UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Bioquímica y Biología Molecular I

CETOGÉNESIS EN ASTROCITOS: CARACTERIZACIÓN, REGULACIÓN Y POSIBLE PAPEL CITOPROTECTOR

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Bajo la dirección del doctor

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TESIS DOCTORAL

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ABREVIATURAS:

ACC	Acetil-CoA carboxilasa
ACC-265	Isoforma de ACC de 265 kDa
ACC-280	Isoforma de ACC de 280 kDa
AMPK	Proteína quinasa activada por 5´-AMP
cAMP	AMP cíclico
CAPK	Proteína quinasa activada por ceramida
CAPP	Proteína fosfatasa activada por ceramida
CPT-I	Carnitina palmitoiltransferasa I
CPT-II	Carnitina palmitoiltransferasa II
ERK	Proteína quinasa activada por señales extracelulares
GABA	Ácido γ-aminobutírico
GFAP	Proteína glial fibrilar ácida
HMG-CoA	3-hidroxi-3-metilglutaril-coA
KSR	Quinasa supresora de Ras
L-CPT-I	Isoforma hepática de CPT-I
LDH	Lactato deshidrogenasa
M-CPT-I	Isoforma muscular de CPT-I
PKA	Proteína quinasa dependiente de cAMP
PKB	Proteína quinasa B
PKC	Proteína quinasa C
PPAR	Receptor activado por proliferadores de peroxisomas
SM	Esfingomielina
SMasa	Esfingomielinasa
SP	Esfingosina
SPT	Serina palmitoiltransferasa

1. INTRODUCCIÓN

La Introducción de este trabajo no pretende ser una revisión exhaustiva de los distintos temas que se incluyen en ella, sino una puesta al día de los antecedentes para permitir al lector una mejor comprensión del enfoque del trabajo, diseño experimental y los resultados obtenidos.

1.1 Astrocitos

GENERALIDADES

Los astrocitos son las células no neuronales mayoritarias del sistema nervioso central, y se agrupan junto con los oligodendrocitos y la microglía en el término general de células gliales (Travis, 1994; Porter y McCarthy, 1997; Araque et al., 1999; Albright et al., 2000). Tanto las neuronas como la macroglía (astrocitos y oligodendrocitos) se originan a partir de precursores en las capas germinales del cerebro en desarrollo, concretamente en las zonas ventricular y subventricular. Las células de origen neurales multipotenciales están presentes en estas regiones durante el desarrollo. No obstante, hace unos años se demostró la existencia de precursores separados para neuronas y células gliales (Raff et al.,1983; Luskin et al., 1988; Mayer-Proschel et al.,1997; Rao et al.,1998; Shi et al., 1998).

Durante mucho tiempo se pensó que los progenitores que originan los linajes de neuronas y macroglía iban siendo segregados progresivamente durante el desarrollo, de manera que estas células madre estarían presentes en etapas tempranas de la histogénesis del cerebro (de Vellis y Carpenter, 1999). Sin embargo, la neurogénesis y la gliogénesis continúan a lo largo de la vida puesto que también se han aislado células precursoras multipotenciales en cerebro adulto. Se trata de células que poseen la capacidad de autorrenovarse y diferenciarse en neuronas, astrocitos y oligodendrocitos (Doetsch et al., 1999; Laywell et al., 2000). Así, se han identificado recientemente en el cerebro dos tipos celulares como precursores iniciales neurales. Ambos tipos celulares son células gliales: las células ependimales (Johansson et al., 1999) y los astrocitos de la zona subventricular (Doestch et al., 1999). Las células ependimales se diferencian mayoritariamente a células gliales, las cuales bordean la superficie luminal de la zona ventricular en el cerebro adulto y provienen de un grupo de células de la zona ventricular (Barres, 1999) . Johansson et al. (1999), sin embargo, no fueron capaces de observar división de células ependimales ni *in vivo* ni *in vitro*. Por otro lado, los astrocitos de la zona subventricular también son células precursoras neurales multipotenciales y expresan la estructura, morfología y características antigénicas típicas de los astrocitos (Doestch et al., 1999; Magavi et al., 2000). Así, existe acuerdo en que al menos un grupo de células gliales son así mismo células progenitoras neurales, y difieren en cuanto a si las células progenitoras son células ependimales o astrocitos de la zona subventricular.

Además de éstos, se han identificado otros precursores celulares en el cerebro del adulto, como por ejemplo el precursor de oligodendrocitos. Así, en 1985, se demostró la existencia de un "progenitor celular O-2A", que se puede diferenciar a oligodendrocitos o a astrocitos tipo 2: en cultivo y en presencia de suero se diferencia a astrocitos de tipo 2, mientras que en un medio químicamente definido lo hace a oligodendrocitos (Temple y Raff, 1985). Curiosamente, estas células progenitoras pueden incluso proliferar indefinidamente si las condiciones del medio son las adecuadas (Tang et al., 2001). Hoy en día, los astrocitos se detectan generalmente mediante la utilización de anticuerpos anti-GFAP (proteína glial fibrilar ácida), que es marcador específico de astrocitos. En concreto, los astrocitos tipo 1 expresan GFAP, glicoproteína Ran-2, no expresan ganglósido GD3 (=inmunorreactividad frente al anticuerpo A2B5), tienen aspecto poligonal y no derivan de las células O-2A. Por el contrario, los astrocitos tipo 2 expresan GFAP, no expresan Ran-2, expresan GD3, tienen forma estrellada y derivan de las células O-2A (Lee et al., 2000).

Más recientemente se ha identificado un tercer precursor glial con inmunorreactividad frente a A2B5 que difiere del precursor O-2A en su respuesta a factores de crecimiento y en su capacidad de diferenciación (Rao et al., 1998). Este precursor se denominó GRP, y puede dar lugar al diferenciarse a astrocitos tipo 1, astrocitos tipo 2 y oligodendrocitos (Lee et al., 2000).

El desarrollo del sistema nervioso central implica una interacción dinámica entre las neuronas y las células de soporte, incluyendo la macroglía y la microglía. En concreto, los

astrocitos realizan un gran número de funciones que permiten un adecuado funcionamiento del cerebro y desempeñan un papel fundamental en el desarrollo de los patrones de organización anatómicos finales que caracterizan el cerebro adulto (Travis, 1994; Porter y McCarthy, 1997; Araque et al., 1999; Albright et al., 2000). Las células de astroglía son también responsables de la homeostasis de iones (especialmente K^+) y aminoácidos (como glutamato y ácido γ-aminobutírico, GABA) en el medio extracelular, regulan el pH y participan en la recaptura de determinados neurotransmi-sores; además, la astroglía posee una notoria capacidad de regulación del volumen celular, pudiendo de esta forma influir indirectamente en el volumen extracelular. En el desarrollo, durante la formación del tubo neural, los astrocitos dirigen la migración de las neuronas y producen factores neurotróficos y de crecimiento esenciales para el desarrollo y supervivencia neuronal. Los astrocitos configuran el espacio sináptico, mantienen una comunicación bidireccional con las neuronas a través de los neurotransmisores y participan en la formación de sinapsis (Ullian et al., 2001). Además, y en el contexto de este trabajo, los astrocitos son células que proporcionan sustratos metabólicos esenciales para el metabolismo neuronal (Magistretti y Pellerin, 1996; Giaume et al., 1997) (Fig. 1). Todas estas funciones son posibles gracias a que los astrocitos poseen receptores de membrana para un gran número de neurotransmisores y neurohormonas, y están equipados con sistemas específicos de transducción de señales y de transportadores para la captura de los neurotransmisores.

METABOLISMO EN ASTROCITOS

- *Degradación de glucosa*

La glucosa está considerada como el principal nutriente para las células del sistema nervioso adulto (Wiesinger et al., 1997; Clarke y Sokoloff, 1999). Cada tipo de célula nerviosa utiliza sus propios sustratos; además, algunas células nerviosas son capaces de producir compuestos que actúan como fuente de energía para otros tipos celulares. Con respecto a esto, los astrocitos desempeñan un papel fundamental en el flujo de sustratos energéticos a las neuronas debido tanto a su localización estratégica como a su versatilidad metabólica (Magistretti y Pellerin, 1996; Deitmer 2000) (Fig. 1). La particular morfología de los astrocitos, con sus terminaciones alrededor de los capilares del parénquima cerebral, en los cuales se encuentra la fuente de glucosa, hace que los astrocitos sean la primera barrera celular que la glucosa ha de atravesar para penetrar en el cerebro. Esta situación privilegiada apoya el papel que desempeñan los astrocitos en la distribución de nutrientes desde la sangre hasta otras células cerebrales (Magistretti y Pellerin, 1999ab) (Fig. 1).

La utilización de la glucosa por los astrocitos implica mayoritariamente la producción de lactato y piruvato, los cuales no tienen que ser necesariamente metabolizados a través del ciclo de los ácidos tricarboxílicos, sino que, en su mayoría, son liberados al medio extracelular. La glucosa, además, puede incorporarse en lípidos, aminoácidos y glucógeno, y es precursor de algunos neurotransmisores como el GABA, el glutamato y la acetilcolina (Wiesinger et al., 1997; Hertz et al., 1999; Deitmer, 2000). Ciertos intermediarios metabólicos, bajo circunstancias particulares, pueden sustituir a la glucosa y actuar como sustratos alternativos del metabolismo energético cerebral (Magistretti y Pellerin, 1996, 1999ab). Así, el ayuno prolongado, la diabetes o la lactancia en neonatos producen un incremento en los niveles plasmáticos de los cuerpos cetónicos acetoacetato y 3-hidroxibutirato, que pueden ser utilizados por el cerebro como sustratos metabólicos y pueden preservar la integridad y excitabilidad neuronal, particularmente en el desarrollo (Robinson y Williamson, 1980; Edmond et al., 1985; Izumi et al., 1997).

El primer paso en la entrada de glucosa al interior de los astrocitos es el transporte a través de la membrana plasmática, proceso que es llevado a cabo por transportadores de tipo GLUT1 y GLUT5 (Tabernero et al., 1996; Vanucci et al., 1997, Vanucci y Vanucci, 2000). A continuación tiene lugar su fosforilación, siendo ésta a su vez una de las etapas limitantes en su metabolismo. Las células de astroglía expresan (mayoritariamente en la mitocondria) hexoquinasa 1, que transforma la glucosa en glucosa 6-fosfato. Ésta puede entrar en la ruta glicolítica, en la ruta de las pentosas fosfato, en la ruta del sorbitol, o bien almacenarse en forma de glucógeno (Wiesinger et al., 1997). La cantidad de glucosa metabolizada por la ruta de las pentosas fosfato en células de astroglía en cultivo es generalmente pequeña comparada con la

metabolizada vía glicolítica. Además, la actividad glicolítica de células de astroglía en cultivo es predominantemente anaerobia y se considera el lactato como el principal producto metabólico (Wiesinger et al., 1997; Magistretti y Pellerin 1999ab). De hecho, el lactato mantiene la función sináptica en ausencia de glucosa en cortes de hipocampo (Schurr et al., 1988, 1999) y puede también mantener la función cognitiva en hipoglucemia (Maran et al., 1994; Magistretti y Pellerin, 1999ab) e hipoxia (Schurr y Rigor, 1998). El piruvato también mantiene la actividad sináptica y la morfología neuronal durante la carencia de glucosa o cuando se administra iodoacetato, un inhibidor de la glicolisis (Izumi et al., 1994; 1997). Así, la astroglía proporciona a las neuronas vecinas monocarboxilatos (lactato y piruvato) durante situaciones de elevado requerimiento energético o carencia de glucosa. La utilización de éstos como fuente de energía requiere metabolismo oxidativo, el cual queda comprometido en los casos de anoxia, pero puede tener lugar si la glicolisis está inhibida. Con bajos niveles de glucosa los astrocitos son capaces de mantener la actividad neuronal por un largo período de tiempo vía liberación de monocarboxilatos (Izumi et al., 1997; Schurr y Rigor, 1998).

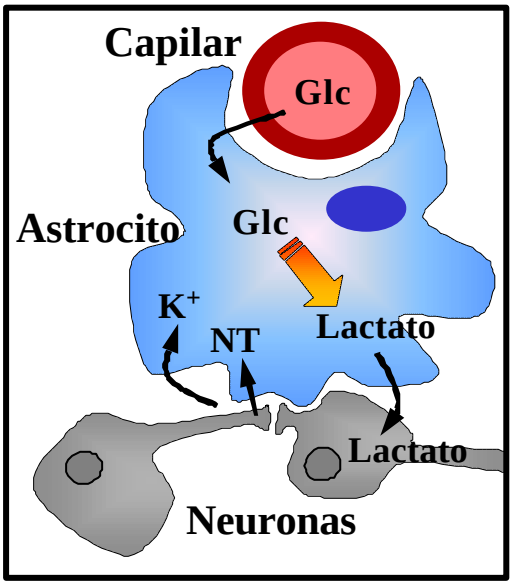

Fig. 1. Representación esquemática de la relación citológica entre los astrocitos, las neuronas y los vasos sanguíneos. Abreviaturas: Glc, glucosa; NT, neurotransmisores.

Para poder ser utilizado como sustrato celular, el lactato ha de transportarse a través de las membranas mediante transportadores específicos. Se han identificado al menos dos transportadores de monocarboxilatos en cerebro con actividad frente a lactato, piruvato, y los cuerpos cetónicos 3-hidroxibutirato y acetoacetato: MCT1 (expresado en células endoteliales vasculares y astrocitos) y MCT2 (expresado fundamentalmente en neuronas de hipocampo y corticales) (Gerhart et al., 1997; Bröer et al., 1997; Pellerin et al., 1998). A continuación, el lactato ha de convertirse en piruvato, constituyendo así una fuente metabólica eficiente de ATP, puesto que una molécula de piruvato proporciona 15 moléculas de ATP tras su oxidación a través de la piruvato deshidrogenasa y el ciclo de los ácidos tricarboxílicos. La enzima responsable de esta conversión es la lactato deshidrogenasa (LDH), cuya expresión e isoformas varían en los distintos tejidos. Bittar et al. (1996) observaron que la isoforma LDH-5, que favorece la producción de lactato, se encuentra mayoritariamente en astroglía, mientras que la LDH-1, isoforma que favorece la utilización de lactato, se encuentra mayoritariamente en las neuronas. Estos datos apoyan la idea de que los astrocitos procesarían la glucosa vía glicolisis generando lactato, el cual, una vez liberado al medio y posteriormente capturado por las neuronas, sería transformado a piruvato, que tras entrar en el ciclo de los ácidos tricarboxílicos proporcionaría la energía necesaria para la célula (Magistretti y Pelllerin, 1999ab).

En relación con este proceso, Magistretti y colaboradores han observado que el glutamato, el principal neurotransmisor excitatorio del sistema nervioso central, estimula la captura de glucosa y la liberación de lactato por astrocitos. La captura del glutamato ocurre a través de transportadores, dos de los cuales son predominantemente, si no exclusivamente, específicos de células gliales. Se trata de los transportadores GLT-1 (=EAAT2) y GLAST (=EAAT1) (Robinson y Dowd, 1997; Araque et al., 1999). Este transporte de glutamato en astrocitos está dirigido por un gradiente electroquímico de Na^+: una molécula de glutamato es cotransportada con tres iones Na^+ hacia el interior celular y como consecuencia, un ión K^+ sale al exterior. Esto conduce a un incremento de la concentración intracelular de Na^+, que es equilibrado a través de la Na^+/K^+-ATPasa. Este proceso consume ATP e implica la activación de la glicolisis y la producción de lactato (Pellerin y Magistretti, 1994, 1997; Takahashi et al., 1995; Magistretti y Pellerin,

1999ab) (Fig. 2). Un gran número de evidencias experimentales apoya que este efecto del glutamato depende exclusivamente de sus transportadores y no de sus receptores (Pellerin y Magistretti, 1994, 1997; Takahashi et al., 1995).

De esta manera se consigue un acoplamiento entre actividad neuronal, liberación de glutamato, activación de la glicolisis en astrocitos y aporte de lactato como fuente energética para las neuronas (Fig. 2).

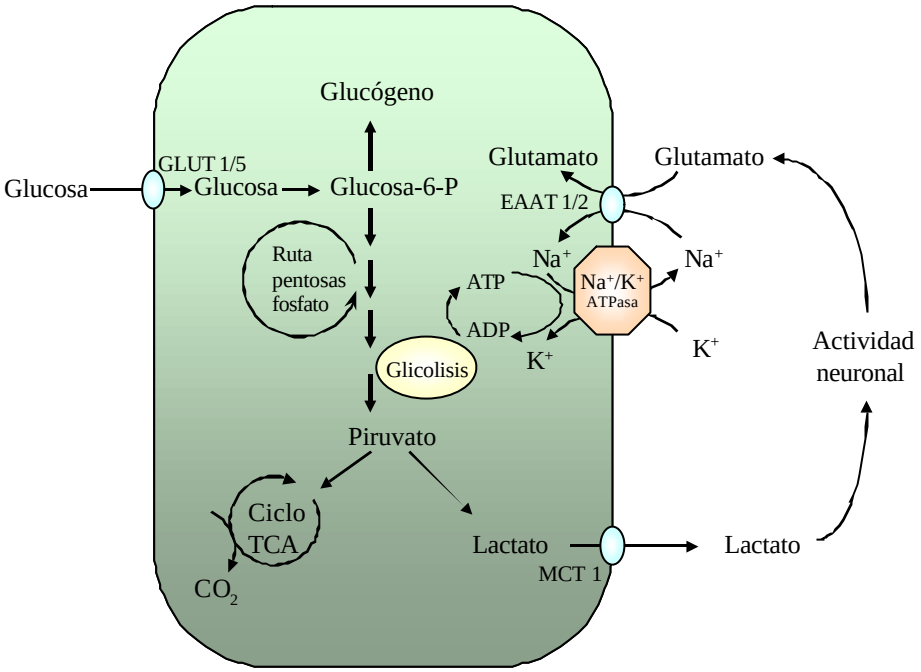

Fig. 2. Esquema de las rutas metabólicas de la glucosa en astrocitos y de la activación de la glicolisis por glutamato en condiciones de activación neuronal.

- *Metabolismo del glucógeno*

El glucógeno constituye la principal reserva energética del cerebro y está mayoritariamente localizado en los astrocitos, aunque también está presente en células del plexo coroideo y ependimal, así como en algunas neuronas del tallo cerebral. El cerebro puede considerarse como un órgano almacenador de glucógeno, cuya función podría ser proveer unidades de glucosa durante la actividad fisiológica. De hecho, el recambio de glucógeno es extremadamente rápido en los astrocitos y se correlaciona con la actividad de las neuronas vecinas (Magistretti y Pellerin, 1996; Choi et al., 1999; Deitmer, 2000; Wender et al., 2000). La degradación de glucógeno astroglial resulta activada por diversos neurotransmisores (entre los que se encuentran péptido intestinal vasoactivo, glutamato, noradrenalina, serotonina e histamina) vía cAMP y Ca^{2+} (Magistretti y Pellerin, 1996; Pellerin et al., 1997, Hamai et al., 1999). Una disminución de los niveles de glucosa del medio causa una pérdida en el glucógeno acumulado (Dringen y Hamprecht, 1992, 1993), mientras que la insulina y factores de crecimiento análogos incrementan los niveles de glucógeno almacenado en los astrocitos (Pellerin et al., 1997; Hamai et al., 1999).

El aspecto quizás más importante de la degradación del glucógeno es el destino de los residuos glicosílicos. Los residuos liberados no aparecen mayoritariamente como glucosa libre en el medio, sino que los astrocitos liberan principalmente ácido láctico (Dringen et al., 1993; Magistretti y Pellerin, 1996). Alternativamente, la glucosa procedente de la degradación del glucógeno puede metabolizarse vía ruta de las pentosas fosfato para generar NADPH y proteger a los astrocitos frente al estrés oxidativo (Dringen, 2000; Rahman et al., 2000). Aunque se ha detectado actividad, mRNA y proteína de glucosa

6-fosfatasa en astrocitos (Forsyth et al., 1993), estudios posteriores no han sido capaces de reproducir estos hallazgos (Magistretti y Pellerin, 1999ab; Gotoh et al., 2000).

- *Gluconeogénesis*

En los astrocitos también se da el proceso de gluconeogénesis hasta glucosa 6-fosfato, y de hecho se ha descrito la presencia de tres enzimas implicadas exclusivamente en la ruta (piruvato carboxilasa, fosfoenolpiruvato carboxiquinasa y fructosa 1,6-*bis*fosfatasa) en estas células (Wiesinger et al., 1997). La gluconeogénesis en astrocitos podría actuar como mecanismo tamponador, controlando la osmolaridad del fluido extracelular y reciclando el lactato generado por las células vecinas, derivándolo hacia rutas como la síntesis de glucógeno (Schmoll et al., 1995; Wiesinger et al., 1997; Jitrapakdee y Wallace, 1999).

1.2 Cetogénesis

GENERALIDADES

La mayoría de los estudios sobre cetogénesis y oxidación de ácidos grasos realizados hasta el momento se han llevado a cabo en el hígado. La producción de cuerpos cetónicos por las mitocondrias del hígado es un proceso complejo y finamente regulado (Guzmán y Geelen, 1993; Zammit, 1994). En primer lugar, la célula debe capturar ácidos grasos. Aunque tradicionalmente se ha asumido que los ácidos grasos atraviesan la membrana plasmática siguiendo un proceso no saturable de difusión simple (Cooper et al., 1989), durante los últimos años se ha ido acumulando una cierta evidencia que apoya la existencia de un sistema saturable de transporte facilitado de ácidos grasos. En concreto, se ha identificado una proteína de membrana ligante de ácidos grasos de 40 kDa, que podría actuar como transportador de ácidos grasos y que podría constituir un primer punto de control de la entrada de ácidos grasos al interior de la célula (Stremmel et al., 1992; Glatz y van der Vusse, 1996; Hamilton, 1998).

El tráfico intracelular de ácidos grasos de cadena larga está mediado por proteínas ligantes de ácidos grasos (van Nieuwenhoven et al., 1996). Tras su activación a acil-CoA (Watkins, 1997), éstos se unen a proteínas ligantes de acil-CoA, que podrían estar implicadas en la regulación del aporte de sustrato a las enzimas que utilizan acil-CoA (Gossett et al., 1996) .

La membrana mitocondrial interna es impermeable a los acil-CoA de cadena larga, por lo que, como se verá más adelante, existe un sistema de transporte dependiente de carnitina que transloca los acil-CoA a la matriz mitocondrial. En el interior de la mitocondria, los acil-CoA son escindidos en fragmentos de acetil-CoA a través de la ruta de la β-oxidación, los cuales a su vez pueden ser completamente oxidados a CO_2 y H_2O a través de la acción subsecuente del ciclo de los ácidos tricarboxílicos y la cadena respiratoria transportadora de electrones o bien ser convertidos en cuerpos cetónicos (Sugden et al., 1989). El principal punto de control de todo este proceso es la reacción catalizada por la carnitina palmitoiltransferasa I (CPT-I), pudiendo la 3-hidroxi-3-metilglutaril-CoA (HMG-CoA) sintasa mitocondrial desempeñar un papel regulador adicional (ver más adelante). Aunque la β-oxidación de ácidos grasos comprende dos etapas oxidativas (las reacciones catalizadas por la acil-CoA-deshidrogenasa y la 3-hidroxiacil-CoA-deshidrogenasa), que pueden ser reguladas por la relación entre las concentraciones de NADH y NAD^+ en la mitocondria, y existe una serie de efectores que puedan actuar *in vitro* a distintos niveles en la β-oxidación (Eaton et al. 1996), no se considera que esta ruta posea un papel regulador importante en la cetogénesis (Zammit, 1994).

La formación de cuerpos cetónicos constituye el destino metabólico principal del acetil-CoA producido por la β-oxidación de ácidos grasos en el hígado. Así, la contribución del ciclo de los ácidos tricarboxílicos a la utilización de este acetil-CoA es bastante reducida (menos del 10%) en situaciones en las cuales la disponibilidad de glucosa es la adecuada, y prácticamente nula en estados catabólicos tales como el ayuno y la diabetes, en los que los ácidos grasos oxidados en el hígado se desvían prácticamente en su totalidad hacia la síntesis de cuerpos cetónicos (McGarry y Foster, 1980; Sugden et al., 1989; Brown, 1992; Zammit, 1994).

El primer paso de la formación de cuerpos cetónicos es la condensación de dos moléculas de acetil-CoA para generar acetoacetil-CoA, en una reacción catalizada por la acetil-CoA acetiltransferasa (Fig. 3), que puede ser inhibida por uno de sus productos, la

CoA libre, que disminuye la afinidad de la enzima por su sustrato, el acetil-CoA (Guzmán y Geelen, 1993; Zammit, 1994). El acetoacetil-CoA es un importante metabolito implicado en el control del metabolismo mitocondrial de ácidos grasos en el hígado. Así, el acetoacetil-CoA inhibe *in vitro* a la acil-CoA deshidrogenasa, la primera enzima implicada en la β-oxidación de ácidos grasos, y ejerce inhibición por producto sobre la acetil CoA-acetiltransferasa y por sustrato sobre HMG-CoA sintasa (Hegardt, 1999). La HMG-CoA sintasa mitocondrial cataliza la síntesis de HMG-CoA para la formación de cuerpos cetónicos y parece representar la principal enzima limitante del flujo en la cetogénesis a partir de acetil-CoA en hepatocitos aislados (Quant et al., 1993; Hegardt, 1999). Sin embargo, estudios de control metabólico indican que su potencial regulador quedaría supeditado al de la CPT-I en la producción de cuerpos cetónicos a partir de ácidos grasos (Drynan et al., 1996; Spurway et al., 1997).

En astrocitos, un primer estudio concluyó que la actividad de esta enzima apenas es detectable y, por tanto, la síntesis de cuerpos cetónicos tras la formación de acetoacetil-CoA podría proseguir vía acetoacetil-CoA deacilasa (que deacila el acetoacetil-CoA para formar acetoacetato) y/o 3-oxoácido-CoA transferasa (que cataliza la transferencia de CoA desde el acetoacetil-CoA al succinato, liberando acetoacetato y succinil-CoA) (Auestad et al., 1991) (Fig. 3). Sin embargo, en otros estudios posteriores se ha detectado en astrocitos cultivados en presencia de suero o dexametasona la presencia de mRNA para la HMG-CoA sintasa, proponiéndose que en la síntesis de cuerpos cetónicos por estas células actuaría fundamentalmente esta enzima, formándose como metabolito intermediario HMG-CoA y posteriormente acetoacetato (Cullingford et al., 1998ab).

Una vez producido el acetoacetato, éste se reduce en la matriz mitocondrial a 3-hidroxibutirato por la acción de la 3-hidroxibutirato deshidrogenasa (Fig. 3).

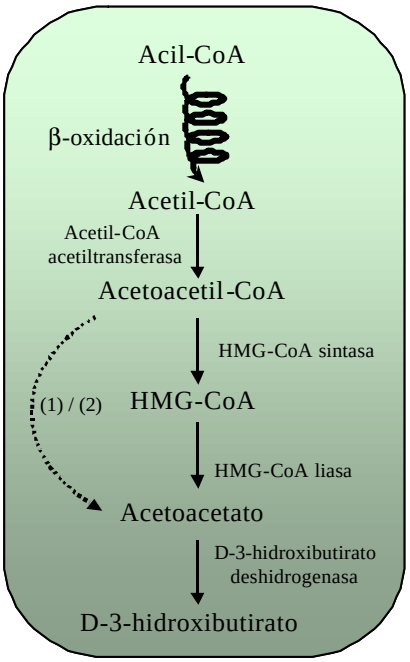

Fig. 3. Esquema de la ruta cetogénica mitocondrial que transcurre vía HMG-CoA. En la ruta alternativa para la formación de acetoacetato a partir de acetoacetil-CoA estarían implicadas la acetoacetil-CoA deacilasa (1) y/o la 3-oxoácido-CoA transferasa (2).

CARNITINA PALMITOILTRANSFERASA I

Como se ha mencionado anteriormente, los ácidos grasos de cadena larga no pueden atravesar la membrana mitocondrial interna. Así, el primer paso para su translocación a la matriz mitocondrial consiste en su activación en la membrana externa de la mitocondria por una acil-CoA sintetasa de cadena larga. A continuación, y previamente a la β-oxidación, los acil-CoA pasan al interior de la mitocondria por la acción del sistema de transporte dependiente de carnitina. Este sistema implica a tres proteínas, la CPT-I, la carnitina:acilcarnitina translocasa y la carnitina palmitoiltransferasa II (CPT-II), cada una de ellas con distinta localización submitocondrial (McGarry y Brown, 1997; Kerner y Hoppel, 2000). En una primera etapa, los acil-CoA son convertidos en acilcarnitinas por un proceso de transesterificación catalizado por la CPT-I, situada en la membrana mitocondrial externa; a continuación, el complejo acilcarnitina difundiría a través del espacio intermembrana o a través de los puntos de contacto entre las dos membranas mitocondriales (Kerner y Bieber, 1990; Fraser y Zammit, 1998; Hoppel et al., 1998) hasta llegar a la cara externa de la membrana mitocondrial interna, donde se translocaría al interior de la mitocondria por una reacción de intercambio catalizada por la translocasa de carnitina:acilcarnitina. En la matriz mitocondrial, las acilcarnitinas son entonces reconvertidas en sus respectivos acil-CoA por la acción de la CPT-II, enzima que se encuentra asociada a la cara interna de la membrana mitocondrial interna (McGarry y Brown, 1997; Kerner y Hoppel, 2000) (Fig. 4).

- *Estructura e isoformas*

La caracterización de la CPT-I fue difícil por tratarse de una enzima estrechamente asociada a la membrana mitocondrial externa y por la pérdida de actividad catalítica que experimenta cuando se separa de su entorno nativo (Declercq et al., 1987; Woeltje et al., 1987; Zammit et al., 1989). Una vez desarrolladas las técnicas adecuadas para su caracterización, se aisló un clon que permitió predecir para el hígado de rata una proteína (L-CPT-I) de 773 aminoácidos y 88 kDa de peso molecular, además de presentar una actividad inhibible por malonil-CoA y sensible al tratamiento con detergentes (Esser et al., 1993). Previamente el mismo grupo había clonado la CPT-II de hígado de rata (Woeltje et al., 1990) y unos años después se clonó la CPT-I de músculo de rata (M-CPT-I) (Esser et al., 1996).

De acuerdo con su papel fundamental en la oxidación mitocondrial de ácidos grasos, la CPT-I existe en al menos dos isoformas distintas. Las dos isoformas están codificadas por genes localizados en diferentes cromosomas y exhiben distinta distribución tisular y diferentes propiedades cinéticas y reguladoras (McGarry y Brown, 1997; Kerner y Hoppel, 1998; Jackson et al., 2000) (Tabla 1). Existen además isoformas de CPT-I generadas por procesamiento alternativo del mRNA de la CPT-I de músculo (Yu et al., 1998).

Estudios de topología han demostrado que la CPT-I se localiza en la membrana mitocondrial externa; está formada por dos fragmentos hidrofóbicos transmembranales, así como un corto dominio N-terminal y un largo dominio C-terminal situados hacia la cara citoplasmática de la membrana mitocondrial externa. Además, presenta un corto bucle de 27 aminoácidos que une los dominios transmembranales y que sobresale hacia el espacio intermembrana (Fraser et al., 1997; van der Leij et al., 1999). Se han realizado además experimentos con malonil-CoA y octanoil-CoA (inhibidor y sustrato de la CPT-I respectivamente) asociados a agarosa, para evitar que atraviesen la membrana mitocondrial externa, que indican que el dominio catalítico y regulador de la CPT-I están localizados hacia la cara citosólica (Fraser et al., 1997).

Características	L-CPT I	M-CPT I	CPT II
Peso molecular	88 kDa	88 kDa	67 kDa
IC_{50} para malonil-CoA	2.5 µM	0.03 µM	--
K_m para carnitina	30 µM	500 µM	120 µM
Cromosoma humano	11q13	22q13.3	1p32
Expresión			
Hígado	++++	--	+
Músculo esq.	(+)	++++	+
Corazón	+	+++	+
Riñón	++++	(+)	+
Adiposo	+	+++	+
Cerebro	++++	--	+

Tabla 1. Características de las CPT mitocondriales**.** Los niveles relativos de expresión en cada órgano están basados en análisis por *Northern blot* (y en algunos casos por marcaje con [^{3}H]etomoxir-CoA). (+) Sólo trazas en comparación con la isoforma alternativa. (-) Indetectable. Los datos de expresión en tejidos se refieren a rata. La K_m para la carnitina de la CPT-II fue determinada tras solubilización de las mitocondrias en octilglucósido y por tanto no debe ser comparada directamente con los valores de CPT-I (McGarry y Brown, 1997).

