxic threshold responsible for DA degeneration. Our experiments suggest that in such a scenario, intervention of the AA metabolism through 12-LOX pathway may be beneficial to individuals suffering from PD. The model predicts that under GSH decreased conditions such as PD, any stimuli that increase non-esterified AA availability will also contribute to cell death through 12-LOX pathway. In this way, pro-inflammatory cytokines (IL-1, TNF-α and IFN-γ) which are increased in PD brains (16), have been shown to induce activation and increased synthesis of cytosolic PLA2 (61).

The signalling pathway AA/12-LOX/12-HPETE/GC/PKG may be important in several pathologies in which GSH depletion has been documented, like PD (62). The potentiating effect of NO over such signalling pathway, may be of relevance as part of the cascade of events leading to and sustaining nerve cell death.

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GSH and NO interact on 12-LOX pathway to induce cell death

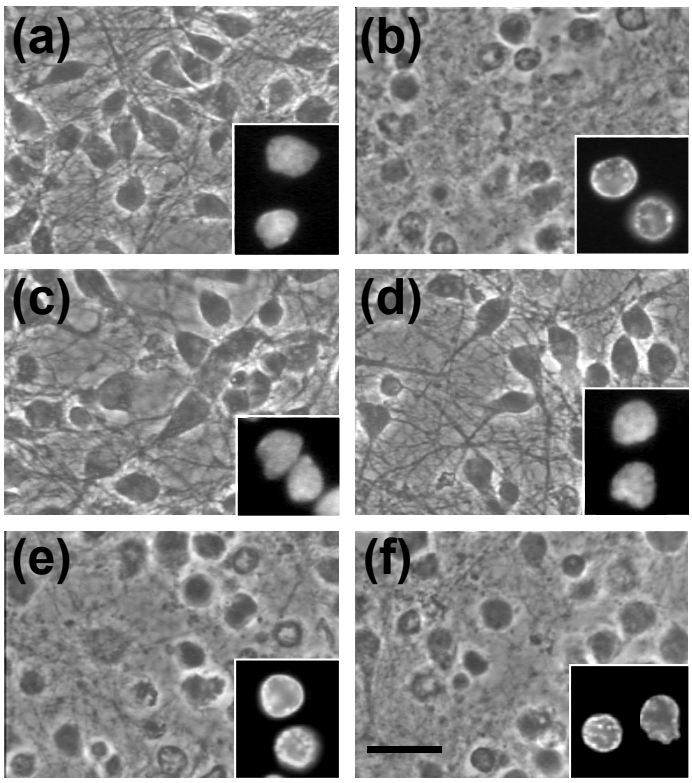

Fig. 1. Effect of inhibitors for LOX, COX and epoxygenase pathways of AA metabolism in the toxicity of NO in GSH-decreased midbrain cultures. Phase-contrast photomicrographs showing: (a) Control cultures. (b) Cultures treated on the 4th day *in vitro* with 20 μM BSO and on the 5th day with 50 μM DEA/NO for additional 24 h. (c-f) Cultures treated with 0.5 μM NDGA (c), 2.5 μM baicalein (d), 25 μM indomethacin (e) or 5 μM clotrimazole (f), 30 min before DEA/NO addition in BSO-pretreated cultures. Insets show the morphology of bis-benzimide stained nuclei in each experimental condition. Scale bar = 25 μm.

GSH and NO interact on 12-LOX pathway to induce cell death

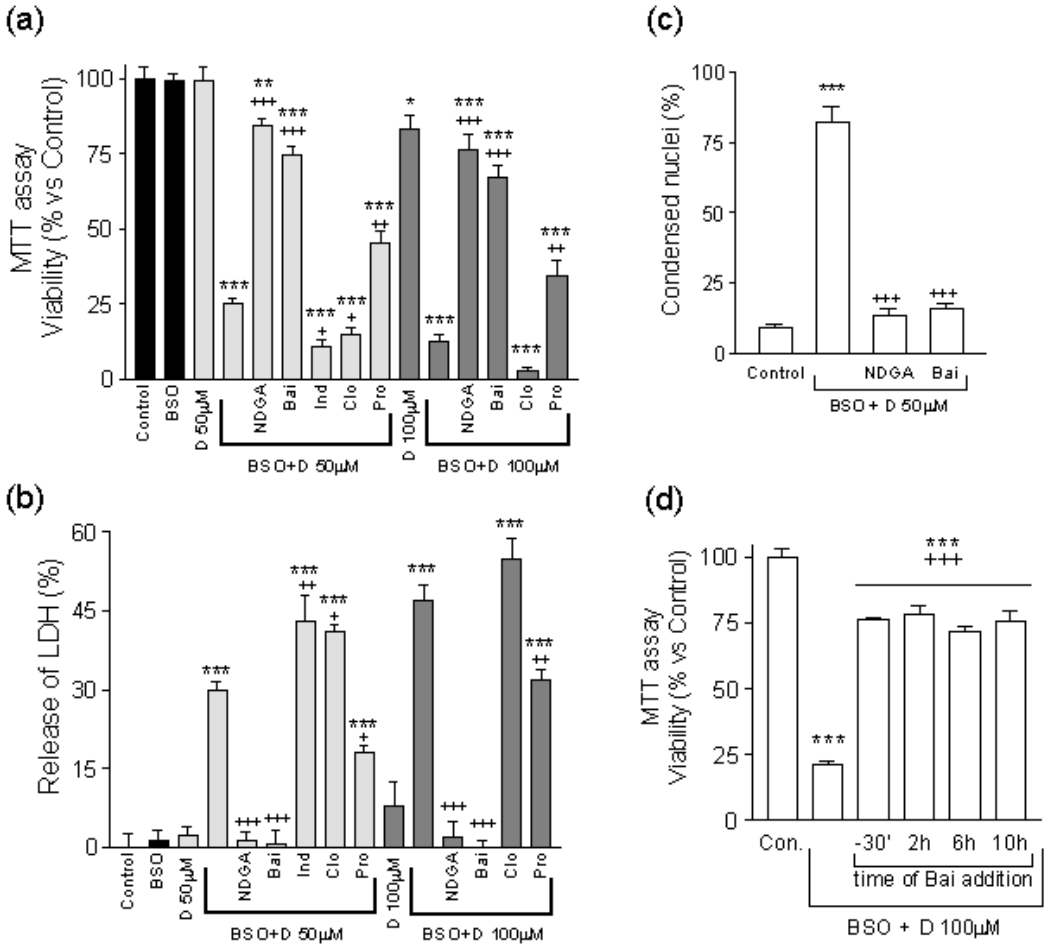

Fig. 2. 12-Lipoxygenase inhibition protects from NO-induced toxicity in GSH decreased midbrain cultures. After 4 days *in vitro*, the cultures were treated with 20 µM BSO, then on the 5th day, preestablished groups were treated with NDGA (0.5 µM), baicalein (Bai; 2.5 µM), indomethacin (Ind; 25 µM), clotrimazole (Clo; 5 µM), proadifen (Pro; 10 µM) or vehicle and 30 min later with DEA/NO (D; 50-100 µM) for 24 h. (a) Cell viability measured by MTT assay and presented as a percentage *vs.* controls. (b) Cell cytotoxicity measured by LDH activity in the culture medium. (c) Chromatin condensed nuclei stained with bis-benzimide and expressed as a percentage *vs.* total cell number. (d) Baicalein (Bai, 2.5 µM) was added to the cultures 30 min before DEA/NO treatment or up to 10 h later. After 24 h of DEA/NO addition, cell viability was measured by MTT assay and is expressed as a percentage *vs.* controls. Values are expressed as the mean ± SEM from n=4-5 replicates. Similar results were obtained in 3-4 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. *p<0.05, **p<0.01, *** p<0.001 *vs.* controls; +p<0.05, ++p<0.01, +++p<0.001 *vs.* BSO + DEA/NO.

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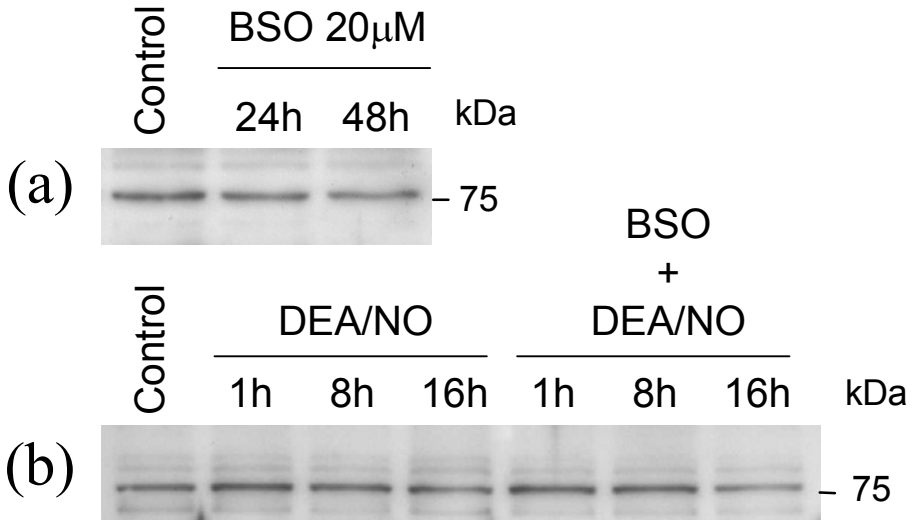

Fig. 3. Effect of BSO and DEA/NO treatments on 12-LOX protein levels. (a) Midbrain cultures were treated for 24 and 48h with 20 μM BSO and 12-LOX protein analysed by western blot. (b) Time course effect of 100 μM DEA/NO on 12-LOX protein levels in vehicle or 20 μM BSO pretreated cultures. Western blot analysis shows a 75 kDa band corresponding to 12-LOX protein. β-actin has been used as a control of charge. These experiments have been replicated twice with identical results.

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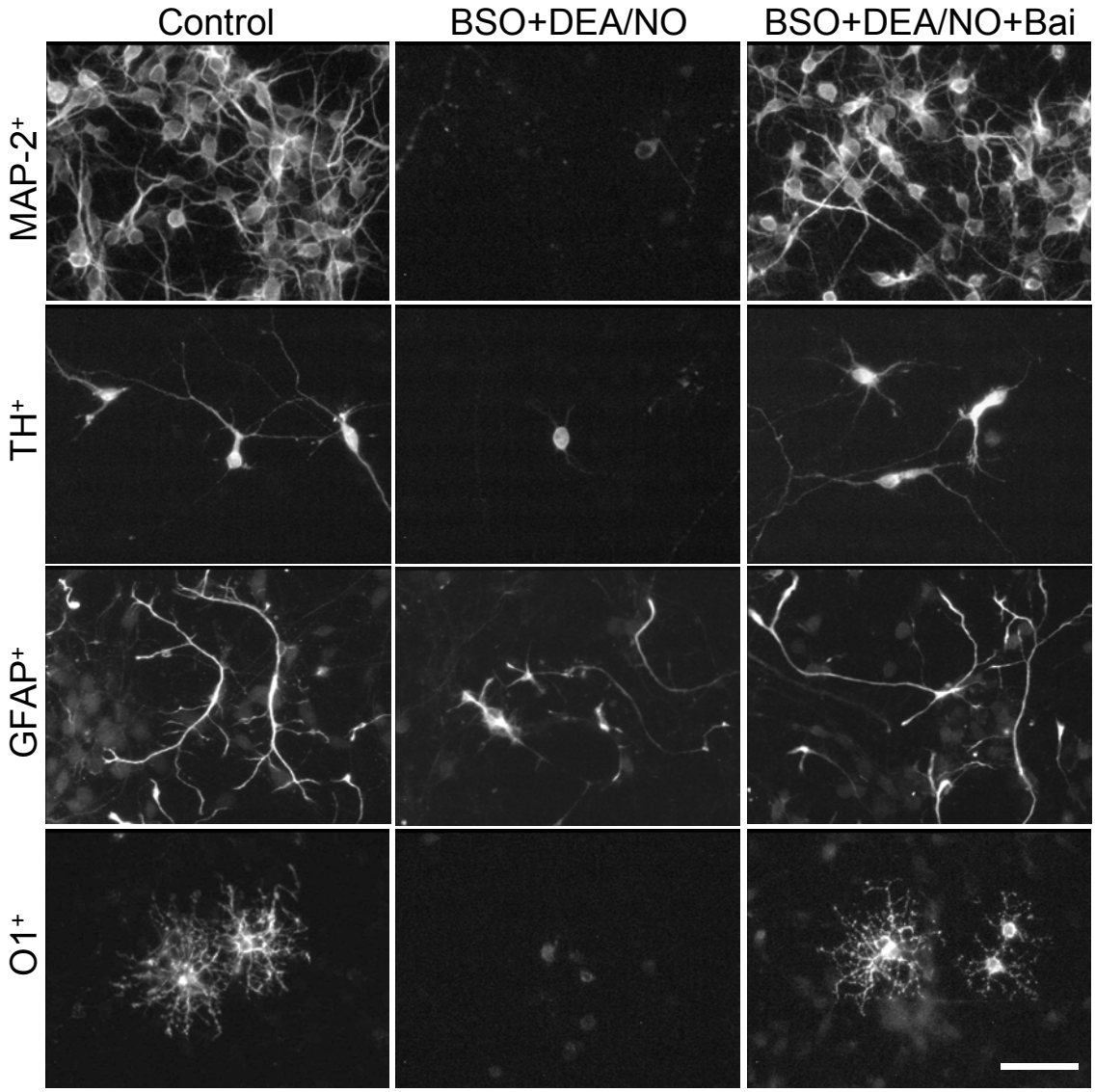

Fig. 4. Cell type selectivity of BSO + DEA/NO-induced toxicity and cell protection by the 12-LOX inhibitor baicalein (Bai). After 4 days *in vitro*, the cultures were treated with 20 μM BSO, then on the 5th day, preestablished groups were treated with 2.5 μM Bai or vehicle and 30 min later with 50 μM DEA/NO for additional 24 h. Photomicrographs show total neurons (MAP-2^{+}), DA neurons (TH^{+}), astrocytes (GFAP^{+}) and oligodendrocytes (O1^{+}). Scale bar = 50 μm.

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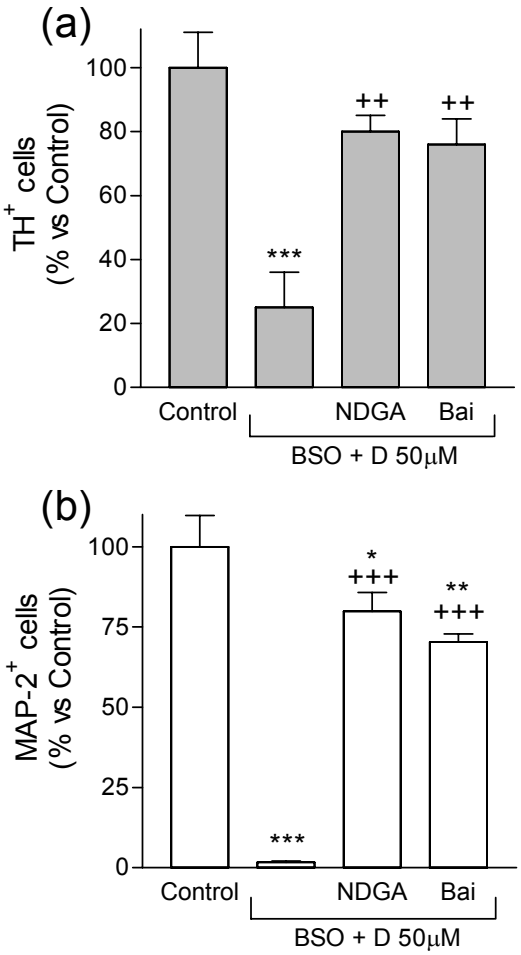

Fig. 5. Lipoxygenase inhibitors protect DA cells and midbrain neurons from BSO + DEA/NO-induced toxicity. After 4 days *in vitro*, the cultures were treated with 20 μM BSO, then on the 5th day, preestablished groups were treated with NDGA (0.5 μM), baicalein (Bai, 2.5 μM) or vehicle and 30 min later with 50 μM DEA/NO for additional 24 h. Number of (a) DA neurons (TH^+) and (b) total neurons ($MAP\text{-}2^+$) are expressed as a percentage *vs.* controls. Values are expressed as the mean ± SEM from n=6 replicates. Similar results were obtained in 2 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. *p<0.05, **p<0.01, *** p<0.001 *vs.* controls; ++p<0.01, +++p<0.001 *vs.* BSO + DEA/NO.