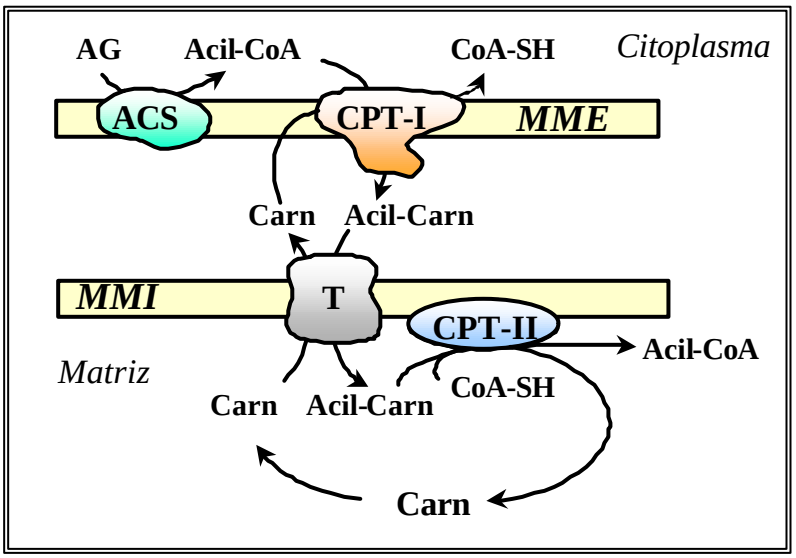

Fig. 4. Translocación de ácidos grasos a la matriz mitocondrial**.** Abreviaturas: ACS, acil-CoA sintetasa; AG, ácido graso; Carn, carnitina; MME, membrana mitocondrial externa; MMI, membrana mitocondrial interna; T, translocasa de carnitina:acilcarnitina.

- *Regulación*

La CPT-I cataliza el primer paso en la oxidación mitocondrial de los ácidos grasos y desempeña un papel fundamental en la regulación de todo el proceso. Los mecanismos por los cuales se controla la actividad CPT-I incluyen, entre otros, cambios en la concentración de malonil-CoA (regulación a corto plazo) y cambios en los niveles de enzima y en su sensibilidad a inhibición por malonil-CoA (regulación a largo plazo). También se ha propuesto la existencia de un mecanismo agudo de regulación de la actividad CPT-I independiente de los niveles de malonil-CoA..

Regulación por malonil-CoA

Hígado

La CPT-I es inhibida por malonil-CoA, el producto de la reacción catalizada por la acetil-CoA carboxilasa (ACC), que constituye a su vez la etapa limitante de la síntesis de ácidos grasos (McGarry et al., 1977; Geelen et al., 1980). De esta manera, se consigue un control coordinado de la síntesis y la oxidación de ácidos grasos en el hígado (Zammit, 1994). Los niveles de malonil-CoA en la célula son dependientes de la actividad ACC y, por tanto, se ven afectados notablemente por cambios a corto plazo que diferentes mecanismos, como pueden ser alteraciones en el estado hormonal y nutricional del animal, producen sobre dicha actividad enzimática (McGarry y Foster, 1980; Zammit, 1994). Especialmente determinante es el cociente insulina/glucagón en plasma: cuando este cociente es alto los niveles de malonil-CoA también lo son y la actividad CPT-I está inhibida, y viceversa (Zammit, 1996). Recientemente se ha propuesto que la malonil-CoA descarboxilasa, que descarboxila el malonil-CoA a acetil-CoA, podría determinar así mismo los niveles de malonil-CoA y, por tanto, la actividad CPT-I en hepatocitos (Dyck et al., 2000; Saha et al., 2000).

Músculo esquelético y corazón

Diferentes tejidos no lipogénicos, como músculo esquelético y corazón, también contienen malonil-CoA, cuyos niveles oscilan en función del grado nutricional del organismo. Este hecho podría indicar un papel regulador del malonil-CoA en estos tejidos (Lopaschuk et al., 1994). La isoforma de CPT-I mayoritariamente presente en músculo cardíaco y esquelético (M-CPT I) es mucho más sensible a malonil-CoA que la isoforma hepática (McGarry y Brown, 1997). Diversos estudios indican que los niveles de malonil-CoA en corazón dependen principalmente de la disponibilidad de acetil-CoA citosólico, el sustrato de la ACC. Puesto que además apenas existe ácido graso sintasa en músculo y corazón, el papel del malonil-CoA sería el de regulador de la CPT-I más que el de precursor para la síntesis de ácidos grasos (McGarry y Brown, 1997).

Por estudios de inmunofluorescencia se ha visto que la ACC-280 (isoforma de ACC de 280 kDa, expresada mayoritariamente en músculo y corazón) está asociada a la mitocondria, a diferencia de la ACC-265 (isoforma de ACC de 265 kDa, expresada predomi-nantemente en tejidos lipogénicos), que tiene localización citosólica. Esta asociación de la ACC-280 a la mitocondria apoya la hipótesis de su implicación en la regulación de la oxidación de ácidos grasos por la mitocondria a través de la inhibición de la CPT-I por el malonil-CoA sintetizado (Abu-Elheiga et al., 2000).

Célula β

La regulación de la CPT-I mediada por malonil-CoA parece tener un papel fisiológico importante en las células β del páncreas. Se ha propuesto que, tras un incremento de la concentración de glucosa en sangre, este azúcar es metabolizado por la célula β a través de la ruta glicolítica, de forma que parte del piruvato sería desviado hacia la síntesis de malonil-CoA. Este malonil-CoA inhibiría a la CPT-I, lo que provocaría una acumulación de acil-CoA de cadena larga en el citoplasma de la célula β. Los acil-CoA podrían actuar estimulando la exocitosis de los gránulos de insulina acumulados en estas células (Newgard y McGarry, 1995; Zammit, 1999).

Regulación a corto plazo independiente de malonil-CoA

Durante más de dos décadas se ha considerado la inhibición de la CPT-I por malonil-CoA como el mecanismo principal de regulación de la cetogénesis hepática; sin embargo, observaciones más recientes indican que la CPT-I también está regulada a corto plazo por mecanismos independientes de malonil-CoA (Guzmán et al., 2000). Así,

mediante el empleo de hepatocitos permeabilizados, se ha observado la existencia de cambios en la actividad CPT-I independientes de malonil-CoA (Guzmán y Geelen, 1993; Guzmán et al., 1994). Utilizando moduladores del estado de agregación y polimerización del citoesqueleto se propuso la posibilidad de un papel regulador de componentes del citoesqueleto sobre la actividad CPT-I, identificándose posteriormente los filamentos intermedios, y específicamente las citoqueratinas 8 y 18, como los principales candidatos a mediar esta regulación (Velasco et al., 1998ab). Además, el empleo de inhibidores específicos de proteína quinasas y fosfatasas, junto con experimentos de reconstitución y estudios de fosforilación de citoqueratinas en hepatocitos intactos, permitió implicar a la proteína quinasa dependiente de Ca^{2+}-calmodulina de tipo II y a la proteína quinasa activada por AMP (AMPK) como enzimas que afectan al estado de agregación del citoesqueleto y, como consecuencia de ello, a la actividad CPT-I. Así, la activación de la Ca^{2+}-calmodulina quinasa II o de la AMPK induce la fosforilación de las citoqueratinas 8 y 18, lo que supone la desagregación de los filamentos intermedios, con lo que se pierde la interacción entre la CPT-I y los componentes del citoesqueleto y la CPT-I se activa (Velasco et al.,1998ab). A su vez, la activación de las dos quinasas también regula la actividad CPT-I a través de un mecanismo dependiente de malonil-CoA, puesto que fosforilan e inactivan a la ACC produciendo un descenso en los niveles de malonil-CoA y, por tanto, la activación indirecta de la CPT-I (Guzmán et al., 2000).

Estudios llevados a cabo por Sleboda et al. (1999) también apoyan esta regulación de la CPT-I independiente de malonil-CoA por componentes celulares extramitocondriales. Así mismo, se ha observado en cardiomiocitos de rata neonata que la hipoxia produce una estimulación significativa en la actividad CPT-I por un mecanismo estable a nivel de regulación post-traduccional (Wang et al., 1998).

Regulación de la sensibilidad a malonil-CoA

El malonil-CoA, por sí mismo, no sólo inhibe a la actividad CPT-I, sino que también podría determinar la sensibilidad de la CPT-I a inhibición por malonil-CoA. Así, en diferentes situaciones fisiopatológicas, la sensibilidad de la CPT-I al malonil-CoA se ve modificada. Por ejemplo, los cambios inducidos por la dieta y el estado hormonal en el flujo a través de la ruta oxidativa de los ácidos grasos están normalmente acompañados de variaciones paralelas en la sensibilidad al malonil-CoA de la CPT-I de hígado. En estados cetóticos como el ayuno, la diabetes y el hipertiroidismo, la sensibilidad de la CPT-I a inhibición por malonil-CoA disminuye, mientras que en estados lipogénicos como el hipotiroidismo o la realimentación tras el ayuno ocurre lo contrario (Zammit, 1994). Este cambio en la sensibilidad a malonil-CoA sólo se ha observado en la isoforma hepática, no en la CPT-I de corazón ni en la de músculo.

Aún no se conoce el mecanismo por el cual se desensibiliza la CPT-I, pero se ha observado que la cardiolipina produce un incremento y recuperación en la sensibilidad al malonil-CoA en mitocondrias de hígado de rata en ayuno. Puesto que la cardiolipina es un componente minoritario de la membrana mitocondrial externa, se ha sugerido la posibilidad de que pueda formar un microambiente de membrana específico para la CPT-I (Kerner y Hoppel, 2000). De acuerdo con esto, preparaciones de membrana mitocondrial externa de ratas en ayuno, diabéticas o en estados cetóticos presentan una relación inversa entre la sensibilidad de la CPT-I a malonil-CoA y la fluidez de la membrana (Zammit et al., 1998), lo que se ha comprobado así mismo por experimentos de reconstitución (McGarry y Brown, 2000).

Regulación de los niveles de enzima

Existen bastantes estudios acerca de cómo diversos factores nutricionales y hormonales pueden afectar a la expresión de las CPT de hígado de rata. Diferentes situaciones que estimulan la cetogénesis en hígado, como el ayuno, la diabetes, el hipertiroidismo o el tratamiento con agentes proliferadores de peroxisomas, inducen tanto un aumento en los niveles de proteína inmunorreactiva como de mRNA de CPT-I y CPT-II (Kolodziej et al., 1992; Park et al., 1995; McGarry y Brown, 1997; Jensen et al., 2000). Otra situación fisiológica que afecta de manera notable a la expresión de los genes de CPT-I y CPT-II es el desarrollo postnatal. En el hígado fetal, tanto los niveles de mRNA como la actividad CPT-I son muy bajos. Sin embargo, ambos aumentan bruscamente durante el primer día de vida extrauterina y permanecen elevados durante todo el periodo de lactancia hasta el

destete (Thumelin et al., 1994; Asins et al., 1995), lo que se corresponde con el mayor porcentaje en grasas de la alimentación durante el periodo de lactancia.

Se están empezando a conocer los factores que regulan la transcripción de los genes de las isoformas de CPT-I. Así, se ha caracterizado el promotor del gen de la L-CPT-I, en el cual se han identificado dos sitios de unión a los factores de transcripción Sp1 y Sp3, así como también al factor nuclear Y (NF-Y) (Steffen et al., 1999). Por otro lado, la expresión de la M-CPT-I, está regulada a nivel transcripcional por la unión de receptores activados por proliferadores de peroxisomas (PPAR) a su promotor (Mascaró et al., 1998), mientras que la regulación transcripcional de la L-CPT-I parece ser independiente de PPAR (Louet et al., 2001).

1.3 Lipoapoptosis

GENERALIDADES

La apoptosis es un mecanismo esencial en el desarrollo del sistema nervioso de vertebrados que está implicado en procesos de remodelación tisular, embriogénesis, respuesta inmune y en determinadas situaciones patológicas como enfermedades autoinmunes y neurodegenerativas (Desagher y Martinou, 2000; Hengartner, 2000; Strasser et al., 2000). Se trata de un proceso de muerte celular caracterizado porque las células se encogen, se condensa la cromatina, la integridad (no la asimetría) de la membrana plasmática se mantiene hasta el último momento y se produce la activación de caspasas y la fragmentación internucleosómica del DNA. Aunque existen formas de muerte con características intermedias entre necrosis y apoptosis (Sperandio et al., 2000), todo ello diferencia a la primera de la segunda, que cursa con hinchamiento celular, degradación no específica del DNA, lisis de la célula e inflamación del tejido circundante.

Como se verá a lo largo de esta memoria, existen numerosos estudios de señalización celular sobre esfingolípidos (especialmente ceramida) como mediadores intracelulares de apoptosis (Kolesnick y Krönke, 1998; Hannun y Luberto, 2000; Radin, 2001). ¿Cómo podría influir la CPT-I en este proceso? Paumen et al. (1997) observaron que en líneas celulares hematopoyéticas la CPT-I podría proteger de la apoptosis inducida por ácidos grasos a través de una inhibición de la síntesis *de novo* de esfingolípidos; así, observaron que la inhibición farmacológica de la CPT-I incrementaba la muerte celular inducida por palmitato y los niveles de ceramida sintetizada *de novo* a partir de este ácido graso. Posteriormente, Shimabukuro et al. (1998) descubrieron el mismo fenómeno en células β de páncreas, y acuñaron el término "lipoapoptosis" para definir este proceso de muerte celular vía síntesis *de novo* de ceramida a partir de ácidos grasos. Existen otros estudios que han demostrado que el palmitato está implicado en la inducción de apoptosis. Así, en cardiomiocitos de ratas neonatas la incubación en presencia de concentraciones fisiológicas de palmitato induce acumulación de ceramida, inhibición de la AMPK, inhibición de la CPT-I y, en último término, apoptosis (Hickson-Bick et al., 2000). En suma, a la vista de estos estudios se puede pensar que la CPT-I tendría un papel regulador del metabolismo lipídico y del destino celular puesto que, en función de su actividad, los ácidos grasos de cadena larga se dirigirán hacia la ruta de la β-oxidación o bien hacia la ruta de síntesis de ceramida.

CERAMIDA

Durante la última década se ha investigado intensamente la posibilidad de que los esfingolípidos sean mediadores importantes en la señalización celular. Los esfingolípidos están implicados en la regulación de la mayoría de los aspectos fundamentales de la biología celular, incluyendo procesos de crecimiento, diferenciación, arresto del ciclo celular, apoptosis y oncogénesis en células nerviosas y no nerviosas (Goswami y Dawson, 2000; Hannun y Luberto, 2000). En concreto, la ceramida es un segundo mensajero esfingolipídico que presenta una rápida capacidad de translocación de la superficie celular al interior de la membrana y que regula directa o indirectamente un gran número de enzimas. Se genera como respuesta a la estimulación de muy diversas vías implicadas en muerte celular, como por ejemplo aquellas ligadas a citoquinas proinflamatorias, agentes utilizados en quimioterapia, radiación ionizante y agentes oxidantes. No obstante, no debe olvidarse que la interpretación de algunos datos de la bibliografía es confusa debido a factores como la cinética variable de generación de

ceramida, el alto número de enzimas implicadas en dicho proceso, la compartimentalización subcelular de la ceramida y el uso de análogos no fisiológicos de ceramida (Hoffmann y Dixit, 1998; Kolesnick y Hannun, 1999; Levade y Jaffrézou, 1999; Siskind y Colombini, 2000; Venkataraman y Futerman, 2000).

- *Generación de ceramida*

La ceramida puede formarse a través de diversas rutas. En el contexto de la presente memoria destaca sin duda la ruta de síntesis *de novo* (Fig. 5). Ésta comienza en el retículo endoplásmico con la condensación de palmitoil-CoA (o estearoil-CoA) y serina para formar 3-cetodihidroesfingosina en una reacción catalizada por la serina palmitoiltransferasa (SPT). A continuación se produce la reducción a esfinganina por una reductasa dependiente de NADPH. Por una reacción de acilación llevada a cabo por una aciltransferasa (la dihidroceramida sintasa) se forma la dihidroceramida y, por último, una desaturasa introduce un doble enlace en las posiciones 4 y 5 del esqueleto esfingoide de la molécula, formándose la *N*-acilesfingosina o ceramida (Kolter y Sandhoff, 1999; Luberto y Hannun, 1999).

La ceramida puede ser sintetizada alternativamente por acilación de la esfingosina por una aciltransferasa (Luberto y Hannun, 1999) que podría tratarse de una ceramidasa neutra que cataliza una reacción reversible de acilación-desacilación (Tani et al., 2000). Además, la ceramida constituye un punto intermedio en la formación de la mayoría de los esfingolípidos con importancia biológica (Fig. 6).

Se ha considerado hasta el momento que la generación de ceramida implicada en procesos apoptóticos tiene lugar por la hidrólisis de esfingomielina catalizada por esfingomielinasas. Estas enzimas hidrolizan el enlace fosfodiéster de la esfingomielina, que preferentemente se encuentra en la membrana plasmática de las células de mamíferos, produciendo ceramida y fosforilcolina (Kolesnick y Krönke, 1998; Levade y Jaffrézou, 1999). Existen diversas esfingomielinasas con características de regulación diferentes y con distinto pH óptimo. A grandes rasgos existen esfingomielinasas ácidas (pH óptimo 4.5-5.0), que se localizan en compartimentos celulares ácidos como lisosomas y endosomas, y esfingomielinasas neutras (dependientes e independientes de Mg^{2+}), que actúan en la membrana plasmática y abundan en los *rafts* lipídicos. Ambos tipos de esfingomielinasas responden generalmente a estímulos que incrementan los niveles de ceramida a tiempos cortos, del orden de segundos a minutos, por lo que se las principales responsables de la producción de ceramidas en las señales de transducción tempranas (Kolesnick y Krönke, 1998; Levade y Jaffrézou, 1999). Sin embargo, la cuestión de cuántas esfingomielinasas existen y cuál está implicada en cada pico de ceramida está aún sin resolver (Chatterjee et al., 1999; Levade y Jaffrézou, 1999; Bernardo et al., 2000; Fensome et al., 2000; Hofmann et al., 2000).

Por el contrario, la ruta de síntesis *de novo* de ceramidas ha ido ganando progresivamente importancia como forma alternativa de generar un *pool* apoptótico de ceramida, y compuestos como la L-cicloserina (un inhibidor de la SPT) y la fumonisina B1 (un inhibidor de la dihidroceramida sintasa) (Fig. 5) previenen la acumulación de ceramida y la muerte por apoptosis inducida por ácidos grasos en células hematopoyéticas y células β del páncreas (ver más arriba). Así mismo, la exposición a determinadas drogas utilizadas en quimioterapia como la doxorrubicina, la daunorrubicina y el etopósido podrían inducir apoptosis por un incremento en la síntesis *de novo* de ceramida (Bose et al., 1995; Perry et al., 2000) acompañado de la estimulación de la hidrólisis de esfingomielina (Jaffrézou et al., 1996; Andrieu-Abadie et al., 1999). Otros estudios han demostrado la importancia de la síntesis *de novo* de ceramida en la apoptosis producida por angiotensina A-II (Lehtonen et al., 1999) y el gen de la ataxia-talangiectasia (Liao et al., 1999), así como en la apoptosis que tiene lugar durante la diferenciación neuronal (Herget et al., 2000). Por todo ello se hará más adelante una especial mención a la enzima clave de la ruta, la SPT.

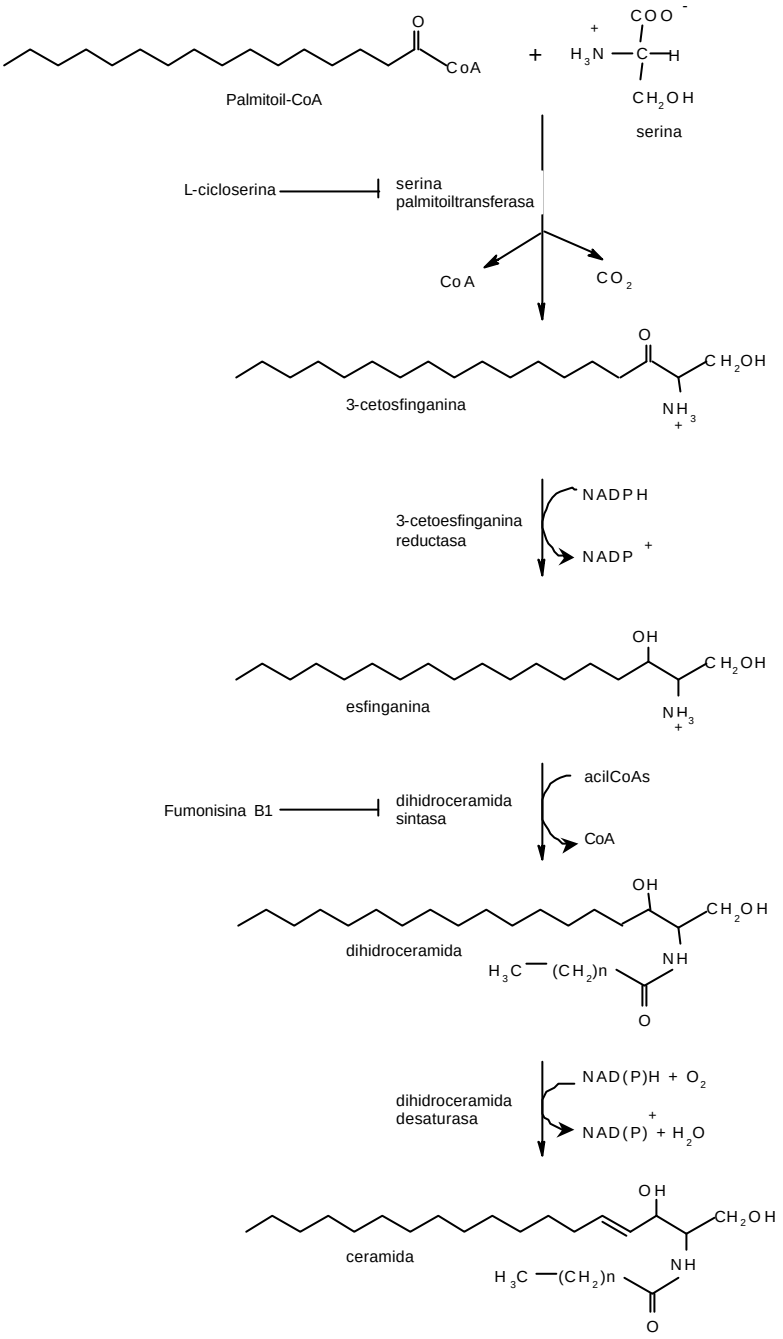

Figura 5. Esquema de la síntesis *de novo* de ceramida. Se incluyen los dos inhibidores de la ruta empleados en este trabajo.

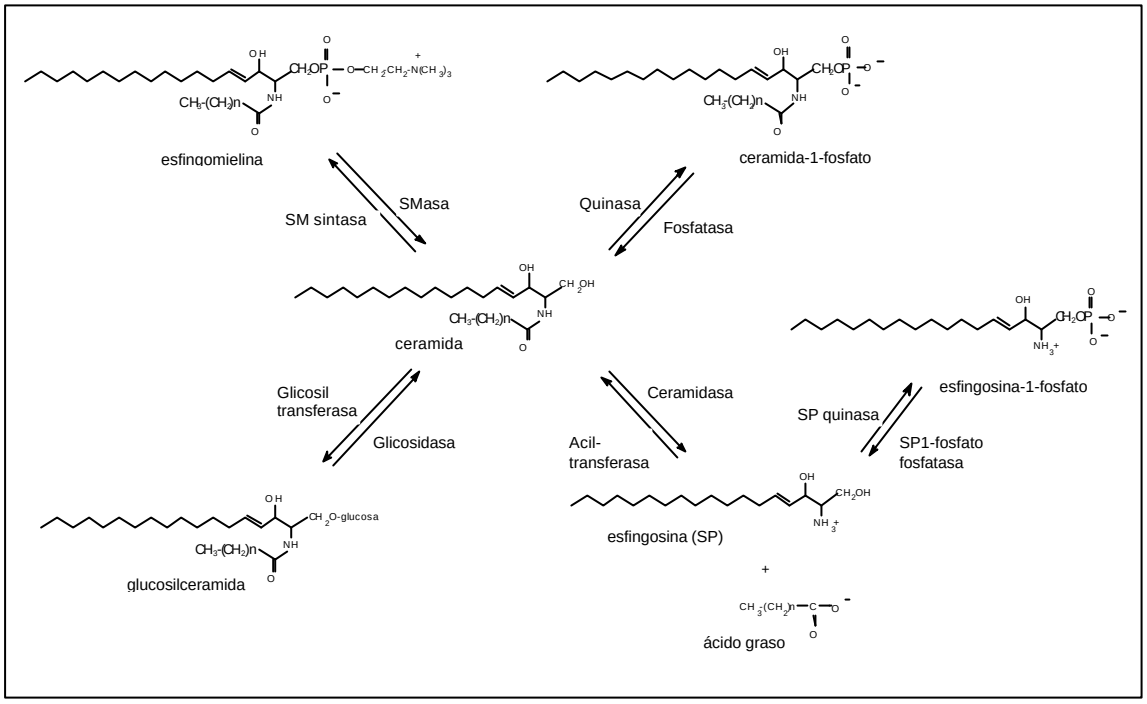

Figura 6. Esquema del papel central de la ceramida en el metabolismo de esfingolípidos.

- *Mecanismo de acción*

La ceramida regula indirectamente la actividad de un gran número de enzimas y componentes de señalización, entre los que se incluyen quinasas, fosfatasas, fosfolipasas, factores de transcripción y caspasas. Sin embargo, los blancos primarios de la acción de la ceramida son aún poco conocidos.

Una de las posibles dianas de la ceramida es la proteína quinasa activada por ceramida (CAPK). La CAPK, caracterizada por el grupo de Kolesnick como quinasa supresora de Ras (KSR) (Zhang et al., 1997), es capaz de fosforilar y activar Raf-1, provocando así la activación de la cascada de proteína quinasas activadas por señales extracelulares (ERK) (Yao et al., 1995; Xing y Kolesnick, 2001). Se ha sugerido que KSR podría estar implicada en la apoptosis inducida por ceramida (Basu et al., 1998; Xing et al., 2000). Sin embargo, otros autores no han sido capaces de demostrar esto (Michaud et al., 1997; Müller et al., 1998). Huwiler et al. (1996) publicaron que Raf-1 podría ser una quinasa activada directamente por ceramida, lo que podría explicar cómo la ceramida activa la cascada ERK. Sin embargo, Müller et al. (1998) no fueron capaces de reproducir este efecto.

La proteína fosfatasa activada por ceramida (CAPP), una fosfatasa de tipo 2A, también se ha identificado como posible diana de la ceramida (Dobrowsky et al., 1993). Aunque el grupo de Hannun ha trabajado afanosamente en esta fosfatasa (Chalfant et al., 1999; Hannun y Luberto, 2000), su papel señalizador es dudoso, aunque parece mediar la desfosforilación e inactivación de la proteína quinasa B (PKB) (Schubert et al., 2000). Sin embargo, esta relación entre ceramida, CAPP y PKB no es tan clara (Stratford et al., 2001).

Una tercera proteína, la proteína quinasa C ζ (PKCζ), puede unir directamente ceramida y resultar activada (Lozano et al., 1994; Galve-Roperh et al., 1997; Bourbon et al., 2000). El ácido araquidónico compite con la ceramida, inhibiendo así la activación que ésta ejerce sobre la PKCζ (Müller et al., 1995).

SERINA PALMITOILTRANSFERASA

La SPT es la enzima clave en la biosíntesis de ceramida; se encuentra situada en el retículo endoplásmico y cataliza la condensación de palmitoil-CoA y serina para generar 3-cetoesfinganina en una reacción dependiente de piridoxal 5´-fosfato (Dickson et al., 1999).

- *Estructura*

Se han estudiado numerosas propiedades de esta enzima y, sin embargo, su total purificación no se ha conseguido aún debido a que presenta una actividad bastante baja. La caracterización de la SPT se realizó primeramente en levaduras; la enzima está codificada por dos genes, LCB1 y LCB2, que presentan un 22 % de homología y codifican dos proteínas (Lcb1p y Lcb2p, de 62 y 63 kDa respectivamente) con actividad 5-aminolevulinato sintasa, 2-amino-oxobutirato-CoA ligasa y 8-amino-7-oxononanoato sintasa; ambos genes son requeridos para que la enzima exhiba actividad. Además, se ha caracterizado un tercer gen, TSC3, requerido para el ensamblado adecuado de la SPT. Este gen codifica para una proteína de 14 kDa, que actuaría post-traduccionalmente y se asociaría a las dos subunidades estimulando la actividad SPT (Dickson et al., 1999; Gable et al., 2000).

Se ha llevado a cabo el aislamiento y caracterización en ratón y humano de los cDNA de LCB1 y LCB2; de la expresión de estos genes se obtuvieron dos proteínas de 53 kDa y 63 kDa respectivamente, que además no contenían péptido señal ni se glicosilaban. A diferencia de la SPT de levaduras, para que la enzima sea activa sólo es necesaria la expresión de la subunidad LCB2. Por *Northern blot* se determinó el tamaño de los mRNAs de LCB1 y LCB2 (2.9 kb y 2.3 kb, respectivamente) y su distribución tisular (Tabla 2). Parece que el residuo Lys 397 de la subunidad catalítica LCB2 es esencial para la actividad catalítica de la SPT (Weiss y Stoffel, 1997; Hanada et al., 1998; Dickson et al., 1999).

Características.	*Subunidades*	
	mLCB1	*mLCB2*
Peso molecular	53 kDa	63 kDa
K_m para serina	0.6 mM	
K_m para palmitoil-CoA	0.05 mM	
K_m para piridoxal fosfato	2.5 µM	
Expresión		
Hígado	+	+
Músculo	+++	++
Corazón	+++	++++
Riñón	+++	++++
Pulmón	++++	++
Cerebro	+++	++

Tabla 2. Características de la SPT de ratón. n.d. no determinado. Los signos + indican de un modo cualitativo la abundancia de las dos subunidades en los distintos tejidos.

- *Regulación*

Existen pocos estudios acerca de la regulación de la SPT. La angiotensina II, al unirse al receptor AT_2, induce apoptosis en células PC12 por un incremento en la actividad de la SPT y, por tanto, en los niveles de ceramida. Esta acumulación de ceramida y muerte por apoptosis es inhibida por fumonisina B1 y β-cloro-L-alanina (inhibidores de la ruta de síntesis *de novo*), así como por la toxina pertúsica (inhibidora del sistema de transducción acoplado al receptor AT_2) (Lehtonen et al., 1999). El etopósido, una droga quimioterapeútica, es capaz de activar la ruta de síntesis *de novo* de esfingolípidos. Esta sustancia induce apoptosis en células leucémicas humanas produciendo un incremento en la formación de ceramida por un aumento en la actividad de la SPT a nivel post-transcripcional, probablemente por alguna modificación covalente o por regulación alostérica de la enzima, pero no por cambios en los niveles de mRNA de la misma (Perry et al., 2000). Este mismo tipo de regulación ha sido también observada por Herget et al. (2000) en el proceso de diferenciación neuronal por ácido

retinoico. En el caso de la radiación ultravioleta, ésta incrementa la actividad de la SPT y la síntesis de esfingolípidos en queratinocitos por un aumento en los niveles de mRNA y proteína de las subunidades LCB1 y LCB2 (Farrell et al., 1998). De esta forma, diversos agentes actúan regulando la enzima clave de la ruta de síntesis *de novo* de esfingolípidos tanto a nivel transcripcional como post-traduccional.

Se han identificado una serie de inhibidores de la SPT, que constituyen herramientas muy útiles para el estudio y regulación de la ruta, la posible implicación de la enzima y el origen de los productos obtenidos. Estos inhibidores son por ejemplo la L-cicloserina y las β-haloalaninas, tales como la β-cloro-L-alanina y la β-fluoro-L-alanina, aunque estas últimas no son necesariamente específicas de la SPT y pueden afectar también a otras enzimas que utilizan piridoxal 5´-fosfato como cofactor. Se han descrito además diversos inhibidores con mayor especificidad como son las esfingofunginas, la ISP1/myriocina, la lipoxamicina y las viridiofunginas (Dickson et al., 1999; Kolter y Sandhoff, 1999).

1.4 Objetivos

Como ya se ha comentado anteriormente, está ampliamente aceptado que los astrocitos desempeñan un papel muy activo en la regulación del metabolismo de la glucosa en el cerebro (Magistretti y Pellerin, 1996; Wiesinger et al., 1997). Así, los astrocitos capturan y metabolizan glucosa con gran actividad. Uno de los principales productos del metabolismo de la glucosa es el lactato, que podría cederse a las neuronas y ser utilizado por éstas como sustrato de rutas biosintéticas y catabólicas. Además, el glucógeno constituye una importante fuente de energía para el cerebro; el glucógeno cerebral se almacena principalmente en los astrocitos, y su tasa de recambio es muy alta y está coordinada con la actividad sináptica.

Los estudios realizados hasta el momento sobre metabolismo de astrocitos se han centrado principalmente en el metabolismo de glucosa y muy pocos de ellos han profundizado en el metabolismo de ácidos grasos. La glucosa es la principal fuente de energía del cerebro, pero bajo determinadas circunstancias los cuerpos cetónicos procedentes de la oxidación hepática de ácidos grasos pueden reemplazar a la glucosa como fuente energética (Zammit, 1994). Se cree que el hígado es el órgano que mayoritariamente aporta cuerpos cetónicos a los tejidos extrahepáticos. Sin embargo, los ácidos grasos pueden ser oxidados en el cerebro (Auestad et al., 1991; Edmond, 1992). Los astrocitos son precisamente las únicas células del cerebro que pueden oxidar ácidos grasos y que además muestran preferencia por éstos como combustible sobre la glucosa y los cuerpos cetónicos (Edmond et al., 1987; Edmond, 1992; Staub et al., 1995). Es más, los astrocitos en cultivo son capaces de producir cuerpos cetónicos a partir de ácidos grasos (Auestad et al.,1991) y leucina (Bixel y Hamprecht, 1995). Todo esto plantea la cuestión de si los astrocitos podrían proveer a las neuronas *in vivo* con cuerpos cetónicos como fuente de energía alternativa a la glucosa.

Puesto que aún no se conocen los mecanismos que controlan la ruta cetogénica en astrocitos, el primer objetivo global de este trabajo fue la caracterización de la ruta cetogénica en astrocitos, así como el estudio de los posibles mecanismos que la regulan, poniendo especial énfasis en la CPT-I, enzima reguladora de la oxidación de ácidos grasos en otros tejidos. Para ello se utilizó como modelo experimental cultivos primarios de astrocitos de rata, y como posibles mecanismos reguladores de la cetogénesis los mediados por la proteína quinasa dependiente de cAMP (PKA) y la AMPK, así como los dependientes de ceramida. Además, y a la vista de los últimos estudios que se han llevado a cabo sobre la apoptosis inducida por ácidos grasos en distintos tipos celulares, nos propusimos como segundo objetivo global el estudio de este proceso en nuestro modelo experimental, así como su posible regulación por la AMPK con el fin de desvelar un posible papel fisiológico.

1.5 BIBLIOGRAFÍA

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2. RESULTADOS Y DISCUSIÓN I
CETOGÉNESIS EN ASTROCITOS: CARACTERIZACIÓN Y REGULACIÓN

ROLE OF CARNITINE PALMITOYLTRANSFERASE I IN THE CONTROL OF KETOGENESIS IN PRIMARY CULTURES OF RAT ASTROCYTES

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Abbreviations used: ACC, acetyl-CoA carboxylase; ACC-265, 265-KDa isoform of ACC; ACC-280, 280-KDa isoform of ACC; CPT-I, carnitine palmitoyltransferase I; TDGA, tetradecylglycidic acid.

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ABSTRACT: The role of carnitine palmitoyltransferase I (CPT-I) in the control of ketogenesis was studied in primary cultures of rat astrocytes. Ketone bodies were the major product of [^{14}C]palmitate oxidation by cultured astrocytes, whereas CO_2 made a minor contribution to total oxidation product. Using tetradecylglycidate as a specific, cell-permeable inhibitor of CPT-I, a flux control coefficient of 0.77±0.07 was calculated for CPT-I over the flux of [^{14}C]palmitate to ketone bodies. CPT-I from astrocytes was sensitive to malonyl-CoA (IC_{50} = 3.4±0.8 μM) and cross-reacted on Western blots with an antibody raised against liver CPT-I. On the other hand, astrocytes expressed significant acetyl-CoA carboxylase (ACC) activity and consequently they contained considerable amounts of malonyl-CoA. Western blot analysis of ACC isoforms showed that ACC in astrocytes -like in neurons, liver and white adipose tissue- mostly comprised the 265-KDa isoform, whereas the 280-KDa isoform -which was highly expressed in skeletal muscle- showed much lower abundance. Forskolin was used as a tool to study the modulation of the ketogenic pathway in astrocytes. Thus, forskolin decreased in parallel ACC activity and intracellular malonyl-CoA levels, whereas it stimulated CPT-I activity and [^{14}C]palmitate oxidation to both ketone bodies and CO_2. Results show that in cultured astrocytes (i) CPT-I exerts a very high degree of control over ketogenesis from palmitate, (ii) the ACC/malonyl-CoA/CPT-I system is similar to that of liver, and (iii) the ACC/malonyl-CoA/CPT-I system is regulable by cAMP. *Key words:* Astrocytes; Fatty acids; Ketone bodies; Carnitine palmitoyltransferase I; Acetyl-CoA carboxylase; Malonyl-CoA.

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It is currently well established that astrocytes play an active role in the regulation of brain glucose metabolism (Magistretti and Pellerin, 1996). Thus, astrocytes take up and metabolize glucose at higher rates than neurons (Magistretti and Pellerin, 1996; Vanucci et al., 1997). One of the major products of glucose metabolism in astrocytes is lactate, which may be delivered to neurons and used by these cells as a substrate for biosynthetic and catabolic pathways (Magistretti and Pellerin, 1996; Wiesinger et al., 1997). In addition, glycogen is the single largest energy reserve of the brain, and is mainly localized in astrocytes (Magistreti and Pellerin, 1996; Wiesinger et al., 1997); glycogen turnover in the brain is extremely rapid and finely coordinated with synaptic activity (Magistretti and Pellerin, 1996). Accruing evidence shows that glucose utilization by astrocytes is sensitive to a great number of mediators such as neurotransmitters (e.g. vasoactive intestinal peptide, norepinephrine) and cytokines (e.g. TNFα, interleukin-1a) (Pellerin et al., 1997).

Although the pathways for glucose metabolism in astrocytes have received considerable attention, fewer studies have focussed on fatty acid metabolism in these cells. Glucose is the primary fuel for the brain, but ketone bodies may replace glucose as the major source of neuronal energy metabolism in patho-physiological

situations in which energy deprivation ensues (Zammit, 1994; Magistretti and Pellerin, 1996). The liver is generally believed to be the major organ that supplies extra-hepatic tissues with ketone bodies as a glucose-replacing fuel (Zammit, 1994; McGarry and Brown, 1997). However, numerous studies have demonstrated that fatty acids are readily taken up and oxidized by the brain (cf. Auestad et al., 1991; Edmond, 1992). Among the different brain cell populations, astrocytes are the only one that can oxidize fatty acids and that exhibit a preference for fatty acids over glucose or ketone bodies as their primary metabolic fuel (Edmond et al., 1987; Edmond, 1992; Staub et al., 1995). Furthermore, astrocytes in culture are actually able to produce ketone bodies from fatty acids (Auestad et al., 1991; Edmond, 1992) as well as from branched-chain amino acids (Bixel and Hamprecht, 1995). This raises the intriguing question of whether astrocytes might provide neurons with ketone bodies as a glucose-replacing fuel in vivo.

Ketone body production by liver mitochondria is a complex, finely regulated process (Guzmán and Geelen, 1993; Zammit, 1994). It is well established that carnitine palmitoyltransferase I (CPT-I), the carnitine palmitoyltransferase located in the the mitochondrial outer membrane, catalyzes the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix and is a key regulatory site of long-chain fatty acid oxidation overall (Guzmán and Geelen, 1993; Zammit, 1994; McGarry and Brown, 1997). CPT-I is subject to long-term regulation in response to alterations in the nutritional and hormonal status of the animal (Zammit, 1994; McGarry and Brown, 1997). Short-term control of CPT-I activity involves inhibition by malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase (ACC) (McGarry and Foster, 1980; McGarry and Brown, 1997). Since the latter enzyme is a key regulatory site of fatty acid synthesis *de novo* (cf. Guzmán and Geelen, 1993; Zammit, 1994), malonyl-CoA inhibition of CPT-I allows an elegant explanation for the coordinate control of the partition of hepatic fatty acids into esterification and oxidation. More recent studies have also put forward the existence of a malonyl-CoA-independent mechanism of acute control of rat liver CPT-I activity. This novel mechanism involves the modulation of interactions between cytoskeletal components and the mitochondrial outer membrane (Velasco et al., 1996; 1997ab).

The mechanisms that control the flux through the ketogenic pathway in astrocytes are as yet unknown. In addition, the potential patho-physiological importance of astroglial ketogenesis could be clearly reinforced by showing that it is a process subject to regulation by extra- and intracellular modulators. However, as far as we know, this possibility has not been studied to date. The present work was therefore undertaken to study the potential regulatory mechanisms that control the rate of ketogenesis in astrocytes in culture, with especial emphasis on CPT-I.

MATERIALS AND METHODS

Materials

[1-^{14}C]palmitic acid, L-[*Me*-^{3}H]carnitine, [1-^{14}C]acetyl-CoA and [^{35}S]-streptavidin were from Amersham International (Amersham, Bucks, UK). Tetradecylglycidic acid (TDGA) was kindly donated by Dr. J.M. Lowenstein (Brandeis University, Waltham, MA). The anti rat-liver CPT-I polyclonal antibody was kindly given by Dr. V.A. Zammit (Hannah Research Institute, Ayr, United Kingdom). The anti rat-liver ACC polyclonal antibody was kindly given by Dr. Math J.H. Geelen (Utrecht University, The Netherlands). Digitonin was from Sigma Chemical Co. (St. Louis, MO, USA). Forskolin and okadaic acid were from Calbiochem (San Diego, CA, USA). Foetal calf serum and all plastic material for cell cultures were from Nunc (Roskilde, Denmark).

Astrocyte and neuron cultures

Cortical astrocytes were derived from 1-2 day-old rats and cultured as described by Galve-Roperh et al. (1997). Briefly, cells were seeded at a density of ca. $3x10^4$ cells/cm^2 on plastic plates previously coated with 5 µg/ml L-polyornithine in water. Cells were cultured for 3 weeks in a mixture of DMEM medium and Ham's F12 medium (1:1, v/v) supplemented with 0.66% (w/v) glucose, 5 µg/ml streptomycin, 5 U/ml penicillin and 10% FCS. Cell cultures consisted of at least 95% astrocytes as judged by immunocytochemical staining of glial fibrilary acidic protein (Galve-Roperh et al., 1997). All the animal protocols followed the guidelines of the Spanish Ministry of Health.

For all the experimental determinations performed (see below), 48 h before the experiment the FCS-containing medium was removed and cells were

transferred to a chemically-defined, serum-free medium consisting of DMEM/Ham's F12 (1:1, v/v) supplemented with 25 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, 30 nM sodium selenite and 1.0% (w/v) defatted and dialyzed bovine serum albumin. Stock solutions of TDGA, forskolin and okadaic acid were prepared 1000X in Me_2SO. Then, control incubations had the corresponding Me_2SO content. No significant influence of Me_2SO on any of the parameters determined was observed at the final concentration used (0.1%, v/v). Experiments were performed at a cell density of ca. $6x10^4$ cells (=7 mg protein)/cm^2.

Primary cultures of newborn-rat cortical neurons were used in some experiments for comparative purposes. Neurons were cultured essentially as described by O'Malley et al. (1993). Briefly, cells were seeded at a density of ca. $3x10^5$ cells/cm^2 on plastic plates previously coated with 50 μg/ml L-polylysine in water. Cells were cultured in a mixture of DMEM and Ham's F12 medium (1:1, v/v) supplemented with 5 μg/ml streptomycin, 5 U/ml penicillin and 15% FCS. After 24 h, the FCS-containing medium was removed and cells were further cultured in a chemically-defined, serum-free medium consisting of DMEM and Ham's F12 medium (1:1, v/v) supplemented with 6 mg/ml glucose, 25 μg/ml insulin, 100 μg/ml human transferrin, 20 nM progesterone, 60 μM putrescine and 30 nM sodium selenite. Cells were maintained in this medium for 6 days and then used for the experiments.

Determination of the rate of [^{14}C]palmitate oxidation

Astrocytes were incubated as described above in 25-cm^2 flasks. Reactions were started by the addition of 0.15 mM (final concentration) albumin-bound [1-^{14}C]palmitate (0.5 μCi) plus 0.5 mM (final concentration) L-carnitine to the cell cultures, and stopped with 0.3 ml of 2 M $HClO_4$ after 2 h (standard assay). At the same time, 0.15 ml of benzethonium hydroxide (1 M in methanol) were injected in a center well containing filter paper. Samples were allowed to equilibrate for 12 additional h, and the center well (with the $^{14}CO_2$ fixed as bicarbonate) was transferred to vials for radioactive counting (Sánchez et al., 1997). The cell precipitates were neutralized with K_2CO_3 and used to quantitate ketone bodies as acid-soluble products (Guzmán and Geelen, 1992). Total oxidation products were determined as the sum of CO_2 plus acid-soluble products.

Assay of CPT-I activity

CPT-I activity was determined in digitonin-permeabilized astrocytes as the TDGA-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine by an adaptation of the method routinely used in our laboratory for cultured hepatocytes. Thus, astrocytes (cultured in P6 plates) were preincubated for 45 min in the absence or in the presence of 20 μM TDGA, a specific irreversible inhibitor of CPT-I (Guzmán and Geelen, 1992; McGarry and Brown, 1997). The medium was then aspirated and cells were washed twice with PBS. Cells were subsequently permeabilized with 700 μl of a medium containing 50 mM imidazole, pH 7.1, 70 mM KCl, 80 mM sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithioerythritol, 1mM KCN, 1 mM ATP and 0.1% (w/v) defatted and dialyzed bovine serum albumin (medium A), supplemented with 100 μl of 0.2 mg/ml digitonin (Spurway et al., 1997). The medium was aspirated after 3 min and reactions were started by the addition of 700 μl of medium A supplemented with 100 μl of 0.4 mM palmitoyl-CoA plus 4 mM L-[*Me*-^{3}H]carnitine (0.3 μCi). After 4 min (standard assay) at 37°C, reactions were stopped with 0.8 ml of 2 M HCl and [^{3}H]palmitoylcarnitine product was extracted with n-butanol (Guzmán and Geelen, 1992). When the inhibitory effect of malonyl-CoA was tested (Fig. 5), cells were permeabilized and subsequently preincubated for 3 min with 700 μl of medium A containing the indicated concentrations of malonyl-CoA before the enzyme substrates were added.

Calculation of the flux control coefficient of CPT-I

Flux control coefficients were calculated as the ratio of the initial slope of the inhibition curve of ketogenesis by TDGA to the initial slope of the inhibition curve of CPT-I by TDGA (Drynan et al., 1996; Spurway et al., 1997). Initial slopes were calculated from the straight lines fitted to data obtained at low (≤ 100 nM) concentrations of TDGA using linear least squares regression.

Western blot analysis of CPT-I

Astrocytes were cultured in P100 plates. The medium was aspirated, and cells were washed twice with PBS and scraped in 500 μl of a medium containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM EGTA, 10 mM

2-mercaptoethanol, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml benzamidine. Cells were sonicated (2x5 s) on ice and the particulate fraction was obtained after centrifugation at 40,000 *g* for 60 min (Galve-Roperh et al., 1997). Samples were subjected to SDS-PAGE using 1.0-mm thick 10% polyacrylamide gels. Stacking and resolving buffer pH values were adjusted to 6.8 and 8.8, respectively. Proteins were transferred from SDS gels onto nitrocellulose membranes. The blots were then blocked with 5% fat-free dried milk in PBS supplemented with 0.1% Tween 20. They were subsequently incubated with the anti-CPT-I antibody (1:10,000) in PBS/Tween 20 for 2 h at 4°C, and washed thorougly. The blots were then incubated with anti-sheep peroxidase-conjugated secondary antibody (1:15,000) for 1 h at room temperature, and finally subjected to luminography with an ECL detection kit. For comparative purposes, mitochondria from rat liver, skeletal muscle and white adipose tissue -isolated according to Guzmán et al. (1994)- as well as membrane fractions (40,000 *g* supernatants) of newborn-rat cortical neurons were used in parallel.

Assay of ACC activity

ACC activity was determined in digitonin-permeablized astrocytes as the incorporation of radiolabelled acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction by an adaptation of the method routinely used in our laboratory for cultured hepatocytes. This method avoids the number of interferences inherent to the classical bicarbonate-fixation assay of ACC activity (Bijleveld and Geelen, 1987). Thus, astrocytes (cultured in P6 plates) were washed twice with PBS. Reactions were subsequently started by the addition of 250 μl of PBS plus 250 μl of assay mixture containing 126 mM Hepes, pH 7.5, 20 mM NaCl, 4.2 mM $MgCl_2$, 1.0 mM citrate, 20 mM $NaHCO_3$, 4.0 mM ATP, 1.0 mM NADPH, 1.0 mM dithioerythritol, 2.0% (w/v) bovine serum albumin (defatted and dialyzed), 80 μM [1-^{14}C]acetyl-CoA (0.05 μCi), 80 μM butyryl-CoA, 3.0 mU of purified rat liver fatty acid synthase and 0.05 mg/ml digitonin. After 5 min (standard assay) at 37°C, reactions were stopped with 0.25 ml of 10 M NaOH and fatty acids were extracted for radioactive counting (Guzmán et al., 1995).

Western blot analysis of ACC isoforms

Astrocytes cultured in P100 plates were treated exactly as described above for the Western blot analysis of CPT-I. After sonication, samples were centrifuged at 20,000 *g* for 15 min. The resulting post-mitochondrial supernatants were used for Western blot analysis of ACC isoforms, which was performed by a previously described procedure which involves immunoprecipitation, incubation with [^{35}S]streptavidin, SDS-PAGE, immunoblotting, autoradiography, and scanning of the autoradiograms (Guzmán et al., 1995). As in the case of CPT-I, post-mitochondrial supernatants of rat liver, skeletal muscle, white adipose tissue and newborn-rat cortical neurons were used in parallel for comparative purposes.

Other analytical procedures

Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid extracts by a radioenzymatic method (Guzmán et al., 1995). The activities of lactate dehydrogenase and glutamate dehydrogenase were calculated by standard spectrophotometric methods (Bergmeyer, 1985).

Statistical analysis

Results shown represent the means±S.D. of the number of experiments indicated in every case. Five-six different replicates of the various conditions included in each experiment were routinely performed. Statistical analysis was performed by the Student t-test.

RESULTS

[^{14}C]palmitate oxidation by cultured astrocytes

Astrocytes were incubated with [^{14}C]palmitate and the time-course of both acid-soluble product and CO_2 formation was determined. As shown in Fig. 1, acid-soluble products made a major contribution (85-90%) to total oxidation products, whereas CO_2 made only a minor contribution (10-15%). Two methods were used to determine the nature of the acid-soluble products present in the medium after incubation of cultured astrocytes with [^{14}C]palmitate in the conditions described above: (i) ^{14}C-labelled acid-soluble products were characterized by anion-exchange chromatography exactly as described by Lopes-Cardozo and Klein (1982). (ii) The amount of 3-hydroxybutyrate and acetoacetate in total acid-soluble products was also determined by

standard spectrophotometric methods using 3-hydroxybutyrate dehydrogenase (Bergmeyer, 1995). In line with Auestad et al. (1991), the two methods showed that ketone bodies accounted for more than 90% of total acid-soluble products (results not shown). Therefore, in subsequent experiments ketogenesis was determined as the rate of acid-soluble products. A reaction time of 2 h was chosen for subsequent standard determinations.

Properties of CPT-I in cultured astrocytes

Experiments were carried out to design an assay of CPT-I activity in permeabilized astrocytes. Permeabilizing cells with digitonin opens the possibility of investigating intracellular enzymes in a more or less natural environment and alleviates the necessity of preparing cellular fractions for enzyme assay (cf. Bijleveld and Geelen, 1987; Guzmán and Geelen, 1988). As mitochondria stay within the cell at the concentrations of digitonin used in the present study (cf. Guzmán and Geelen, 1988; 1992), CPT-I activity is assayed in situ. The use of this procedure may be especially important for CPT-I, since it has been shown that the activity of rat liver CPT-I is affected by extra-mitochondrial cell components that are lost upon isolation of mitochondria (e.g. Guzmán et al., 1994).

In a first series of experiments, the optimal concentration of digitonin for measuring CPT-I activity in cultured astrocytes was determined. As shown in Fig. 2, exposure of astrocytes to digitonin at 0.025 mg/ml produced a rapid and considerable permeabilization of the plasma membrane, as determined by the release of lactate dehydrogenase, a cytosolic marker enzyme. The integrity of mitochondria was proved by the fact that less than 3% of total glutamate dehydrogenase, a mitochondrial-matrix marker enzyme, was liberated from the permeabilized cells during the first 3 min of exposure to digitonin. Carnitine palmitoyltransferase activity followed the same behaviour as GDH activity (Fig. 2). However, after 3 min of exposure of the cells to that concentration of digitonin a low but significant release of both glutamate dehydrogenase and CPT-I was evident (Fig. 2). Higher digitonin concentrations produced a serious damage of mitochondria, whereas lower digitonin concentrations failed to significantly permeabilize the plasma membrane (results not shown). Therefore, astrocyte permeabilization was achieved in subsequent experiments by exposing cells to 0.025 mg/ml digitonin for 3 min.

Fig. 3 shows CPT-I activity in the permeabilized-cell procedure as a function of time of assay. The time course of the reaction shows that exposure of the enzyme to the substrates must be very quick since no lag phase was observed. The reaction was linear at least for up to 4 min, enzyme activity slightly declining between 4 and 6 min. Further assays were carried out for 4 min.

The assay of CPT-I activity in permeabilized astrocytes was used to study the role of CPT-I in the control of the flux through the ketogenic pathway. TDGA is a specific, irreversible, cell-permeable inhibitor of CPT-I that may be used to titrate CPT-I activity in whole-cell systems (Guzmán and Geelen, 1992) and therefore it allows determination of the flux control coefficient of CPT-I over the ketogenic pathway (Drynan et al., 1996; Spurway et al., 1997). As shown in Fig. 4, the pattern of inhibition of CPT-I by increasing concentrations of TDGA closely resembled that of the rate of ketogenesis from [^{14}C]palmitate. Thus, the value of the flux control coefficient of CPT-I, calculated from the initial slopes of the plots in Fig. 4, was very high (0.77±0.07).

Fig. 5 shows the plot of CPT-I inhibition by malonyl-CoA in digitonin-permeabilized astrocytes. As in other types of cells (McGarry and Brown, 1997), CPT-I from astrocytes was sensitive to malonyl-CoA. The IC_{50} value of malonyl-CoA inhibition was 3.4±0.8 µM, a value similar to that displayed by the liver CPT-I isoform (McGarry and Brown, 1997). To confirm that astrocytes express the liver isoform of CPT-I, Western blot analyses of CPT-I were conducted. A major band of 88 KDa, which corresponds to the molecular weight of rat liver CPT-I (McGarry and Brown, 1997), was observed in the blots. As shown in Fig. 6, the antibody raised against rat liver CPT-I recognized the enzyme from astrocytes and neurons. The specificity of the antibody was proved by the fact that it recognized liver CPT-I but not skeletal muscle CPT-I (Fig. 6). Adipose tissue expresses low levels of the liver-type CPT-I (Brown et al., 1997; McGarry and Brown, 1997), and therefore a very weak band was evident in the luminograms (Fig. 6).

Properties of ACC in cultured astrocytes

ACC is the enzyme responsible for malonyl-CoA synthesis, and therefore its activity determines that of CPT-I (Zammit, 1994; McGarry and Brown, 1997). Experiments were carried out to design an assay of ACC

activity in permeabilized astrocytes. Since ACC is mostly cytosolic and it leaks from permeabilized cells at the concentrations of digitonin used (Bijleveld and Geelen, 1987), cell permeabilization and assay of enzyme activity were simultaneously performed. Fig. 7 shows the time course of ACC activity in the permeabilized-cell assay. The reaction was linear for up to 5 min and -like in the case of CPT-I- did not show any lag phase. Further assays of ACC activity were carried out for 5 min.

The presence of significant ACC activity in cultured astrocytes indicate that malonyl-CoA must be present in these cells. This was demonstrated by measuring intracellular malonyl-CoA levels in astrocyte extracts. Thus, Table 1 shows that malonyl-CoA was indeed present in astrocytes at significant levels.

Two different ACC isoforms with distinct kinetic/regulatory properties and tissue distribution have been characterized to date, *viz.* a 265-KDa isoform (ACC-265) and a 280-KDa isoform (ACC-280) (Bianchi et al., 1990; Lopaschuk et al., 1994; Ha et al., 1996). Fig. 8 shows the separation pattern of the two ACC isoforms from astrocytes. Astrocytes expressed mostly ACC-265 (90-95% of total ACC) together with a small amount of ACC-280 (5-10% of total ACC). This ACC isoform pattern was similar to that of neurons (Fig. 8). In line with previous reports, ACC-265 was also the major ACC isoform in liver (75-80% of total ACC) and adipose tissue (95-100% of total ACC), whereas skeletal muscle exclusively expressed ACC-280 (Bianchi et al., 1990; Guzmán et al., 1995).

Modulation of the ACC/malonyl-CoA/CPT-I system by forskolin

Fatty acid oxidation is modulated by the cAMP signalling pathway in many cell types (Guzmán and Geelen, 1993; Zammit, 1994). In addition, glucose uptake and utilization by astrocytes in culture is sensitive to changes in the intracellular concentration of cAMP (Pellerin et al., 1997; Wiesinger et al., 1997). Hence, forskolin, a cell-permeable activator of adenylyl cyclase, was used as a tool to test whether ketone body production by cultured astrocytes may be modulated by an elevation of intracellular cAMP levels (Table 1). Forskolin decreased in parallel ACC activity and the intracellular levels of malonyl-CoA. This was concomitant to a remarkable stimulation of [^{14}C]palmitate oxidation to both ketone bodies and CO_2. Although cell permeabilization leads to the leakage of cytosolic metabolites -including malonyl-CoA- in the extracellular medium (Guzmán and Geelen, 1988; Guzmán et al., 1994), and this is removed before the assay of CPT-I activity is performed, an increase in CPT-I activity was observed after challenge of astrocytes to forskolin. Likewise, okadaic acid, an inhibitor of protein phosphatases 1 and 2A which stimulates hepatic CPT-I (Guzmán and Geelen, 1992), produced a 53±16% stimulation of CPT-I in cultured astrocytes when enzyme activity was determined by the permeabilized-cell procedure.

DISCUSSION

Previous studies on metabolic regulation in cultured astrocytes have mostly focussed on carbohydrate (e.g. Magistretti and Pellerin, 1996; Wiesinger et al., 1997) and amino acid metabolism (e.g. Sonnewald et al., 1997; Tsacopulos et al., 1997). Instead, the present study focussed on the regulation of ketone body production by astrocytes. The general conclusion that may be inferred from our data is that astrocytes closely resemble hepatocytes (paradigmatic ketogenic cells) in many aspects concerning ketone body production. This makes astroglial ketogenesis a metabolic pathway with potential physiological relevance. The common properties of hepatic and astroglial ketogenesis are discussed below.

Ketone body production in astrocytes compared to hepatocytes

Cultured astrocytes produce ketone bodies at rates similar to those of hepatocytes (e.g. Guzmán and Geelen, 1992; Guzmán et al., 1995). In addition, ketone bodies constitute the bulk of the oxidation products in astrocytes (the present report) and hepatocytes (Zammit, 1994). Moreover, both astrocytes (Edmond, 1992) and hepatocytes (Zammit, 1994) exhibit a preference for fatty acids, compared with glucose, as their primary metabolic fuel. Some studies performed in liver cells have put forward the notion of substrate channeling between the enzymes of the β-oxidation spiral and the ketogenic route as well as between pyruvate oxidation and the tricarboxylic acid cycle, i.e. acetyl-CoA produced by β-oxidation of fatty acids would be preferentially diverted into the ketogenic pathway, whereas that formed by pyruvate dehydrogenase would be mostly employed by the tricarboxylic acid cycle (cf. Zammit, 1994; Eaton et al.,

1996). Other studies have shown that in liver the ketogenic pathway may serve as an effective drain of acetyl-CoA, since the activity of the tricarboxylic acid cycle in liver mitochondria under basal conditions is relatively close to saturation (cf. Guzmán and Geelen, 1993; Zammit, 1994). The present data indicate that this preferential diversion to the ketogenic pathway of the excess of acetyl-CoA produced by fatty acid β-oxidation could also occur in astrocytes.

Regulatory role of CPT-I in astrocytes compared to hepatocytes

The control exerted by CPT-I over the flux through the ketogenic pathway is consistently high in both astrocytes (the present report) and hepatocytes (Drynan et al., 1996; Spurway et al., 1997). Regulation of ketogenesis from long-chain fatty acids is a highly integrated process that comprises a number of potential pace-setting steps (Guzmán et al., 1993; Zammit, 1994; Eaton et al., 1996). However, recent determination of flux control coefficients of the enzymes involved in hepatic long-chain fatty acid oxidation shows that CPT-I catalyzes the pace-setting step of hepatic ketogenesis under different substrate concentrations and patho-physiological states (Drynan et al., 1996; Spurway et al., 1997). In addition to CPT-I, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase has been suggested by some authors to be a putative regulatory site of hepatic ketogenesis from acetyl-CoA in situations such as the administration of diets containing medium-chain triacylglycerols (Pégorier et al., 1988), the suckling period (Serra et al., 1993) or the starved-to-fed transition (Casals et al., 1992). However, the role of this enzyme in ketone body production by astrocytes is still unclear. On one hand, careful studies conducted by Auestad et al. (1991) with radiolabelled tracers in cultured astrocytes have shown that acetoacetate is primarily formed by the action of 3-oxoacid-CoA transferase and/or acetoacetyl-CoA deacylase instead of by the action of 3-hydroxy-3-methylglutaryl-CoA synthase. In line with this observation, these authors showed that the activity of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in astrocytes was barely detectable (Auestad et al., 1991). In this respect ketone body production in astrocytes would differ from that in hepatocytes in that hepatic ketogenesis mostly relies on the 3-hydroxy-3-methylglutaryl-CoA cycle (Zammit, 1994). On the other hand, more recent studies have provided evidence for the existence of the mRNA encoding the three enzymes of the 3-hydroxy-3-methylglutaryl-CoA cycle (including mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase) in primary cultures of newborn-rat cortical astrocytes (Cullingford et al., 1998). Further research is therefore necessary to clarify the possible involvement of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in the control of ketogenesis in astrocytes.

Properties of CPT-I in astrocytes compared to hepatocytes

CPT-I from rat liver and from rat astrocytes seems to be the same enzyme. (i) Compared to the muscle CPT-I isoform, which is hypersensitive to malonyl-CoA ($IC_{50} \approx 0.03$ μM), the liver CPT-I isoform has a lower sensitivity to malonyl-CoA (IC_{50} in the low μM range, i.e. like astrocyte CPT-I) (Brown et al., 1997; McGarry and Brown, 1997). Likewise, the sensitivity of CPT-I to the synthetic inhibitor TDGA is similar in astrocytes and in hepatocytes (IC_{50} about 0.2 μM) (Guzmán and Geelen, 1992; Drynan et al., 1996). (ii) Relative to total cell protein, CPT-I activity expressed by astrocytes (the present report) is similar to that of liver (e.g. Guzmán et al., 1992; Velasco et al., 1997ab), which expresses values of CPT-I activity intermediate between those of lipogenic tissues, which have low CPT-I activity (e.g. white adipose tissue, lactating mammary gland), and those of oxidative tissues, which have high CPT-I activity (e.g. skeletal muscle, heart). (iii) The short-term stimulation of hepatic (Guzmán and Geelen, 1993; Guzmán et al., 1994; Velasco et al., 1997ab) and astroglial CPT-I (the present report) by extracellular modulators seems to rely in part on a malonyl-CoA-independent mechanism. It should be kept in mind out that, in order to measure CPT-I activity, we have permeabilized the plasma membrane and this has caused the cytosol to leak out of the cell, leading to a large dilution of cytosolic components including malonyl-CoA (cf. Guzmán and Geelen, 1988; 1992). In addition, the extracellular medium is removed from the astrocyte cultures before CPT-I activity is determined. Thus, although inhibition of CPT-I by malonyl-CoA is a well-described property of the enzyme (McGarry and Foster, 1980; McGarry and Brown, 1997), a more stable regulatory mechanism may be also involved in the short-term control of CPT-I activity by cellular effectors. This mechanism seems to rely on the modulation of the interactions between mitochondria and cytoskeletal

components (Velasco et al., 1996; 1997ab). (iv) CPT-I from rat hepatocytes (McGarry and Brown, 1997) and rat astrocytes exhibit the same electrophoretic mobility (M_r = 88 KDa) and recognition by antibodies. The hepatic CPT-I isoform is also expressed in the kidney, another tissue with ketogenic capacity (McGarry and Brown, 1997).