GSH and NO interact on 12-LOX pathway to induce cell death

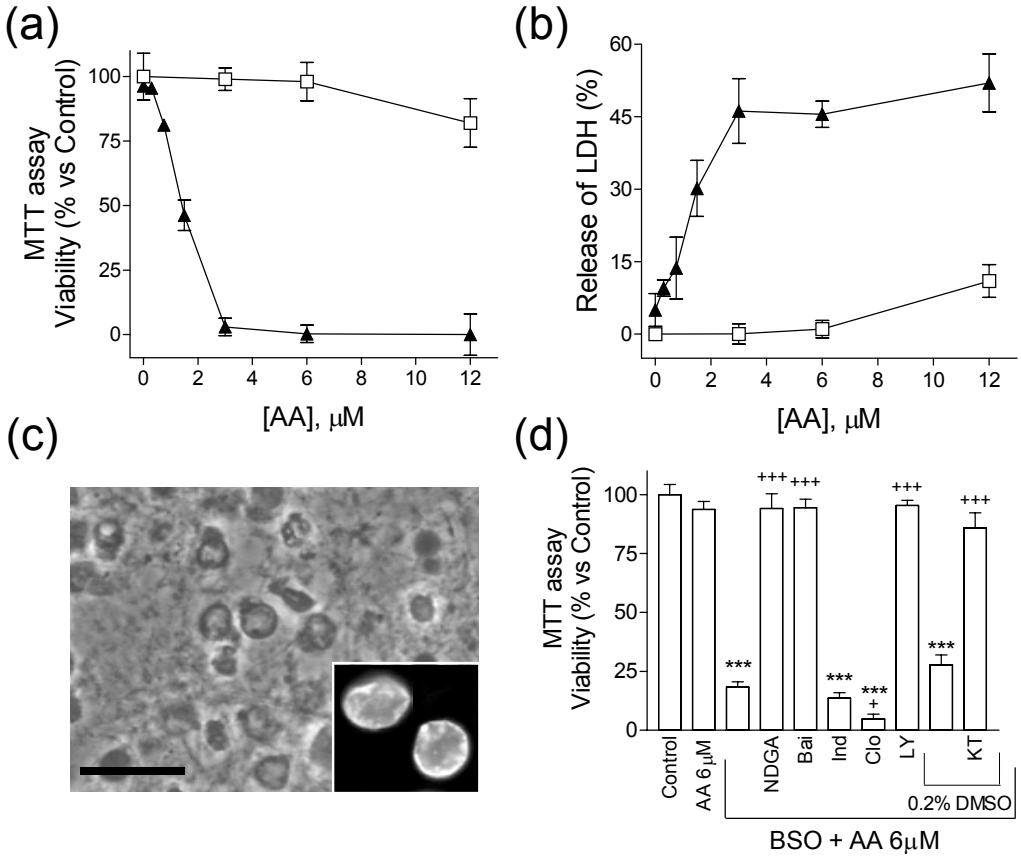

Fig. 6. Arachidonic acid (AA) supplementation to GSH depleted midbrain cultures precipitates a cell death process that is indistinguishable from that initiated by NO. After 4 days *in vitro*, the cultures were treated with 20 μM BSO (filled triangles in (a) and (b)) or vehicle (open squares in (a) and (b)), then on the 5th day, preestablished groups were treated with indicated doses of AA, for additional 24 h. Cell viability was investigated by (a) MTT assay, (b) LDH activity in the culture medium and (c) phase-contrast microscopy. Inset in (c) shows peripherally-condensed chromain in nuclei stained with bis-benzimide. Scale bar = 25 μm. (d) Inhibitors of LOX (NDGA 0.5 μM and baicalein 2.5 μM; Bai), COX (indomethacin 25 μM; Ind), epoxygenase (clotrimazole 5 μM; Clo), GC (LY83583 0.2 μM; LY) and PKG (KT5823 1 μM; KT) were added 30 min before AA in BSO pretreated cultures and cell viability was measured by MTT assay after 24h. As previously reported, 0.2 % DMSO used as solvent for KT, slightly interferes with the model. The AA concentration in (c) and (d) was 6 μM. Values are expressed as the mean ± SEM from n=4-5 replicates. Similar results were obtained in 3 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. *** $p<0.001$ *vs.* controls; +$p<0.05$, +++$p<0.001$ *vs.* BSO + AA alone.

GSH and NO interact on 12-LOX pathway to induce cell death

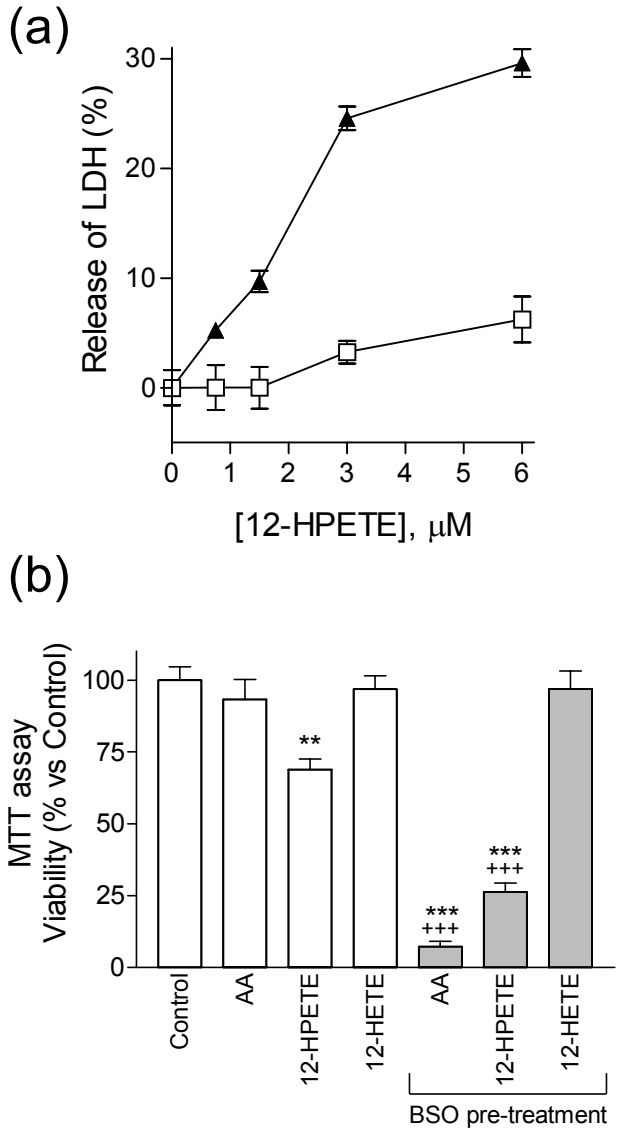

Fig. 7. Effect of 12-LOX products, 12-HPETE and 12-HETE, on cell viability in primary midbrain cultures. After 4 days *in vitro*, the cultures were treated with 20 μM BSO (filled triangles in (a)) or vehicle (open squares in (a)), then on the 5th day, preestablished groups were treated with indicated doses of 12-HPETE (a) or AA, 12-HPETE and 12-HETE 6μM each (b), for additional 24 h. Cell death in the culture was measured by LDH activity in the culture medium (a) and MTT assay (b). Values are expressed as the mean ± SEM from n=4 replicates. Similar results were obtained in 3 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. **p<0.01, *** p<0.001 *vs.* controls; +++p<0.001 *vs.* AA or 12-HPETE.

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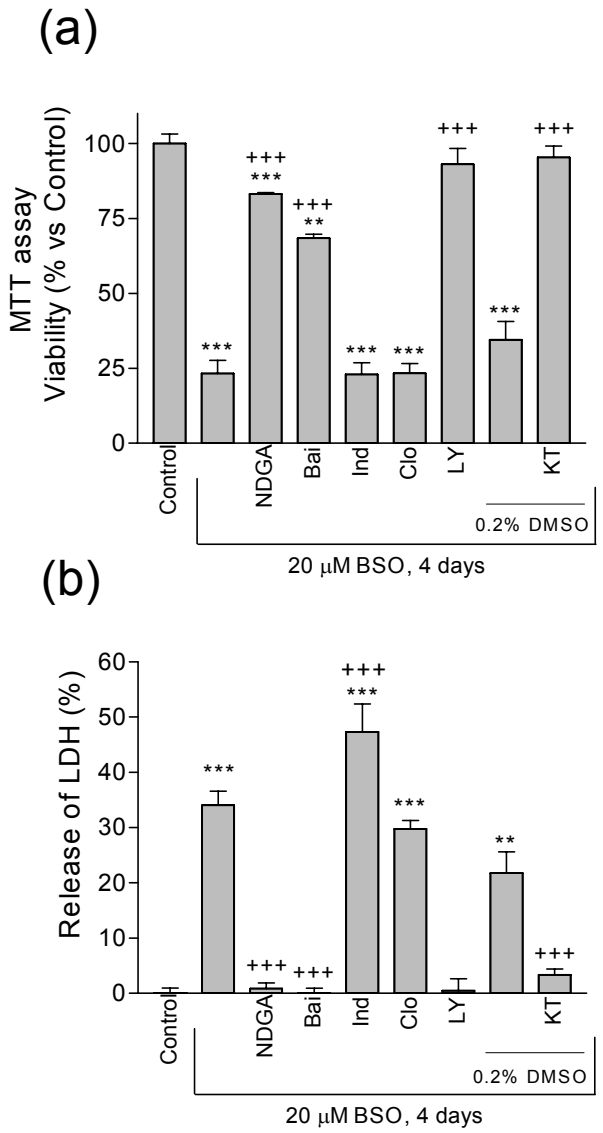

Fig. 8. Persistent GSH depletion induces cell death in midbrain cultures: participation of 12-LOX, GC and PKG. After 4 days *in vitro*, the cultures were treated with 20 μM BSO, then on the 5th day, preestablished groups were treated with NDGA (0.5 μM), baicalein (Bai, 2.5 μM), indomethacin (Ind, 25 μM), clotrimazole (Clo, 5 μM), LY83583 (LY, 0.2 μM), KT5823 (KT, 1 μM) or vehicle and the treatment proceeded for an additional 72 h. (a) Cell viability measured by MTT assay and presented as a percentage *vs.* controls. (b) Cell cytotoxicity measured by LDH activity in the culture medium. As previously reported, 0.2 % DMSO used as solvent for KT, slightly interferes with the model. Values are expressed as the mean ± SEM from n=4 replicates. Similar result were obtained in 3 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. **p<0.01, *** p<0.001 *vs.* controls; +++p<0.001 *vs.* BSO.

Trabajo 6

Selective and persistent activation of extracellular signal-regulated protein kinase by nitric oxide in glial cells induces neuronal degeneration in glutathione depleted midbrain cultures

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Selective and Persistent Activation of Extracellular Signal-Regulated Protein Kinase by Nitric Oxide in Glial Cells Induces Neuronal Degeneration in Glutathione Depleted Midbrain cultures

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Intracellular glutathione (GSH) levels determine whether nitric oxide (NO) is neurotrophic for dopamine neurons or neurotoxic in primary midbrain cultures. We have investigated herein the role of the extracellular-signal regulated protein kinase (ERK)-1/2 pathway in this GSH switching effect. The short lived NO donor DEA/NO induces a transient activation of ERK-1/2 that totally disappears 2h after NO administration. The depletion of GSH increases and the supplementation of GSH, but not ascorbate, suppresses the ERK-1/2 activation peak 1h after NO treatment. More interestingly, GSH depletion changes the kinetic of phosphorylation leading to a second prolonged phase of ERK-1/2 activation, from 2 to 16h after NO addition. This change of kinetic is ultimately responsible for NO-neurotoxicity under GSH depleted conditions, since selective blockade of the second and persistent phase of activation, by MEK-1/2 inhibitors PD98059 and U0126, prevents cell death. In addition, the only transient ERK activation, induced by NO under normal GSH conditions, did not cause ERK-dependent cell death. Immunocytochemical co-localization studies demonstrate that ERK activation in response to NO takes place exclusively in astrocytes, oligodendrocytes and glial progenitors. Furthermore, glial cell elimination from the culture, by gliotoxic drugs, abrogates NO-induced ERK activation. Finally, we demonstrate that active ERK-1/2 translocate into the nucleus during the first transient phase, as well as the second sustained phase of the activation.
Our results indicate that neurotrophism of NO switches into neurotoxicity after GSH depletion, due to persistent activation of the ERK-1/2 signaling pathway in glial cells. The implication of these results in pathological conditions like Parkinson's disease, where GSH depletion and NO overproduction have been documented, are discussed.

Key Words: MAPK, ERK-1/2, nitric oxide, glutathione, midbrain cultures, cell death, dopamine neurons, glial cells, Parkinson's disease

Introduction

Decreased levels of the thiolic antioxidant glutathione (GSH) have been documented in several forms of cell death and associated with neurodegenerative diseases like Parkinson's disease (PD) (Perry et al., 1982; Riederer et al., 1989; Sian et al., 1994; Weber, 1999). GSH depletion is the earliest biochemical alteration shown to date in PD brains. It seems to appear before neurodegeneration in incidental Lewy bodies' disease, considered as the presymptomatic manifestation of PD (Riederer et al., 1989). However, data from animal models show that GSH depletion by itself is not sufficient to induce nigral degeneration (Wullner et al., 1996; Toffa et al., 1997; Toffa et al., 1997); though, reduction of GSH levels may became deleterious in combination with other non-toxic stimuli or enhance the susceptibility of dopamine (DA) cells to the toxicity of other insults (Pileblad et al., 1989; Wullner et al., 1996; Nakamura et al., 1997; Canals et al., 2001a), and thus, promote the neurodegenerative process.

We have previously described in midbrain cultures, that the short lived nitric oxide (NO) donor diethyl-amine/nitric oxide complexed sodium (DEA/NO) at doses of 25 and 50μM exerts a DA cell-specific neurotrophic effect (Canals et al., 2001b). Interestingly, this neurotrophism of NO disappears when the GSH content is lowered to 50% by L-buthionine-(S,R)-sulfoximine (BSO) pretreatment (Canals et al., 2001a), a GSH depletion similar to that occurring in PD (Perry et al., 1982; Riederer et al., 1989; Sian et al., 1994; Weber, 1999; Riederer et al., 1989; Sian et al., 1994). In addition, under GSH-decreased conditions, neurotrophic doses of NO trigger a programmed cell death characterized by an early step of free radicals production, followed by a late requirement of guanylate cyclase (GC) and cyclic GMP-dependent protein kinase (PKG) activation (Canals et al., 2001a).

Nitric oxide and reactive oxygen species contribute to cell death in several experimental models by acting on cellular signaling cascades including the mitogen-activated protein kinase (MAPK) pathway (Jiménez et al., 1997; Lander, 1997; Turner et al., 1998; Srivastava et al., 1999; Ghatan et al., 2000; Taimor et al., 2001; Kulich and Chu, 2001). In the present work, we focus on MAPKs as a possible mediator of cell death triggered by NO in GSH depleted midbrain cultures. MAPKs are a family of related serine/threonine protein kinases that becomes catalytically active upon tyrosine and threonine phosphorylation by MAPK kinases (Boulton et al., 1991; Davis, 1993; Chang and Karin, 2001) and participates in a wide range of processes, ranging from cell division and differentiation to cell survival and degeneration (Seger and Krebs, 1995). Extracellular signal regulated kinases (ERK)-1/2, p38 MAPK, c-Jun N-terminal kinase or stress-activated

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protein kinase (JNK/SAPK) and ERK5 comprise the four major classes of MAPKs. Important features of the MAPKs function, that finally determines their biological effects, include the subcellular localization of the active kinase and the precise kinetic parameters of the activation (Chang and Karin, 2001; Hazzalin and Mahadevan, 2002).

The results reported in this study demonstrate that in midbrain cultures ERK-1/2 are activated in response to a pulse of NO and that GSH levels determine whether such activation is neurotoxic or not, in virtue of the kinetic parameters. Interestingly, this decision take place in glia, since we also demonstrate that NO-induced and GSH-modulated ERK-1/2 activation in midbrain cultures exclusively occur in glial cells.

Materials and Methods

Materials

Culture media: Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5g/l), Ham's F12 nutrient mixture, Eagle's minimal essential medium (EMEM) with Earl's salts and Leibovitz's L-15 medium, all of which were supplemented with L-glutamine, fetal calf serum (FCS), sodium pyruvate and L-glutamine, were purchased from Gibco BRL (Paisley, Scotland, UK). Glucose 45%, insulin, putrescine, progesterone and sodium selenite were from Sigma (Madrid, Spain) and human transferrin, 30% iron-saturated, from Boehringer-Mannheim (Barcelona, Spain).

Antibodies: anti-tyrosine hydroxylase (TH) antibodies made in mouse and rabbit were from Chemicon (Temecula, CA, USA); O1, O4 and A2B5 were obtained from hybridoma supernatants (Raff et al., 1979; Raff et al., 1983); polyclonal anti-GFAP antibody, raised in rabbit, was from DAKO (Glostrup, Denmark); antibody against β-Tubulin (βIII) raised in rabbit was from Babco (Richmond, CA, USA), isolectin B_4 from *Bandeiraea simplicifolia* FITC labelled, anti-mitogen activated protein kinase, anti-phospho-ERK1 and ERK2 antibody, anti-microtubule-associated protein 2a + 2b (MAP-2) antibody and anti-rabbit IgG conjugated with tetramethylrhodamine (TRITC) were purchased from Sigma (Madrid, Spain), anti-mouse Ig fluorescein was from Jackson (PA, USA) and anti-mouse IgM Alexa Fluor® 594, anti-mouse IgG Alexa Fluor® 568 and anti-rabbit IgG Alexa Fluor® 488 were from Molecular Probes (Eugene, OR, USA).

Chemicals: poly-D-lysine, p-phenylenediamine, bis-Benzimide, L-buthionine-[S,R]-sulfoximine (BSO), dimethyl sulphoxide (DMSO), 5,5'-dithio-*bis*2-nitrobenzoic acid (DTNB), reduced and oxidised forms of glutathione, L-α-aminoadipic acid and fluoroacetate were from Sigma (Madrid, Spain). Diethylamine/nitric oxide complexed sodium (DEA/NO) SB203580 and PD98059 were from Alexis (Carlsbad, CA, USA). NADPH, the cytotoxicity detection kit (LDH), cell proliferation kit I (MTT) and GSH reductase (GR) were from Boehringer-Mannheim (Barcelona, Spain); 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ) was from Biomol (Plymouth, PA, USA); KT5823, baicalein, nordihydroguaiaretic acid (NDGA) and 1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene (U0126) were from Calbiochem (Darmstadt, Germany). The BCA protein assay kit was from Pierce (Rockford, Ill, USA). All other reagents were of the highest purity commercially available from Merck or Sigma.

Neuronal culture

Animal use was in accordance with the European Convention for Animal Care and Use of Laboratory Animals and was approved by the Ramón y Cajal University Hospital Animal Care Committee. Neuronal-enriched cultures from embryonic Sprague-Dawley rat midbrain E-14 (crown-rump length 10-12 mm) were obtained and prepared as previously described (Mena et al., 1993; Pardo et al., 1997). The cells were seeded in DMEM with 15% fetal calf serum (DMEM-FCS) at a density of 10^5 cells/cm^2 in multiwells or glass cover slides previously coated with poly-D-lysine, 4.5 μg/cm^2, in 0.1 M borate buffer, pH 8.4. The cultures were kept in a humidified chamber at 37°C in a 5% CO_2 atmosphere. Twenty-four hours after plating, the cells were changed to serum-free defined medium (EF12) as reported elsewhere (Mena et al., 1993; Pardo et al., 1997). EF12 consisted of a 1:1 (v/v) EMEM and nutrient mixture of Ham's F-12, supplemented with D-glucose (6 mg/ml), insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (60 μM), progesterone (20 nM) and sodium selenite (30 nM).

With the above protocol, neuronal-enriched midbrain cultures consist of 85-90 % neuronal cells (β-tubulin$^+$ or MAP-2$^+$ cells) of which around 3-5 % are dopaminergic neurons (TH$^+$ cells), and 10-15 % glial cells composed mainly by astrocytes (GFAP$^+$), glial progenitors (A2B5$^+$) and oligodendrocytes (O1$^+$ and O4$^+$). Microglial cells are rarely present in the culture and when detected (OX42$^+$ or lectin labelled cells) they represent less that 0.05 % of total cells.

Experimental treatments

After 4 days in culture, cells were treated with 20 μM BSO or vehicle, and then on the fifth day, pre-established groups were exposed to the NO donor DEA/NO (50 or 100 μM) for different time periods; 1-16 h for western blot and immunocytochemistry analysis and 24 h for viability assays. Enzyme inhibitors or their corresponding solvents, were routinely added 30 min before DEA/NO treatment or up to 10 h later. To study the potential role of ERK-1/2 inhibition on NO-induced toxicity in BSO-untreated cultures, we used higher doses of DEA/NO (200 and 400 μM) for 24h, which induce apoptotic as well as necrotic cell death in midbrain cultures (Canals et al., 2001b).

To selectively kill glial cells in the culture, we used two gliotoxins, L-α-aminoadipic acid (L-α-aa) and fluoroacetate (FA). L-α-aa (10-30 μM) was added to the culture 48h after plating and maintained in the culture media to the end of the experiment (O'Malley et al., 1994). For FA treatment two protocols were used: pre-incubation of 1h with 10 mM FA before NO treatment or 4h with 1mM FA before NO addition.

Immunocytochemistry

Active phosphorylated ERK-1/2 was immunocytochemically detected with the anti-phospho-ERK1/2 antibody (1:200). DA neurons were characterized by immunostaining with a mouse monoclonal anti-TH antibody (1:100) or a rabbit policlonal anti-TH antibody (1:500), astrocytes with a rabbit policlonal anti-GFAP antibody (1:500), oligodendrocytes with monoclonal anti-O1 and anti-O4 antibodies (1:10) (Sommer and Schachner, 1981), glial progenitors with anti-A2B5 (1:10) (Raff et al., 1979; Raff et al., 1983) and microglial cells were identified with isolectin B4 FITC-labelled (Streit and Kreutzberg, 1987; Ashwell, 1991). To detect all neurons in the culture, a mouse monoclonal anti-MAP-2 antibody (1:250) or

rabbit policlonal anti-β-Tubulin (βIII) (1:1000) were used. For TH, GFAP and MAP-2 immunostaining, cultures were fixed with 4% paraformaldehyde, washed in 0.1 M Tris-buffered saline, pH 7.4 (TBS), permeabilized with ethanol-acetic acid (19:1) and incubated over night at 4ºC with primary antibodies diluted in TBS containing 10% normal goat serum (NGS). Alexa Fluor®-conjugated secondary antibodies were employed to visualize positive cells under fluorescent or confocal microscopy. Co-localization of active ERKs with TH^+ and $GFAP^+$ cells was made as above but including anti-phospho-ERK1/2 during overnight incubation. For β-Tubulin and phospho-ERK co-localization, paraformaldehyde fixed cultures were incubated overnight with appropriate antibodies in TBS containing 10% NGS and 0.01% Triton X-100, at 4ºC. Different fixation protocols (paraformaldehyde + glutardialdehyde) and permeabilizations (higher Triton concentrations or ethanol-acetic acid) were also used with similar results. For O1, O4 and A2B5 staining, antibodies were directly added to living cells and incubated for 15 min at room temperature, washed in DMEM and fixed with 4% paraformaldehyde previous to anti-mouse IgM Alexa Fluor® 488 development. For co-localization studies, after O4 or A2B5 staining has been completed, cells were incubated overnight with anti-phospho-ERK1/2 antibody in TBS containing 10% NGS, 0.01% Triton X-100 and the next day developed with FITC-conjugated anti-mouse IgG (Jackson). Phospho-ERK1/2 and microglial cells co-localization was performed in 4% paraformaldehyde, 1% glutardialdehyde fixed cultures, by overnight incubation with 12.5 mg/L isolectin B4 and anti-phospho-ERK1/2 in TBS with 1% Triton X-100, at 4ºC. To eliminate glutardialdehyde auto-fluorescence, after fixation cover slides were treated with 4 mg/ml $NaBH_4$ in TBS for 10 min at room temperature. The number of immunoreactive cells was counted in 1/7 of the total area of the cover slides by random sampling.

Cell viability measurements

Mitochondrial activity was measured with the MTT assay. Cells were grown on 24-well culture plates with 500 µL defined medium and treated with various reagents according to the experimental design. The MTT assay measures the ability of cells to metabolize 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). At the end of the treatment period, 300 µL of culture medium were removed from each well and 20 µL of MTT solution (5 mg/mL) were added and incubated for 1 h. At this time, 200 µL of solubilisation solution (10% SDS in HCl 0.01M) were then added to the wells and after 24 h of incubation at 37ºC, 100 µL were transferred into 96-well microtiter plates, and the absorption value at 540 nm was measured in an automatic microtiter reader (Spectra Fluor, Tecan).

Chromatin condensation was assesed by DNA staining with bisBenzimide (Hoechst 33342). Cells growing on cover slides were fixed in 4% paraformaldehyde and nuclei were stained with bisBenzimide added in the anti-fading solution ($3x10^{-6}$ M final concentration) (Hilwig and Gropp, 1975; Pardo et al., 1997).

For necrotic cell death determination, lactate dehydrogenase (LDH) activity was measured in the culture medium by using a cytotoxicity detection kit (Decker and Lohmann-Matthes, 1988), and expressed as a percentage *vs.* detergent-extracted controls (100% cytotoxicity). In our system, LDH release to the culture medium correlates with cell death measured by trypan blue dye exclusion assay (Canals et al., 2001b).

Western blot analysis

Primary midbrain cultures were homogenized with a sonicator in buffer containing 20 mM TrisHCl, 10 mM AcK, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, leupeptin, aprotinin, pepstatin 5 µg/mL each, 10 mM FNa, 2 mM sodium molibdate, 10 mM β-glicerophosphate, 0.2 mM ortovanadate, 0.25% NP-40, pH 7.4, and then centrifuged at 12.000 x g for 30 min at 4°C. The supernatant was used for protein determination by the BCA protein assay kit and for electrophoretical separation. Samples (30 µg) were added to SDS sample loading buffer, electrophoresed in 10% SDS-polyacrilamide gels and then electroblotted to 0.45 µm nitrocellulose membranes. For immunolabeling, the blots were blocked with TTBS (20 mM Tris-HCl pH 7.6, 137 mM NaCl plus 0.1% (v/v) Tween-20 and 5% dry skimmed milk) for 1 h at room temperature. After blocking non-specific binding, the membranes were incubated with mouse anti-phospho-ERK1/2 (1:5000) in blocking solution overnight at 4°C. The blots were developed by chemiluminiscence detection using a commercial kit (Amersham) and quantified by computer-assisted videodensitometry. Rabbit anti-ERK1/2 (1:20000) was employed as a control of charge after stripping nitrocellulose membrane.

Glutathione measurements

Total glutathione levels were measured by the method of Tietze (1969). Briefly, 1 x 10^5 cells were washed with PBS, lysed in 100 µL of 3% perchloric acid (PCA) for 30 min at 4ºC, centrifuged, and the supernatants were neutralised with 4 vol of 0.1 M NaH_2PO_4, 5 mM EDTA, pH 7.5. Fifty µL of resulting supernatants were mixed with DTNB (0.6 mM), NADPH (0.2 mM) and glutathione reductase (1U) and the reaction monitored in a P96 automatic microtiter reader at 412 nm for 6 minutes. Oxidised glutathione (GSSG) was measured in the cells by the method of Griffith (1980). Briefly, after PCA extraction and pH neutralisation, GSH was derivatized with 2-vinylpyridine at room temperature for 1 hour and the reaction carried out as above. GSH was obtained by subtracting GSSG levels from total glutathione levels.

Uptake studies

[^{3}H]DA uptake was measured after incubation of the cells with 10^{-8} M [^{3}H]DA (70 Ci/mmol), in the presence of pargyline 10^{-5} M, and ascorbic acid 10^{-3} M, at 37º C for 30 min. Non-specific uptake/binding was calculated in the presence of 10^{-5} M mazindol and represented ≤ 5% (Beart and McDonald, 1980). [^{3}H]GABA uptake was performed in the presence of 10^{-5} M aminooxyacetic acid and 10^{-3} M ascorbic acid and incubated for 4 min with 10 nM [^{3}H]GABA (90 Ci/mmol). Non-specific uptake/binding was calculated by incubating cultures at 0ºC and represented ≤ 7% of the total (Michel and Hefti, 1990). Proteins were measured by the BCA protein assay kit.

Statistical analysis

The results were statistically evaluated for significance with one-way analysis of variance followed by the Newman-Keuls multiple comparison test as a post-hoc evaluation. Differences were considered statistically significant when $p<0.05$.

Results

Nitric oxide activates ERK-1/2 in primary midbrain cultures: intracellular GSH levels regulate the amplitude and kinetic of that activation.

We have previously described a primary midbrain cell culture model in which the decrease of intracellular GSH content, transforms a NO neurotrophic effect, selective for DA neurons, into a toxic effect inducing extensive cell death in the culture (Canals et al., 2001a). To investigate in that model the activation of EKR-1/2 in response to NO and/or GSH depletion, we first studied the phosphorylation state of these proteins by western blot analysis. Midbrain cultures treated with the GSH synthesis inhibitor L-buthionine-(S,R)-sulfoximine (BSO) at 20 μM, decrease their GSH content from 19.4 ± 0.5 μg/mg of protein to 3.9 ± 0.1 μg/mg of protein (≈20 % vs control) after 24h and to 2.0 ± 0.1 μg/mg of protein (≈10 % vs control) after 48h of treatment, but this rate of GSH synthesis inhibition did not change the phosphorylation state of ERK-1/2 (Fig. 1A and 2B), indicating that GSH decrease did not induce ERK activation in midbrain cultures at least for 40 h of depletion. By contrast, the NO donor diethylamine/nitric oxide-complexed sodium (DEA/NO) dose-dependently increases the active phosphorylated state of ERK-1/2 in the culture (Fig. 1A and B) and in accordance with reported data in Jurkat T cells (Lander et al., 1996), this activation is 2 fold increased after GSH depletion (24h of 20 μM BSO pre-treatment).

The NO-induced phosphorylation of ERK and the potentiation afforded by GSH depletion is dose-dependently blocked by GSH and its cell permeable analogue, glutathione ethyl ester (GSH_{EE}) (Fig. 1C and D) but not by the non-thiolic antioxidant ascorbic acid in concentrations of up to 0.5 mM (Fig. 1D). In addition, neither the GC inhibitor 1H-[1,2,4]oxadiazolo[4,3a]quinoxaline-1-one (ODQ, 1-10 μM), nor the PKG inhibitor KT5823 (1 μM), added 30 min before DEA/NO treatment, blocked NO-induced ERK activation (data not shown). Since arachidonic acid metabolism through the 12-lipoxygenase (12-LOX) pathway is implicated in the cell death process triggered by NO under GSH depleted conditions (S. Canals, M.J. Casarejos, S. de Bernardo, E. Rodríguez-Martín and M.A. Mena, unpublished observations), and 12-LOX metabolites induce ERK-1/2 activation (Stanciu et al., 2000), we tried to block ERK activation in our model by inhibiting the 12-LOX pathway. The general LOX inhibitor nordihydroguaiaretic acid (NDGA) and the 12-LOX specific inhibitor, baicalein, were both unable to prevent NO-induced ERK activation in GSH depleted cultures, when the phosphorylation state of ERK was measured 1 and 8h after DEA/NO addition (data not shown).

We next studied the kinetic of ERK activation in response to NO (Fig. 2). BSO or vehicle pretreated cultures were exposed to 100 μM DEA/NO and the phosphorylation state of ERK-1/2 was analyzed 1, 2, 4, 8 and 16 h after NO addition. The maximal ERK activation was observed 1h after NO exposure in both control (vehicle-treated) or BSO treated cultures. In control conditions, DEA/NO induced a transient ERK activation that totally disappeared after 2h. By contrast, in BSO pretreated cultures the maximal peak of ERK activation decreased but maintained an augmented active steady-state for at least 16h after NO addition. Therefore, GSH depletion not only potentiates NO induced peak ERK activation but also changes the kinetic of phosphorylation leading to a more sustained active state.

MEK-1/2 inhibitors, U0126 and PD98059, block NO triggered cell death in GSH decreased conditions.