Properties of ACC in astrocytes compared to hepatocytes

The properties of ACC, the malonyl-CoA-synthesizing enzyme, are similar in astrocytes and hepatocytes. (i) Relative to total cell protein, ACC activity expressed by astrocytes (the present report) is similar to that of hepatocytes (Bijleveld and Geelen, 1987; Velasco et al., 1997a); consequently, malonyl-CoA levels are similar in astrocytes (the present report) and hepatocytes (Guzmán and Geelen, 1992; Guzmán et al., 1995). (ii) The ACC isoform pattern in astrocytes (the present report) is similar to that of hepatocytes (Bianchi et al., 1990; Guzmán et al., 1995) in that the two types of cells express high levels of ACC-265 and low levels of ACC-280. However, ACC-280 is more abundant in liver than in astrocytes. ACC-280 is less sensitive than ACC-265 to allosteric activation by citrate and is especially abundant in tissues with a high fatty acid-oxidative capacity (e.g. heart and skeletal muscle) (Lopaschuk et al., 1994). These and other observations have led to the suggestion that ACC-265 might synthesize malonyl-CoA to be channeled through FAS to lipogenesis, whereas ACC-280 could be involved in the production of malonyl-CoA for CPT-I inhibition (Lopaschuk et al., 1994; Ha et al., 1996). However, experiments performed with eluates obtained after selective damage of the periportal and the perivenous zone of the liver (Witters et al., 1994) or with isolated periportal and perivenous hepatocytes (Guzmán et al., 1995) have shown that no differences in the ACC-265:ACC-280 ratio exist in the two regions of the liver in a number of patho-physiological situations such as starvation, refeeding after starvation and cold exposure, in which the rate of fatty acid oxidation changes. Alternatively, Winz et al. (1994) have suggested a possible role of ACC-280 in controlling the aggregation state of ACC. In any case, the precise role of the two ACC isoforms is as yet unknown.

Possible physiological role of ketogenesis in astrocytes

Astrocytes express a number of receptors (e.g. β-adrenergic receptors, vasoactive intestinal peptide receptors) that are coupled to activation of adenylyl cyclase (Porter and McCarthy, 1997). An increase in the intracellular concentration of cAMP in cultured astrocytes has been shown to stimulate glycogen mobilization, glycolysis, and lactate output to the extracellular medium (Magistretti and Pellerin, 1996; Pellerin et al., 1997). This process has been suggested to play a role in the supply of lactate for neuronal oxidative metabolism in situations in which synaptic activity is enhanced (Magistretti and Pellerin, 1996; Izumi et al., 1997). In this respect it is worth noting that a saturable, specific, high-affinity transport system for monocarboxylates -e.g. lactate- exists in neurons (Dringen et al., 1993). In contrast, the mechanism of lactate transport in astrocytes is still unclear, and both a carrier-mediated mechanism (Tildon et al., 1993) and a simple diffusion process (Dringen et al., 1995) have been suggested to occur. Interestingly, Bittar et al. (1996) have shown that lactate dehydrogenase-5, an isoform that favors lactate production, is found in astrocytes, whereas lactate dehydrogenase-1, an isoform that favors lactate use, is found in neurons. In short, a net flux of lactate from astroglial glycolytic production to neuronal utilization is assumed to exist in brain during physiological activation (Magistretti and Pellerin, 1996).

In line with the aforementioned observations, the ACC/malonyl-CoA/CPT-I system in astrocytes -like in hepatocytes (Guzmán and Geelen, 1993)- is sensitive to acute changes in intracellular cAMP concentration. In particular, forskolin was able to stimulate CPT-I and ketogenesis in astrocytes. Although the extent to which ketone body formation by astrocytes occurs in vivo remains to be determined, it could be suggested that ketone bodies potentially supplied from astrocytes to neurons in situ may be a relevant source of carbon (i) for neuronal oxidative metabolism in situations such as prolonged fasting or enhanced synaptic activity, and (ii) for neuronal biosynthetic processes during postnatal development (Lopes-Cardozo et al., 1986; Edmond, 1992). In addition, fatty acid uptake and utilization by astrocytes may serve as a mechanism for handling elevated concentrations of non-esterified fatty acids occuring in brain in pathological situations such as trauma, ischemia or octanoic acidemia (cf. Edmond, 1992).

We are aware nonetheless that astrocytes and neurons express similar amounts and isoform patterns of CPT-I and ACC. Then why the former are ketone body-producing cells whereas the latter are ketone body-consuming cells? A number of factors additional to those studied in the present report may explain this apparent dilemma. For example, the enzyme activities expressed by neurons regarding fatty acid β-oxidation as well as ketone body synthesis and utilization allow these cells oxidize -rather than produce- ketone bodies (cf. Edmond, 1992). In this respect it is interesting that neuronal CPT-I has been suggested to be actively involved in the remodeling of triglyceride and phospholipid acyl moieties rather than in shuttling acyl groups into mitochondrial oxidative metabolism (Arduini et al., 1994). In addition, ACC activity (and therefore malonyl-CoA levels) inside the cell is dependent e.g. on the intracellular concentration of allosteric activators (e.g. citrate) and inhibitors (e.g. long-chain acyl-CoA), as well as on the activities of the protein kinases and phosphatases involved the covalent modification of the enzyme (cf. Bijleveld and Geelen, 1987; Zammit, 1994). Moreover, irrespective of the mechanism by which long-chain fatty acids enter the brain (cf. Magret et al., 1996), the localization of astrocytes in the blood-brain barrier most likely make these cells the primary site of fatty acid entrance in the brain.

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TABLE 1. *Effect of forskolin on fatty acid oxidation in cultured astrocytes*

Parameter	Additions	
	None	Forskolin
Rate of [^{14}C]palmitate oxidation		
Ketone bodies	57.4±8.8	101.0±9.2*
CO_2	9.1±1.0	16.5±3.0*
Total oxidation product	66.5±7.9	117.5±12.7*
[Malonyl-CoA]	87±4	47±8*
ACC activity	0.24±0.04	0.13±0.02*
CPT-I activity	2.85±0.67	3.99±0.54**

Astrocytes were incubated in the absence or in the presence of 20 μM forskolin for 30 min. Then, cells were used for measurement of [^{14}C]palmitate oxidation, ACC and CPT-I activities, as well as malonyl-CoA concentration. Rates of fatty acid oxidation are expressed as nmol [1-^{14}C]palmitate oxidized/h per mg cell protein. Malonyl-CoA levels are expressed as pmol/mg cell protein. Enzyme activities are expressed as nmol product/min per mg cell protein. Results correspond to 6 different cell preparations. Significantly different from incubations with no additions: *$P<0.01$. ** $P<0.05$.

FIGURES

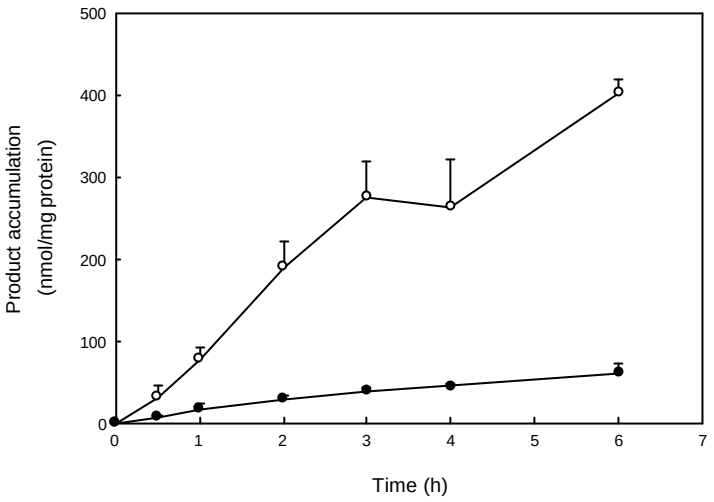


Fig. 1

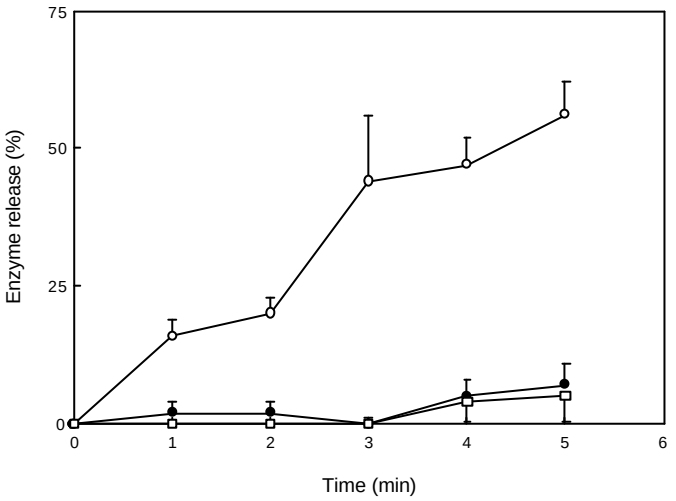
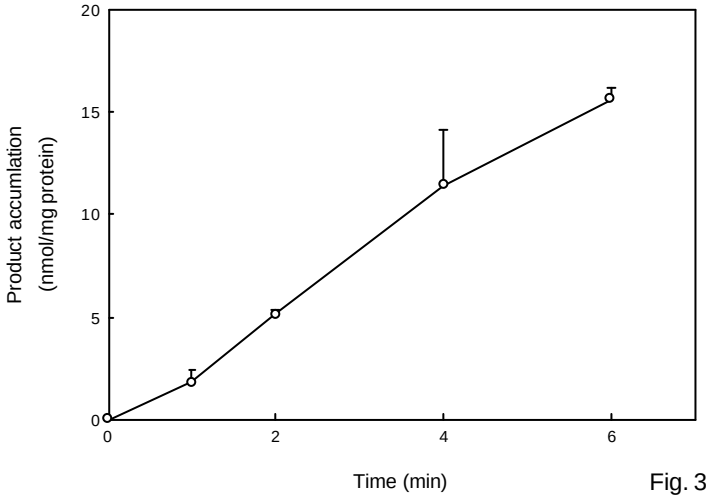


Fig. 2



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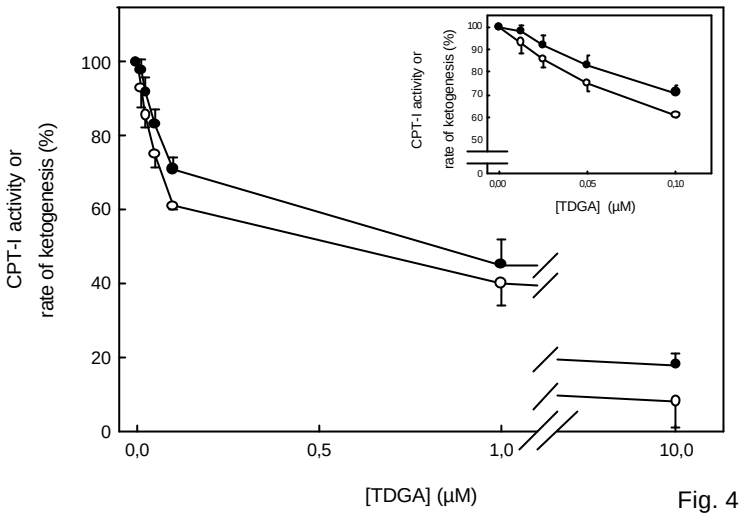

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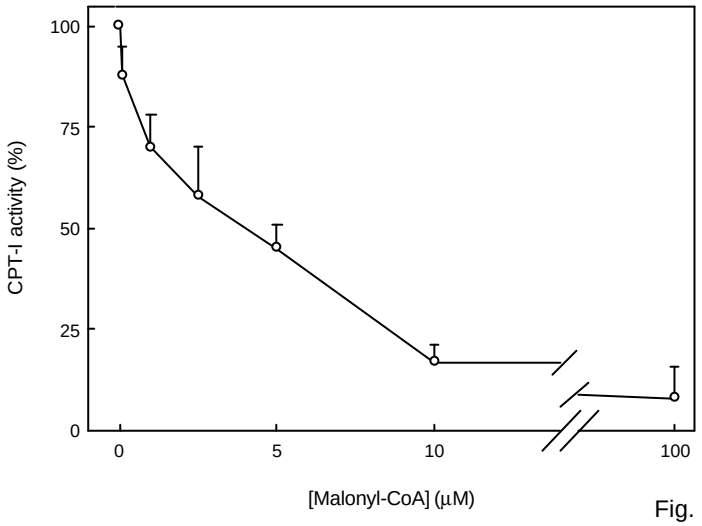

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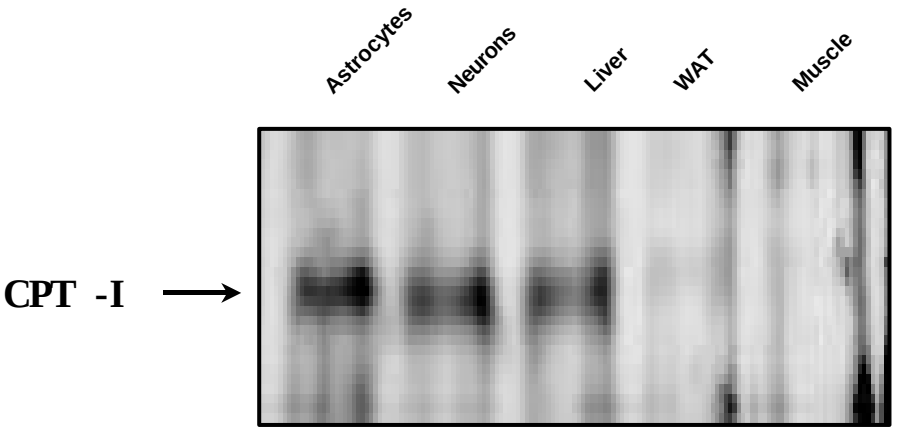

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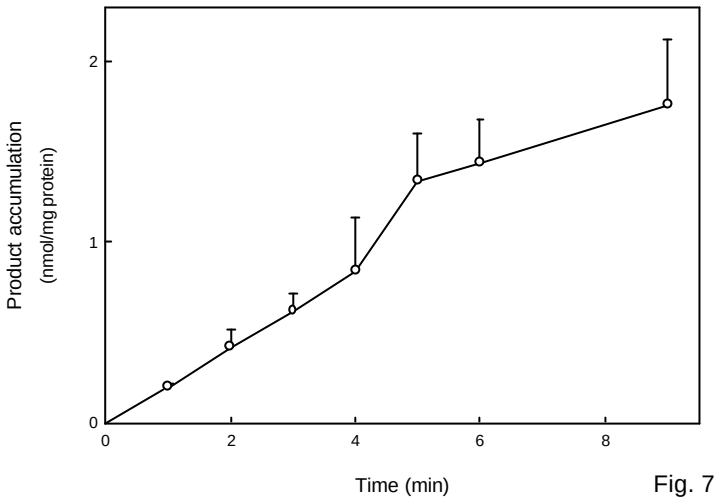
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(nmol/mg protein)
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1
2
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4
6
8
Time (min)

Fig. 7

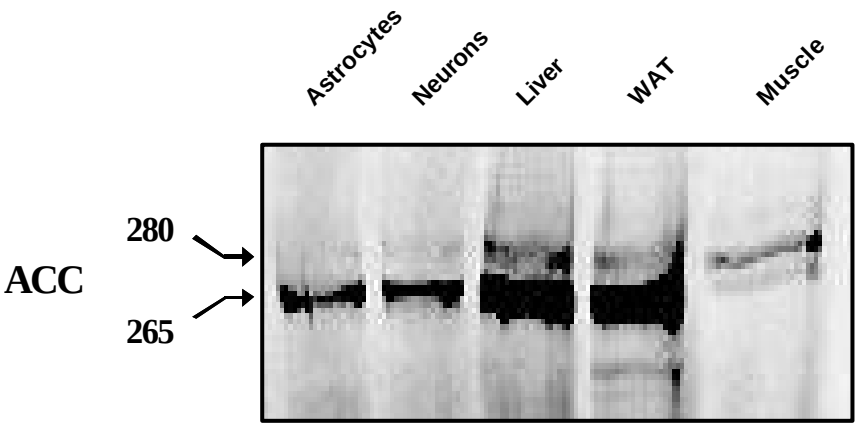
Astrocytes
Neurons
Liver
WAT
Muscle
ACC
280
265

Fig. 8

LEGENDS TO FIGURES

FIG. 1. Time course of [^{14}C]palmitate oxidation to acid-soluble products (o) and CO_2 (•) in cultured astrocytes. Results correspond to 4 different cell preparations.

FIG. 2. Release of lactate dehydrogenase (o), glutamate dehydrogenase (•) and carnitine palmitoyltransferase ( ) from astrocytes permeabilized with 0.025 mg/ml digitonin. Results correspond to 4 different cell preparations.

FIG. 3. CPT-I activity in permeabilized astrocytes as a function of reaction time. Astrocytes were permeabilized by exposure to 0.025 mg/ml digitonin for 3 min and CPT-I activity was subsequently determined at the times indicated. Results correspond to 4 different cell preparations.

FIG. 4. Effect of TDGA on CPT-I activity and on the rate of ketogenesis from [^{14}C]palmitate. Astrocytes were exposed to the indicated concentrations of TDGA for 30 min and then CPT-I activity (o) and the rate of ketogenesis from [^{14}C]palmitate (•) were monitored. Results correspond to 6 different cell preparations. The inset shows the data corresponding to 12 different experiments performed at low TDGA concentrations that were used for the calculation of the flux control coefficient of CPT-I.

FIG. 5. Sensitivity of CPT-I to malonyl-CoA in permeabilized astrocytes. Permeabilized astrocytes were preincubated for 3 min in medium A supplemented with the indicated concentrations of malonyl-CoA before reactions were started by the addition of palmitoyl-CoA and L-[*Me*-^{3}H]carnitine. Results correspond to 4 different cell preparations.

FIG. 6. Western blot analysis of CPT-I from cultured astrocytes. Total membrane fractions (10 µg total protein per lane) from astrocytes and neurons, or isolated mitochondria (5 µg total protein per lane) from liver, white adipose tissue (WAT) and skeletal muscle were subjected to Western blotting with an antibody raised against rat liver CPT-I. A representative luminogram is shown. Essentially identical results were obtained in three other experiments.

FIG 7. ACC activity in permeabilized astrocytes as a function of reaction time. Astrocytes were permeabilized with 0.025 mg/ml digitonin and ACC activity was determined at the times indicated. Results correspond to 4 different cell preparations.

FIG. 8. Western blot analysis of ACC isoforms from cultured astrocytes. Post-mitochondrial supernatants (1.0 mg total protein) of the indicated tissues or cell types were used for immunoprecipitation with an antibody raised against rat liver ACC. A representative autoradiogram is shown. Essentially identical results were obtained in three other experiments. WAT: white adipose tissue.

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The Stimulation of Ketogenesis by Cannabinoids in Cultured Astrocytes Defines Carnitine Palmitoyltransferase I as a New Ceramide-Activated Enzyme

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Abstract: The effects of cannabinoids on ketogenesis in primary cultures of rat astrocytes were studied. Δ^9-Tetrahydrocannabinol (THC), the major active component of marijuana, produced a malonyl-CoA-independent stimulation of carnitine palmitoyltransferase I (CPT-I) and ketogenesis from [^{14}C]palmitate. The THC-induced stimulation of ketogenesis was mimicked by the synthetic cannabinoid HU-210 and was prevented by pertussis toxin and the CB_1 cannabinoid receptor antagonist SR141716. Experiments performed with different cellular modulators indicated that the THC-induced stimulation of ketogenesis was independent of cyclic AMP, Ca^{2+}, protein kinase C, and mitogen-activated protein kinase (MAPK). The possible involvement of ceramide in the activation of ketogenesis by cannabinoids was subsequently studied. THC produced a CB_1 receptor-dependent stimulation of sphingomyelin breakdown that was concomitant to an elevation of intracellular ceramide levels. Addition of exogenous sphingomyelinase to the astrocyte culture medium led to a MAPK-independent activation of ketogenesis that was quantitatively similar and not additive to that exerted by THC. Furthermore, ceramide activated CPT-I in astrocyte mitochondria. Results thus indicate that cannabinoids stimulate ketogenesis in astrocytes by a mechanism that may rely on CB_1 receptor activation, sphingomyelin hydrolysis, and ceramide-mediated activation of CPT-I. **Key Words:** Astrocytes—Ketone bodies—Carnitine palmitoyltransferase I—Ceramide—Cannabinoids.
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Cannabinoids, the active components of marijuana, exert a wide spectrum of effects such as alterations in cognition and memory, analgesia, anticonvulsion, antiinflammation, and alleviation of both intraocular pressure and emesis (Abood and Martin, 1992; Pertwee, 1997*a*). It is currently established that cannabinoids exert their effects by binding to specific plasma membrane receptors (Howlett, 1995; Pertwee, 1997*a*). To date, two different cannabinoid receptors have been characterized and cloned from mammalian tissues: CB_1 and CB_2 (Howlett, 1995; Pertwee, 1997*a*). The CB_1 receptor is mainly distributed in the CNS, whereas the CB_2 receptor is mostly expressed in cells of the immune system (Pertwee, 1997*a*). The recent discovery of a family of endogenous ligands of cannabinoid receptors (Hillard and Campbell, 1997; Pertwee, 1997*a*) and the potential therapeutic applications of several cannabinoid ligands (Pertwee, 1997*b*) have focused much attention on cannabinoids during the last few years.

Astrocytes, the major class of glial cells in the mammalian brain, play a pivotal role in the regulation of brain energy metabolism by providing neurons with anaplerotic metabolites and substrates for generation of energy. Studies on metabolic regulation in cultured astrocytes have mostly focused on carbohydrate metabolism (see, e.g. Magistretti and Pellerin, 1996; Wiesinger et al., 1997). However, ketone bodies may replace glucose as the major source of neuronal energy metabolism in pathophysiological situations in which energy deprivation ensues (Edmond, 1992; Zammit, 1994). Although the liver is generally believed to be the major organ that supplies extrahepatic tissues with ketone bodies (Zammit, 1994), astrocytes in culture are able to produce large amounts of ketone bodies from fatty acids (Auestad et al., 1991; Blázquez et al., 1998) as well as from branched-chain amino acids (Bixel and Hamprecht, 1995). This raises the interesting possibility that astrocytes may provide neurons with ketone bodies as a glucose-replacing fuel in situ.

The existence of a direct and specific action of cannabinoids on glial cells is supported by some recent observations. Thus, astrocytes express the CB_1 receptor mRNA (Bouaboula et al., 1995*a*) and protein (Sánchez et al., 1998*a*). Astrocytes in culture have also been shown to bind and take up anandamide, a putative endogenous ligand of the CB_1 receptor (Di Marzo et al., 1994). In

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Abbreviations used: cAMP, cyclic AMP; CPT-I, carnitine palmitoyltransferase I; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MAPK, mitogen-activated protein kinase; PMA, 4β-phorbol 12β-myristate 13α-acetate; THC, Δ^9-tetrahydrocannabinol.

astrocytoma cells, cannabinoids lead to the stimulation of the mitogen-activated protein kinase (MAPK) cascade and to the induction of the immediate-early gene *krox-24* (Bouaboula et al., 1995*a*,*b*). It is thus conceivable that cannabinoids may perturb the metabolic functions of the astroglia. In fact, Δ^9-tetrahydrocannabinol (THC), the major active component of marijuana, has been shown to affect glucose metabolism in rat glioma cells in vitro (Sánchez et al., 1997) and in rat (Megulies and Hammer, 1991) and human (Volkow et al., 1996) brain in vivo. These observations stress the importance of the study of the metabolic effects of cannabinoids in primary astrocytes. In addition, the mechanism by which cannabinoids exert their metabolic effects is as yet unknown. The present study was therefore designed to address two questions by using ketogenesis as a model pathway: (a) determining whether cannabinoids directly exert metabolic effects on astrocytes and (b) unraveling the mechanism by which cannabinoids may affect astroglial metabolism.

MATERIALS AND METHODS

Materials

SR141716 and SR144528 were kindly given by Sanofi Recherche (Montpellier, France). HU-210 was kindly given by Prof. R. Mechoulam (Hebrew University, Jerusalem, Israel). Tetradecylglycidic acid was kindly given by Dr. J. M. Lowenstein (Brandeis University, Waltham, MA, U.S.A.). All the radioisotopes, the [^{3}H]cyclic AMP ([^{3}H]cAMP) assay kit, the ECL detection kit, and the p42/p44 MAPK assay components were from Amersham (Bucks, U.K.). THC and neutral sphingomyelinase (from *Staphylococcus aureus*) were from Sigma (St. Louis, MO, U.S.A.). Forskolin, pertussis toxin, A23187, BAPTA acetoxymethyl ester (BAPTA/AM), 4β-phorbol 12β-myristate 13α-acetate (PMA), GF109203X, wortmannin, PD098059, SB203580, and *N*-acetylsphingosine were from Calbiochem (San Diego, CA, U.S.A.). Fetal calf serum and all plastic material for cell cultures were from Nunc (Roskilde, Denmark).

Astrocyte cultures

Cortical astrocytes were derived from 1–2 day-old rats and cultures exactly as previously described (Blázquez et al., 1998). For all the experimental determinations performed (see below), 48 h before the experiment the serum-containing medium was removed, and the cells were transferred to a chemically defined, serum-free medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 (1:1, vol/vol) supplemented with 25 μg/ml insulin, 50 μg/ml human transferrin, 20 n*M* progesterone, 50 μ*M* putrescine, 30 n*M* sodium selenite, and 1.0% (wt/vol) defatted and dialyzed bovine serum albumin.

Determination of rate of ketogenesis

Reactions were started by addition to the astrocyte cultures of 0.15 m*M* (final concentration) albumin-bound [1-^{14}C]-palmitic acid or [1-^{14}C]octanoic acid (0.3 μCi per flask) plus 0.5 m*M* (final concentration) L-carnitine and stopped with 0.3 ml of 2 *M* $HClO_4$ after 2 h. Ketone bodies were extracted and quantified exactly as described before (Blázquez et al., 1998). In some experiments, the mass of ketone bodies was determined by a standard spectrophotometric method (Blázquez et al., 1998).

Assay of carnitine palmitoyltransferase I (CPT-I) activity

CPT-I activity was routinely determined in digitonin-permeabilized astrocytes as the tetradecylglycidate-sensitive incorporations of radiolabeled L-carnitine into palmitoylcarnitine by a modification of the previously described procedure (Blázquez et al., 1998). Thus, cultured astrocytes were preincubated for 45 min in the absence or in the presence of 20 μ*M* tetradecylglycidate, a specific irreversible inhibitor of CPT-I (Guzmán and Geelen, 1992; McGarry and Brown, 1997). The different modulators were subsequently added. After 20 min, the medium was aspirated, and cells were washed twice with phosphate-buffered saline. Reactions were started by addition of 800 μl of a medium containing 50 m*M* imidazole (pH 7.1), 70 m*M* KCl, 80 m*M* sucrose, 1 m*M* EGTA, 2 m*M* $MgCl_2$, 1 m*M* dithioerythritol, 1 m*M* KCN, 1 m*M* ATP, 1 mg/ml defatted and dialyzed bovine serum albumin, 50 μ*M* palmitoyl-CoA, 0.5 m*M* L-[*methyl*-^{3}H]carnitine (1.0 μCi), and 0.025 mg/ml digitonin. After 1 min at 37°C, reactions were stopped with 0.8 ml of 2 *M* HCl, and [^{3}H]palmitoylcarnitine product was extracted with *n*-butanol (Guzmán and Geelen, 1992).

In some experiments (see Fig. 7), CPT-I activity was determined in isolated mitochondria as the malonyl-CoA-sensitive incorporation of radiolabeled L-carnitine into palmitoylcarnitine by a previously described procedure (Guzmán et al., 1994; Velasco et al., 1998*b*).

Assay of mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase activity

Mitochondrial HMG-CoA synthase activity was determined in astrocytes as the incorporation of radiolabeled acetyl-CoA into HMG-CoA by an adaptation of the methods used in our laboratory for liver mitochondria (Guzmán et al., 1995) and digitonin-permeabilized hepatocytes (C. Sánchez and M. Guzmán, unpublished data). Thus, the medium of the astrocytes (cultures in P6 plates) was aspirated, and cells were washed twice with phosphate-buffered saline. Cells were subsequently permeabilized with 700 μl of a medium containing 50 m*M* imidazole (pH 7.1), 70 m*M* KCl, 80 m*M* sucrose, 1 m*M* EGTA, 2 m*M* $MgCl_2$, 1 m*M* dithioerythritol, 1 m*M* KCN, 1 m*M* ATP, and 1 mg/ml defatted and dialyzed bovine serum albumin (medium A), supplemented with 100 μl of 0.2 mg/ml digitonin. Permeabilized cells were subsequently washed twice with 800 μl of medium A. This procedure allows release and washing of cytosolic enzymes, whereas mitochondria remain intact within the permeabilized cells (cf. Guzmán and Geelen, 1992; Blázquez et al., 1998). Reactions were started by addition of 250 μl of phosphate-buffered saline supplemented with 2% (vol/vol) Triton X-100, plus 250 μl of assay mixture containing 100 m*M* Tris-HCl (pH 8.0), 5 m*M* EDTA, 2 m*M* dithioerythritol, 20 μ*M* acetoacetyl-CoA, and 0.1 m*M* [1-^{14}C]acetyl-CoA (0.2 μCi). After 3 min at 37°C, reactions were stopped with 0.2 ml of concentrated HCl, and the amount of [^{14}C]HMG-CoA product was determined (Guzmán et al., 1995).

Determination of sphingomyelin levels

Astrocytes were cultured in P6 plates. Forty-eight hours before the experiment, cells were transferred to chemically defined medium (see above) supplemented with 1 μCi of [*methyl*-^{3}H]choline per well. Reactions were started by addition of the different modulators and were terminated by aspiration of the medium and addition of 1.0 ml of methanol. Lipids were extracted and saponified, and sphingomyelin was extracted exactly as described before (Galve-Roperh et al., 1998).

Determination of ceramide levels

Astrocytes were cultured as described above for determination of sphingomyelin levels but using 1 μCi of [9,10-^{3}H]palmitate instead of radiolabeled choline. Lipids were extracted and saponified (Galve-Roperh et al., 1998), and ceramide was resolved by TLC on silica-gel G60 plates with chloroform/methanol/water (100:42:6, by volume) as the developing system until the front reached two-thirds of the plate. The solvent was then evaporated, and plates were subsequently chromatographed with chloroform/methanol/acetic acid (94:1:5, by volume) until the front reached the top of the plate.

Other analytical procedures

Acetyl-CoA carboxylase activity was determined in digitonin-permeabilized astrocytes as the incorporation of [1-^{14}C]-acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction (Blázquez et al., 1998). Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid extracts by a radioenzymatic method (Blázquez et al., 1998). Intracellular levels of cAMP were determined in astrocyte lysates with a [^{3}H]cAMP assay kit (Sánchez et al., 1997). p42/p44 MAPK activity was determined in astrocyte extracts as the incorporation of ^{32}P from [γ-^{32}P]ATP into a MAPK substrate peptide (Galve-Roperh et al., 1997).

Statistical analysis

Results shown represent mean ± SD values of the number of experiments indicated in every case. Five or six replicates of the various conditions included in each experiment were routinely performed. Statistical analysis was performed by Student's *t* test. ANOVA was used when more than two sets of data were compared.

RESULTS

THC produces a malonyl-CoA-independent stimulation of ketogenesis

The effect of cannabinoids on ketone body production was studied in primary cultures of newborn rat astrocytes. THC, the major active component of marijuana, induced a significant dose-dependent stimulation of ketogenesis from [^{14}C]palmitate (Fig. 1). Half-maximal stimulation of ketogenesis was observed at ~40 n*M* THC (Fig. 1), which is in the range of the binding affinity of THC for cannabinoid receptors in whole-cell systems (Howlett, 1995). Because the maximal effect of THC occurred at ~1 μ*M* (Fig. 1), further experiments were conducted with this standard dose of THC.

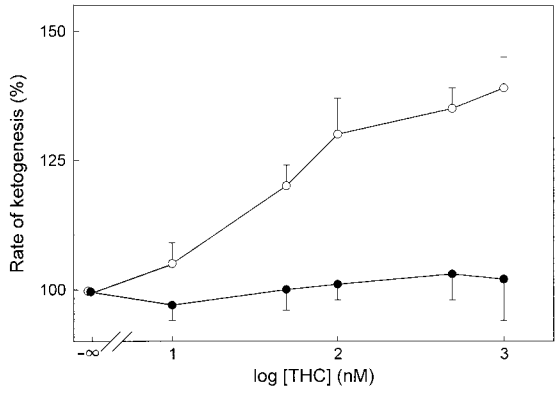

FIG. 1. Dose-dependent effect of THC on ketogenesis in cultured astrocytes. Cells were incubated with different concentrations of THC for 20 min. Then either [^{14}C]palmitate (○) or [^{14}C]octanoate (●) was added, and the rate of ketogenesis was determined. Results are expressed as percentages of incubations with no additions and were obtained from four different cell preparations. Data are mean ± SD (bars) values. Log (-∞) [THC] denotes incubations with no THC.