We and others have previously demonstrated that a GSH depletion of 80-90% vs. controls for up to 72h, did not induce cell death in neuron enriched primary midbrain cultures (Marshall et al., 1999; Mytilineou et al., 1999; Canals et al., 2001a). Nevertheless, under these GSH depleted conditions (24h pre-treatment with 20 μM BSO), treatment of midbrain cultures for an additional 24h with DEA/NO at doses previously shown to be neurotrophic for DA neurons and non-toxic for the rest of the culture (50 μM) or slightly toxic (100 μM), results in extensive cell death (Figs. 3B, 4A and Canals et al., 2001b). To investigate the role of the above described GSH-modulated and NO-dependent ERK activation in the cell death process, we used U0126 and PD98059, two selective inhibitors of the ERK-activating kinases, MEK-1 and MEK-2 (MEK-1/2). As shown in figure 3, these inhibitors prevent both ERK-1/2 activation and cell death induced by NO in GSH-depleted conditions. Added to the culture 30 min before NO treatment, U0126 preserves all cell viability parameters studied, being 10 μM the concentration required for maximal protection (Fig. 4). In the same way, PD98059 preserves cell viability in the culture, with maximal protection observed at 20 μM (Fig. 5). Both inhibitors protect from cell death without interfering with BSO induced GSH depletion (data not shown) and have a low, but significant, effect on cell viability by itself (Figs. 4B and 5A-B). Overall, these results demonstrate that ERK-1/2 activation is a key step for NO actions on cell death process when GSH synthesis is altered. In contrast, when SB203580 a specific inhibitor of p38 MAPK, was used at concentrations of up to 20 μM, no protection from cell death was observed (Fig. 5C).

Immunocytochemical characterization of cell death induced by NO in GSH decreased conditions showed that $MAP\text{-}2^+$ cells (neurons), TH^+ cells (DA neurons) and $O1^+$ cells (oligodendrocytes) were highly affected by the treatment at 24h, being $GFAP^+$ cells (astrocytes) the most resistant cell type in the culture. In addition, when the ability of PD98059 to protect from cell death was studied for different cell populations, we observed that all damaged cell types in the culture were protected from toxicity (Fig. 6).

MEK inhibitors protect GSH-decreased midbrain cultures from NO toxicity even when added after NO treatment (Fig. 7). The ability of PD98059, given following NO treatment, to protect cells from toxicity diminished over time but was still apparent 10h after cells were initially exposed to 100 μM DEA/NO. When the MEK inhibitor was added to the culture just after the initial peak of ERK activity (2h after NO, see Fig. 2) and thus blocking only the delayed phase of the activation, the cell survival at 24h was similar to that afforded by pre-treating cells with the inhibitor before NO addition. This indicate that the early transient phase of ERK activation has a low impact on viability, being the delayed and persistent activation crucial for cell death induction.

We have previously described the neurotoxic effects of higher doses of DEA/NO (200-400 μM) for midbrain cultures (Canals et al., 2001b). To test if ERK-1/2 activation participates in NO-induced toxicity when the GSH system is not altered, we pretreated midbrain cultures with MEK inhibitors prior to 100, 200 and 400 μM DEA/NO treatment.

As previously shown, 24h of 100 μM DEA/NO results in slight loss of viability (Fig. 4A) and the toxicity increases in parallel with the DEA/NO concentration, reaching viability values of 64 ± 2 % vs. control (MTT assay) for the highest DEA/NO dose. In these conditions, MEK inhibitors did not confer any protection from NO-induced toxicity at any of the DEA/NO doses tested (data not shown). ERK-1/2 activation by NO in not implicated in cell death when the GSH system is not altered, reinforcing the notion that sustained but not transient ERK activation participates in cell death induced by NO in midbrain cultures.

Nitric oxide-induced ERK activation in control and GSH-depleted primary midbrain cultures is restricted to glial cells.

To investigate if NO induced ERK phosphorylation occurs in concrete cell types or ubiquitously in the culture, we performed immunocytochemical techniques for fluorescent and confocal microscopy examination. In control conditions, we found less than 1 % of cells showing a very light phospho-ERK positive (p-ERK$^+$) staining (Fig. 8). This proportion of basal p-ERK$^+$ cells did not vary with GSH depletion. By contrast, after 100 μM DEA/NO treatment, p-ERK$^+$ staining become more evident and there was a 10 fold increase in the number of positive cells (Fig. 8). Phospho-ERK labelled cells were diverse in morphology and size. BSO pre-treatment greatly increased the intensity of p-ERK$^+$ staining in response to NO, in accordance to western blot results, but the net amount of positive cells in the culture did not vary (Fig. 8). Similarly, there was increased intensity of p-ERK$^+$ staining with higher DEA/NO doses (1 mM) but no changes in the morphology and number of responsive cells were observed (Fig. 8A, 8B and Fig. 9). All these data indicate that there is a concrete subset of cells in the culture that represents 10 % of the population, in which NO is able to activate ERK-1/2.

The anti-phospho-ERK1/2 antibody used for the immunocytochemical study was the same antibody that specifically recognizes the 42 and 44 kDa bands of active ERK-1 and ERK-2 in western blot studies (Figs. 1 and 2). The specificity of the staining technique is further supported because pre-incubating cells with MEK-1/2 inhibitors before NO addition, eliminates p-ERK labelling in the culture (data not shown). In addition, the temporal course of p-ERK staining in the culture, strongly correlates with that observed with western blot analysis. The number and intensity of positive cells decrease over time but are still evident after 8 h of NO treatment in GSH depleted conditions (Fig. 8B last panel and Fig. 9I), whereas no p-ERK staining is observed in BSO-untreated, DEA/NO-treated cultures at this time (data not shown).

To identify the midbrain culture cell types in which NO activates ERK, co-localization studies were performed. The basal level of p-ERK$^+$ cells in control conditions were identified as β-tubulin positive (β-Tub$^+$) cells (neurons) (Figs. 9A and 9H) and GFAP$^+$ cells (astrocytes) (Fig. 9H). Unexpectedly, the basal level of β-Tub$^+$/p-ERK$^+$ cells in the culture did not vary under any of the studied treatments: 1, 4 and 8 h of 100 μM DEA/NO under GSH depleted conditions (Figs. 9B, C and I), 1h of 100 μM or 1 mM DEA/NO in BSO-untreated cultures (data not shown) and different immunocytochemistry protocols (see methods). This result indicates that NO released from DEA/NO is unable to activate ERK-1/2 in primary midbrain neurons (β-Tub$^+$ nor TH$^+$ cells). By contrast, the number of p-ERK labelled astrocytes greatly increased 1h after NO treatment and persisted for more than 8h in GSH decreased cultures (Fig. 9D and I). In addition to astrocytes, A2B5$^+$ (glial progenitors) and O4$^+$ cells (oligodendrocytes) also co-localized p-ERK in response to NO (Fig 9E and F). The relative contributions of each cell population to the total number of p-ERK$^+$ cells are presented in the figure 9H. Despite the low proportion of microglial cells in our cultures (see methods) we studied the ability of NO to induce ERK phosphorylation in this cell type. As shown in figure 9G a proportion of microglial cells (which represent less than 0.03 % of total cells in the culture) responded to NO treatment by activating ERK-1/2.

If ERK-1/2 activation in response to NO occurs selectively in glial cells, then elimination of these cells from the culture must prevent NO-dependent EKR phosphorylation. To test this hypothesis, we eliminated glial cells in midbrain cultures by using the specific gliotoxin L-α-aminoadipic acid (L-α-aa;10-30 μM) (O'Malley et al., 1994) and then we analyzed the activation of ERK in response to NO by western blot. At concentrations of L-α-aa that eliminated detectable GFAP$^+$ cells in the culture (Fig. 10A), NO was unable to activate ERK-1/2 (Fig. 10C). L-α-aa treatment did not alter the morphology of MAP-2$^+$ and TH$^+$ labeled neurons (Fig. 10A and data not shown) nor the high affinity ^{3}H-DA and ^{3}H-GABA uptake (Fig. 10B), suggesting that neuronal cells are preserved from L-α-aa toxicity. To corroborate the results obtained with L-α-aa we used another gliotoxic drug, fluoroacetate (FA), an aconitase inhibitor (once metabolized into fluorocitrate) that is selectively uptaken by glial cells and rapidly depletes intracellular ATP levels (Peters, 1963; Keyser and Pellmar, 1994; Waniewski and Martin, 1998). After overnight incubation with 1 mM or 1h incubation with 10 mM FA, NO was unable to activate ERK-1/2 in midbrain cultures (Fig. 10C). The concentrations and protocols used for FA treatment showed altered glial morphology without changes in the morphology of neuronal population (Fig. 10A) and neurotransmitters uptake (Fig. 10B), indicating a glial-specific effect of the treatment.

Overall, co-localization experiments and gliotoxins studies demonstrate that in primary midbrain cultures, NO, alone or in combination with GSH depletion, specifically activates ERK-1/2 in glial cells.

Active ERK-1/2 translocates into the nucleus after NO treatment

An important feature of ERK function is its translocation into the nucleus to phosphorylate and regulate the activity of several transcription factors. We studied the subcellular distribution of p-ERK$^+$ staining in the above experimental conditions and we found that a proportion of cells in the culture at every time analyzed (1 to 8h after 100 μM DEA/NO in GSH depleted cultures) translocate active ERK into the nucleus (see arrows in Fig. 8B). This translocation did not depend on GSH levels since it was also observed 1h after DEA/NO addition in BSO-untreated cultures (see arrow in Fig. 8B). Co-localization studies showed that nuclear ERK translocation occurs in astrocytes, oligodendrocytes, as well as in glial progenitors (Fig. 9D-F).

Discussion

The major finding of this study is the demonstration that in primary midbrain cultures, NO selectively activates ERK-1/2 in glial cells and that such activation becomes persistent and induces extensive neuronal death when intracellular GSH is diminished. We have previously reported in primary midbrain cultures that GSH depletion from 50% switches a DA neuron-specific trophic effect of NO into neurotoxic (Canals et al., 2001a). This effect is mediated by the GC/cGMP/PKG signaling cascade and requires *de novo* mRNA and protein synthesis and caspase activation (Canals et al., 2001a). Recent studies in our laboratory also implicates arachidonic acid metabolism through 12-LOX as an upstream step in the cell death cascade triggered by NO under GSH depleted conditions (S. Canals, M.J. Casarejos, S. de Bernardo, E. Rodríguez-Martín and M.A. Mena, unpublished observations). In the present work we demonstrate that the NO action in the above cell death process is mediated by ERK-1/2 activation and that this kinase represents an interaction point between NO and GSH. The amplitude of NO-induced ERK activation is increased by GSH depletion, but more important, GSH depletion changes the kinetic of such activation leading to a more sustained active state. This change of kinetic is ultimately responsible for cell death induction. Finally, we demonstrate that NO-dependent and GSH-modulated ERK-1/2 activation takes place selectively in glial cells in the primary midbrain culture. This suggests that in the above conditions, sustained ERK activation in glial cells initiates a process that finally causes cell death in the culture, mainly neurons (including DA cells) and oligodendrocytes.

To the best of our knowledge, this is the first report showing glial cell specific activation of ERK-1/2 in response to NO in a primary culture containing both neurons and glia. This conclusion is based on: (1) there is a fixed subset of cells in the culture that represents a 10 % of the total population, in which NO is able to activate ERK-1/2. The proportion of responsive cells did not vary by increasing the NO concentration in the culture, nor after GSH depletion. (2) Immunocytochemical co-localization studies demonstrates that NO activates ERK-1/2 exclusively in astrocytes, glial progenitors, oligodendrocytes and microglia, identifying the NO-responsive population as glial cells. (3) Glial cell elimination from midbrain cultures or selective glial metabolic impairment, by using gliotoxic drugs, eliminates ERK-1/2 activation in response to NO. The gliotoxins used, L-α-aa and FA, have been widely employed to discriminate glial-cell mediated actions in different models, including mixed neuron-glia cultures (O'Malley et al., 1994; Kenigsberg and Mazzoni, 1995; Krieglstein and Unsicker, 1997), brain slice preparations (Keyser and Pellmar, 1994; Alvarez-Maubecin et al., 2000) and in vivo studies (Largo et al., 1996). In all this reports, as well as in the present study, L-α-aa and FA have demonstrated high specific glial actions with low or no effect on neuronal population. Specific MAPK activation in glial cells has been reported with other stimuli; for example, the transforming growth factor α, a member of the epidermal growth factor family, activates ERK-1/2 in astrocytes but not neurons in cortical cultures (Gabriel et al., 2002). A recent report in hippocampal slices (Berkeley and Levey, 2003) demonstrates that cell type specific activation of ERK-1/2 in the CA1 region depends on heterotrimeric G protein subtypes. G(q)-coupled receptors activate ERK-1/2 in CA1 pyramidal neurons, G(i/o)-coupled receptors activate ERK-1/2 in glia scattered throughout CA1 and G(s)-coupled receptors activate ERK-1/2 in scattered interneurons. Investigation of the mechanism underlying NO dependent glial cell-specific ERK activation in our system may be of relevance to understand the specificity of NO signaling.

Nitric oxide can activate MAPK through GC dependent (Parenti et al., 1998; Callsen et al., 1998; Yamazaki et al., 2001) and independent mechanisms (Lander et al., 1996; Yun et al., 1998). Preliminary results indicate that ERK activation in our model is GC independent since the soluble GC inhibitor ODQ and the PKG inhibitor KT5823, did not block NO-induced ERK activation. The GC-independent mechanism consists in the S-nitrosylation of a critical cysteine residue (Cys 118) in Ras protein (Lander et al., 1997) that stimulates guanine nucleotide exchange, resulting in an active Ras form that finally activates its downstream effectors ERK-1/2, JNK/SAPK and p38 (Lander et al., 1995a; Lander et al., 1996). Interestingly, Ras dependent ERK-1/2 activation in response to NO is potentiated by BSO pretreatment in Yurkat T cells (Lander et al., 1996), but no data of GSH depletion effects on the kinetics of ERK activation are reported in that study. Further investigation will be necessary to clarify the exact mechanism of ERK activation in our model.

Here we show that in primary midbrain cultures, BSO treatment increases 2-fold the ERK activation peak 1h after NO treatment, in agreement with previously reported data (Lander et al., 1996). More interestingly, GSH depletion changes the kinetic of phosphorylation leading to a second prolonged phase of ERK-1/2 activation, from 2 to 16h after NO addition, which is finally responsible for cell death induction. The BSO effect on the early ERK activation peak may be explained by decreased competition for NO due to GSH depletion. Both GSH, which is unable to cross cell membrane and its cell permeable analog GSH_{EE}, blocked NO-induced ERK activation, as well as the BSO potentiating effect on the activation. The fact that extracellular GSH blocks NO action suggests a direct interaction between NO and GSH, possibly through the formation of GSNO (Rodríguez-Martín et al., 2002). Since BSO inhibits the γ-glutamylcysteine syntethase, a possible increase in intracellular GSH concentration after cell culture supplementation with impermeable GSH, due to transpeptidase and dipeptidase-dependent increase in cysteine availability, is discarded. In addition, a general burst in free radicals due to GSH depletion is not behind the BSO potentiating effect, since supplementation of the culture medium with 0.5 mM ascorbate before NO addition in BSO-pretreated cultures, did not decrease ERK activation. Nevertheless, the way in which GSH influences ERK-1/2 activation and principally its kinetic parameters, requires more investigation. Possible mechanisms also include: (1) the facilitation of denitrosylation events in exposed Cys 118 of ras protein (Stamler et al., 2002), (2) the S-glutathiolation of cysteins involved in lipid modifications of ras protein (Mallis et al., 2001), (3) the restoration of the protein phosphatase function under oxidative or nitrosative conditions (Chiarugi et al., 2001; Lu et al., 2001; Rao and Clayton, 2002) and (4) oxidative stress-mediated sustained ERK-1/2 activation (Lander et al., 1995b).

In addition to the above possibilities, two independent reports (Stanciu et al., 2000; Satoh et al., 2000) demonstrate that in primary immature cortical neurons and the HT22

hippocampal nerve cell line, glutamate initiates a persistent and delayed ERK-1/2 activation that induces cell death. This process is known as oxidative glutamate toxicity and is produced by glutamate competition of cystine transport and subsequent GSH depletion (Murphy et al., 1989). Persistent ERK-1/2 activation by oxidative glutamate toxicity in HT22 cells is produced by 12-LOX derived arachidonic acid metabolites, since the 12-LOX inhibitor baicalein blocked it (Stanciu et al., 2000). Furthermore, arachidonic acid metabolites are responsible for both ERK and JNK/SAPK activation in response to oxidative stress in primary cultured astrocytes (Thomas et al., 1997). Although 12-LOX participates in the cell death process observed in our model, its participation in ERK-1/2 activation is discarded since baicalein or NDGA, a general LOX inhibitor, are unable to block the first transient, or the second sustained phase of ERK activation in response to NO. Interestingly, 12-LOX inhibitors blocked cell death induced by NO in GSH-depleted midbrain cultures, without interfering with ERK activity, suggesting that ERK activation is necessary but not sufficient to induce cell death in our model.

Although numerous reports have implicated ERK in neuronal cell survival (Xia et al., 1995; Yujiri et al., 1998), ERK does not appear to act always in this way. ERK-1/2 activation has been demonstrated after focal cerebral ischemia in mice and MEK1 protein kinase inhibition protects against damage in this model (Namura et al., 1999). Sustained activation of ERK-1/2, brought about by protein phosphatase inhibition, induced neuronal cell death in hippocampal slice cultures (Runden et al., 1998). Furthermore, cell death by oxidative glutamate toxicity in immature cortical cultures and in HT22 cells needs persistent activation and nuclear retention of ERK-1/2 (Stanciu et al., 2000; Stanciu and DeFranco, 2002). Finally, $ONOO^-$ induces apoptosis in human DA neuroblastoma SH-SY5Y cells through ERK-1/2 and p-38 MAPK pathways (Oh-hashi et al., 1999). It has been proposed that the precise parameters of ERK-1/2 activation ultimately determine whether the kinase participates in cell death-promoting or cell survival pathways (Chang and Karin, 2001). Distinct kinetics of ERK-1/2 activation differentially activates transcription factors of the AP1 family (Cook et al., 1999) suggesting that unique sets of genes can be expressed in response to concrete times of ERK-1/2 activation (Marshall, 1995; Hazzalin and Mahadevan, 2002). Here, we demonstrate that prolonged ERK-1/2 activation is critical for cell death induction in primary midbrain cultures, since the selective blockade of the second and persistent phase of activation accounted for almost 90% of the protective effect obtained by MEK inhibition, and the only transient ERK activation induced by NO under normal GSH conditions, did not cause ERK-dependent cell death. In addition we demonstrate active ERK-1/2 translocation into the nucleus of glial cells during the first transient phase, as well as the second sustained phase of the activation.

We previously demonstrated *de novo* mRNA and protein synthesis requirement for cell death induction in our model (Canals et al., 2001a). This suggests the possibility that nuclear targets of ERK in glial cells, regulate the transcription of genes implicated in this cell death process. In addition to nuclear targets, cytoplasmic targets of active ERK-1/2, like cPLA2, may be of relevance in the death cascade since we demonstrated the participation of arachidonic acid metabolites in the process. It has been shown that ERK-1/2 activation in murine primary astrocytes induces cPLA2 phosphorylation and regulates arachidonic acid release (Xu et al., 2002).

Regardless of the mechanism, our results indicate that NO and GSH depletion interact in glial cells on the MAPK signaling cascade to induce neuronal cell death and suggest a process in which glial cells could actively participate in neuronal degeneration in pathological conditions. Since GSH depletion in neurons and glial cells, as well as increased NO production and iNOS expression in glial cells, have been demonstrated in the *sustantia nigra* of PD patients, the biochemical conditions for the above described NO-GSH interaction are present in the brain of patients. In combination, NO overproduction and GSH depletion may interact, reaching a toxic threshold responsible for the DA-cell degeneration. The identification of nuclear and cytoplasmic targets of sustained ERK-1/2 activation may be of relevance in the understanding of NO and GSH functions in brain physiology and pathology.

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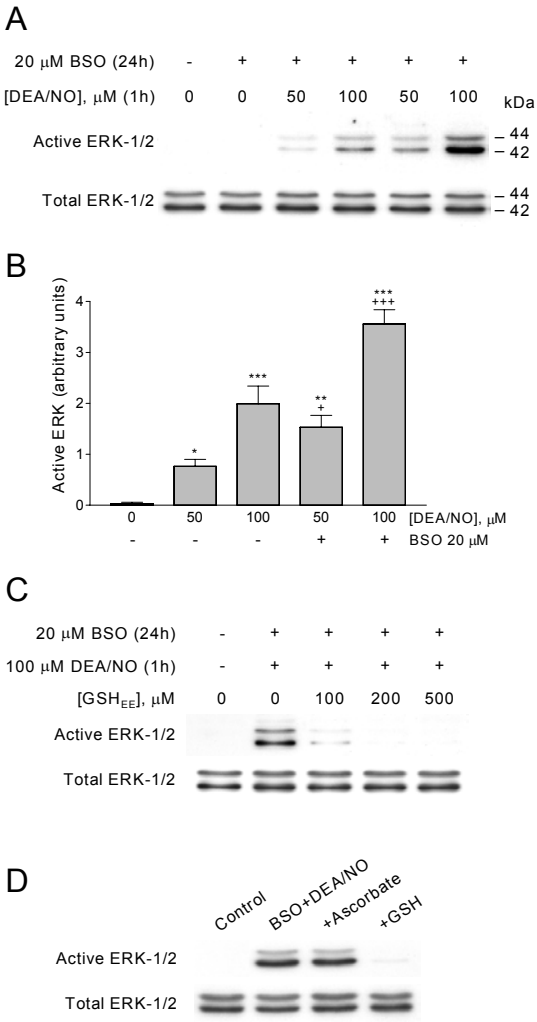

Fig. 1. Nitric oxide activates ERK-1/2 in primary midbrain cultures and that activation is potentiated by GSH depletion and blocked by GSH supplementation. A, After 4 days *in vitro*, the cultures were treated with 20 μM BSO or vehicle, then on the 5th day, preestablished groups were treated with the indicated doses of DEA/NO for 1 h. Cells were then harvested and lysed, and active or total ERK-1/2 were visualized by Western blot analysis. Relative active ERK levels were determined from densitometric scanning of enhanced chemiluminescence-exposed films and corrected for charge differences with total ERK-1/2 (B). Values are expressed as the mean ± SEM from n=3-6 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. *p<0.05, **p<0.01, *** p<0.001 *vs.* control; +p<0.05 *vs.* 50 μM DEA/NO alone; +++p<0.001 *vs.* 100 μM DEA/NO alone. C, Cultures were treated as in (A) but the cell permeable GSH analog, GSH ethyl ester (GSH_{EE}), was added to the culture 30 min before DEA/NO treatment. D, Ascorbate or GSH 0.5 mM each were added 30 min before DEA/NO treatment. Experiments in (C) and (D) have been replicated three times with similar results.

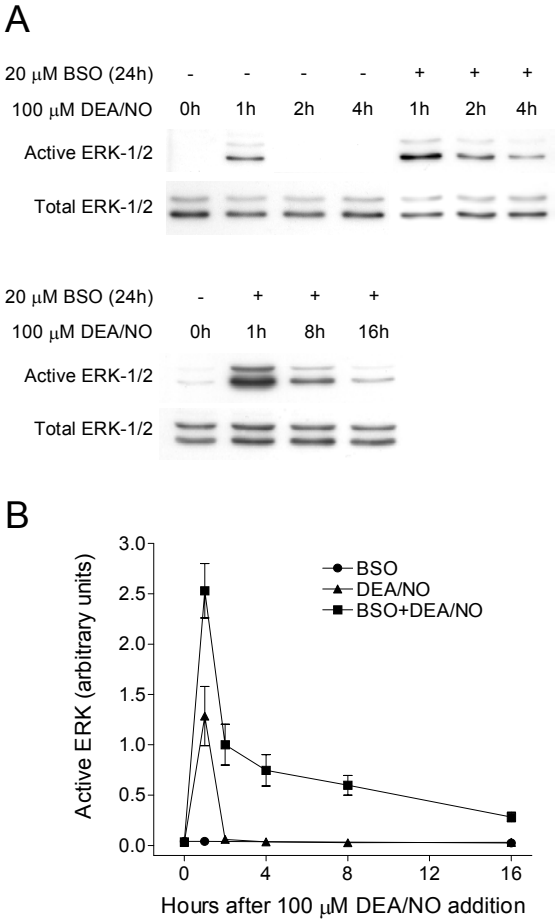

Fig. 2. GSH depletion changes the kinetic of NO-induced ERK-1/2 phosphorylation, leading to a more persistent active state. After 4 days *in vitro*, the cultures were treated with 20 μM BSO (circles and squares) or vehicle (triangles), then on the 5th day, preestablished groups were treated with 100 μM DEA/NO (triangles and squares) for various lengths of time (1-16 h). Cells were then harvested and lysed, and active or total ERK-1/2 were visualized by Western blot analysis (A). Relative active ERK levels were determined from densitometric scanning of enhanced chemiluminescence-exposed films and corrected with total ERK-1/2 (B). Values are expressed as the mean ± SEM from n=3 independent experiments.

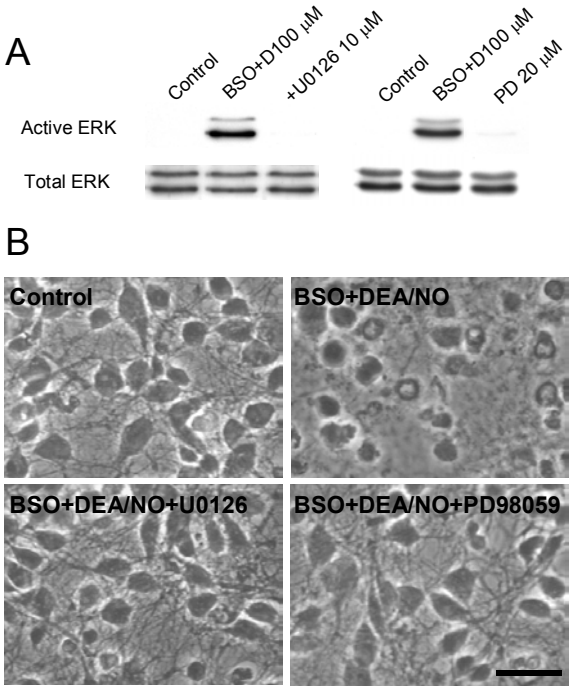

Fig. 3. ERK-1/2 activation in response to NO induces cell death in GSH-depleted conditions. After 4 days *in vitro*, the cultures were treated with 20 μM BSO or vehicle, then on the 5th day, preestablished groups were treated with the MEK-1/2 inhibitors, U0126 (10μM) and PD98059 (20μM), or vehicle and 30 min later with 100 μM DEA/NO. A, One hour after DEA/NO treatment cells were harvested and lysed, and active or total ERK-1/2 were visualized by Western blot to corroborate ERK inhibition. Cultures in (B) were mantained 24 h after DEA/NO treatment and cell viability was inspected by phase-contrast microscopy. Scale bar = 25 μm. Experiments have been replicated three times with similar results.

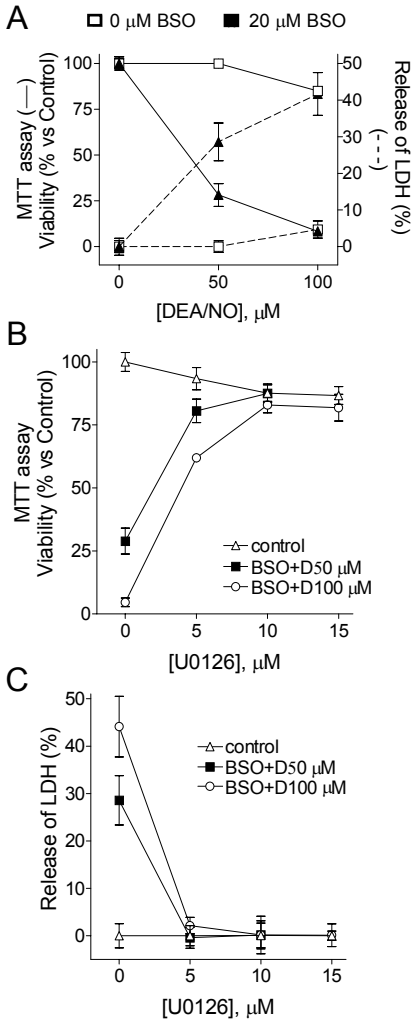

Fig. 4. Dose-dependent protective effect of MEK-1/2 inhibitor U0126, against NO-induced toxicity in GSH depleted cultures. A, After 4 days *in vitro*, the cultures were treated with 20 μM BSO (filled triangles) or vehicle (open squares), then on the 5th day, preestablished groups were treated with indicated doses of DEA/NO for additional 24 h. Cell viability was measured by MTT assay and presented as a percentage *vs.* controls (solid line) and by LDH activity in the culture medium (broken line). B-C, Cultures were treated with 5, 10 or 15 μM U0126 alone (triangles) or 30 min before DEA/NO addition in BSO pretreated cultures (squares and circles), and cell viability was measured 24h later by MTT assay (B) or LDH release to the culture medium (C). Values are expressed as the mean ± SEM from n=4 replicates. Similar results were obtained in 3-5 independent experiments.

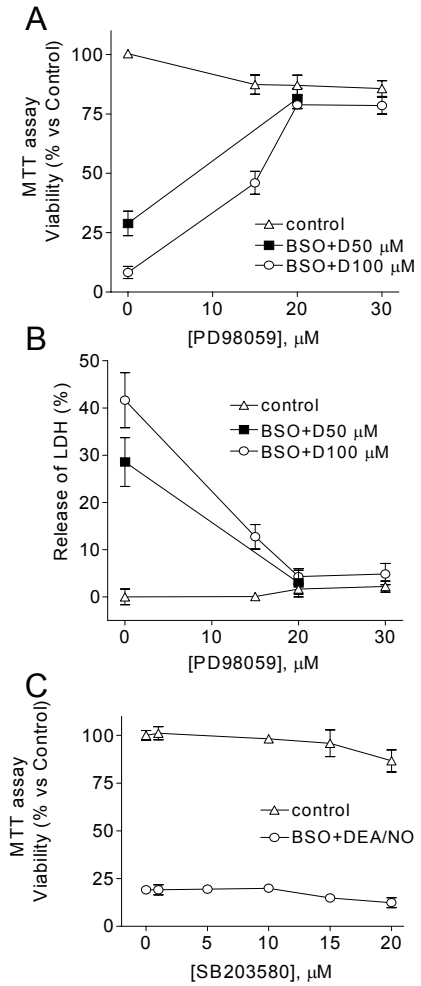

Fig. 5. Dose-dependent protective effect of MEK-1/2 inhibitor PD98059, against NO-induced toxicity in GSH depleted cultures. After 4 days *in vitro*, the cultures were treated with 20 μM BSO (squares and circles) or vehicle (triangles), then on the 5th day, preestablished groups were treated with 15-30 μM PD98059 (A and B) or 1-20 μM p38 MAPK inhibitor, SB203580 (C), and 30 min later with 50 (squares) and 100 (circles) μM DEA/NO, for additional 24 h. Cell viability was measured by MTT assay (A and C) or LDH release to the culture medium (B). Values are expressed as the mean ± SEM from n=4 replicates. Similar results were obtained in 3-5 independent experiments.