TABLE 1. *Malonyl-CoA-independent stimulation of ketogenesis by THC in cultured astrocytes*

Parameter	Addition	
	None	1 μ*M* THC
Ketogenesis from		
[^{14}C]Palmitate	59.8 ± 9.2	83.1 ± 7.8[a]
[^{14}C]Octanoate	93.3 ± 15.7	95.2 ± 9.3
CPT-I activity	1.98 ± 0.26	2.97 ± 0.28[a]
Mitochondrial HMG-CoA synthase activity	0.37 ± 0.03	0.36 ± 0.04
Acetyl-CoA carboxylase activity	0.23 ± 0.04	0.23 ± 0.05
[Malonyl-CoA]	108 ± 19	118 ± 26

Astrocytes were incubated in the absence or presence of THC for 20 min. Then cells were used for measurement of the rate of ketogenesis from ^{14}C-fatty acids, enzyme activities, and malonyl-CoA concentration. Rates of ketogenesis are expressed as nanomoles of ^{14}C-fatty acid oxidized per hour per milligram of cellular protein. Enzyme activities are expressed as nanomoles of product per minute per milligram of cellular protein. Malonyl-CoA levels are expressed as picomoles per milligram of cellular protein. Results were obtained from six different experiments, except for mitochondrial HMG-CoA synthase activity, which was obtained from four different experiments. Data are mean ± SD values.

[a] $p < 0.01$, significantly different from incubations with no additions.

The stimulatory effect of THC on ketogenesis from palmitate was also evident when the mass of ketone bodies was determined by a spectrophotometric assay. Thus, levels of acetoacetate plus 3-hydroxybutyrate production from 0.15 m*M* palmitate were 221 ± 28 (incubations with no additions) and 303 ± 47 (incubations with 1 μ*M* THC) nmol of ketone bodies/h/mg of cellular protein (n = 4). These values are in line with those shown in Table 1 for [^{14}C]palmitate oxidation, considering that the complete oxidation of 1 mol of palmitate yields 4 mol of ketone bodies.

Unlike ketogenesis from [^{14}C]palmitate, ketogenesis from [^{14}C]octanoate was not significantly affected by THC (Fig. 1). It is well established that long-chain fatty acids such as palmitate are transported into mitochondria by a carnitine-dependent process, whereas medium-chain fatty acids such as octanoate may enter mitochondria independently of carnitine (Zammit, 1994). Therefore, this observation suggests that the target for THC action might be CPT-I, the key regulatory enzyme of the transport of long-chain fatty acids into mitochondrial oxidative metabolism in many cell types, including astrocytes (Guzmán and Geelen, 1993; Zammit, 1994; McGarry and Brown, 1997; Blázquez et al., 1998). As shown in Table 1, CPT-I activity was enhanced on incubation of

TABLE 2. *Involvement of the CB_1 cannabinoid receptor in stimulation of ketogenesis by THC in cultured astrocytes*

Addition	Ketogenesis from [^{14}C]palmitate (%)
None	100 ± 11
1 μM THC	139 ± 11[a]
0.1 μM HU-210	141 ± 6[a]
1 μM THC + 25 ng/ml pertussis toxin	104 ± 3
1 μM THC + 1 μM SR141716	103 ± 8
1 μM THC + 1 μM SR144528	145 ± 8[a]

Astrocytes were incubated with the additions indicated for 20 min. Then [^{14}C]palmitate was added, and the rate of ketogenesis was determined. Results are expressed as percentages of incubations with no additions and were obtained from four different experiments. Data are mean ± SD values.

[a] $p < 0.01$, significantly different from incubations with no additions.

astrocytes with THC. Moreover, the magnitude of this stimulation was quantitatively similar to that exerted by THC on ketogenesis from [^{14}C]palmitate.

It is widely accepted that malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase, is the physiological inhibitor of CPT-I (Zammit, 1994; McGarry and Brown, 1997). However, THC was unable to affect either acetyl-CoA carboxylase activity or intracellular malonyl-CoA levels in cultured astrocytes (Table 1), indicating that the THC-induced stimulation of CPT-I occurs by a malonyl-CoA-independent mechanism.

Astrocytes express mitochondrial HMG-CoA synthase, an enzyme that has been suggested to play a regulatory role in ketogenesis (Cullingford et al., 1998*a*,*b*). However, THC was unable to affect mitochondrial HMG-CoA synthase activity in astrocytes (Table 1).

The CB_1 cannabinoid receptor is involved in regulation of ketogenesis

Most of the effects of cannabinoids in the CNS described so far are believed to be mediated by the CB_1 cannabinoid receptor (Howlett, 1995; Pertwee, 1997*a*). Hence, several experiments were performed to test whether the THC-induced stimulation of ketogenesis in astrocytes was also dependent on this receptor. Like THC, the synthetic cannabinoid HU-210 was able to stimulate ketogenesis (Table 2). Moreover, the stimulatory effect of THC on ketogenesis was abolished by treatment of cells with pertussis toxin, which prevents the dissociation of G_i/G_o protein, and with SR141716, a selective CB_1 receptor antagonist (Table 2). Neither pertussis toxin nor SR141716 at the concentrations used had any significant effect on ketogenesis (data not shown).

It has been reported that cerebellar granule cells and the cerebellum express not only the CB_1 receptor but also the CB_2 receptor (Skaper et al., 1996). In addition, SR141716 at 1 μM may also bind to the CB_2 receptor (Showalter et al., 1996). However, as shown in Table 2, the selective CB_2 antagonist SR144528 (Rinaldi-Carmona et al., 1998) was unable to prevent the ketogenic effect of THC, pointing to a lack of involvement of the CB_2 receptor in this cannabinoid action.

The THC-induced stimulation of ketogenesis is independent of cAMP, Ca^{2+}, protein kinase C, and MAPK

In a first attempt to elucidate the mechanism of the cannabinoid-induced stimulation of ketogenesis, the possible involvement of cAMP was studied. It is widely accepted that the CB_1 cannabinoid receptor is coupled to inhibition of adenylyl cyclase through G_i protein (Howlett, 1995). However, Jung et al. (1997) have observed that the CB_1 receptor in cultured astrocytes seems to be uncoupled from adenylyl cyclase. Hence, we determined the effect of THC on the intracellular concentration of cAMP. THC partially antagonized the forskolin-induced elevation of intracellular cAMP concentration (Fig. 2), pointing to a G_i protein-mediated inhibition of adenylyl cyclase by cannabinoids in our experimental system. The effect of THC on the forskolin-induced elevation of cAMP levels was not evident when SR141716 was present in the culture medium (Fig. 2), indicating the involvement of the CB_1 receptor. However, THC per se was unable to affect basal cAMP levels (Fig. 2). In addition, forskolin—like THC—stimulated ketogenesis (Table 3). These observations therefore indicate that the THC-induced stimulation of ketogenesis is independent of cAMP.

Studies using transfected cells have shown that the CB_1 receptor is coupled to inhibition of N-type Ca^{2+} channels (Pan et al., 1996). In addition, the endogenous cannabinoid ligand anandamide inhibits intercellular Ca^{2+} signaling in cultured astrocytes (Venance et al., 1995). However, the THC-induced stimulation of ketogenesis in astrocytes was not affected either by the intracellular Ca^{2+} chelator BAPTA/AM or by the Ca^{2+}

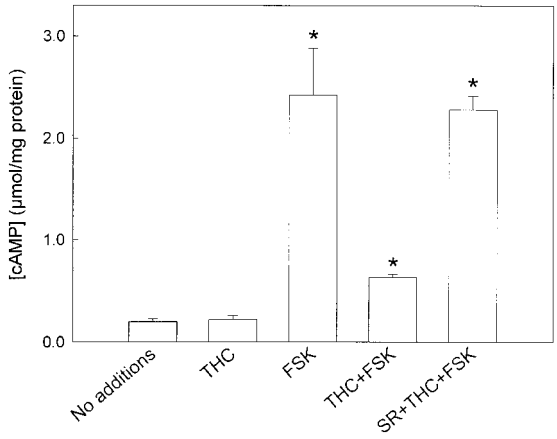

FIG. 2. Effect of THC on intracellular cAMP concentration in cultured astrocytes. Cells were incubated with or without 1 μM SR141716 (SR) for 20 min, then with or without 1 μM THC for an additional 20 min, and finally with or without 20 μM forskolin (FSK) for an additional 20 min. Results were obtained from four different experiments. Data are mean ± SD (bars) values. *$p < 0.01$, significantly different from incubations with no additions.

TABLE 3. *Effect of different cellular effectors on ketogenesis in cultured astrocytes*

Addition	Ketogenesis from [^{14}C]palmitate (%)
None	100 ± 10
1 μM THC	140 ± 9[a]
20 μM forskolin	176 ± 16[a]
10 μM A23187	102 ± 9
50 μM BAPTA/AM	98 ± 8
1 μM THC + 10 μM A23187	142 ± 11[a]
1 μM THC + 50 μM BAPTA/AM	140 ± 10[a]
1 μM PMA	93 ± 12
1 μM THC + 2 μM GF109203X	143 ± 11[a]
1 μM THC + 50 μM PD098059	139 ± 6[a]
1 μM THC + 10 μM SB203580	148 ± 7[a]
1 μM THC + 0.1 μM wortmannin	144 ± 14[a]
0.2 U/ml SMase	145 ± 12[a]
0.2 U/ml SMase + 50 μM PD098059	141 ± 10[a]
0.2 U/ml SMase + 10 μM SB203580	150 ± 7[a]
0.2 U/ml SMase + 1 μM THC	153 ± 9[a]

Astrocytes were incubated with the additions indicated for 20 min. Then [^{14}C]palmitate was added, and the rate of ketogenesis was determined. Results are expressed as percentages of incubations with no additions and were obtained from four different experiments. Data are mean ± SD values. SMase, sphingomyelinase.

[a] $p < 0.01$, significantly different from incubation with no additions.

ionophore A23187 (Table 3). In addition, neither BAPTA/AM nor A23187 per se exerted any significant effect on ketogenesis (Table 3). Hence, ketogenesis in astrocytes seems to be independent of changes in intracellular Ca^{2+} concentration.

Protein kinase C might play a role in cannabinoid action. Thus, cannabinoids have been shown to activate brain protein kinase C in vitro (Hillard and Auchampach, 1994). In addition, the CB_2 cannabinoid receptor-mediated induction of the *krox-24* gene seems to rely on protein kinase C activation (Bouaboula et al., 1996). We therefore studied whether protein kinase C may be involved in the THC-induced stimulation of ketogenesis in astrocytes. As shown in Table 3, acute exposure of astrocytes to the protein kinase C activator PMA had no effect on ketogenesis. Likewise, longer treatment (12 h) of astrocytes with PMA did not affect ketogenesis from palmitate (data not shown). In addition, the protein kinase C inhibitor GF109203X did not prevent the THC-induced stimulation of ketogenesis. Protein kinase C seems therefore to play no modulatory role in astroglial ketogenesis.

Stimulation of the p42/p44 MAPK cascade occurs on activation of the CB_1 and CB_2 cannabinoid receptors in transfected cells and astrocytoma cells (Bouaboula et al., 1995*a*,*b*, 1996). The possibility that cannabinoids stimulate MAPK in primary astrocytes was therefore tested. THC was observed to stimulate significantly p42/p44 MAPK activity in astrocytes. Thus, relative values of p42/p44 MAPK activity (n = 4) were 100 ± 20 (incubations with no additions), 386 ± 88 (incubations with 1 μM THC for 20 min), and 103 ± 24 (incubations with 1 μM SR141716 for 20 min followed by 20 min with 1 μM THC). However, the p42/p44 MAPK cascade-specific inhibitor PD098059 was unable to antagonize the THC-induced stimulation of ketogenesis (Table 3). Likewise, the p38 MAPK-specific inhibitor SB203580 did not prevent the effect of THC on ketogenesis (Table 3). Moreover, although the CB_1 receptor-induced stimulation of the MAPK cascade has been shown to be prevented by the phosphoinositide 3′-kinase inhibitor wortmannin (Bouaboula et al., 1997), this compound did not antagonize the stimulation of ketogenesis evoked by THC (Table 3). Therefore, the THC-induced stimulation of ketogenesis seems to be independent of MAPK.

Because negative results were obtained with A23187, BAPTA/AM, PMA, GF109203X, PD098059, SB203580, and wortmannin, it might be argued that these agents were not used at effective concentrations. However, both our previous observations (Galve-Roperh et al., 1997; Sánchez et al., 1998*b*) and data from the literature (cf. Berridge, 1995; Hinterding et al., 1998) show that the concentrations at which those compounds were used in the present study are effective in the modulation of metabolism in various types of cells, including astrocytes.

The THC-induced stimulation of ketogenesis may be mediated by a ceramide-dependent activation of CPT-I

Sphingomyelin hydrolysis and subsequent ceramide generation are key processes in the control of many physiological events related to signal transduction and cellular regulation (Hannun, 1996; Kolesnick and Krönke, 1998). Hence, the possible effect of THC on sphingomyelin hydrolysis was tested. As shown in Fig. 3, THC induced a dose-dependent stimulation of sphingomyelin hydrolysis. The half-maximal effect of THC

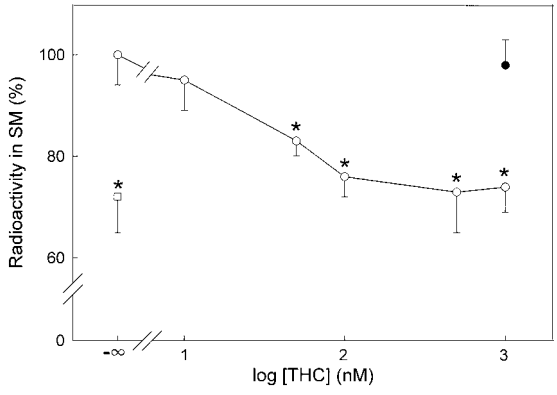

FIG. 3. Dose-dependent stimulation of sphingomyelin (SM) hydrolysis by THC in cultured astrocytes. Cells were exposed for 20 min to different concentrations of THC (○) or to 0.2 U/ml sphingomyelinase (□). Incubations were also carried out in the presence of 1 μM SR141716 for 20 min followed by an additional 20 min with 1 μM THC (●). Results are expressed as percentages of incubations with no additions and were obtained from six different experiments. Data are mean ± SD (bars) values. Log (-∞) [THC] denotes incubations with no THC. *$p < 0.01$, significantly different from incubations with no additions.

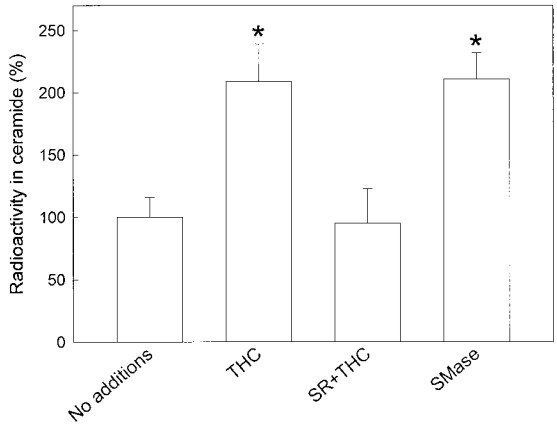

FIG. 4. Stimulation of ceramide formation by THC in cultured astrocytes. Cells were incubated with or without 1 μM THC, 0.2 U/ml sphingomyelinase (SMase), and 1 μM SR141716 as described in Fig. 4. Results are expressed as percentages of incubations with no additions and were obtained from four different experiments. Data are mean ± SD (bars) values. *$p < 0.01$, significantly different from incubations with no additions.

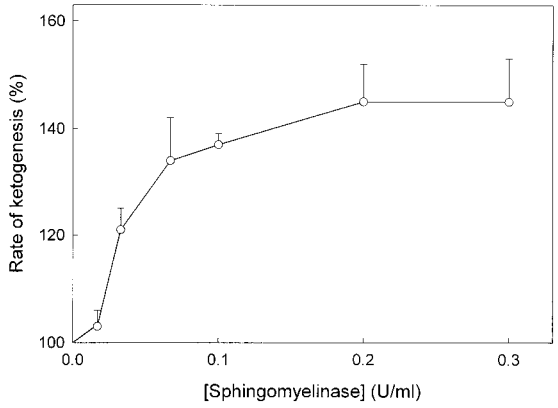

FIG. 5. Dose-dependent stimulation of ketogenesis by sphingomyelinase in cultured astrocytes. Cells were incubated with different concentrations of sphingomyelinase for 20 min. Then [^{14}C]palmitate was added, and the rate of ketogenesis was determined. Results are expressed as percentages of incubations with no additions and were obtained from six different cell preparations. Data are mean ± SD (bars) values.

occurred at a concentration of ~30 nM, i.e., in the same range as the THC-induced stimulation of ketogenesis. The THC-evoked sphingomyelin breakdown was concomitant with a remarkable elevation of the intracellular levels of ceramide, the product of sphingomyelin hydrolysis by sphingomyelinase (Fig. 4). Moreover, the effect of THC on sphingomyelin breakdown (Fig. 3) and ceramide generation (Fig. 4) in astrocytes seemed to rely on a CB_1 receptor-dependent process as it was prevented by SR141716.

Experiments were subsequently conducted to study whether sphingomyelin breakdown may be linked to the THC-induced stimulation of ketogenesis in astrocytes. It is well known that exogenous sphingomyelinase cleaves sphingomyelin molecules in the outer leaflet of the plasma membrane and that the ceramide molecules formed may translocate through the plasma membrane to gain access to the inside of the cell. Addition of exogenous sphingomyelinase is therefore a widely used method to increase intracellular ceramide levels in vitro (cf. Hannun, 1996; Kolesnick and Krönke, 1998). Sphingomyelin breakdown and intracellular ceramide accumulation were thus triggered by addition of exogenous neutral sphingomyelinase to the astrocyte incubation medium (Figs. 3 and 4). As shown in Fig. 5, exogenous sphingomyelinase was able to activate ketogenesis from [^{14}C]palmitate in a dose-dependent manner. The sphingomyelinase-induced stimulation of ketogenesis was not prevented by either PD098059 or SB203580 and was not additive to that exerted by THC, pointing to a common mechanisms of action (Table 3).

The THC-induced stimulation of ketogenesis might be therefore dependent on sphingomyelin hydrolysis and ceramide generation. In any case, this modulation of ketogenesis seems to be independent of MAPK and to involve a malonyl-CoA-independent stimulation of CPT-I (see above). Hence, the possibility that CPT-I was directly activated by ceramide was tested. As shown in Fig. 6, the synthetic ceramide *N*-acetylsphingosine was able to induce a dose-dependent stimulation of CPT-I in astrocyte mitochondria.

DISCUSSION

Data presented in this report indicate that cannabinoids modulate ketogenesis in astrocytes by a mechanism that may rely on the cascade CB_1 receptor activation → sphingomyelin breakdown → ceramide generation → CPT-I activation → stimulation of ketogenesis. The different steps of this novel mechanism of cannabinoid action are discussed below.

Receptor dependency of the effects of cannabinoids

THC stimulated ketogenesis in primary astrocytes at doses similar to those found in plasma from humans who

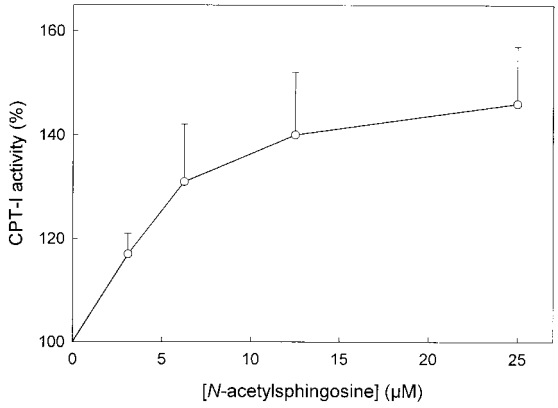

FIG. 6. Dose-dependent stimulation of CPT-I by *N*-acetylsphingosine. Mitochondria from astrocytes were preincubated with different concentrations of *N*-acetylsphingosine for 5 min, and then CPT-I activity was determined. Results were obtained from four different experiments. Data are mean ± SD (bars) values.

had smoked marijuana or from laboratory animals that were injected with THC (Abood and Martin, 1992). It is well established that THC is able to cross the blood–brain barrier and gain access to the brain (Schou et al., 1977; Pertwee, 1997*a*,*b*). In addition, the effect of THC occurred at concentrations close to its binding affinity for cannabinoid receptors in whole-cell systems (Howlett, 1995). All this supports a possible physiological relevance of the metabolic effects of cannabinoids described herein.

On the basis of the antagonism exerted by pertussis toxin and especially SR141716, the cannabinoid-induced stimulation of ketogenesis in astrocytes seems to be a CB_1 receptor-mediated process. This is in line with the observations that primary rat astrocytes express CB_1 receptor mRNA (Bouaboula et al., 1995*a*) and protein (Sánchez et al., 1998*a*). Nevertheless, the existence in rat brain of two different isoforms of the central cannabinoid receptor, i.e., CB_1 and CB_{1A}, should be kept in mind. Both CB_1 and CB_{1A} bind SR141716 and are coupled to activation of the MAPK cascade through a pertussis toxin-sensitive G protein (Rinaldi-Carmona et al., 1996).

Induction of sphingomyelin breakdown and ceramide accumulation by cannabinoids

The sphingomyelin cycle has been shown to play a pivotal role in the regulation of cell function in the CNS (Ariga et al., 1998). Thus, changes in the activity of the sphingomyelin cycle, which may in turn be related to the induction of apoptotic cell death, have been shown to occur during brain development as well as in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, epilepsy, and ischemia/stroke (Ariga et al., 1998; Leist and Nicotera, 1998). Likewise, exposure of neural cells to physical, e.g., ultraviolet radiation, chemical, e.g., tumor necrosis factor-α, bacterial, e.g., lipopolysaccharide, or viral, e.g., human immunodeficiency virus 1, stimuli may stimulate sphingomyelin breakdown and therefore evoke changes in the cell survival/cell death decision (Ariga et al., 1998; Kolesnick and Krönke, 1998).

The possibility that cannabinoids may control the activity of the sphingomyelin cycle (the present study) and the MAPK cascade (Bouaboula et al., 1995*a*,*b*) points therefore to a general role of these compounds as modulators of cell fate. In this respect, we have recently observed that the cannabinoid-induced accumulation of ceramide in astrocytes leads to the activation of the MAPK cascade through Raf-1 (Sánchez et al., 1998*b*). The Raf-1/MAPK cascade is known to play an important role in the cell proliferation/cell death decision (Morrison and Cutler, 1997; Ariga et al., 1998; Kolesnick and Krönke, 1998). This is in line with the recent observations that the cannabinoid-induced apoptosis of C6 glioma cells (Sánchez et al., 1998*a*) is prevented by PD098059 (C. Sánchez and M. Guzmán, unpublished data). It is also noteworthy that oleoylethanolamide, an acylethanolamide that is coreleased with anandamide by neurons (Di Marzo et al., 1994; Pertwee, 1997*a*), is an inhibitor of acid ceramidase and should be able therefore to increase intracellular ceramide levels (McKay, 1997). Our research is currently focused on the mechanism of control of the sphingomyelin cycle by cannabinoids.

Stimulation of CPT-I by ceramide

Evidence has accumulated during the last 2 decades highlighting the physiological importance of malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase, in the regulation of CPT-I activity and long-chain fatty acid oxidation in different tissues (Guzmán and Geelen, 1993; Zammit, 1994; McGarry and Brown, 1997). Because acetyl-CoA carboxylase is a key regulatory enzyme of fatty acid synthesis de novo (cf. Guzmán and Geelen, 1993; Zammit, 1994), malonyl-CoA inhibition of CPT-I allows an elegant explanation for the coordinate control of fatty acid synthesis and oxidation. However, a malonyl-CoA-independent mechanism of control of CPT-I activity was put forward several years ago. Studies using permeabilized hepatocytes showed that various agents exert acute effects on CPT-I activity in parallel with changes in the rate of ketogenesis from palmitate (Guzmán and Geelen, 1992, 1993). These malonyl-CoA-independent changes of CPT-I activity have been shown to require extramitochondrial cell components (Guzmán et al., 1994) and seem to rely on the modulation of interactions between mitochondria and cytoskeletal components (Velasco et al., 1996, 1998*a*).

A second malonyl-CoA-independent mechanism of control of CPT-I activity is presented in this report. Thus, cannabinoids and exogenous sphingomyelinase may stimulate CPT-I by evoking sphingomyelin breakdown and intracellular ceramide accumulation. Although ceramide has been reported to modulate MAPK cascades (Kolesnick and Krönke, 1998), the stimulatory effect of cannabinoids and sphingomyelinase on ketogenesis was not prevented by either PD098059 or SB203580. Moreover, ceramide was able to stimulate CPT-I in isolated mitochondria. Ceramide has been reported to regulate the activity of several enzymes and signaling components (Kolesnick and Krönke, 1998). However, the interpretation of the published data is hampered by the fact that most of them come from studies in which intact cells were treated for long intervals with synthetic ceramides. Hence, the observed effects of ceramide (a) may reflect an indirect action of ceramide-activated protein kinase(s) or phosphatase(s) on the enzyme under study and (b) may not reflect the rapid and transient elevation of intracellular ceramide levels resulting from sphingomyelin breakdown induced by physiological mediators. Although the molecular mechanism by which ceramide activates CPT-I is as yet unknown, as far as we know CPT-I is the first enzyme described so far distinct from a protein kinase or a protein phosphatase that seems to be directly activated by ceramide (cf. Kolesnick and Krönke, 1998).

The notion that CPT-I is a ceramide-activated enzyme is interesting in view of the possible implication of this

enzyme in ceramide-mediated apoptosis. As palmitate is a precursor for ceramide synthesis de novo, pharmacological inhibition of CPT-I leads to accumulation of palmitate in the cytoplasm, increased ceramide synthesis, and apoptosis (Paumen et al., 1997*b*). Likewise, expression of high CPT-I activity may help cells to withstand palmitate-induced apoptosis (Paumen et al., 1997*b*; Velasco et al., 1998*b*). Our data suggest the existence of a regulatory loop in which elevated ceramide levels occurring on CPT-I inhibition might be a signal for CPT-I reactivation. The observation that CPT-I directly interacts with the antiapoptotic protein Bcl-2 in the mitochondrial outer membrane (Paumen et al., 1997*a*) and the well-established role of mitochondria in the onset of apoptosis (Kolesnick and Krönke, 1998) point to a general role of CPT-I as a regulator of apoptosis.

Stimulation of ketogenesis by cannabinoids

The activation of CPT-I by cannabinoids in astrocyte cultures was concomitant with a quantitatively similar stimulation of ketogenesis from palmitate. This is consistent with recent determinations of flux control coefficients of the enzymes involved in long-chain fatty acid oxidation, which show that CPT-I catalyzes the pacesetting step of ketogenesis in astrocytes and hepatocytes (cf. Blázquez et al., 1998). In addition to CPT-I, mitochondrial HMG-CoA synthase has been suggested to be a putative important site of control of ketogenesis from acetyl-CoA in astrocytes (Cullingford et al., 1998*a*,*b*) and hepatocytes (cf. Guzmán and Geelen, 1993; Zammit, 1994). However, in the present study the THC-evoked stimulation of ketogenesis from palmitate was not accompanied by any change in mitochondrial HMG-CoA synthase activity. In addition, THC had no effect on ketogenesis from octanoate. Hence, the regulation of astroglial ketogenesis by THC seems to be independent of HMG-CoA synthase.

Astrocytes play an active role in the regulation of brain energy metabolism (Magistretti and Pellerin, 1996). Accruing evidence shows that glucose utilization by astrocytes is sensitive to mediators such as neurotransmitters and cytokines (Pellerin et al., 1997; Wiesinger et al., 1997). Although astrocytes possess an enzymatic equipment capable of synthesizing ketone bodies (Auestad et al., 1991), exhibit a preference for fatty acids over glucose as their primary metabolic fuel (Edmond, 1992), and produce ketone bodies as the bulk of their fatty acid oxidation product (Blázquez et al., 1998), the regulatory mechanisms of astroglial ketogenesis are largely unknown. The acetyl-CoA-carboxylase/malonyl-CoA/CPT-I system seems to be involved in the regulation of ketogenesis by cAMP in astrocytes (Blázquez et al., 1998). The direct stimulation of CPT-I by ceramide provides an additional regulatory mechanism of ketogenesis in astrocytes. Recent observations indicate that ceramide also modulates glucose uptake and metabolism in adipocytes (David et al., 1998; Wang et al., 1998) and astrocytes (Sánchez et al., 1998*b*). Ceramide may be therefore a general modulator of intermediate metabolism.

As far as we know, this is the first time in which a connection between stimulation of a signal transduction system and a metabolic response is established for the action of cannabinoids. Nevertheless, we are aware that the metabolic implications of the present findings are not obvious. The endogenous cannabinoid system, a potential novel neuromodulatory system (Hillard and Campbell, 1997), might regulate the amount of ketone bodies supplied from astrocytes to neurons as a source of carbon for neuronal biosynthetic processes, e.g., myelination, and/or oxidative metabolism, e.g., synaptic activity (cf. Edmond, 1992). In addition, the Ca^{2+}-evoked production of brain endogenous cannabinoids (Hillard and Campbell, 1997) might be a buffering mechanism for handling elevated concentrations of nonesterified fatty acids occurring in stroke/ischemia (cf. Edmond, 1992). It is clear, anyway, that further research is required to understand the role of cannabinoids in the modulation of astroglial functions.

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The AMP-Activated Protein Kinase Is Involved in the Regulation of Ketone Body Production by Astrocytes

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Abstract: The possible role of the AMP-activated protein kinase (AMPK), a highly conserved stress-activated kinase, in the regulation of ketone body production by astrocytes was studied. AMPK activity in rat cortical astrocytes was three times higher than in rat cortical neurons. AMPK in astrocytes was shown to be functionally active. Thus, incubation of astrocytes with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a cell-permeable activator of AMPK, stimulated both ketogenesis from palmitate and carnitine palmitoyltransferase I. This was concomitant to a decrease of intracellular malonyl-CoA levels and an inhibition of acetyl-CoA carboxylase/fatty acid synthesis and 3-hydroxy-3-methylglutaryl-CoA reductase/cholesterol synthesis. Moreover, in microdialysis experiments AICAR was shown to stimulate brain ketogenesis markedly. The effect of chemical hypoxia on AMPK and the ketogenic pathway was studied subsequently. Incubation of astrocytes with azide led to a remarkable drop of fatty acid β-oxidation. However, activation of AMPK during hypoxia compensated the depression of β-oxidation, thereby sustaining ketone body production. This effect seemed to rely on the cascade hypoxia → increase of the AMP/ATP ratio → AMPK stimulation → acetyl-CoA carboxylase inhibition → decrease of malonyl-CoA concentration → carnitine palmitoyltransferase I deinhibition → enhanced ketogenesis. Furthermore, incubation of neurons with azide blunted lactate oxidation, but not 3-hydroxybutyrate oxidation. Results show that (a) AMPK plays an active role in the regulation of ketone body production by astrocytes, and (b) ketone bodies produced by astrocytes during hypoxia might be a substrate for neuronal oxidative metabolism.
Key Words: Astrocyte —Ketone bodies—Hypoxia—AMP-activated protein kinase —Carnitine palmitoyltransferase I—Neuroglial interactions.
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Astrocytes play an active role in the regulation of brain glucose metabolism. Thus, lactate produced by astrocytes may be delivered to and used by neurons as an efficient energy substrate, therefore contributing to maintain synaptic transmission, particularly during periods of enhanced neuronal activity (Tsacopoulos and Magistretti, 1996; Izumi et al., 1997). In addition, glycogen is the single largest energy reserve of the brain, and is localized mainly in astrocytes; glycogen turnover in the brain is extremely rapid and finely coordinated with synaptic activity (Tsacopoulos and Magistretti, 1996; Wiesinger et al., 1997). Accruing evidence shows that glucose utilization by astrocytes is sensitive to modulation by a number of mediators, including neurotransmitters (Pellerin et al., 1997), cytokines (Yu et al., 1995), and xenobiotics (Sánchez et al., 1998).