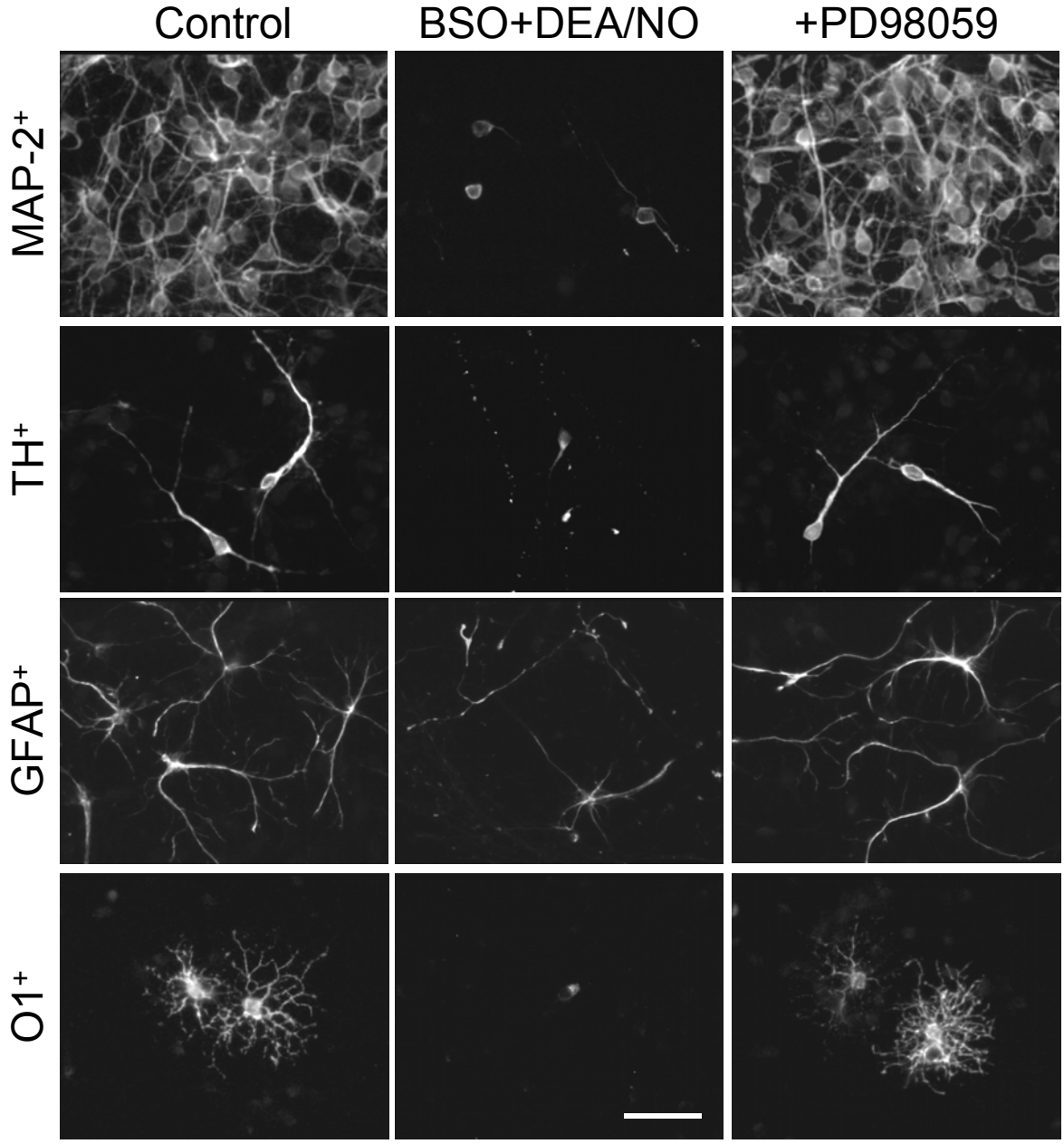

Fig. 6. Cell type selectivity of BSO + DEA/NO-induced toxicity and cell protection by the MEK-1/2 inhibitor PD98059. After 4 days *in vitro*, the cultures were treated with 20 μM BSO, then on the 5th day, preestablished groups were treated with 20 μM PD98059 or vehicle and 30 min later with 100 μM DEA/NO for additional 24 h. Photomicrographs show total neurons (MAP-2$^+$), DA neurons (TH$^+$), astrocytes (GFAP$^+$) and oligodendrocytes (O1$^+$). Scale bar = 50 μm.

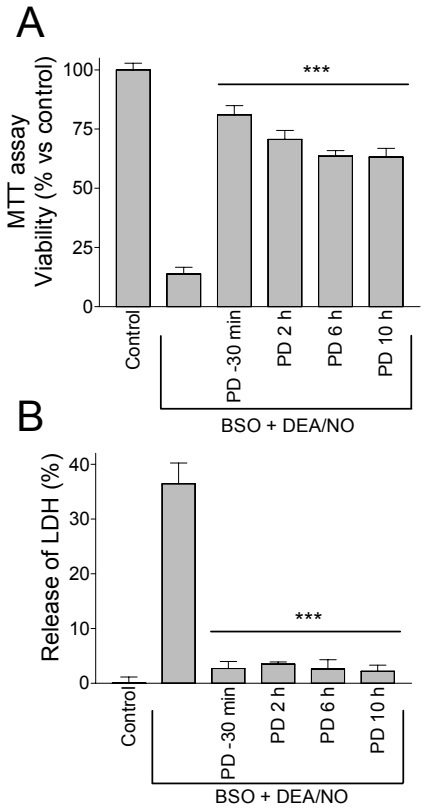

Fig. 7. MEK-1/2 inhibitor PD98059, protects GSH-depleted midbrain cultures from NO toxicity when added after NO treatment. After 4 days *in vitro*, the cultures were treated with 20 µM BSO or vehicle, then on the 5th day, preestablished groups were treated with 100 µM DEA/NO, and 20 µM PD98059 was added to the culture thirty min before or 2, 6 and 10 h after DEA/NO treatment. Cell death was measured in all groups 24 h after DEA/NO addition by MTT assay (A) and LDH release to the culture medium (B). Values are expressed as the mean ± SEM from n=4 replicates. Similar results were obtained in 3 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. *** $p<0.001$ *vs.* BSO + DEA/NO alone.

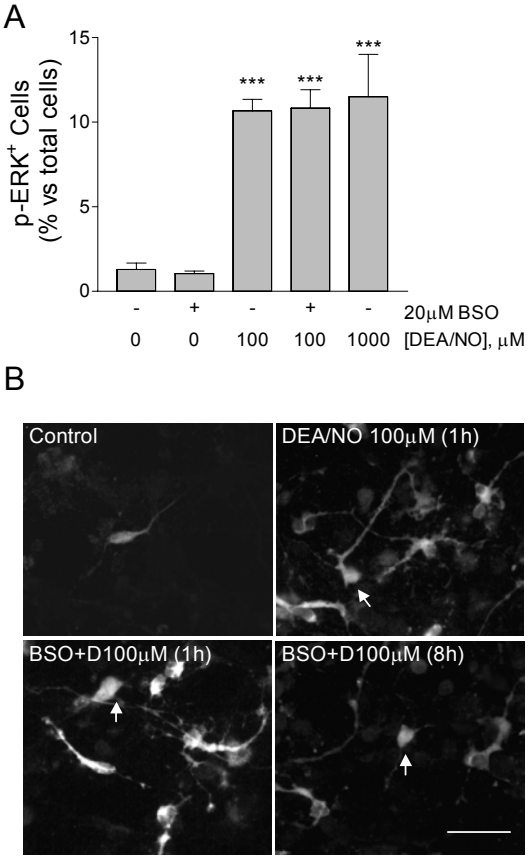

Fig. 8. There is a constant subpopulation of cells in the culture in which NO is able to activate ERK-1/2. After 4 days *in vitro*, the cultures were treated with 20 μM BSO, then on the 5th day, preestablished groups were treated with 100 μM or 1mM DEA/NO. A, 1 h after DEA/NO addition, the cultures were fixed and immunostained using an anti-phospho(p)-ERK antibody. Labeled cells were counted and presented as a % *vs.* control. Values are expressed as the mean ± SEM from n=3 replicates. Similar results were obtained in 3 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. *** $p<0.001$ *vs.* control. B, Representative photomicrographs of p-EKR$^+$ cells in control (untreated) conditions, in cultures treated 1h with 100 μM DEA/NO alone or after 24h of BSO pretreatment, and cultures 8h after 100 μM DEA/NO in BSO pretreated cultures. Note that the intensity of p-ERK$^+$ staining 1h after DEA/NO addition but not the number of p-ERK$^+$ cells, was increased by BSO pretreatment. Arrows indicate p-ERK$^+$ staining in the cellular nuclei. Scale bar = 50 μm.

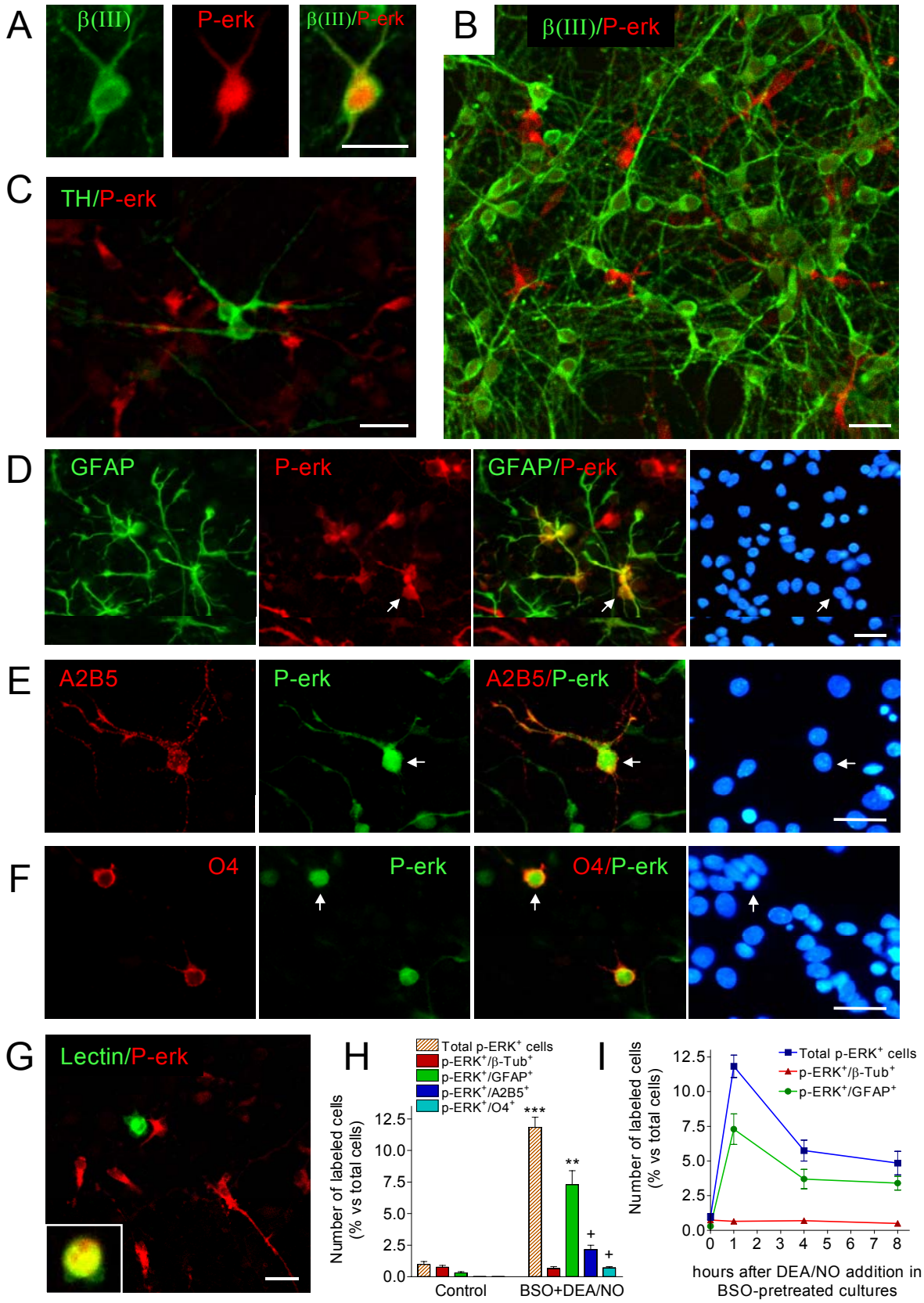
A
β(III)
P-erk
β(III)/P-erk
B
β(III)/P-erk
C
TH/P-erk
D
GFAP
P-erk
GFAP/P-erk
E
A2B5
P-erk
A2B5/P-erk
F
O4
P-erk
O4/P-erk
G
Lectin/P-erk
H
Total p-ERK+ cells
p-ERK+/β-Tub+
p-ERK+/GFAP+
p-ERK+/A2B5+
p-ERK+/O4+
Number of labeled cells (% vs total cells)
12.5
10.0
7.5
5.0
2.5
0.0
***
**
+
+
Control
BSO+DEA/NO
I
Total p-ERK+ cells
p-ERK+/β-Tub+
p-ERK+/GFAP+
Number of labeled cells (% vs total cells)
12.5
10.0
7.5
5.0
2.5
0.0
0 1 2 3 4 5 6 7 8
hours after DEA/NO addition in BSO-pretreated cultures

Fig. 9. Nitric oxide selectively activates ERK-1/2 in glial cells in primary midbrain cultures. A, Double-staining immunocytochemistry showing a neuron (β-Tub$^+$ cell) with cytoplasmic and nuclear distribution of active ERK (p-ERK) in control (untreated) conditions. B-G, After 4 days *in vitro*, the cultures were treated with 20 μM BSO, and on the 5th day, received 100 μM DEA/NO for 1 h. At the end of the incubation time, cultures were fixed and doubled-stained with p-ERK antibody and: β-Tub (B), TH (DA neurons; C), GFAP (astrocytes; D), A2B5 (glial progenitors; E), O4 (oligodendrocytes; F) and lectin (microglia, G). Arrows in D-F show active ERK translocation into the nucleus. Right panels in D-F correspond to total nuclei stained with bis-benzimide. In (G) two different microglial cells are shown, one of which (inset) is p-ERK labeled. H, Double-stained cells in control and BSO + DEA/NO groups were quantified and expressed as a percentage *vs.* total cells. I, Temporal course of the number of total p-ERK$^+$ (blue squares), p-ERK$^+$/β-Tub$^+$ (red triangles) and p-ERK$^+$/GFAP$^+$ (green circles) cells in BSO + DEA/NO treated cultures. Values are expressed as the mean ± SEM from n=5 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test. ** $p<0.01$, *** $p<0.001$ *vs.* control; +, neither p-ERK$^+$/A2B5$^+$ nor p-ERK$^+$/O4$^+$ cells were found in control cultures. Scale bar in all photomicrographs = 25 μm.

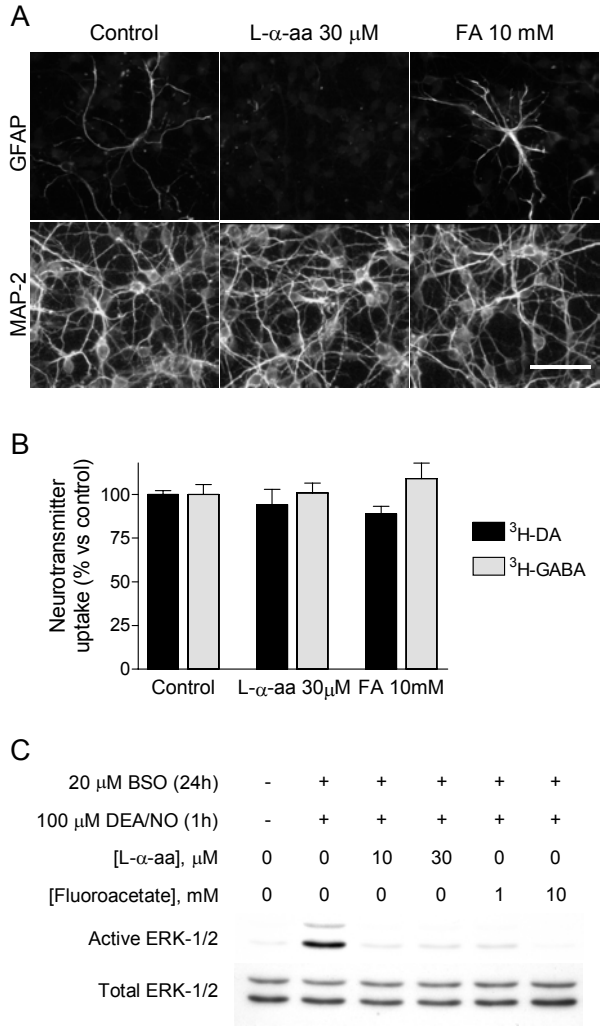

Fig. 10. The gliotoxins, L-α-aminoadipic acid (L-α-aa) and fluoroacetate (FA), block NO-induced ERK activation in midbrain cultures. For L-α-aa experiments, the cultures after 48h in vitro received 10-30 μM L-α-aa, then on the 4th day, cultures were treated with 20 μM BSO or vehicle and on the 5th day with 100 μM DEA/NO for 1h. For FA treatment, cultures were treated on the 4th day with BSO and 12h or 1h before DEA/NO addition received 1 or 10 mM FA, respectively. At the end of the incubation time cultures were processed for GFAP and MAP-2 immunocytochemistry (A, Scale bar = 50 μm); for high affinity ^{3}H-DA and ^{3}H-GABA uptake (B); and for Western blot analysis of active and total ERK-1/2 (C). Values are expressed as the mean ± SEM from n=4 replicates. Similar results were obtained in 2 independent experiments. Statistical analysis was performed by ANOVA followed by the Newman-Keuls multiple comparison test.

RESUMEN DE RESULTADOS

Trabajo 1

En cultivos primarios de mesencéfalo de rata, el óxido nítrico (NO) tiene un efecto bifásico que depende de su concentración. Dosis de 25-50 μM del donador de NO sodio-(Z)-1-(N,N-dietilamina) diazen-1-ium-1,2-diolato (DEA/NO) muestran un efecto neurotrófico, selectivo para la función dopaminérgica (DA), caracterizado por la expresión *de novo* de células tirosina hidroxilasa positivas, aumento de los procesos neuríticos y un incremento en los niveles endógenos de DA y en su captación de alta afinidad. Dosis superiores del donador (200-400 μM) inducen la muerte celular apoptótica y necrótica, selectiva de neuronas (incluidas las DA) y oligodendrocitos, quedando preservados del daño los astrocitos y progenitores gliales. Ambos efectos del NO, trófico y tóxico, son independientes de la activación de la guanilato ciclasa (GC) y correlacionan, respectivamente, con un incremento y disminución en los niveles de glutation (GSH). El efecto tóxico del NO es totalmente prevenido por factores solubles liberados por la glía.

Trabajo 2

El NO muestra un efecto bifásico dosis-dependiente en la funcionalidad DA de la línea celular catecolaminérgica humana, NB69. Dosis de 50-125 μM del donador de NO S-nitroso-N-acetilpenicilamina (SNAP), potencian la función DA, incrementando la actividad del enzima tirosina hidroxilasa a nivel postraduccional y la captación de alta afinidad de dopamina, así como la extensión de neuritas. Dosis superiores del donador (500-750 μM) detienen la replicación celular y disminuyen todos los parámetros DA, sin inducir muerte celular. Ambos efectos del NO son independientes de la activación de la GC.

Trabajo 3

El efecto tóxico del NO (DEA/NO 200-400 μM) en cultivos primarios de mesencéfalo, se previene totalmente con antioxidantes tiólicos como el GSH y la N-acetil-L-cisteína (L-NAC). Por el contrario, el antioxidante no tiólico, ácido ascórbico (200-400 μM), no solo no protege de la muerte celular inducida por altas dosis de NO, sino que potencia su toxicidad. El ácido úrico (300-600 μM), otro antioxidante no tiólicos con similar capacidad que el ácido ascórbico y el GSH, para detoxificar $ONOO^-$, tampoco protege de la toxicidad del NO. Así mismo, ni el atrapador de radicales $O2^{\cdot -}$, tiron (1-2 mM), ni el sistema

enzimático detoxificante, superóxido dismutasa (100 U/mL) + catalasa (100 U/mL), previenen la muerte celular.

El efecto protector del GSH frente a la toxicidad del NO, correlaciona con la formación de S-nitrosoglutation (GSNO) en el medio extracelular.

Trabajo 4

La disminución de la concentración intracelular de GSH, mediante la inhibición de su síntesis con L-butionina-(S,R)-sulfoximina (BSO), elimina la capacidad neurotrófica del NO sobre las células DA y la transforma en neurotóxica. El efecto tóxico es dosis dependiente entre un 50% y un 90% de depleción de GSH. La reducción en la concentración de GSH por debajo del 10%, no altera por sí sola la viabilidad celular durante el experimento (48 horas).

La toxicidad consiste en un proceso de muerte celular programada, dependiente de síntesis de macromeléculas y activación de caspasas, caracterizado por la condensación de la cromatina sin fragmentación del ADN y por la ruptura de la membrana plasmática. Tanto el ácido ascórbico, como la inhibición de la guanilato ciclasa (GC) y la proteína kinasa dependiente de GMPc (PKG), previenen la muerte celular. Los inhibidores de la GC y la PKG mantienen esta cualidad hasta 10 horas después del tratamiento con NO, sin embargo, el ácido ascórbico la pierde a partir de las 2 horas.

Trabajo 5

En cultivos primarios de mesencéfalo, la depleción severa (≅ 90%) y prolongada de los niveles de GSH, induce muerte celular al cuarto día de tratamiento con BSO. En estas condiciones, la toxicidad se previene con inhibidores de la GC y la PKG, así como inhibidores de la lipoxigenasa-12 (12-LOX), no siendo efectiva la inhibición de rutas alternativas del metabolismo del ácido araquidónico (AA), como las ciclooxigenasas y las epoxigenasas.

El NO acelera la toxicidad debida a la depleción de GSH, precipitando la muerte celular en 24 horas, tras el tratamiento con el donador. Este efecto del NO se previene también con inhibidores de la 12-LOX. De igual forma que el NO, el AA precipita la toxicidad debida a la depleción de GSH. El proceso de muerte celular no se distingue del iniciado por NO, ni en su morfología, ni en su curso temporal, ni en la dependencia de 12-LOX, GC y PKG. Finalmente, el 12-HPETE, primer metabolito del AA en la ruta de la 12-LOX, induce

muerte celular en el cultivo de mesencéfalo y su toxicidad se incrementa tras la depleción de GSH.

Trabajo 6

En cultivos primarios de mesencéfalo, el NO activa las MAPKs (ERK-1/2), selectivamente en las células gliales. Dicha activación es modulada por los niveles intracelulares de GSH, de forma que su disminución produce un incremento tanto en la amplitud, como en la duración de la activación. La activación de ERK-1/2 es independiente de GC y se bloquea totalmente suplementando el medio de cultivo con GSH. Los inhibidores de ERK-1/2, U0126 y PD98059, previenen la muerte celular hasta 10 horas después del tratamiento con NO. El cambio en la cinética de activación de ERK-1/2 que se produce en condiciones de depleción de GSH, es necesario para que el NO precipite la muerte neuronal.

DISCUSIÓN GENERAL

Existe cierta controversia en la literatura científica del óxido nítrico (NO) acerca de sus efectos sobre las neuronas dopaminérgicas (DA). Mientras algunos datos indican su participación en procesos de muerte celular *in vitro* e *in vivo*, otros investigadores han demostrado un papel neuroprotector frente al estrés oxidativo. La doble funcionalidad del NO ha sido descrita en diversos paradigmas experimentales y para distintos tipos celulares (Revisado en Yun et al., 1996; Chiueh, 1999), los estudios realizados son numerosos y bien contrastados, de forma que la pregunta que surge es: ¿Qué factor o factores determinan el efecto final del NO?

En algunos modelos, como el de la neurotoxicidad del glutamato asociada a la activación de receptores NMDA, se han dado respuestas satisfactorias a la pregunta anterior. Mientras algunos autores demostraban que la toxicidad del NMDA se previene, *in vivo* e *in vitro*, con inhibidores de la NOS (Dawson et al., 1991; Nowicki et al., 1991), otros argumentaban que el tratamiento con NO protegía de la toxicidad del NMDA, disminuyendo la corriente de Ca_2^+ a través del canal, por un mecanismo que implicaba la S-nitrosilación de un residuo/os tiólicos en el sitio de modulación redox del receptor (Lei et al., 1992). Esta aparente paradoja se resolvió con los trabajos de Lipton y colaboradores (1993) en cultivos primarios de corteza, donde demostraron que las acciones del NO, estaban relacionadas con su estado redox. En un ambiente reductor, conseguido añadiendo ácido ascórbico o compuestos tiólicos al medio de cultivo (L-NAC y cisteína), se favorece la reacción del NO ($NO^{\cdot}$) y el $O_2^{\cdot -}$, con la consiguiente formación de $ONOO^-$ y pérdida de viabilidad celular. Por el contrario, estados redox alternativos del NO (equivalentes NO^+ como RS-NO y Fe-nitrosil), mediaban la S-nitrosilación del receptor de NMDA y con ello la prevención de la toxicidad. Estas observaciones pioneras centraron la cuestión de la influencia del microambiente celular en los efectos del NO y resolvieron la contradicción creada entorno a los efectos del NO en la toxicidad por glutamato. No obstante, en otros modelos experimentales, los mecanismos por los que el NO y el ambiente interaccionan para determinar los efectos biológicos finales, no se conocen bien. Se han descrito efectos tóxicos del NO independientes de la formación de $ONOO^-$ (Clementi et al., 1998), efctos protectores del $ONOO^-$ (Garcia-Nogales et al., 2003) y efectos protectores del NO que solo pueden ser mediados por $NO^{\cdot}$ y no por NO^+, como la terminación de reacciones en cadena de radicales libres (Chiueh and Rauhala, 1999).

Neurotrofismo y toxicidad del NO sobre las neuronas DA: papel del GSH

En cultivos primarios de mesencéfalo de rata y en la línea DA de neuroblastoma humano NB69, hemos estudiado la influencia de la concentración del NO en su efecto final, cuando se aplica en un tratamiento agudo. Utilizamos para ello dos donadores de NO de liberación rápida, el DEA/NO ($t_{1/2}$ de desintegración de 3,5 ± 0,2 min) y el SNAP ($t_{1/2}$ de desintegración de 37,2 ± 13,8 min). Los resultados, discutidos en los trabajos 1 y 2, demuestran la importancia de la concentración del NO, en la dualidad de sus efectos sobre las neuronas DA. Dosis bajas de NO resultan neurotróficas, selectivamente para las neuronas DA en el cultivo de mesencéfalo, y aumentan la funcionalidad DA de las células NB69. Por el contrario, dosis altas de NO, aproximadamente un orden de magnitud con respecto a las dosis neurotróficas, inducen muerte celular por apoptosis y necrosis en el cultivo primario y parada de la replicación celular con disminución de la funcionalidad DA, en la línea celular. Ambos efectos, trófico y tóxico, son independientes de la activación de la GC y correlacionan, en el cultivo primario, con un incremento y disminución, respectivamente, en los niveles intracelulares de GSH.

Nuestros resultados muestran que la inhibición del enzima limitante en la síntesis de GSH, la γ-glutamilcisteina sintetasa, previa al tratamiento con NO, elimina la capacidad neurotrófica dopaminérgica de éste y la transforma en neurotóxica (trabajo 4). Si bien estos resultados no demuestran que el aumento de GSH sea el responsable del neurotrofismo del NO, sí nos permiten concluir que la correcta homeostasis del GSH es esencial para que se produzca. Por su parte, la pérdida de viabilidad celular inducida por altas dosis de NO en cultivos primarios de mesencéfalo, se previene totalmente con GSH, GSH_{EE} (análogo permeable del GSH) y L-NAC. Por el contrario, la toxicidad no se previene con los antioxidantes no tiólicos, ácido ascórbico y ácido úrico, ni con atrapadores de O_2^- o sistemas detoxificantes, como el constituído por SOD/CAT (trabajo 3). Esto indica en primer lugar, que solo los compuestos tiólicos previenen de la muerte celular inducida por NO en cultivos de mesencéfalo y en segundo lugar, que el $ONOO^-$ tendría una escasa participación en la toxicidad observada en nuestro sistema.

En conjunto, los resultados sugieren un modelo en el que el GSH condiciona el efecto final del NO. El GSH contrarrestaría continuamente los efectos tóxicos del NO, de forma que si la concentración de NO supera un cierto umbral o el GSH disminuye por debajo de un nivel crítico, o ambos, el sistema se desborda. Cuando se sobrepasa la capacidad "protectora" del GSH, se produce una inflexión en el efecto del NO y los efectos neurotóxicos se manifiestan. Los mecanismos por los que el GSH puede actuar como neuroprotector incluyen: (1) detoxificación de especies nitrosilantes, (2) regeneración de proteínas nitrosiladas via transnitrosilación y (3) reacción con el NO para formar GSNO (Clementi et al., 1998; Chiueh and Rauhala, 1999). Aunque no se conoce el mecanismo exacto por el que NO y GSH forman GSNO a pH fisiológica (Yun et al., 1996), este compuesto ha sido identificado *in vivo* e *in vitro* y puede participar en la protección de las neuronas DA, en nuestro modelo, frente a dosis altas de NO (trabajo 3). Otros grupos han demostrado un efecto neuroprotector del GSNO para las neuronas DA *in vivo* (Rauhala et al., 1996; Rauhala et al., 1998; Mohanakumar et al., 1998) frente al estrés oxidativo. De hecho, el GSNO es aproximadamente 100 veces más potente que el GSH en la supresión de la generación de radicales $OH^{\cdot}$ y la peroxidación lipídica en el cerebro (Rauhala et al., 1998). Además, comparado con el NO, la mayor estabilidad del GSNO supone que el GSH, por medio de su conjugación con NO, actuaría como tampón frente a un incremento agudo y potencialmente tóxico, en los niveles de NO. Por otro lado, el GSNO puede servir como vehículo de transporte de los efectos biológicos del NO a mayor distancia dada su estabilidad y como medio de transferencia de GSH entre células, dada su permeabilidad (Chiueh and Rauhala, 1999).

Otro factor importante en la dualidad de los efectos del NO es la duración del estímulo, siendo diferente la respuesta celular frente a una exposición aguda o crónica. Por ejemplo, tiempos cortos de exposición a NO disminuyen reversiblemente la actividad de los complejos mitocondriales I y IV, regulando fisiológicamente la respiración mitocondrial (Clementi et al., 1998). Por el contrario, si el tiempo de exposición aumenta, se produce una inhibición irreversible del complejo I mitocondrial. Además, la conversión de NO en $ONOO^{-}$ no es necesaria para esta inhibición patológica del enzima. Resulta interesante que la inhibición del complejo I se hace progresivamente persistente a medida que disminuye la concentración de GSH intracelular y que la inhibición de la síntesis de GSH con BSO, precipita el proceso. Por el contrario, el tratamiento con GSH o agentes

reductores como el DTT (ditiotreitol), restablecen totalmente la actividad enzimática, sugiriendo la nitrosilación como mecanismo de inhibición del complejo I mitocondrial (Clementi et al., 1998).

Los datos anteriores indican que, en la mitocondria, el GSH contrarresta los efectos del NO para que sean transitorios, probablemente previniendo o revirtiendo la nitrosilación de residuos clave de cisteína en el complejo I mitocondrial. Como hemos visto hasta el momento, tanto la concentración como el tiempo de exposición, son fundamentales a la hora de determinar la función fisiológica o patológica del NO. No obstante, el punto de inflexión en esta dualidad, parece estar determinado por la concentración de GSH.

Toxicidad asociada a la depleción de GSH: papel del NO

El modelo anterior, en el que el GSH condiciona los efectos del NO, se complica enormemente si tenemos en cuenta que la disminución de GSH tiene por sí misma numerosos efectos, algunos de los cuales son, en esta ocasión, influidos por el NO. Los resultados discutidos en el trabajo 5, demuestran que el metabolismo del AA en cultivos primarios de mesencéfalo, se ve afectado por variaciones en la concentración de GSH. La actividad del enzima 12-LOX incrementa con la disminución intracelular de GSH, por un mecanismo postraduccional no conocido (en correlación con los datos publicados por Shornick y Holtzman, 1993). El aumento de actividad 12-LOX inicia una cascada de muerte celular que requiere síntesis de macromoléculas y actividad caspasa y que se bloquea con inhibidores de la GC y la PKG, preservando totalmente la viabilidad celular. Como ha sido previamente discutido (trabajo 5), la toxicidad asociada a la depleción de GSH en diversos modelos experimentales, está estrechamente ligada a la activación de 12-LOX y GC. Esto sugiere una ruta precisa de señalización de muerte, más que un fenómeno generalizado de estrés oxidativo, debido a la pérdida de capacidad antioxidante de la célula.

El efecto de la disminución de GSH intracelular en la viabilidad neuronal, se ha estudiado intensamente en diversos modelos experimentales de enfermedades neurodegenerativas. Especial atención han recibido los modelos relacionados con la EP, ya que en los pacientes de esta enfermedad se ha descrito una severa depleción de GSH en la

substantia nigra (SN) (Perry et al., 1982; Riederer et al., 1989; Sian et al., 1994). No obstante, los resultados con animales de experimentación indican que la simple depleción de GSH, incluso con decrementos de GSH superiores al 70%, no induce la degeneración *nigro-estriatal* (Wullner et al., 1996; Toffa et al., 1997). Nuestros datos indican que la tolerancia de las neuronas DA a la depleción de GSH, desaparece tras el tratamiento con dosis no tóxicas de NO (neurotróficas cuando la síntesis de GSH no está alterada). La toxicidad por depleción de GSH en cultivos primarios de mesencéfalo, no se manifiesta hasta el cuarto día de depleción con BSO. Sin embargo, cuando se trata simultáneamente con BSO y NO, la muerte celular se precipita en menos de 24h tras el tratamiento con el donador, de forma 12-LOX/GC/PKG dependiente.