Although studies on metabolic regulation in cultured astrocytes have focused mostly on carbohydrate metabolism, ketone bodies may replace glucose as the major source of brain energy in pathophysiological situations in which glucose deprivation ensues (Edmond, 1992; Zammit, 1994). The liver is generally believed to be the major organ that supplies extrahepatic tissues with ketone bodies (Zammit, 1994). However, astrocytes in culture are able to produce ketone bodies from fatty acids (Auestad et al., 1991; Blázquez et al., 1998), as well as from branched-chain amino acids (Bixel and Hamprecht, 1995), raising the interesting possibility that astrocytes may provide neurons with ketone bodies as a glucose-replacing fuel in situ. Carnitine palmitoyltransferase I (CPT-I), the carnitine palmitoyltransferase located in the mitochondrial outer membrane, catalyzes the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix and is a key regulatory site of ketogenesis in hepatocytes (Guzmán and Geelen, 1993; McGarry and Brown, 1997) and astrocytes (Blázquez et

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Abbreviations used: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CPT-I, carnitine palmitoyltransferase I; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PBS, phosphate-buffered saline; PDH, pyruvate dehydrogenase.

al., 1998). CPT-I is subject to inhibition by malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase (ACC) (McGarry and Brown, 1997). Because the latter is a key regulatory enzyme of fatty acid synthesis, malonyl-CoA inhibition of CPT-I allows the coordinate control of fatty acid synthesis and ketogenesis in hepatocytes (McGarry and Brown, 1997) and astrocytes (Blázquez et al., 1998).

Modulation of ACC activity is therefore essential for the control of CPT-I and ketogenesis. Although several protein kinases can phosphorylate and inactivate purified ACC in vitro, there is good evidence demonstrating that in intact hepatocytes and in the liver in vivo the AMP-activated protein kinase (AMPK) is the major protein kinase responsible for the inactivation of ACC by phosphorylation and therefore for the activation of CPT-I and ketogenesis (Hardie and Carling, 1997; Velasco et al., 1997*a*, 1998*b*). AMPK is activated by AMP and by phosphorylation by an upstream kinase, which is itself activated by AMP (Hardie et al., 1998). Once activated, AMPK phosphorylates and inactivates a number of regulatory enzymes involved in biosynthetic pathways. The AMPK cascade seems to have evolved to monitor the energy status of the cell and to initiate appropriate energy-conserving mechanisms in response to ATP depletion during metabolic stress (Hardie et al., 1998; Ponticos et al., 1998). Although AMPK has been shown to be present in brain (Gao et al., 1996; Stapleton et al., 1996; Woods et al., 1996), its function in this organ is as yet unknown. The present study was undertaken therefore to test whether AMPK is involved in the response of astrocytes to metabolic stress, with special emphasis on the regulation of ketone body production during hypoxia.

MATERIALS AND METHODS

Materials

Radiochemicals were from Amersham (Buckinghamshire, U.K.) except 3-[1-^{14}C]hydroxybutyric acid, which was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Tetradecylglycidic acid was kindly donated by Dr. J. M. Lowenstein (Brandeis University, Waltham, MA, U.S.A.). 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal calf serum and all plastic material for cell cultures were from Nunc (Roskilde, Denmark).

Cell cultures

Cortical astrocytes were derived from 24-h-old Wistar rats and cultured exactly as described by Galve-Roperh et al. (1997). For all the experimental determinations performed (see below), 48 h before the experiment the serum-containing medium was removed and cells were transferred to a chemically defined, serum-free medium consisting of Dulbecco's modified Eagle's medium/Ham's F-12 (1:1, vol/vol) supplemented with 5 μg/ml insulin, 50 μg/ml human transferrin, 20 n*M* progesterone, 50 μ*M* putrescine, 30 n*M* sodium selenite, and 1.0% (wt/vol) defatted and dialyzed bovine serum albumin.

Cortical neurons were obtained and cultured exactly as described before (Blázquez et al., 1998).

Rate of ketogenesis

Reactions were started by the addition to the astrocyte cultures of 0.15 m*M* (final concentration) albumin-bound [1-^{14}C]palmitate or [1-^{14}C]octanoate (0.3 μCi/flask) plus 0.5 m*M* (final concentration) L-carnitine, and stopped with 0.3 ml of 2 *M* $HClO_4$ after 1 h (standard assay). Ketone bodies were extracted and quantified exactly as described before (Blázquez et al., 1998).

Rate of fatty acid and cholesterol synthesis

Astrocytes were incubated with 4 m*M* [1-^{14}C]acetate (1.0 μCi/flask). After 1 h, reactions were stopped with 0.2 ml of 10 *M* NaOH, and samples were saponified in 0.3 *M* NaOH in methanol (45 min at 80°C). Cholesterol was extracted with light petroleum ether and subsequent digitonin precipitation, whereas fatty acids were obtained after acidification of the samples and extraction with light petroleum ether (Bijleveld and Geelen, 1987).

Rate of CO_2 production

Neurons were incubated with a 2 m*M* concentration (0.5 μCi/flask) of either [U-^{14}C]lactate or 3-[1-^{14}C]hydroxybutyrate. After 1 h, reactions were stopped with 0.3 ml of 2 *M* $HClO_4$ and, at the same time, 0.15 ml of benzethonium hydroxide (1 *M* in methanol) was injected in a center well containing filter paper. Samples were allowed to equilibrate for an additional 12 h, and the center wells (with the $^{14}CO_2$ fixed as bicarbonate) were transferred to vials for radioactive counting (Sánchez et al., 1998).

Microdialysis procedures

Male Wistar rats (350–450 g) were used throughout. The microdialysis probe (CMA, Stockholm, Sweden; 4 mm length and 0.5 mm diameter, polystyrene, cutoff 100 kDa) was implanted stereotactically into the striatum (coordinates: A 1.0 from bregma; L 3.0; V −6.5; with bregma and lambda horizontal) of rats anesthetized with Equithesin (3.5 ml/kg). Perfusion was carried out with 0.15 m*M* (final concentration) albumin-bound [1-^{14}C]palmitate (30 Ci/mol) in artificial cerebrospinal fluid (in m*M:* 126.5 NaCl, 2.4 KCl, 0.5 NaH_2PO_4, 1.1 $CaCl_2$, 0.85 $MgCl_2$, and 27.5 $NaHCO_3$, pH 6.5) at a flow of 2 μl/min. Body temperature was maintained at 37°C with a heating pad controlled by a rectal thermometer. Thirty-minute (60-μl) fractions were collected after a 30-min stabilization period. Following collection of four baseline samples, the perfusion fluid was switched to one supplemented with 0.5 m*M* AICAR, and four further samples were collected before returning to basal conditions (i.e., AICAR-free medium), in which two more fractions were collected. Collection tubes contained $HClO_4$ (0.4 *M* final concentration) to avoid degradation of ketone bodies and were immediately frozen at −80°C until analyzed. The recovery of the probes was determined for each experiment, and the values obtained were corrected for that recovery. The average relative recovery was 31 ± 4% (n = 6). Blanks were determined with the probe immersed in a vial containing distilled water, instead of being placed in the brain, and were subtracted from the values of ketogenesis experimentally determined.

AMPK activity

AMPK activity was determined essentially as described before (Ponticos et al., 1998; Velasco et al., 1998*b*) with some modifications. Thus, the medium of the cells was aspirated and cells were washed with ice-cold phosphate-buffered saline (PBS). Cells were then scraped in buffer A, consisting of (in m*M*) 50 Tris-HCl, pH 7.5, 50 NaF, 1 EDTA, 5 sodium pyro-

phosphate, and 1 dithiothreitol, together with 10% (vol/vol) glycerol. This medium was supplemented with the following proteinase inhibitors (in μg/ml): 175 phenylmethylsulfonyl fluoride, 5 leupeptin, 2 aprotinin, 10 soybean trypsin inhibitor, and 10 benzamidine. Cell extracts were sonicated (2 × 5 s) on ice, and the soluble fraction was obtained after centrifugation at 40,000 *g* for 60 min. AMPK was assayed in buffer A supplemented with (in m*M*): 0.2 AMP, 0.2 [γ-^{32}P]ATP (2 μCi/assay), 5 $MgCl_2$, and 0.2 SAMS peptide. Negative controls were performed in the absence of cell extract, whereas endogenous phosphorylation was monitored by omitting the SAMS peptide from the incubations. After incubation for 15 min at 30°C, aliquots of the samples were spotted onto P81 papers. The P81 papers were washed five times with 1% (vol/vol) H_3PO_4 and once with acetone, and were finally transferred to vials for radioactive counting.

CPT-I activity

Astrocytes were preincubated for 45 min in the absence or presence of 10 μ*M* tetradecylglycidate, a specific irreversible inhibitor of CPT-I (Guzmán and Geelen, 1992; McGarry and Brown, 1997). The different modulators were added subsequently. After the times indicated, the medium was aspirated and cells were washed twice with PBS. CPT-I activity was determined in digitonin-permeabilized astrocytes as the tetradecylglycidate-sensitive incorporation of [*methyl*-^{3}H]carnitine into palmitoylcarnitine by two different methods. In method A ("one-step assay"), cell permeabilization and assay of enzyme activity were performed simultaneously (Blázquez et al., 1999). In method B ("two-step assay"), cells were first permeabilized and washed, and then enzyme activity was determined in the cell ghosts (Blázquez et al., 1998).

Other enzyme activities

ACC activity was determined in digitonin-permeabilized astrocytes as the incorporation of [1-^{14}C]acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction (Blázquez et al., 1998).

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase activity was determined in digitonin/Triton X-100-permeabilized astrocytes as the incorporation of [1-^{14}C]acetyl-CoA into HMG-CoA (Guzmán et al., 1995; Blázquez et al., 1999).

HMG-CoA reductase activity was determined in digitonin-permeabilized astrocytes as the incorporation of [3-^{14}C]HMG-CoA into mevalonate by a procedure based on that reported for permeabilized hepatocytes (Geelen et al., 1991). Thus, the medium was aspirated and cells were washed twice with PBS. Reactions were started by the addition of 300 μl of PBS plus 100 μl of assay mixture containing (in m*M*) 50 imidazol, pH 7.2, 1 EDTA, 1 EGTA, 150 KF, 120 glucose 6-phosphate, 10 $NADP^+$, 6 dithiothreitol, and 1 [3-^{14}C]HMG-CoA (0.5 μCi), together with 2.5 U of glucose-6-phosphate dehydrogenase and 30 μg of digitonin. After 6 min, reactions were stopped by the addition of 20 μl of concentrated HCl, and [^{14}C]mevalonate product was extracted (Geelen et al., 1991).

Other analytical procedures

Intracellular levels of malonyl-CoA were determined in perchloric acid cell extracts by a radioenzymatic method (Guzmán et al., 1995).

Intracellular levels of ATP, ADP, and AMP were determined in perchloric acid cell extracts by standard spectrophotometric methods (Bergmeyer, 1985).

Statistical analysis

Results shown represent the means ± SD of the number of experiments indicated in every case. Five or six different replicates of the various conditions within every experiment were performed routinely. Statistical analysis was performed by the Student *t* test.

RESULTS

Activation of AMPK stimulates ketogenesis in cultured astrocytes

AMPK has been shown to be present in brain (Gao et al., 1996; Stapleton et al., 1996; Woods et al., 1996), and its cellular distribution within the brain has been reported recently (Turnley et al., 1999). In a first set of experiments, we determined AMPK activity in extracts of cultured astrocytes and neurons. Rat cortical astrocytes were shown to express three times more AMPK activity than rat cortical neurons. Thus, values of AMPK activity (in nmol of phosphate incorporated into SAMS peptide/min/mg of cellular protein) were 0.44 ± 0.07 (astrocytes) and 0.15 ± 0.04 (neurons) ($n = 4$, $p < 0.01$).

AICAR is a selective cell-permeable activator of AMPK, which has been used widely to demonstrate the implication of this kinase in the regulation of cellular processes (Hardie and Carling, 1997). The effect of AICAR on lipid metabolism was studied therefore in astrocyte cultures. AICAR produced a time- and dose-dependent stimulation of ketogenesis from [^{14}C]palmitate (Fig. 1). CPT-I catalyzes the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix in many cell types (Guzmán and Geelen, 1993; Zammit, 1994; McGarry and Brown, 1997), including astrocytes (Blázquez et al., 1998). Three series of observations indicate that the AICAR-induced stimulation of ketogenesis from palmitate relies on the malonyl-CoA-dependent modulation of CPT-I activity:

(a) It is widely accepted that malonyl-CoA, the product of the reaction catalyzed by ACC, is the main physiological inhibitor of CPT-I (Zammit, 1994; McGarry and Brown, 1997). Thus, incubation of astrocytes with AICAR produced a depression of ACC activity, intracellular malonyl-CoA concentration, and rate of fatty acid synthesis de novo (Table 1). This was accompanied by an inhibition of HMG-CoA reductase activity and cholesterol synthesis de novo (Table 1).

(b) CPT-I activity may not be solely dependent on intracellular malonyl-CoA levels. It has been shown recently that rat liver CPT-I is also regulated by a malonyl-CoA-independent mechanism that involves inhibitory interactions with cytoskeletal components (Velasco et al., 1998*a*). A number of studies have shown that assaying CPT-I activity in extensively washed permeabilized cells, in which malonyl-CoA is removed from the medium (i.e., method B in the present report), allows detection of stable, malonyl-CoA-independent changes of enzyme activity. In contrast, assaying CPT-I activity by a rapid procedure in which permeabilization of the plasma membrane and assay of enzyme activity are per-

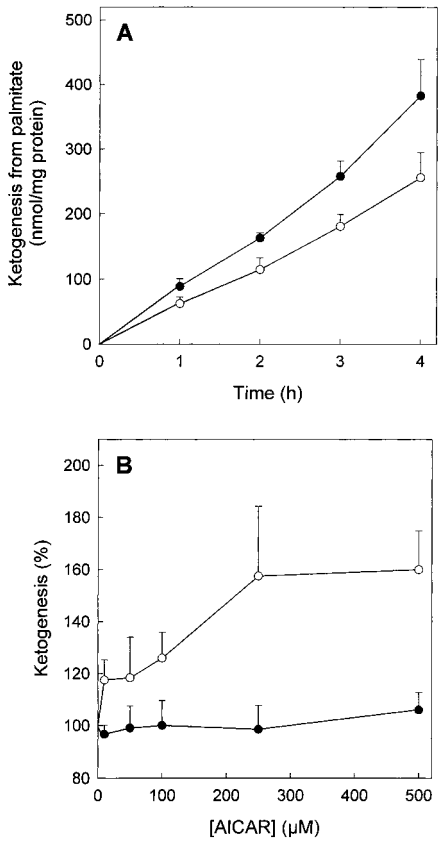

FIG. 1. AICAR stimulates ketogenesis in cultured astrocytes. **A:** Time course. Cells were incubated with [^{14}C]palmitate in the absence (○) or presence (●) of 0.5 m*M* AICAR for the times indicated. **B:** Dose–response curves. Cells were incubated with different concentrations of AICAR for 30 min. Then either [^{14}C]palmitate (○) or [^{14}C]octanoate (●) was added, and the rate of ketogenesis was determined after 1 h. Results are expressed as percentage of incubations with no additions. In both panels, results were obtained from six different experiments.

formed simultaneously (i.e., method A in the present report) allows retention of both malonyl-CoA-dependent and malonyl-CoA-independent changes of enzyme activity (e.g., Velasco et al., 1997*a*,*b*, 1998*a*). As shown in Table 1, CPT-I activity was enhanced by exposure of astrocytes to AICAR when enzyme activity was determined by method A. Moreover, the magnitude of this stimulation was quantitatively similar to that exerted by AICAR on ketogenesis from [^{14}C]palmitate. However, CPT-I activity was not affected by AICAR when enzyme activity was monitored by method B (Table 1).

(c) It is well established that long-chain fatty acids like palmitate are transported into mitochondria by a carnitine-dependent process, whereas medium-chain fatty acids like octanoate enter mitochondria independently of carnitine (Zammit, 1994). As shown in Fig. 1B, ketogenesis from [^{14}C]octanoate was unaffected by AICAR. Moreover, AICAR did not exert any significant effect on mitochondrial HMG-CoA synthase activity (Table 1), the putative regulatory enzyme of ketone body production from acetyl-CoA in hepatocytes (cf. Guzmán and Geelen, 1993; Zammit, 1994) and astrocytes (Cullingford et al., 1998*a*,*b*).

It is thus conceivable that the AICAR-induced stimulation of ketogenesis from long-chain fatty acids in astrocytes may be mediated by the AMPK-dependent phosphorylation and inactivation of ACC, thereby decreasing intracellular malonyl-CoA levels and deinhibiting CPT-I.

Activation of AMPK stimulates ketogenesis in the brain in vivo

Experiments were conducted subsequently to test whether activation of AMPK in vivo leads to a stimulation of ketone body production by the brain. It has been shown that astrocytes are the only brain cell population that can oxidize fatty acids to yield ketone bodies and that exhibit a preference for fatty acids over glucose or ketone bodies as their primary metabolic fuel (Edmond et al., 1987; Edmond, 1992). Brain ketone body production may be therefore a good reflection of astroglial ketogenesis. Thus, microdialysis experiments were carried out in which [^{14}C]palmitate was perfused into the brain, and the production of ketone bodies was recorded. As shown in Fig. 2, AICAR produced a remarkable stimulation of brain ketone body production in vivo. Furthermore, when AICAR was removed from the perfusion medium, the recovery of ketone bodies in the perfusate returned to basal levels.

TABLE 1. *Effect of AICAR on lipid metabolism in cultured astrocytes*

Parameter	Additions	
	None	AICAR
Rate of ketogenesis		
Ketogenesis from palmitate	56.6 ± 7.9	89.0 ± 12.4[a]
Ketogenesis from octanoate	92.3 ± 15.8	96.0 ± 6.1
Rate of fatty acid synthesis	62.1 ± 5.7	13.1 ± 3.4[a]
Rate of cholesterol synthesis	9.2 ± 1.1	1.5 ± 1.1[a]
ACC activity	0.23 ± 0.04	0.03 ± 0.02[a]
[Malonyl-CoA]	87 ± 8	12 ± 7[a]
HMG-CoA reductase activity	0.50 ± 0.10	0.17 ± 0.08[a]
CPT-I activity		
Method A	3.35 ± 0.60	5.63 ± 0.81[a]
Method B	2.87 ± 0.56	2.85 ± 0.37
HMG-CoA synthase activity	0.41 ± 0.13	0.36 ± 0.14

Astrocytes were incubated in the absence or presence of 0.5 m*M* AICAR for 30 min. Then cells were used for measurement of rates of lipid metabolism, enzyme activities, and malonyl-CoA concentration. Rates of ketogenesis are expressed as nmol of ^{14}C-fatty acid oxidized/h/mg of cellular protein. Rates of fatty acid and cholesterol synthesis are expressed as nmol of acetyl units into lipid/h/mg of cellular protein. Enzyme activities are expressed as nmol of product/min/mg of cellular protein. Malonyl-CoA levels are expressed as pmol/mg of cellular protein. Results were obtained from six different experiments.

Significantly different from incubations with no additions: [a] $p < 0.01$.

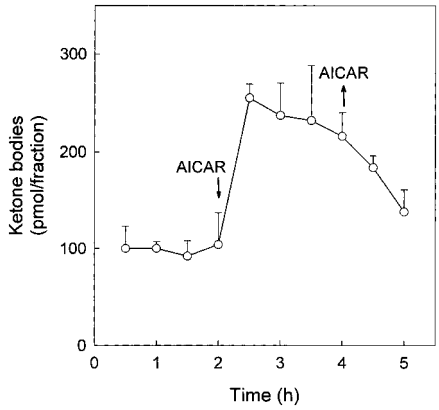

FIG. 2. AICAR stimulates ketogenesis in the brain in vivo. Microdialysis experiments with [^{14}C]palmitate were performed as described in Materials and Methods. [^{14}C]Palmitate was perfused for 2 h. Then [^{14}C]palmitate supplemented with 0.5 m*M* AICAR was perfused for an additional 2 h, after which [^{14}C]palmitate alone was perfused again for 1 h more. Results are expressed as pmol of ^{14}C-fatty acid oxidized/fraction and were obtained from six different experiments.

Activation of AMPK sustains ketogenesis in astrocytes during hypoxia

Because the nervous system has one of the highest rates of oxygen metabolism in the body and lacks tissue oxygen stores, it depends on a continuous exogenous supply of oxygen (Tsacopoulos and Magistretti, 1996). Pathological conditions such as ischemia/hypoxia that diminish or arrest oxygen supply may lead therefore to a rapid breakdown of brain energy homeostasis (Obrenovitch, 1995; Swanson et al., 1997). As the brain may use ketone bodies as a glucose-replacing fuel (Edmond, 1992; Zammit, 1994), we tested whether astroglial ketogenesis might play a role in the delivery of substrates to neurons under hypoxia. Azide blocks the oxygen-requiring steps of energy metabolism by inhibition of the respiratory electron transport chain, and is therefore widely used as an experimental model of hypoxia in vitro—the so called "chemical hypoxia" (cf. Swanson et al., 1997).

Fatty acid β-oxidation comprises two oxidative steps (i.e., the reactions catalyzed by acyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase), and hence uncoupling of the respiratory chain by azide might lead to inhibition of β-oxidation by preventing reoxidation of reduction equivalents (Eaton et al., 1996). Thus, incubation of astrocytes with azide depressed intramitochondrial fatty acid metabolism, as evidenced by the inhibition of octanoate oxidation (Fig. 3A). However, ketogenesis from palmitate was not affected significantly by azide (Fig. 3B).

Mitochondrial metabolism of palmitate and that of octanoate differ essentially in that the former, but not the latter, requires CPT-I to enter mitochondria (Zammit, 1994). In addition, pharmacological activation of AMPK enhances CPT-I activity in astrocytes (see above). May AMPK stimulate CPT-I and therefore long-chain fatty acid translocation into mitochondria during hypoxia? As shown in Table 2, azide produced a slight decrease in ATP concentration in astrocytes, which was concomitant to an increase in ADP and particularly AMP levels. AMPK activity was determined subsequently in astrocyte extracts. Thus, hypoxia led to a marked stimulation of astroglial AMPK that was accompanied by a decrease of ACC activity and intracellular malonyl-CoA levels (Table 2). Moreover, CPT-I activity was enhanced in azide-treated astrocytes when method A was used to determine enzyme activity. In contrast, the use of method B to determine CPT-I activity did not reveal any effect of azide (Table 2). All these observations indicate that during hypoxia the stimulation of AMPK may lead to an activation of long-chain fatty acid transport into mitochondria.

Cultured neurons preferentially oxidize 3-hydroxybutyrate over lactate during hypoxia

The sustained production of ketone bodies by astrocytes during hypoxia might provide ketone bodies as substrate for neuronal metabolism. However, it might be

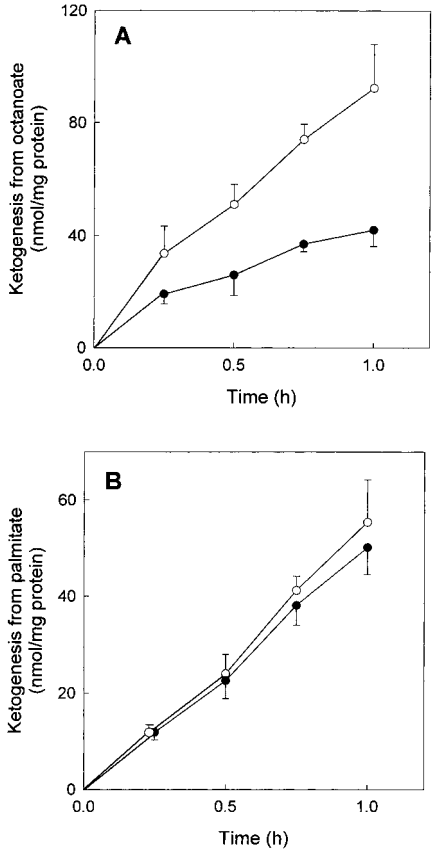

FIG. 3. Differential effect of hypoxia on ketogenesis from octanoate and palmitate in cultured astrocytes. **A:** Ketogenesis from octanoate. **B:** Ketogenesis from palmitate. Cells were incubated in the absence (○) or presence (●) of 1 m*M* NaN_3 for 1 h. Then the ^{14}C-fatty acid was added, and ketogenesis was determined at the times indicated. Results were obtained from six different experiments.

TABLE 2. *Effect of chemical hypoxia on AMPK activity and ketogenesis in cultured astrocytes*

Parameter	Additions	
	None	Azide
Rate of ketogenesis		
Ketogenesis from palmitate	53.2 ± 8.8	49.1 ± 5.9
Ketogenesis from octanoate	93.8 ± 11.6	42.0 ± 5.8[a]
[ATP]	16.1 ± 1.7	13.4 ± 1.4[b]
[ADP]	2.1 ± 0.5	3.4 ± 0.7[b]
[AMP]	0.3 ± 0.01	1.1 ± 0.4[a]
[AMP]/[ATP](%)	1.9 ± 0.5	8.2 ± 1.7[a]
AMPK activity	0.42 ± 0.08	0.87 ± 0.14[a]
ACC activity	0.25 ± 0.04	0.16 ± 0.02[a]
[Malonyl-CoA]	84 ± 7	49 ± 13[a]
CPT-I activity		
Method A	3.04 ± 0.43	4.27 ± 0.35[a]
Method B	2.69 ± 0.31	2.54 ± 0.60

Astrocytes were incubated in the absence or presence of 1 m*M* NaN_3 for 1 h. Then cells were used for measurement of the rate of ketogenesis, enzyme activities, and concentrations of adenine nucleotides and malonyl-CoA. Rates of ketogenesis are expressed as nmol of ^{14}C-fatty acid oxidized/h/mg of cellular protein. Enzyme activities are expressed as nmol of product/min/mg of cellular protein. Nucleotide levels are expressed as nmol/mg of cellular protein. Malonyl-CoA levels are expressed as pmol/mg of cellular protein. Results were obtained from six different experiments.

Significantly different from incubations with no additions: [a] $p < 0.01$; [b] $p < 0.05$.

argued that ketone body oxidative utilization by neurons may be depressed during hypoxia. The effect of azide on neuronal utilization of 3-hydroxybutyrate was compared therefore with that of lactate, a well known substrate that may sustain neuronal activity (Tsacopoulos and Magistretti, 1996; Izumi et al., 1997; Wiesinger et al., 1997). As shown in Fig. 4, under basal conditions neurons oxidized 3-hydroxybutyrate at a much higher rate than lactate. Furthermore, chemical hypoxia produced a significant (37 ± 10%) inhibition of lactate utilization by neurons, whereas 3-hydroxybutyrate oxidation remained unaffected (Fig. 4). Hence, cultured neurons seem to use 3-hydroxybutyrate preferentially over lactate as fuel during hypoxia.

DISCUSSION

AMPK is actively involved in the regulation of ketogenesis in astrocytes

It is widely accepted that the AMPK cascade plays the overall function of controlling metabolism in response to the varying energy status of the cell. The AMPK cascade is believed to respond to changes in the AMP/ATP ratio rather than to changes in AMP levels (Hawley et al., 1995; Hardie et al., 1998). The reaction catalyzed by adenylyl kinase ($2ADP \rightleftarrows ATP + AMP$) makes AMP and ATP concentrations tend to change in opposite directions. Because the equilibrium constant of that reaction is close to one, the AMP/ATP ratio in cells varies approximately as the square of the ADP/ATP ratio (cf. Hardie et al., 1998; the present report). AMP levels in fully energized cells are very low, and AMPK is basically inactive. However, when environmental stress ensues and the ADP/ATP ratio increases, the AMP/ATP ratio enhances much further, allowing the AMPK cascade a high sensitivity to small changes in AMP levels (Hardie et al., 1998). This occurs, for example, when isolated hepatocytes are exposed to heat shock, arsenite, fructose, or cadmium ions (cf. Hardie et al., 1998). However, the precise role of AMPK in liver under the physiological setting is as yet not obvious (Hardie and Carling, 1997). On the basis of in vitro and in vivo experiments, a physiological role for AMPK in the regulation of astroglial ketogenesis during hypoxia is proposed in Fig. 5. Thus, the inhibition of the respiratory electron transport chain during hypoxia may lead to the activation of long-chain fatty acid transport into mitochondria through the cascade increase of the AMP/ATP ratio → AMPK stimulation → ACC inhibition → decrease of malonyl-CoA concentration → CPT-I deinhibition. This might compensate the depression of intramitochondrial fatty acid oxidative metabolism induced by hypoxia, thereby allowing astroglial ketogenesis to be maintained at high rates. Nevertheless, we are aware that it may be difficult to conceive how an increased intramitochondrial concentration of fatty acyl-CoA could lead to a sustained increase in the rate of fatty acyl-CoA oxidation when oxidation power is rate-limiting. In addition, it should be kept in mind that carnitine has been shown to induce a modest stimulation of octanoate oxidation, perhaps through a malonyl-CoA-sensitive carnitine octanoyltransferase, which seems to be the same protein as the soluble peroxisomal carnitine palmitoyltransferase (cf. Zammit, 1994; McGarry and Brown, 1997).

The preferential expression of AMPK activity in astrocytes compared with neurons observed in the present

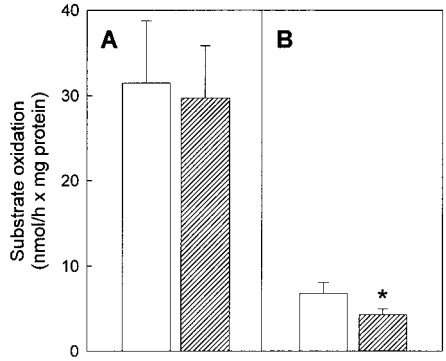

FIG. 4. Differential effect of hypoxia on 3-hydroxybutyrate and lactate oxidation by cultured neurons. **A:** 3-Hydroxybutyrate oxidation. **B:** Lactate oxidation. Cells were incubated in the absence (open columns) or presence (hatched columns) of 1 m*M* NaN_3 for 1 h. Then either 3-[^{14}C]hydroxybutyrate or [^{14}C]lactate was added, and $^{14}CO_2$ production was determined after 1 h. Results were obtained from six different experiments. Significantly different from the respective incubations with no additions: * $p < 0.01$.

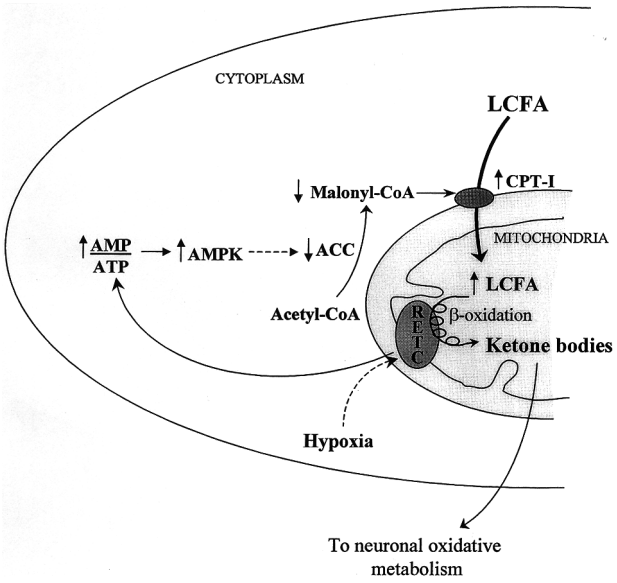

FIG. 5. Proposed model for the role of AMPK in the regulation of ketone body production by astrocytes during hypoxia. Dashed lines indicate inhibition. LCFA, long-chain fatty acids; RETC, respiratory electron transport chain. See Results and Discussion for further details.

work may support a potential physiological role of the kinase in the control of astroglial ketogenesis. However, Turnley et al. (1999) have shown recently that in the brain in vivo the mRNA levels of the AMPK α_1 and α_2 catalytic subunits are higher in neurons than in astrocytes. The reason for this discrepancy may be that cultured cells most likely differ in several respects from cells in vivo. In particular, cultured astrocytes take on many of the characteristics of activated astrocytes, whereas most astrocytes in vivo are quiescent (Ridet et al., 1997; Wu and Schwartz, 1998). This makes a direct comparison of AMPK activity in cultured astrocytes and neurons difficult to interpret. In addition, AMPK activity may not necessarily be directly correlated with the level of expression of AMPK. Thus, AMPK activity inside the cell may depend on the intracellular concentration of allosteric modulators (AMP, ATP), the activity of the protein kinases and phosphatases involved in the covalent modification of AMPK, etc. It is worth noting, however, that Turnley et al. (1999) have reported an up-regulation of AMPK expression, especially of the α_2 subunit, in astrocytes from dysmyelinated mice, which exhibit a remarkable gliosis. This may be in line with data in the present article, as in vivo hypoxia is likely to cause activation of astrocytes (cf. Ridet et al., 1997; Aschner, 1998).