El mecanismo por el que el NO acelera la cascada de muerte celular que se produce en condiciones de bajo GSH, no se conoce con precisión. La toxicidad producida por altas dosis de NO (neurotóxicas) en cultivos primarios de mesencéfalo, no se previene con la inhibición del enzima 12-LOX (trabajo 5), es más, los datos de que se disponen, indican que el NO no afecta (Ma et al., 1996) o inhibe (Maccarrone et al., 1996) la actividad de dicho enzima. Esto sugiere que la potenciación del NO sobre la toxicidad asociada a la disminución de GSH, no se debe a un incremento en la actividad 12-LOX. Nuestros resultados indican que la actividad del enzima está limitada por la disponibilidad de su sustrato, el AA, de forma que concentraciones de AA que no tienen ningún efecto sobre la viabilidad celular, en condiciones de depleción de GSH precipitan el proceso de muerte celular. Dicho proceso no se distingue del iniciado por NO, en su morfología, curso temporal y dependencia de la activación de 12-LOX, GC y PKG. Además, el efecto del NO en este modelo, se incrementa inhibiendo las rutas alternativas de metabolización del AA. En conjunto, los datos sugieren que la potenciación del NO sobre la toxicidad asociada a la disminución de GSH, es debida a un incremento en la disponibilidad de AA no esterificado, para su metaboización por la 12-LOX (discutido en el trabajo 5). La activación de la GC por acción directa del NO, se descarta como mecanismo de potenciación, debido al desfase temporal entre la aplicación del NO y la activación de la GC (discutido en el trabajo 4).

El GSH y el NO aparecen como una pareja funcional en cultivos primarios de mesencéfalo de rata. Los efectos del uno son drásticamente determinados por los niveles intracelulares del otro.

Interacción entre el NO y el GSH: papel de la glía

En un contexto biológico más amplio, podemos hablar del NO y el GSH como parte de un sistema de señalización basado en la modificación postraduccional de proteínas. De igual forma que las kinasas y fosfatasas mantienen un equilibrio entre fosforilación y desfosforilación que regula la actividad de numerosas proteínas, el NO y el GSH participan en fenómenos de nitrosilación y denitrosilación de proteínas tiólicas. Alrededor de 100 ejemplos de proteínas susceptibles de regulación por S-nitrosilación, incluyendo factores de transcripción, canales iónicos y receptores, proteínas G y kinasas, se pueden consultar en http://www.cell.com/cgi/content/full/106/6/675/DC1 (Stamler et al., 2002). La nitrosilación se produce generalmente en un residuo de cisteína localizado en un motivo estructural hidrofóbico, que es además susceptible de ser modificado por GSH y oxígeno (Stamler et al., 2002). En conjunto, podemos hablar de una señalización celular basada en el equilibrio redox, donde el NO y el GSH ocupan papeles principales junto con el oxígeno.

En cultivos primarios de mesencéfalo, el NO activa las proteínas kinasa ERK-1 y ERK-2 (ERK-1/2), y dicha activación es modulada por los niveles intracelulares de GSH (trabajo 6). Aunque el mecanismo exacto de activación de las ERK-1/2 en nuestro modelo, no se conoce, la participación de la GC en el proceso ha sido descartada mediante el uso de inhibidores específicos. Se ha caracterizado una activación independiente de GC de las ERK1/2, en respuesta al NO, (Lander et al., 1995; Lander et al., 1996; Lander, 1997) que consiste en la activación por S-nitrosilación de la proteína Ras y la subsiguiente cascada de señalización hasta la activación de las kinasas ERK-1/2, JNK/SAPK y p38. Los trabajos anteriores en células Yurkat (Lander et al., 1996) y los nuestros en cultivos primarios de mesencéfalo, muestran que la activación de las ERK-1/2 en respuesta al NO, aumenta cuando los niveles intracelulares de GSH están disminuidos farmacológicamente.

En nuestros trabajos se demuestra por primera vez, que los niveles de GSH modulan, no solo la amplitud de la activación de ERK-1/2 por NO, sino también la cinética de dicha activación. De forma que la activación de ERK-1/2, que desaparece al cabo de 2 horas tras el tratamiento con el donador en condiciones control, se mantiene durante al

menos 16 horas, tras la depleción de GSH. Algunos mecanismos por los que el GSH puede modular la actividad de estas kinasas en respuesta al NO, como la alteración de las reacciones de nitrosilación/denitrosilación y la formación de GSNO, se discuten en el trabajo 6.

Como se ha comentado anteriormente, en condiciones de depleción de GSH el NO precipita un proceso de muerte celular que implica activación de 12-LOX/GC/PKG. En el trabajo 6 demostramos que la inhibición de la cascada de las ERK-1/2 previene este efecto neurotóxico del NO. Es más, es el cambio en la cinética de activación de las ERK-1/2, fruto de la interacción entre el NO y la depleción de GSH, lo que determina el disparo de la cascada de muerte celular. Estos resultados están en concordancia con los de otros investigadores, que han propuesto que los parámetros precisos de activación de ERK-1/2, determinan si estas kinasas participan en procesos de supervivencia o muerte celular (Marshall, 1995; Cook et al., 1999; Chang and Karin, 2001). Nuestros resultados indican que la descompensación del equilibio entre NO y GSH, puede alterar las rutas de transducción basadas en el equilibrio redox, transformando un proceso de señalización fisiológico en una cascada de muerte celular, sin necesidad de producir daños oxidativos generalizados.

En conjunto podemos concluir que, en cultivos primarios de mesencéfalo, ERK-1/2 se activan en respuesta a un pulso de NO y que los niveles intracelulares de GSH determinan si esta activación precipita o no una cascada neurotóxica, en virtud de los parámetros cinéticos de la activación. Pero además, en el trabajo 6 demostramos que esta decisión tiene lugar en las células gliales del cultivo. Las kinasas activas se detectan inmunocitoquímicamente solo en este tipo celular y la eliminación de la glía del cultivo o su inhibición metabólica, previene la activación de ERK-1/2 en respuesta al NO. Esto quiere decir, que en condiciones de depleción de GSH, el NO inicia en la glía una cascada de señalización que mata a las neuronas del cultivo. Aunque no se conoce la forma en que la glía ejerce esta influencia sobre las neuronas, debe implicar liberación de sustancias al medio de cultivo capaces de inducir la muerte, y el AA puede ser un buen candidato (discutido en los trabajos 5 y 6). El estudio del mecanismo que subyace a la activación selectiva de ERK-1/2 en las células gliales, puede ser relevante para comprender la especificidad en la señalización por NO, una molécula con capacidad de difusión y un promiscuo espectro de influencia (Stamler et al., 2002).

La reacción glial o gliosis, es una característica neuropatológica en numerosas enfermedades del cerebro, incluida la EP (Vila et al., 2001). Esta respuesta glial puede ser fuente de factores tróficos y antioxidantes y puede rescatar a las neuronas del proceso de muerte. En contraposición con este efecto beneficioso, la reacción glial puede contribuir activamente a la muerte neuronal, liberando radicales libres, citoquinas y prostaglandinas pro-inflamatorias, etc. (Vila et al., 2001; Hunot et al., 2001; Mena et al., 2002). Esta doble funcionalidad de la glía, neurotrófica y tóxica, se pone de manifiesto en nuestros experimentos. La muerte neuronal inducida por altas dosis de NO se previene con factores solubles liberados por astrocitos (trabajo 1), entre los que se incluye el GSH (Mena et al., 2002), cuyo papel protector también ha sido demostrado (trabajo 3). Por el contrario, la activación persistente de las ERK-1/2 en las células gliales (principalmente astrocitos), inducida por NO en condiciones de depleción de GSH, dispara una cascada de muerte neuronal en el cultivo (trabajo 6). Los efectos que la glía ejerce sobre las neuronas en el sistema nervioso pueden depender de diversos factores, pero uno de ellos parece ser el equilibrio redox.

Correlación con los datos in vivo e implicaciones en la enfermedad de Parkinson

El NO participa en la degeneración del sistema DA en modelos experimentales de la EP. En el modelo del MPTP, tanto la inhibición farmacológica como la ablación molecular de los genes de la NOSn o NOSi, atenúan la muerte de las neuronas DA (Schulz et al., 1995; Przedborski et al., 1996; Liberatore et al., 1999). De igual forma, los ratones en los que el gen de la fosfolipasa A_2 (PLA_2) citosólica ha sido eliminado, son resistentes al MPTP (Klivenyi et al., 1998) y el inhibidor general de las PLA_2s, mepacrina, previene la depleción de dopamina en el estriado de la rata, en respuesta a esta toxina (Tariq et al., 2001). Esto quiere decir, que tanto el NO como el AA están implicados en la degeneración del sistema *nigro-estriatal* en roedores. Puesto que el GSH potencia la toxicidad del MPTP, podemos argumentar que las interacciones descritas en nuestros trabajos, entre GSH, NO y AA, pueden ser importantes en la muerte de las neuronas DA en modelos experimentales de la EP.

Los estudios bioquímicos e histológicos *post-mortem* en cerebros de pacientes con EP, demuestran la coexistencia en la SN de algunas de las condiciones que hemos modelizado experimentalmente en nuestro sistema *in vitro*. Una severa depleción en los niveles de GSH y un incremento en la liberación de NO. Además de una prominente gliosis, se ha descrito en la SN de estos enfermos, un marcado incremento en el número de células gliales que expresan NOSi (Hunot et al., 2001). Estos datos sugieren que en la SN de los pacientes con EP, la concentración de NO aumenta en las células gliales y en torno a las neuronas DA, en un microambiente deplecionado de GSH. En combinación, la sobreproducción de NO y la depleción de GSH podrían interaccionar *in vivo*, de forma similar a como lo hacen *in vitro*, precipitándose la cascada de muerte celular. De confirmarse nuestros resultados en modelos experimentales *in vivo*, los elementos de la cascada de señalización descrita, AA/12-LOX/12-HPETE/GC/PKG, así como las ERK-1/2 y sus sustratos (Figura 3), podrían convertirse en dianas terapéuticas interesantes en el tratamiento de la EP y otras patologías asociadas a la desregulación del NO y el GSH.

No hay que olvidar, no obstante, las funciones "buenas" del NO. Además del efecto neurogénico y neurotrófico del NO para las células DA *in vitro* (discutido en los trabajos 1, 2 y 4) y del efecto protector del NO y el GSNO en el sistema *nigro-estriatal in vivo* (Rauhala et al., 1996; Rauhala et al., 1998; Mohanakumar et al., 1998; Chiueh and Rauhala, 1999), recientemente se ha demostrado que la expresión de la NOSi tras una isquemia cerebral focal, estimula la neurogénesis en el giro dentado de ratas y ratones adultos (Zhu et al., 2003), sugiriendo una posible estrategia para la recuperación funcional del sistema nervioso. Todo ello hace deseable y necesario un conocimiento preciso de las rutas de señalización subcelular e intercelular, disparadas por el NO y reguladas por el GSH y *viceversa*, que nos permita diseccionar farmacológicamente los efectos deseables e indeseables, con el fin de alcanzar las terapias más satisfactorias, paliativas o curativas y regeneradoras.

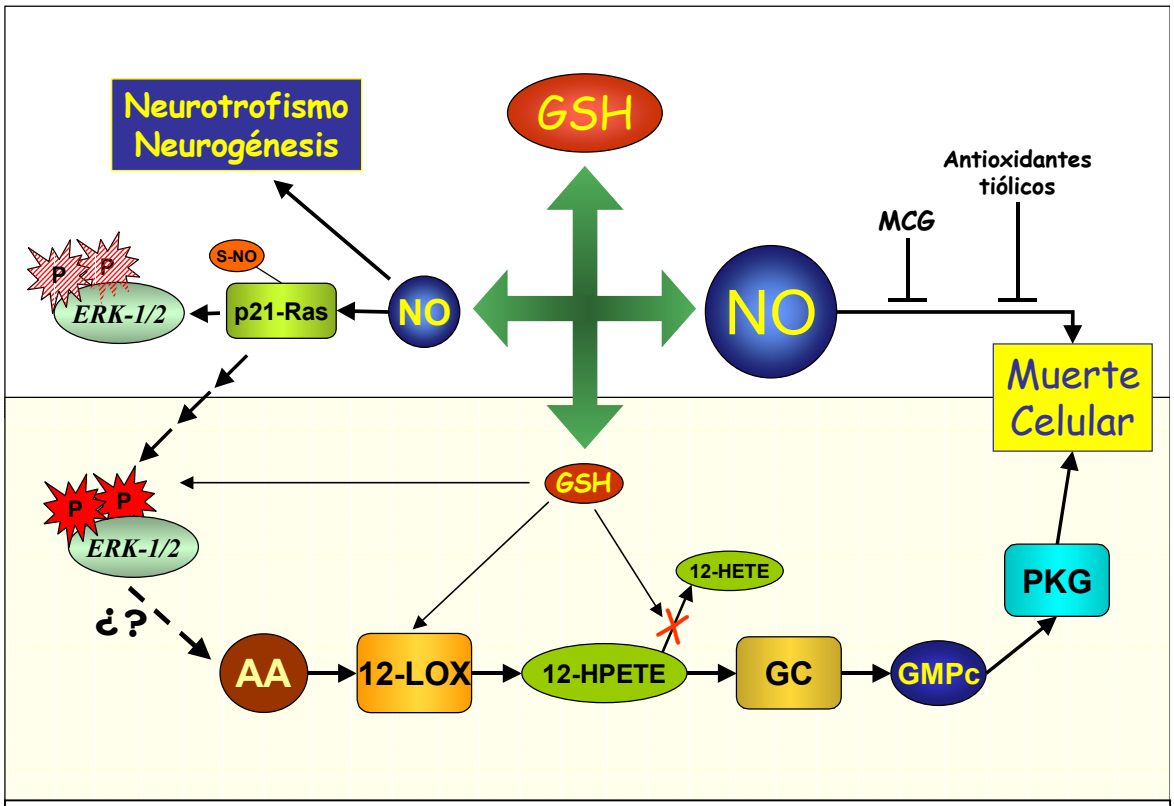

Figura 3: Efecto bifásico del NO en cultivos primarios de mesencéfalo. En primer lugar, la concentración de NO determina si el efecto final, sobre las neuronas DA, es neurotrófico o neurotóxico. En segundo lugar, la concentración intracelular y extracelular de GSH, condiciona el resultado final de la exposición al NO. Cuando la concentración intracelular de GSH disminuye (fondo amarillo), el NO en concentraciones neurotróficas, precipita una cascada de muerte celular. Cuando la concentración de GSH extracelular (u otros antioxidantes tiólicos) aumenta, se previene la toxicidad del NO. Por su parte, la depleción prolongada de GSH, dispara por sí misma, la cascada de muerte celular (fondo amarillo), activando la 12-LOX y disminuyendo la metabolización del 12-HPETE. El NO acelera dicha cascada, actuando en combinación con la disminución de GSH, sobre la ruta de las ERK-1/2 (ver texto).
MCG, medio condicionado de glía.

CONCLUSIONES

1. En cultivos primarios de mesencéfalo de rata y en células DA de neuroblastoma humano, el NO tiene un efecto bifásico que depende de su concentración. Dosis bajas de NO aumentan selectivamente la funcionalidad de las células DA e incrementan la expresión *de novo* de células con este fenotipo. Dosis altas de NO disminuyen la funcionalidad DA en ambos modelos y resultan tóxicas para los cultivos primarios y citostáticas para las células de neuroblastoma. Ambos efectos del NO, trófico y tóxico, son independientes de la activación de la GC.

2. La concentración intracelular y extracelular de GSH condiciona los efectos del NO en cultivos primarios de mesencéfalo. Una disminución de los niveles intracelulares de GSH (a partir de un 50%), elimina la capacidad neurotrófica dopaminérgica del NO y la transforma en neurotóxica. Un incremento en los niveles extracelulares de GSH (u otros antioxidantes tiólicos) previene totalmente la muerte celular en respuesta a dosis altas de NO y preserva la funcionalidad DA.

3. La inhibición persistente de la síntesis de GSH, en cultivos primarios de mesencéfalo, produce muerte celular a largo plazo, en un proceso que implica la activación de los enzimas 12-LOX, GC y PKG. Esta cascada de muerte celular, es acelerada por dosis bajas (neurotróficas) de NO, sugún un mecanismo que podría implicar el aumento en la disponibilidad de AA no esterificado.

4. En cultivos primarios de mesencéfalo, el NO activa selectivamente las ERK-1/2 en células gliales. Esta activación es modulada en amplitud y duración por los niveles intracelulares de GSH, y media la toxicidad del NO en condiciones de depleción de GSH.

5. Las células de la glía son muy resistentes a la toxicidad del NO y desempeñan una doble funcionalidad en los efectos del NO sobre las neuronas. Tienen un papel neuroprotector, previniendo totalmente la muerte neuronal en respuesta a altas dosis de NO, a través de factores solubles liberados al medio extracelular. Por otro lado, cuando la concentración de GSH está disminuida, la glía desempeña un papel neurotóxico induciendo la muerte neuronal en respuesta a bajas dosis de NO.

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