The ACC/malonyl-CoA/CPT-I system regulates ketogenesis in astrocytes

Previous studies on metabolic regulation in cultured astrocytes have investigated mostly carbohydrate (e.g., Tsacopoulos and Magistretti, 1996; Wiesinger et al., 1997) and amino acid metabolism (e.g., Sonnewald et al., 1997; Tsacopoulos et al., 1997). The present study focused instead on the regulation of ketone body production by astrocytes. Like hepatocytes (Zammit, 1994), astrocytes possess an enzymatic equipment capable of synthesizing large amounts of ketone bodies (Auestad et al., 1991; Blázquez et al., 1998). In addition, both astrocytes (Edmond et al., 1987) and hepatocytes (Zammit, 1994) exhibit a preference for fatty acids, compared with glucose, as their primary metabolic fuel. However, the mechanisms that regulate ketogenesis in astrocytes remain largely unknown. Ketogenesis is a highly integrated process that comprises a number of potential pace-setting steps (Guzmán and Geelen, 1993; Zammit, 1994; Eaton et al., 1996). Recent determination of flux control coefficients of the enzymes involved in ketogenesis shows that CPT-I catalyzes the pace-setting step of ketogenesis from long-chain fatty acids in both hepatocytes (Drynan et al., 1996; Spurway et al., 1997) and astrocytes (Blázquez et al., 1998). Data in the present report showing that changes in the rate of ketogenesis upon activation of AMPK rely on parallel changes in CPT-I activity are in agreement with that notion.

In addition to CPT-I, mitochondrial HMG-CoA synthase has been suggested by some authors to be a putative important site of control of ketogenesis from acetyl-CoA in astrocytes (Cullingford et al., 1998*a*,*b*) and hepatocytes (cf. Zammit, 1994; Eaton et al., 1996). However, in the present study, AICAR did not exert any significant effect on ketogenesis from octanoate and mitochondrial HMG-CoA synthase activity. Likewise, the stimulation of ketogenesis by cyclic AMP-raising agents (Blázquez et al., 1998) and ceramide-generating compounds (Blázquez et al., 1999) was shown to be independent of changes in mitochondrial HMG-CoA synthase activity. The regulatory role of mitochondrial HMG-CoA synthase in astrocytes needs therefore to be demonstrated.

Evidence has accumulated during the last two decades highlighting the physiological importance of malonyl-CoA, the product of the reaction catalyzed by ACC, in the regulation of CPT-I activity and ketogenesis in the liver (Guzmán and Geelen, 1993; Zammit, 1994; McGarry and Brown, 1997). As ACC is a key regulatory enzyme of fatty acid synthesis (cf. Guzmán and Geelen, 1993; Zammit, 1994), malonyl-CoA inhibition of CPT-I allows an elegant explanation for the coordinate control of fatty acid synthesis and oxidation. In addition, a malonyl-CoA-independent mechanism of control of rat liver CPT-I activity was put forward several years ago. These malonyl-CoA-independent changes of hepatic CPT-I activity have been shown to rely on the modulation of interactions between mitochondria and cytoskeletal components, most likely keratin intermediate filaments (Velasco et al., 1998*a*). Here we show that the ACC/malonyl-CoA/CPT-I system is solely responsible for the regulation of ketogenesis by AMPK in astrocytes. It is conceivable that the very different composition of intermediate filaments in hepatocytes (mostly cytokeratins 8 and 18) and astrocytes (mostly glial fibrillary

acidic protein) may account for the different mode of regulation of CPT-I in the two cell types.

Ketone bodies and lactate as neuronal fuels during hypoxia

Evidence has accumulated over the last few years indicating that glucose entering the brain is taken up mostly by astrocytes, that lactate is a major product of glucose metabolism by astrocytes, and that lactate is a good substrate for neuronal oxidative metabolism. In addition, glutamate released from neurons during synaptic activity and taken up by astrocytes triggers glucose utilization and lactate production in the latter cells. Moreover, the zonal distribution of monocarboxylate transporters and lactate dehydrogenase isoforms within the brain suggests that astrocytes are lactate "sources," whereas neurons are lactate "sinks." These observations, mostly obtained by Magistretti and co-workers, point to the existence of an activity-dependent astrocyte–neuron lactate shuttle for sustaining neuronal energy metabolism (Magistretti and Pellerin, 1996; Tsacopoulos and Magistretti, 1996; Pellerin et al., 1998).

Glucose utilization by the brain under the physiological setting is nearly matched by CO_2 production. However, when the local glycolytic rate exceeds the local oxidative metabolic rate, lactate may accumulate in brain (Swanson and Benington, 1996; Swanson et al., 1997; Schurr and Rigor, 1998). Accumulation of lactate in the brain is especially notable during pathological conditions of ischemia/hypoxia. In those situations, the anaerobic metabolism of glucose in astrocytes is stimulated, leading therefore to an enhanced output of lactate that might be available for neuronal metabolism (Swanson et al., 1997; Schurr and Rigor, 1998). However, the remarkable inhibition of astroglial glycolysis at low pH on lactate accumulation (Swanson et al., 1997), as well as the inhibition of lactate utilization by neurons on exposure to azide (the present report), may prevent to a certain extent the use of lactate as a neuronal fuel during hypoxia. When energy production in neurons is arrested, transmembrane ion gradients are lost and neuronal death ensues (cf. Cotrina et al., 1998; Rose et al., 1998).

May ketone bodies be a major neuronal fuel during hypoxia? It may be argued that whereas lactate output by astrocytes is enhanced during hypoxia, ketone body production is not stimulated. However, we have not observed any significant depression of ketone body production by astrocytes when the pH of the culture medium was reduced down to 6.6 (C. Blázquez and M. Guzmán, unpublished observations). Furthermore, under the experimental conditions used herein, (a) 3-hydroxybutyrate was preferred over lactate as a substrate for neuronal oxidative metabolism, and (b) 3-hydroxybutyrate oxidation by neurons was not significantly blunted by hypoxia. It should be also kept in mind that after conversion to acetyl-CoA by the action of lactate dehydrogenase and pyruvate dehydrogenase (PDH), 1 mol of lactate can provide 18 mol of ATP via oxidative phosphorylation, whereas after conversion to acetyl-CoA by the reactions catalyzed by 3-hydroxybutyrate dehydrogenase, 3-oxoacid-CoA transferase, and 3-ketoacyl-CoA thiolase, 1 mol of 3-hydroxybutyrate may yield 26 mol of ATP via oxidative phosphorylation. The differential effect of azide on lactate and 3-hydroxybutyrate utilization by neurons may be due to the high sensitivity to hypoxia of the reaction catalyzed by PDH, because elevations of the $NADH/NAD^+$ ratio exert a double inhibitory effect on PDH activity, viz., direct inhibition by a mass action effect and indirect inhibition via activation of PDH kinase, which phosphorylates and inactivates PDH (cf. Sugden et al., 1989; Zaidan et al., 1998). Although the extent to which ketone body formation by astrocytes occurs in vivo remains to be determined, and the role of lactate as a neuronal fuel is supported by solid experimental evidence (see above), our data indicate that ketone bodies may be used together with lactate as substrate for neuronal oxidative metabolism. It is clear anyway that further research is required to understand the role of astroglial ketogenesis in supporting neuronal function.

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SUMARIO

Los astrocitos son las únicas células del cerebro capaces de utilizar los ácidos grasos preferentemente sobre la glucosa y los cuerpos cetónicos como fuente energética. Es más, los astrocitos en cultivo producen cuerpos cetónicos a partir de ácidos grasos y leucina. Sin embargo, los mecanismos que controlan el flujo de la ruta cetogénica en astrocitos son aún desconocidos. Por ello, tratamos de caracterizar el proceso de formación de cuerpos cetónicos en astrocitos en cultivo, realizando un estudio exhaustivo de la CPT-I, puesto que se trata de una enzima reguladora clave de la ruta en hepatocitos. Se puso además especial énfasis en el estudio de algunos de los mecanismos de regulación implicados, concretamente los mediados por PKA, AMPK y ceramida. Por último, estudiamos la importancia de la cetogénesis astroglial en la hipoxia, situación en la cual el estado energético celular se ve comprometido, lo que puede implicar la activación de la cascada de la AMPK.

Los datos obtenidos muestran que los astrocitos producen una gran cantidad de cuerpos cetónicos, que constituyen prácticamente el total de los productos de oxidación de ácidos grasos. Además, la CPT-I cataliza la etapa limitante de la cetogénesis en astrocitos. Estas células expresan la isoforma hepática de CPT-I y un patrón de isoformas de ACC similar al del hígado. La ruta cetogénica en astrocitos es flexible, y se activa vía PKA y AMPK. Ambas quinasas inducen la fosforilación e inactivación de la ACC, de manera que al disminuir los niveles de malonil-CoA se estimularía la CPT-I y por tanto la cetogénesis. La activación de la AMPK en hipoxia podría permitir a los astrocitos suministrar a las neuronas vecinas cuerpos cetónicos como combustible. Por último, el segundo mensajero lipídico ceramida parece ejercer una activación directa de la CPT-I que es independiente del sistema ACC/malonil-CoA/CPT-I.

3. RESULTADOS Y DISCUSIÓN II
CETOGÉNESIS EN ASTROCITOS: POSIBLE PAPEL CITOPROTECTOR

De novo-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase

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ABSTRACT **Recent observations support the importance of ceramide synthesis *de novo* in the induction of apoptosis. However, the downstream targets of *de novo*-synthesized ceramide are unknown. Here we show that palmitate incorporated into ceramide and induced apoptotic DNA fragmentation in astrocytes. These effects of palmitate were exacerbated when fatty acid breakdown was uncoupled and were not evident in neurons, which show a very low capacity to take up and metabolize palmitate. Palmitate-induced apoptosis of astrocytes was prevented by L-cycloserine and fumonisin B1, two inhibitors of ceramide synthesis *de novo*, and by PD098059, an inhibitor of the extracellular signal-regulated kinase (ERK) cascade. Accordingly, palmitate activated ERK by a process that was dependent on ceramide synthesis *de novo* and Raf-1, but independent of kinase suppressor of Ras. Other potential targets of ceramide in the control of cell fate, namely, c-Jun amino-terminal kinase, p38 mitogen-activated protein kinase, and protein kinase B, were not significantly affected in astrocytes exposed to palmitate. Results show that the Raf-1/ERK cascade is the selective downstream target of *de novo*-synthesized ceramide in the induction of apoptosis in astrocytes and also highlight the importance of ceramide synthesis *de novo* in apoptosis of astrocytes, which might have pathophysiological relevance.—Blázquez, C., Galve-Roperh, I., Guzmán, M. *De novo*-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase. *FASEB J.* 14, 2315–2322 (2000)**

Key Words: cell death · sphingolipids · mitogen-activated protein kinases · neural cells

CERAMIDE PLAYS AN important role in the control of cell fate in the central nervous system under different pathophysiological situations. Thus, elevations of intracellular ceramide levels, which may in turn be related to the induction of apoptotic cell death, have been shown to occur in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, epilepsy and ischemia/stroke (1–3). Likewise, exposure of neural cells to physical (e.g., ultraviolet radiation), chemical (e.g., tumor necrosis factor, TNF), bacterial (e.g., lipopolysaccharide), or viral (e.g., human immunodeficiency virus 1) stimuli may increase intracellular ceramide levels and therefore evoke changes in the cell survival/death decision (4, 5). Moreover, changes in ceramide metabolism exert important regulatory effects on neuronal growth and development (6).

In ceramide signaling pathways leading to apoptosis, ceramide generation through sphingomyelin hydrolysis by neutral and/or acid sphingomyelinase is usually considered the norm. The link between receptor activation, sphingomyelinase activation, and ceramide generation is mostly supported by comprehensive studies of the p55 TNF receptor, the p75 neurotrophin receptor, and CD95/Fas (4, 5). However, the *de novo* synthesis pathway has been gaining recognition as an alternative means of generating a signaling pool of ceramide. Thus, compounds such as L-cycloserine, an inhibitor of serine palmitoyltransferase, and fumonisin B1, an inhibitor of ceramide synthase, prevent ceramide accumulation and apoptotic death in hematopoietic (7) and pancreatic β cells (8) exposed to long-chain fatty acids, which are substrates for ceramide synthesis *de novo*. A significant contribution of the *de novo* pathway to ceramide generation and apoptosis has also been reported in endothelial cells exposed to TNF, a paradigmatic example of ligands that are believed to generate ceramide solely through sphingomyelin breakdown (9), and in PC12 pheochromocytoma cells exposed to angiotensin II (10). In addition, the chemotherapeutic drug daunorubicin may induce apoptosis by enhancing ceramide synthesis *de novo* (11) as well as by inducing sphingomyelin breakdown (12, 13).

Despite these recent observations supporting the importance of ceramide synthesis *de novo* in the induction of apoptosis, the characterization of the downstream targets linking *de novo*-synthesized ceramide to apoptosis remains elusive. Moreover, al-

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though ceramide may significantly contribute to cell death in neurological disorders, the possible involvement of ceramide synthesis *de novo* in neural cell death is as yet unknown. The present study was therefore undertaken to address two questions: *1)* Does *de novo*-synthesized ceramide induce apoptosis of neural cells? *2)* If so, which may be downstream targets of *de novo*-synthesized ceramide leading to apoptosis?

MATERIALS AND METHODS

Cell culture

Cortical astrocytes were derived from 24 h Wistar rats and cultured in serum-containing medium as described before (14). For all the experimental determinations performed, the serum-containing medium was removed and cells were transferred to a chemically defined, serum-free medium consisting of DMEM/Ham's F12 (1:1, v/v) supplemented with 5 μg/ml insulin, 50 μg/ml transferrin, 20 nM progesterone, 50 μM putrescine, 30 nM sodium selenite, and 1.0% (w/v) defatted and dialyzed bovine serum albumin. Cortical neurons from 24 h rats were cultured exactly as described before (14).

Cell death

Cell viability was determined by trypan blue exclusion. Oligonucleosomal DNA fragmentation, a characteristic biochemical feature of apoptotic cell death, was measured using a nucleosome DNA enzyme-linked immunoabsorbent assay (Boehringer, Mannheim, Germany), which quantitatively records histone-associated DNA fragments.

Ceramide and sphingomyelin syntheses

Cells were transferred to chemically defined medium. After 24 h, reactions were started by the addition of 1 μCi of L-[U-^{14}C]serine per well together with the different modulators. Reactions were terminated at the times indicated by aspiration of the medium and addition of 1 ml methanol. Lipids were extracted and saponified, and ceramide and sphingomyelin were resolved by thin-layer chromatography in parallel with standards on silica-gel G60 plates with chloroform:methanol:water (100:42:6, v/v/v) as the developing system until the front had reached two-thirds of the plate. The solvent was then evaporated and plates were subsequently run with chloroform:methanol:acetic acid (94:1:5, v/v/v) until the front had reached the top of the plate (15).

Fatty acid uptake and metabolism

Cells were transferred to chemically defined medium. After 24 h, reactions were started by the addition of 0.2 mM (1 μCi) albumin-bound [9,10-^{3}H]palmitate together with the different modulators. At the times indicated, the medium was separated from the cells, and lipids were extracted from the two compartments and subsequently resolved by thin-layer chromatography together with standards. Fatty acid uptake was calculated as the disappearance of [^{3}H]palmitate from the extracellular medium. Nonesterified fatty acids and triacylglycerols were separated on silica-gel G60 plates with chloroform/diethyl ether/acetic acid (70:30:1, v/v/v) as the developing system. Phosphatidylcholine was resolved on silica-gel H60 plates with chloroform/methanol/acetic acid/water (50/25/8/1, v/v/v/v) as developing system. Ceramide and sphingomyelin were resolved as described above.

Mitogen- and stress-activated protein kinase activities

Cells were washed and lysed, and supernatants were obtained as described before (16). Extracellular signal-regulated kinase (ERK) activity was determined as the incorporation of [γ-^{32}P]ATP into a specific peptide substrate (16). The activity of c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) was monitored as the incorporation of [γ-^{32}P]ATP into specific substrates (c-Jun 1–169 and MAPKAP kinase-2 46–600, respectively) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography, and radioactive counting of the excised substrate bands according to manufacturer's instructions (Upstate Biotechnology, Lake Placid, N.Y.) (17).

Raf-1 activity

Raf-1 was immunoprecipitated from cell lysates as described before (16). The kinase reaction was carried out for 30 min at 30°C with 0.7 μg kinase-negative MEK1[97A] (Upstate Biotechnology) and 2 μCi [γ-^{32}P]ATP as substrates in assay buffer containing 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EDTA, 5 mM NaF, 1 mM $NaVO_4$, 1 mM 4-nitrophenylphosphate, and proteinase inhibitors (17, 18). Reactions were stopped with SDS sample buffer, and substrate phosphorylation was determined in the excised bands after SDS-PAGE and autoradiography.

Kinase suppressor of Ras (KSR) activity

KSR was immunoprecipitated from cell lysates with an anti-KSR antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) bound to protein G-Sepharose. The kinase reaction was carried out for 30 min at 30°C, with 0.3 mM synthetic Raf-1 peptide (17, 18) and 2 μCi [γ-^{32}P]ATP as substrates in the assay buffer described above for Raf-1. Phosphorylated peptide was resolved by P81 phosphocellulose paper.

Protein kinase B (PKB) activity

PKB was immunoprecipitated from cell lysates with 2 μg of anti-PKBα antibody bound to protein G-Sepharose (19). PKB activity was determined as the incorporation of [γ-^{32}P]ATP into a specific peptide substrate. Phosphorylated peptide was resolved by P81 phosphocellulose paper (19).

Statistical analysis

Results shown represent the means ± SD of the number of experiments indicated in every case. Five to six different replicates of the various conditions included in each experiment were routinely performed. Statistical analysis was performed by analysis of variance. A *post hoc* analysis was made by the Student-Neuman-Keuls test.

RESULTS

Palmitate signals apoptosis of astrocytes via ceramide synthesis *de novo*

Astrocytes in primary culture were exposed to palmitate at a concentration physiologically relevant in

brain (20), and cell viability was determined at different times. As shown in **Fig. 1*A***, palmitate induced the death of astrocytes in a time-dependent fashion. This effect of palmitate was more pronounced when mitochondrial fatty acid oxidation was blocked with tetradecylglycidic acid (TDGA), a specific inhibitor of carnitine palmitoyltransferase I (CPT-I), the key regulatory enzyme of long-chain fatty acid translocation into mitochondria in astrocytes (14). TDGA alone had no significant effect on cell viability.

The lipid second messenger ceramide is involved in the induction of apoptosis in a number of pathophysiological situations (4, 5). Ceramide is mostly generated by degradation of sphingomyelin or by *de novo* synthesis. Because long-chain fatty acids are biosynthetic precursors of ceramide, the possibility that intracellular ceramide accumulation resulting from enhanced ceramide synthesis mediates palmitate-induced astrocyte death was tested. Palmitate

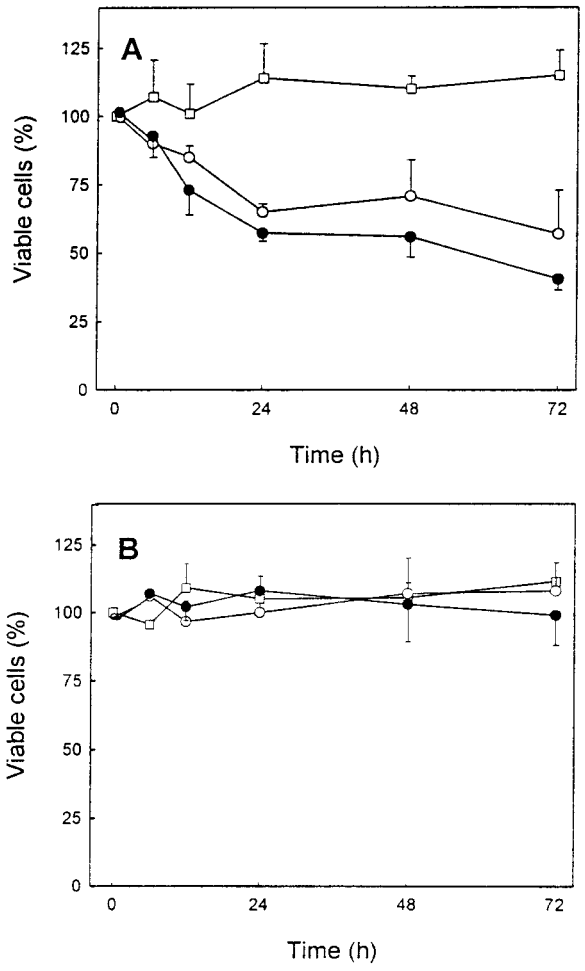

Figure 1. Palmitate-induced death of astrocytes but not of neurons. Cells were incubated for the times indicated with 0.2 mM palmitate in the absence (○) or presence (●) of 20 μM TDGA, or with 20 μM TDGA alone (□). Results are expressed as percentage of incubations with no additions. *A)* Astrocytes (n=6). *B)* Neurons (n=4).

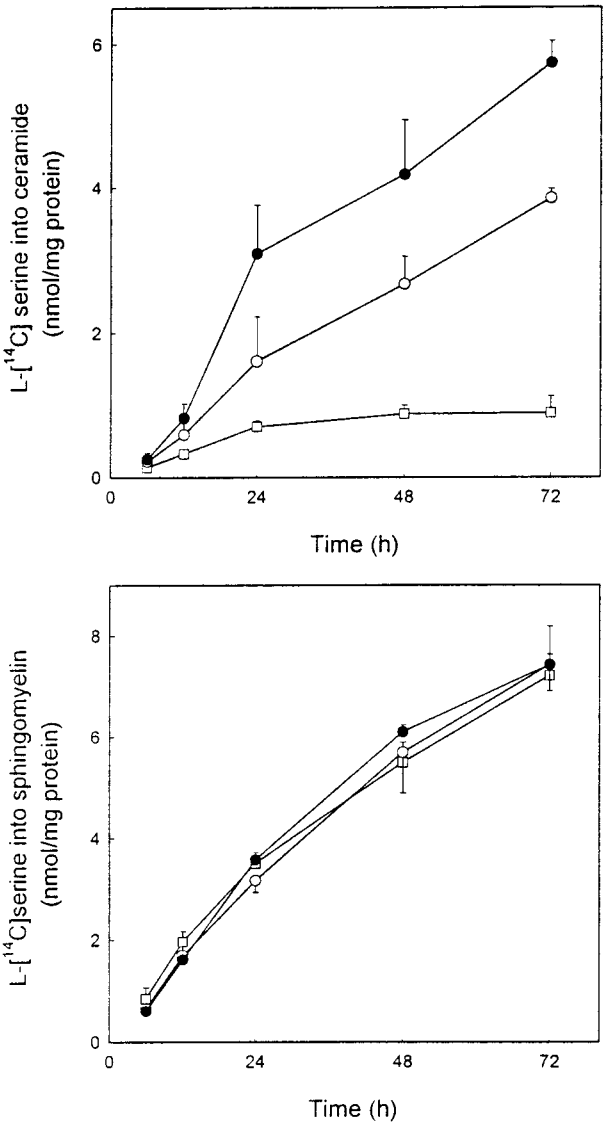

Figure 2. Palmitate-induced synthesis of ceramide but not of sphingomyelin in astrocytes. Cells were incubated for the times indicated with L-[^{14}C]serine, either alone (□), plus 0.2 mM palmitate (○), or plus 0.2 mM palmitate and 20 μM TDGA (●). Results are expressed as percentage of incubations with no additions and were obtained from 6 different experiments. *A)* Ceramide. *B)* Sphingomyelin.

notably increased ceramide synthesis in primary astrocytes (**Fig. 2*A***). By contrast, no significant effect of palmitate on serine incorporation into sphingomyelin was evident (Fig. 2*B*). Coincubation of the cells with palmitate and TDGA exacerbated the effect of the fatty acid on ceramide synthesis (Fig. 2*A*). A similar time course was observed in astrocytes for palmitate-induced death (Fig. 1*A*) and palmitate-induced ceramide synthesis (Fig. 2*A*).

To obtain additional evidence that enhanced ceramide synthesis in palmitate-treated astrocytes reflected *de novo* ceramide formation, cells were incubated with two inhibitors of ceramide biosynthesis: *1)* L-cycloserine, an inhibitor of serine palmitoyltransferase, the first committed step of ceramide synthesis

TABLE 1. *Palmitate-induced ceramide synthesis and astrocyte death: prevention by L-cycloserine and fumonisin B1*[a]

Additions	Viable cells (%)	L-[^{14}C]serine into lipid: Ceramide (%)	L-[^{14}C]serine into lipid: Sphingomyelin (%)
None	100 ± 8	100 ± 23	100 ± 12
C16:0	68 ± 8*	303 ± 43*	105 ± 11
C16:0 + TDGA	56 ± 7*	475 ± 56*	112 ± 6
L-Cycloserine	94 ± 8	51 ± 14*	14 ± 5*
C16:0 + L-cycloserine	94 ± 4	53 ± 9*	8 ± 6*
C16:0 + TDGA + L-cycloserine	96 ± 6	56 ± 6*	11 ± 4*
Fumonisin B1	93 ± 12	18 ± 5*	15 ± 8*
C16:0 + TDGA + fumonisin B1	89 ± 6	13 ± 2*	10 ± 4*

[a] Astrocytes were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0), 20 μM TDGA, 2 mM L-cycloserine, and/or 0.1 mM fumonisin B1. Results are expressed as percentage of incubations with no additions, and were obtained from 6 different experiments.

* Significantly different ($P<0.01$) from the respective incubations with no additions.

de novo; *2)* fumonisin B1, an inhibitor of ceramide synthase, which catalyzes the condensation of sphinganine and acyl-CoA to generate dihydroceramide. As shown in **Table 1**, both L-cycloserine and fumonisin B1 were able to block both palmitate-induced ceramide synthesis and palmitate-induced astrocyte death, even in the presence of TDGA.

Next, we tested whether astrocyte death occurred by a process of apoptosis, as expected for ceramide-mediated cell death. As shown in **Fig. 3**, treatment of astrocytes with palmitate led to a significant increase in oligonucleosomal DNA fragmentation, a hallmark of apoptosis. Again, the effect of palmitate was more remarkable when TDGA was simultaneously present in the incubations. Moreover, the apoptotic effect of palmitate was prevented by L-cycloserine.

De novo-synthesized ceramide signals apoptosis of astrocytes via Raf-1/ERK

It is generally accepted that the ERK cascade promotes cell proliferation. However, recent investigations have begun to define situations in which sustained ERK activation mediates antiproliferative effects (21, 22). We therefore studied the possible involvement of ERK in fatty acid-induced apoptosis of astrocytes. PD098059, a selective inhibitor of the ERK cascade, prevented the decrease in astrocyte viability elicited by palmitate, even in the presence of TDGA. Thus, values of viability of primary astrocytes were 103 ± 10% after 48 h exposure to 25 μM PD098059; 101 ± 11% after 48 h exposure to 0.2 mM palmitate and 25 μM PD098059; and 99 ± 7% after 48 h exposure to 0.2 mM palmitate, 20 μM TDGA

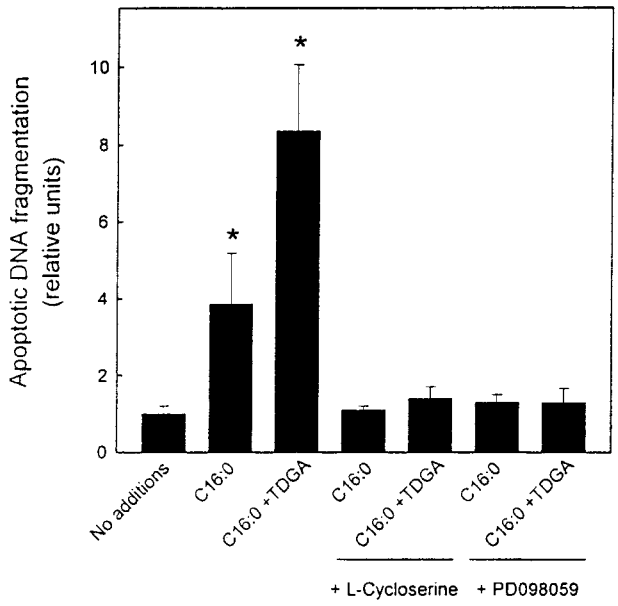

Figure 3. Palmitate-induced apoptotic DNA fragmentation in astrocytes: prevention by L-cycloserine and PD098059. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0), 20 μM TDGA, 2 mM L-cycloserine, and/or 25 μM PD098059. Results are expressed as percentage of incubations with no additions, and were obtained from 4 different experiments. *Significantly different ($P<0.01$) from incubations with no additions.

and 25 μM PD098059 ($n=6$; 100%: incubations with no additions). Likewise, palmitate-induced apoptotic DNA fragmentation in astrocytes was prevented by PD098059 (Fig. 3). The effect of palmitate on ERK activity was subsequently determined. As shown in **Table 2**, palmitate was able to induce a sustained activation of ERK in astrocytes. The stimulatory effect of palmitate was more remarkable when TDGA was simultaneously present in the medium. Moreover, the palmitate-induced stimulation of ERK in

TABLE 2. *Palmitate-induced ERK activation in astrocytes: prevention by L-cycloserine and PD098059*[a]

Additions	ERK activity (%)
None	100 ± 18
C16:0	226 ± 35*
C16:0 + TDGA	250 ± 19*
PD098059	87 ± 17
C16:0 + PD098059	90 ± 14
C16:0 + TDGA + PD098059	83 ± 8
L-Cycloserine	92 ± 5
C16:0 + L-cycloserine	111 ± 10
C16:0 + TDGA + L-cycloserine	113 ± 13

[a] Astrocytes were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0), 20 μM TDGA, 2 mM L-cycloserine, and/or 25 μM PD098059. Results are expressed as percentage of incubations with no additions, and were obtained from 4 different experiments.

* Significantly different ($P<0.01$) from the respective incubations with no additions.

astrocytes was prevented by L-cycloserine and, as expected, by PD098059.

It is widely accepted that Raf-1 represents a pivotal element in the control of cell fate by the ERK cascade (23). Although the molecular link between ceramide accumulation and Raf-1 activation is not well understood and contradictory data have been reported, Kolesnick and co-workers have shown that KSR is a ceramide-activated protein kinase that may phosphorylate and activate Raf-1 (18, 24). The effect of palmitate on Raf-1 and KSR activity in astrocytes was therefore determined. Thus, cell incubation with palmitate induced a significant increase of Raf-1 kinase activity (**Fig. 4*A***). This effect was exacerbated by coincubation with TDGA. Palmitate-induced activation of Raf-1 was prevented by L-cycloserine (Fig. 4*A*), pointing to an involvement of *de novo*-synthesized ceramide. In contrast to Raf-1, KSR activity was not significantly affected by palmitate, either alone or in combination with TDGA (Fig. 4*B*).

Other protein kinases distinct from ERK—namely, JNK, p38 MAPK, and PKB—have been proposed as potential targets of ceramide in the control of cell fate (4, 5). The activity of those kinases was therefore determined. However, exposure of astrocytes to 0.2 mM palmitate (with or without 20 μM TDGA) for 48 h did not significantly affect JNK activity (n=4), p38 MAPK activity (n=3), and PKB activity (n=4).

Neurons are resistant to the apoptotic action of palmitate

In contrast to what was observed in primary astrocytes, the viability of cortical neurons in primary culture was not reduced by palmitate along the 72 h experimental period, even in the presence of TDGA in the medium (Fig. 1*B*). Likewise, palmitate was unable to stimulate ceramide synthesis in neurons, either alone or in combination with TDGA. Thus, incorporation of L-[^{14}C]serine into ceramide in neurons was 97 ± 20% after 48 h exposure to 0.2 mM palmitate and 98 ± 13% after 48 h exposure to 0.2 mM palmitate and 20 μM TDGA (n=4; 100%: incubations with L-[^{14}C]serine alone).

To test whether neurons, unlike astrocytes, possess the ability to prevent exogenous fatty acids from entering the ceramide synthesizing pathway, cells were cultured in the presence of exogenous [^{3}H]palmitate and the metabolic fate of the fatty acid was determined. As shown in **Table 3**, compared to astrocytes, neurons had a very low capacity to take up palmitate, and to incorporate the fatty acid into glycerolipids (phosphatidylcholine and especially triacylglycerols) and sphingolipids (sphingomyelin and especially ceramide). Furthermore, TDGA significantly enhanced palmitate uptake and incorporation into ceramide in astrocytes but not in neurons (Table 3).

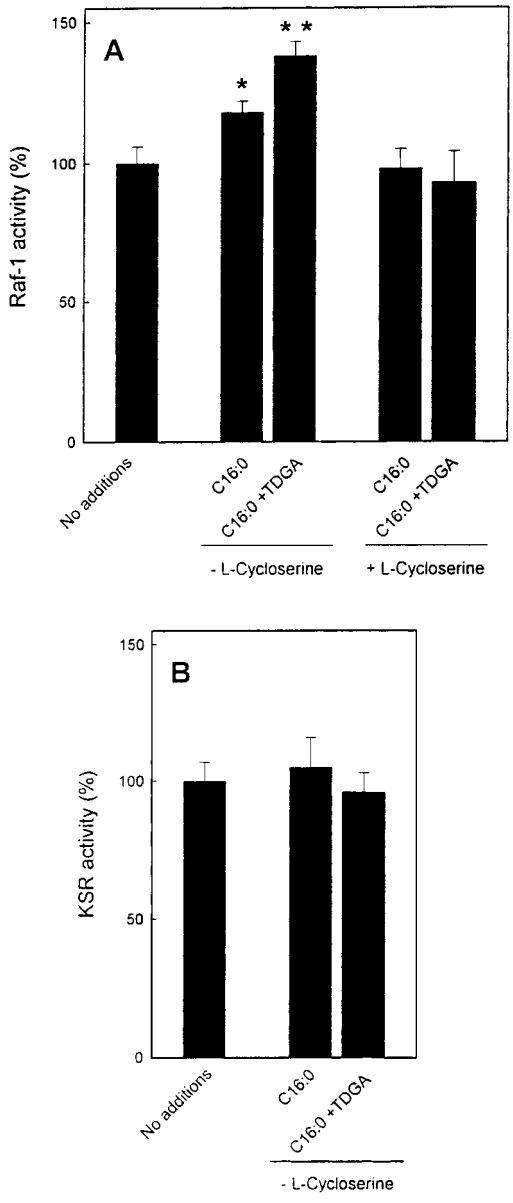

Figure 4. Palmitate-induced activation of Raf-1 but not of KSR in astrocytes: prevention by L-cycloserine. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0), 20 μM TDGA, and/or 2 mM L-cycloserine. Results are expressed as percentage of incubations with no additions and were obtained from 4 different experiments. *A)* Raf-1. *B)* KSR. Significantly different from incubations with no additions: $*P < 0.05$; $**P < 0.01$.

If ERK activation mediates apoptosis induced by *de novo*-synthesized ceramide (see above), then ERK should not be stimulated in palmitate-treated neurons. ERK activity was therefore determined in neurons, and no significant effect of palmitate was observed. Thus, ERK activity in neurons was 112 ± 9% after 48 h exposure to 0.2 mM palmitate and 97 ± 6% after 48 h exposure to 0.2 mM palmitate and 20 μM TDGA (n=4; 100%: incubations with no additions). Likewise, exposure of neurons to 0.2 mM palmitate (with or without 20 μM TDGA) for 48 h

TABLE 3. *Palmitate uptake and incorporation into lipids in astrocytes and neurons*[a]

Parameter	Astrocytes		Neurons	
	−TDGA	+TDGA	−TDGA	+TDGA
Fatty acid uptake	1060 ± 145	1711 ± 169†	291 ± 32*	271 ± 40*
Fatty acid incorporation into				
Phosphatidylcholine	110 ± 26	149 ± 41	24.7 ± 7.2*	20.8 ± 5.1*
Triacylglycerols	254 ± 73	282 ± 24	10.1 ± 2.2*	8.9 ± 1.7*
Ceramide	5.3 ± 0.3	8.4 ± 0.4†	0.4 ± 0.1*	0.4 ± 0.1*
Sphingomyelin	10.4 ± 1.4	12.2 ± 1.7	1.6 ± 0.4*	1.7 ± 0.4*

[a] Astrocytes and neurons were incubated for 24 h with 0.2 mM [^{3}H]palmitate in the absence or presence of 20 μM TDGA. Results are expressed as nmol [^{3}H]fatty acid taken up or incorporated into lipid per 24 h per mg cellular protein and were obtained from 6 different experiments.

* Significantly different ($P<0.01$) from the respective incubations of astrocytes.

† Significantly different ($P<0.01$) from the respective incubations without TDGA.

did not significantly affect JNK activity ($n=4$), p38 MAPK activity ($n=3$), and PKB activity ($n=4$).

DISCUSSION

Importance of ceramide synthesis *de novo* in apoptosis of astrocytes

The hypothesis that ceramide acts as a second messenger in the induction of apoptosis has attracted much attention during recent years. The interpretation of some of the published data is hampered, however, by factors such as the variable kinetics of ceramide generation, the high number of regulatory enzymes involved in ceramide formation, the subcellular compartmentation of ceramide, and the use of nonphysiological short-chain ceramide analogs (4, 5, 25). Nevertheless, ample consensus supports the notion that ceramide plays a pivotal role in the control of neural cell death (1–3) and differentiation (6). Ceramide-induced apoptosis is usually ascribed to ceramide generation through sphingomyelin hydrolysis by neutral and/or acid sphingomyelinase (4, 5, 25). By contrast, the present report demonstrates that ceramide synthesis *de novo* is important in determining the apoptotic outcome of neural cells. Work by Bazan in the early 1970s demonstrated an enhanced breakdown of cellular glycerolipids and a concomitant accumulation of nonesterified fatty acids, including palmitic acid, in a number of models of brain trauma/ischemia (26, 27). The breakdown of membrane phospholipids on trauma/ischemia seems to be the result of Ca^{2+}-induced stimulation of phospholipases and of uncoupling of phospholipid resynthesis due to energy depletion, and may be involved in irreversible damage of membrane structure and function (28). In addition, nonesterified fatty acids exert various detrimental effects on brain structure and function such as uncoupling of oxidative phosphorylation, disruption of plasma membrane and mitochondrial ion fluxes, inhibition of membrane receptors, enzymes, and ion channels, and elevation of synaptic glutamate concentration (28). Both apoptotic and necrotic cell death occur in brain trauma/ischemia (1–3). Our data indicate that under these situations, ceramide synthesized *de novo* from nonesterified fatty acids may contribute to the apoptotic death of astrocytes.

Selective involvement of ERK in apoptosis of astrocytes induced by *de novo*-synthesized ceramide

It is generally accepted that the activation of the ERK cascade leads to cell proliferation (21, 22). However, recent investigations have begun to define situations in which ERK mediates cell cycle arrest (e.g., ref 29), antiproliferation (e.g., ref 30), as well as apoptotic (e.g., ref 31) and nonapoptotic death (e.g., ref 32) in a number of cells, including neural cells. Data in the present work show for the first time that the apoptotic action of *de novo*-synthesized ceramide relies selectively on ceramide-induced Raf-1/ERK activation. This assumption is mostly based on the following observations: *1)* PD098059 prevents palmitate-induced ERK activation and astrocyte death; *2)* blockade of ceramide synthesis *de novo* with L-cycloserine prevents palmitate-induced ceramide accumulation and Raf-1/ERK activation; *3)* TDGA enhances palmitate-induced Raf-1/ERK activation and astrocyte death; *4)* other potential targets of ceramide in the control of cell fate were not significantly affected in astrocytes exposed to palmitate; *5)* unlike astrocytes, neurons are reluctant to palmitate-induced ERK activation and cell death.

Data also show that ceramide-induced activation of Raf-1/ERK in astrocytes occurs independently of KSR, a protein kinase that has been suggested to be involved in the stimulation of Raf-1 by the p55 TNF receptor (18) and in ceramide-induced apoptosis (24). These authors reported that ceramide selectively induces the autophosphorylation of KSR,

thereby enhancing its capacity to phosphorylate and activate Raf-1/ERK. Our observations are in line with those of Huwiler et al. (33), however, who have shown that ceramide directly binds to and activates Raf-1. As a matter of fact, Raf-1 has a ceramide binding motif (4), therefore linking the ceramide pathway with the ERK cascade in the control of cell fate. Nevertheless, others have reported that ceramide binding to Raf-1 does not lead to Raf-1 stimulation (34) and that the activation of Raf-1 by KSR is independent of the kinase activity of the latter (35). The precise role of KSR as a modulator of the ERK cascade is still a matter of debate.

Differential sensitivity of astrocytes and neurons to fatty acid-induced apoptosis

The importance of ceramide and ERK in fatty acid-induced apoptosis of astrocytes is supported by the observation that the absence of palmitate-induced ceramide synthesis and ERK activation in neurons renders these cells reluctant to apoptosis. Inherent differences in fatty acid uptake and metabolism are evident between astrocytes and neurons. Compared to astrocytes, neurons show a very low capacity to take up palmitate and to divert the fatty acid to glycerolipid and sphingolipid synthesis. This different behavior of the two cell types is particularly relevant for triacylglycerol and ceramide biosyntheses, which were ~ 25 and 15 times higher in astrocytes than in neurons, respectively. Triacylglycerols seem to be the major source of the nonesterified palmitic acid released during ischemia (36).

TDGA, a specific inhibitor of CPT-I, exacerbated the effect of palmitate on ceramide accumulation, Raf-1/ERK activation and apoptotic death in astrocytes. Evidence has accumulated during the last two decades highlighting the physiological importance of CPT-I in the control of mitochondrial fatty acid oxidation in many cell types, including astrocytes (14). CPT-I has been implicated in ceramide-mediated apoptosis (7). Because palmitate is a precursor for ceramide synthesis *de novo*, it is conceivable that inhibition of CPT-I leads to accumulation of palmitate in the cytoplasm, increased ceramide synthesis, and apoptosis. This is what actually occurs in astrocytes treated with TDGA. Likewise, expression of high CPT-I activity may help cells to withstand palmitate-induced apoptosis (7, 37). Neural cells have been shown to exhibit a high activity of sphingosine acylation to generate ceramide (38). In addition, the recently purified neutral ceramidase might also be involved in ceramide synthesis *de novo* owing to its ability to catalyze the reverse amidase reaction, i.e., the condensation of the fatty acid with sphingosine to generate ceramide (39). However, the situation may be more complex in that CPT-I in astrocytes is a ceramide-activated enzyme (14), pointing to the existence of a regulatory loop in which elevated ceramide levels occurring on CPT-I inhibition might be a signal for the reactivation of the enzyme. The observation that CPT-I directly interacts with the anti-apoptotic protein Bcl-2 in the mitochondrial outer membrane (40) and the well-established role of mitochondria in the onset of apoptosis (4) point to a general role of CPT-I as a regulator of apoptosis (41). FJ

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The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes

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Abstract Fatty acids induce apoptosis in primary astrocytes by enhancing ceramide synthesis de novo. The possible role of the AMP-activated protein kinase (AMPK) in the control of apoptosis was studied in this model. Long-term stimulation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) prevented apoptosis. AICAR blunted fatty acid-mediated induction of serine palmitoyltransferase and ceramide synthesis de novo, without affecting fatty acid synthesis and oxidation. Prevention of ceramide accumulation by AICAR led to a concomitant blockade of the Raf-1/extracellular signal-regulated kinase cascade, which selectively mediates fatty acid-induced apoptosis. Data indicate that AMPK may protect cells from apoptosis induced by stress stimuli.

Key words: AMP-activated protein kinase; Apoptosis; Ceramide; Extracellular signal-regulated kinase; Astrocyte

1. Introduction

Mammalian AMP-activated protein kinase (AMPK) belongs to a family of protein kinases that has been highly conserved throughout evolution in animals, plants and yeast, and which plays a major role in cell response to metabolic stress [1,2]. AMPK is activated by AMP and by phosphorylation by an upstream kinase, which is itself activated by AMP. Once activated, AMPK phosphorylates and inactivates a number of regulatory enzymes involved in biosynthetic pathways. The AMPK cascade seems to have evolved to monitor the energy status of the cell and to initiate appropiate energy-conserving mechanisms in response to ATP depletion during metabolic stress [1,2]. One of the most intriguing and unexplored actions of AMPK is its possible anti-apoptotic effect. Thus, two previous reports have shown that pharmacological activation of AMPK protects thymocytes from dexamethasone-induced apoptosis [3] and Rat-1 fibroblasts from serum withdrawal-induced apoptosis [4]. However, the potential pathophysiological implications of these findings are hampered by the absolute lack of knowledge of the underlying mechanisms.

The present work was therefore undertaken to study the mechanism of the anti-apoptotic effect of AMPK in neural cells. One of the most important mediators of apoptosis in the central nervous system is ceramide. Thus, ceramide accumulation occurs in cultured neural cells exposed to stress stimuli, as well as in the brain in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, epilepsy and ischemia/stroke [5,6]. Moreover, changes in ceramide metabolism exert important regulatory effects on neuronal growth and development [7]. We have recently developed a model in which exposure of primary astrocytes to fatty acids leads to apoptosis selectively via de novo-synthesized ceramide [8]. By using this model we sought to answer two questions: (a) does AMPK activation prevent ceramide-induced apoptosis?; (b) if so, which may be the targets of AMPK action?

2. Materials and methods

2.1. Cell culture

Cortical astrocytes were derived from 24 h old Wistar rats and cultured as described before [9]. For all the experimental determinations performed, the serum-containing medium was removed and cells were transferred to a chemically defined, serum-free medium consisting of DMEM/Ham's F12 (1:1, v/v) supplemented with 5 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, 30 nM sodium selenite and 1.0% (w/v) defatted and dialyzed bovine serum albumin.

2.2. Cell death

Cell viability was determined by trypan blue exclusion. For Hoechst 33258 staining, cells were grown in glass coverslips and fixed (20 min at room temperature) with 4% paraformaldehyde in phosphate-buffered saline supplemented with 5% sucrose. The dye was applied at a final concentration of 16 μg/ml and cells were examined by fluorescence microscopy.

2.3. Rates of metabolic pathways

(a) Sphingolipid synthesis: reactions were started by the addition of 1 μCi L-[U-^{14}C]serine, and stopped with 1 ml methanol at the times indicated. Lipids were extracted and saponified, and ceramide and sphingomyelin were resolved by thin layer chromatography [8,10]. (b) Fatty acid oxidation: reactions were started by the addition of 0.2 mM (0.3 μCi) albumin-bound [1-^{14}C]palmitate plus 0.5 mM L-carnitine, and stopped with 0.3 ml 2 M $HClO_4$ after 2 h. Oxidation products were extracted and quantified exactly as described before [9]. (c) Fatty acid synthesis: reactions were started by the addition of 4 mM (1.0 μCi) [1-^{14}C]acetate, and stopped with 0.2 ml 10 M NaOH after 6 h. Samples were saponified and fatty acids were extracted with light petroleum ether [11].

2.4. Protein kinase activities

(a) AMPK: cells were lysed, supernatants were obtained, and

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CPT-I, carnitine palmitoyltransferase I; ERK, extracellular signal-regulated kinase; SPT, serine palmitoyltransferase

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AMPK activity was determined as the incorporation of [γ-^{32}P]ATP into the specific SAMS peptide substrate [11]. (b) Extracellular signal-regulated kinase (ERK): cells were lysed, supernatants were obtained, and ERK activity was determined as the incorporation of [γ-^{32}P]ATP into a specific peptide substrate [10]. (c) Raf-1: after Raf-1 immunoprecipitation, Raf-1 kinase activity was determined as the incorporation of [γ-^{32}P]ATP into kinase-negative MEK1(97A) [10].

2.5. Other enzyme activities and levels

(a) Serine palmitoyltransferase (SPT): enzyme activity was determined in digitonin-permeabilized astrocytes as the incorporation of radiolabelled L-serine into ketosphinganine by a new procedure. Thus, the medium was aspirated and cells were washed twice with phosphate-buffered saline. Reactions were started by the addition of 100 mM HEPES, pH 8.3, 200 mM sucrose, 2.5 mM EDTA, 5 mM dithioerythritol, 50 μM pyridoxal phosphate, 1.0 mg/ml defatted and dialyzed bovine serum albumin, 15 μg/ml digitonin, 0.3 mM palmitoyl-CoA and 0.25 mM L-[U-^{14}C]serine (3 μCi/assay). After 45 min, reactions were stopped with 0.5 M NH_4OH and [^{14}C]ketosphinganine product was extracted with chloroform/methanol/1% NaCl [12]. Preliminary experiments defined the optimal concentration of palmitoyl-CoA, serine and digitonin in the assay, as well as its linearity with time. The validity of the assay was proved by the full inhibitory effect exerted by the SPT competitive inhibitor L-cycloserine. Western blot analysis was carried out with a polyclonal antibody raised against hamster SPT LCB2 catalytic subunit [13]. (b) Carnitine palmitoyltransferase I (CPT-I): enzyme activity was determined in digitonin-permeabilized astrocytes as the tetradecylglycidate-sensitive incorporation of L-[*Me*-^{3}H]carnitine into palmitoylcarnitine by method A ('one-step assay') described in [11]. Western blot analysis was carried out with a polyclonal antibody raised against rat liver CPT-I [9]. (c) Acetyl-CoA carboxylase (ACC): enzyme activity was determined in digitonin-permeabilized astrocytes as the incorporation of [1-^{14}C]-acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction [9]. Mass measurement of ACC was performed by avidin-based enzyme-linked immunosorbent assay analysis using a polyclonal antibody raised against rat liver ACC [14].

2.6. Statistical analysis

Results shown represent the means ± S.D. of the number of experiments indicated in every case. Statistical analysis was performed by ANOVA. A post hoc analysis was made by the Student–Neuman–Keuls test.

3. Results

3.1. Long-term AMPK stimulation prevents fatty acid-induced apoptosis

We have previously shown that exposure of primary astrocytes to palmitate leads to apoptosis [8]. Astrocytes were exposed to palmitate at a concentration physiologically relevant in brain, and cell viability was determined at different times. As shown in Fig. 1, palmitate induced the death of astrocytes in a time-dependent fashion. Prolonged exposure to 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a selective cell-permeable activator of AMPK which has been widely used to demonstrate the implication of this kinase in the regulation of cellular processes [1], was able to prevent completely palmitate-induced cell death, as determined by both trypan blue exclusion (Fig. 1A) and Hoechst 33258 staining (Fig. 1B). In our cultured astrocyte system, incubation with 0.2 mM AICAR for 48 h activated AMPK by 47 ± 14% ($n = 3$, $P < 0.01$ vs. incubations with no additions).

3.2. Long-term AMPK stimulation prevents ceramide synthesis de novo and SPT induction

Fatty acid-induced apoptosis of astrocytes occurs selectively by enhanced ceramide synthesis de novo [8]. As shown in Table 1, palmitate notably stimulated ceramide synthesis de

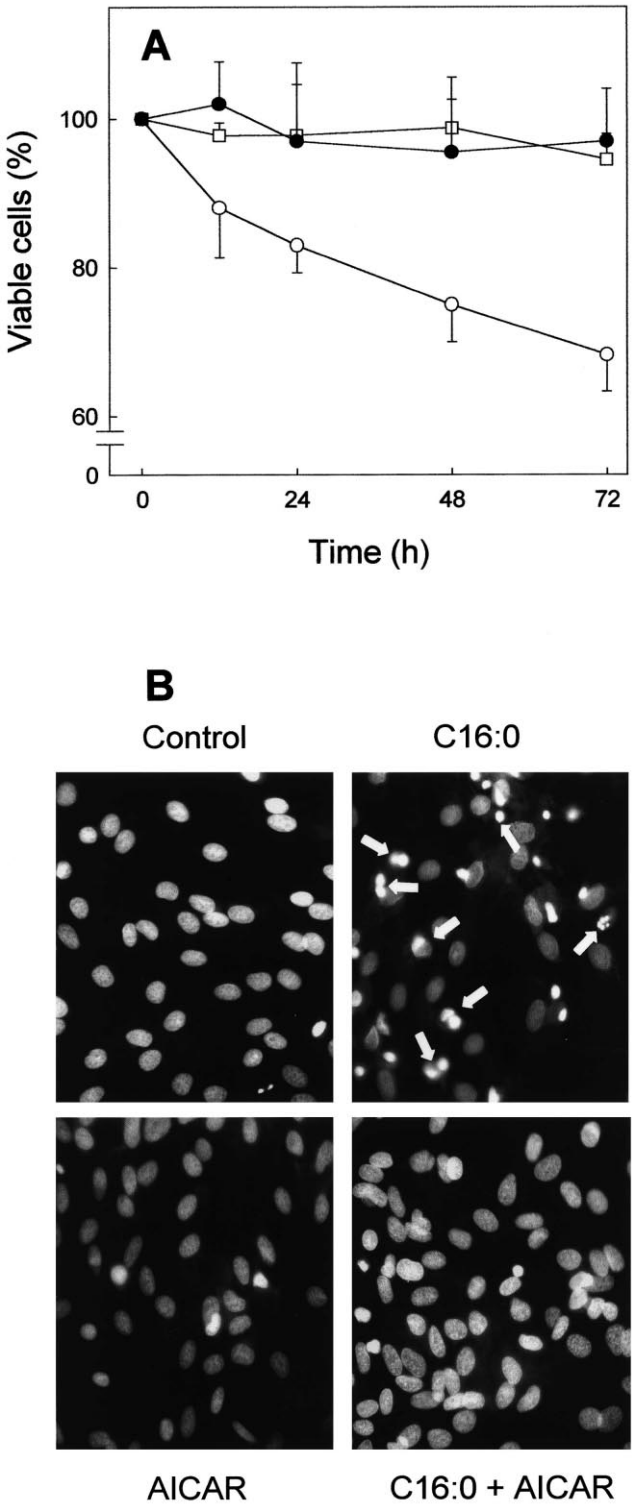

Fig. 1. Palmitate-induced death of astrocytes: prevention by AICAR. (A) Cell viability. Cells were incubated for the times indicated with 0.2 mM palmitate in the absence (○) or presence (●) of 0.2 mM AICAR, or with 0.2 mM AICAR alone (□). Results are expressed as percentage of incubations with no additions, and were obtained from four different experiments. (B) Hoechst 33258 staining. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. A representative experiment is shown. Similar results were obtained in two other experiments. Arrows point to condensed or fragmented nuclei.

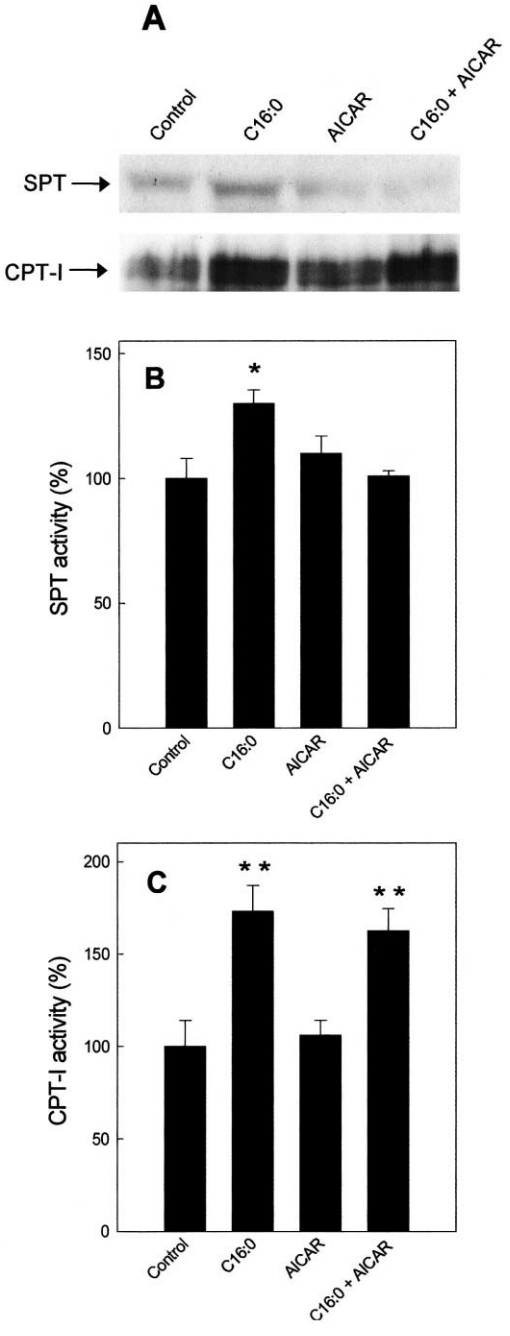

Fig. 2. Palmitate-mediated induction of SPT and CPT-I: differential effect of AICAR. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. (A) SPT and CPT-I expression. A representative Western blot is shown. (B) SPT activity. (C) CPT-I activity. Results in (B) and (C) are expressed as percentage of incubations with no additions, and were obtained from four different experiments. Significantly different from incubations with no additions: $*P < 0.05$; $**P < 0.01$.
←

novo. By contrast, no significant effect of palmitate on serine incorporation into sphingomyelin was evident. AICAR blunted palmitate-induced ceramide generation without exerting any effect on sphingomyelin synthesis. To further support the involvement of AMPK in the control of ceramide synthesis de novo we determined the activity of SPT, which catalyzes the pace-setting step of this pathway. Thus, AICAR prevented palmitate-mediated SPT induction, as determined by both Western blot (Fig. 2A) and assay of enzyme activity (Fig. 2B). Relative levels of SPT expression, as determined by densitometric analysis of the bands in the Western blots, were 1.00 (no additions), 1.37 ± 0.14 (48 h exposure to 0.2 mM palmitate), 1.01 ± 0.09 (48 h exposure to 0.2 mM AICAR), and 0.91 ± 0.15 (48 h exposure to 0.2 mM palmitate and 0.2 mM AICAR) ($n = 4$).

3.3. Long-term AMPK stimulation prevents Raf-1/ERK activation

We have previously shown that de novo-synthesized ceramide signals apoptosis in primary astrocytes via activation of the Raf-1/ERK cascade [8]. The effect of AICAR on the activity of these two kinases was therefore determined. The palmitate-induced activation of ERK (Fig. 3A) and Raf-1 (Fig. 3B) was prevented by AICAR. Although AMPK has been shown to phosphorylate a Raf-1 peptide in vitro [15], incubation of astrocytes with AICAR alone did not affect basal ERK and Raf-1 activities.

3.4. Long-term AMPK stimulation does not affect fatty acid catabolism

AMPK stimulation enhances fatty acid oxidation in astrocytes [11] and other cells such as myocytes and hepatocytes [1,2]. It might therefore be argued that AICAR prevents ceramide accumulation by diverting palmitate into the oxidative pathway. However, this is not the case in our experimental system. Although rapid (minute range) AMPK stimulation with AICAR does enhance fatty acid oxidation and blunt fatty acid synthesis in astrocytes [11], these alterations return to basal levels after prolonged (day range) AICAR challenge.

Table 1
Palmitate-induced ceramide synthesis and astrocyte death: prevention by AICAR

Additions	Viable cells (%)	L-[^{14}C]serine into lipid	
		Ceramide (%)	Sphingomyelin (%)
None	100 ± 8	100 ± 18	100 ± 14
C16:0	72 ± 7[a]	217 ± 36[a]	109 ± 25
AICAR	101 ± 9	93 ± 21	94 ± 8
C16:0+AICAR	95 ± 8	127 ± 34	91 ± 15

Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. Results are expressed as percentage of incubations with no additions, and were obtained from six different experiments. 100% values of L-[^{14}C]serine incorporation into ceramide and sphingomyelin were 0.77 and 3.06 nmol per 24 h per mg cell protein, respectively.
[a]Significantly different ($P < 0.01$) from the respective incubations with no additions.

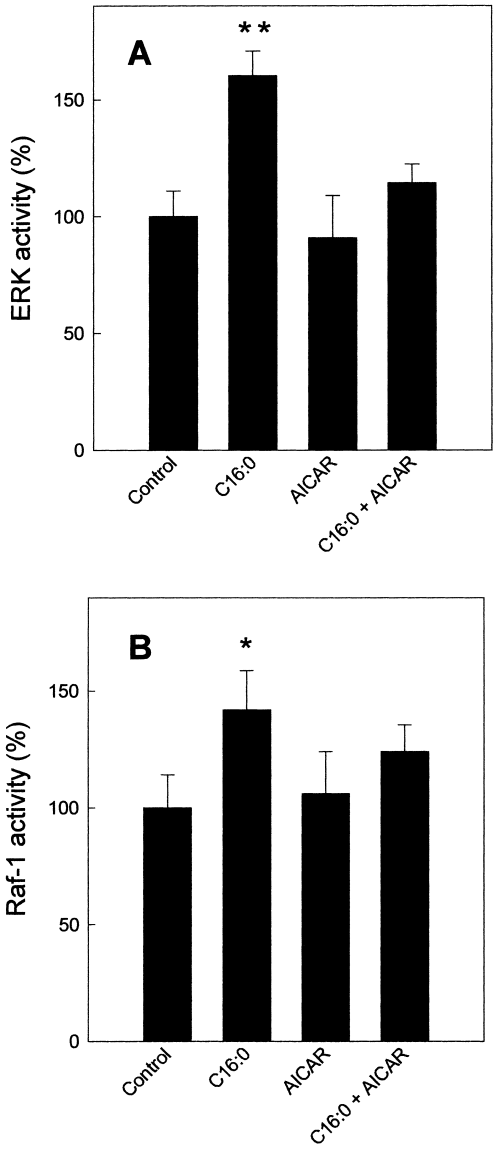

Fig. 3. Palmitate-induced activation of ERK and Raf-1 in astrocytes: prevention by AICAR. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. Results are expressed as percentage of incubations with no additions, and were obtained from four different experiments. (A) ERK activity. (B) Raf-1 activity. Significantly different from incubations with no additions: $*P < 0.05$; $**P < 0.01$.

Thus, no significant effect of 48 h AICAR exposure was evident on the rate of fatty acid oxidation and fatty acid synthesis (data not shown), and on the activity and levels of the pace-setting enzymes of these two pathways, CPT-I (Fig. 2A,C) and ACC (data not shown), respectively. Relative levels of CPT-I expression, as determined by densitometric analysis of the bands in the Western blots, were 1.00 (no additions), 1.61 ± 0.20 (48 h exposure to 0.2 mM palmitate), 1.05 ± 0.14 (48 h exposure to 0.2 mM AICAR), and 1.64 ± 0.11 (48 h exposure to 0.2 mM palmitate and 0.2 mM AICAR) ($n = 4$).

4. Discussion

The hypothesis that ceramide acts as a second messenger in the induction of apoptosis has attracted a lot of attention during the last few years. It is usually considered that ceramide generation through sphingomyelin hydrolysis by neutral and/or acid sphingomyelinase is the norm in ceramide signaling pathways leading to apoptosis. The link between receptor activation, sphingomyelinase stimulation and ceramide generation is mostly supported by comprehensive studies on the p55 tumor necrosis factor receptor, the p75 neurotrophin receptor and CD95/Fas [16,17]. In addition, other stress stimuli of chemical, physical, bacterial and viral origin may induce apoptosis by evoking changes in the sphingomyelin/ceramide cycle [16,17]. However, the de novo synthesis pathway has been gaining appreciation as an alternative means of generating an apoptotic pool of ceramide. Thus, a significant contribution of the de novo pathway to ceramide generation and apoptosis has been reported in a number of paradigms employing cultured neural (e.g. [8,10,18]) and non-neural cells (e.g. [19–21]). In particular, because palmitate is a precursor for ceramide synthesis de novo, it is conceivable that its accumulation may lead to increased ceramide synthesis and apoptosis, as evidenced in hematopoietic cells [22], pancreatic β cells [23] and astrocytes [8].

Work by Bazan in the early 70s demonstrated an enhanced breakdown of cellular glycerolipids and a concomitant accumulation of non-esterified fatty acids – including palmitic acid – in a number of models of brain trauma/ischemia [24,25]. The breakdown of membrane phospholipids on trauma/ischemia seems to be the result of Ca^{2+}-induced stimulation of phospholipases and of impairment of phospholipid resynthesis owing to energy depletion, and may be involved in irreversible damage of membrane structure and function [26]. In addition, non-esterified fatty acids exert various detrimental effects on brain structure and function such as uncoupling of oxidative phosphorylation; disruption of plasma membrane and mitochondrial ion fluxes; inhibition of membrane receptors, enzymes and ion channels; and elevation of synaptic glutamate concentration [26]. Hence our data indicate that ceramide synthesized de novo from non-esterified fatty acids may contribute to the apoptotic death of astrocytes in situations of brain trauma/ischemia. AMPK is expressed in brain [27–29], but its function in this organ is as yet unknown. Because in astrocytes (a) AMPK is stimulated in hypoxic [11] and gliotic states [30], and (b) AMPK activation prevents fatty acid-evoked SPT induction, stimulation of ceramide synthesis de novo and apoptosis (the present report), data also suggest that the balance between pro-apoptotic fatty acid accumulation and anti-apoptotic AMPK stimulation might determine ceramide levels and therefore influence the cell survival/death decision. In line with other studies showing that AMPK controls gene expression [31,32], cytoskeletal dynamics [33] and cell death [3,4], the present report supports the emerging notion that AMPK regulates not only energy metabolism but a much wider array of cellular functions.

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SUMARIO

Suele considerarse que la generación de ceramida con efecto apoptótico tiene lugar a partir de la hidrólisis de esfingomielina por diversas esfingomielinasas. Sin embargo, en los últimos años la síntesis *de novo* ha ido adquiriendo importancia como medio alternativo de generación de un *pool* apoptótico de ceramida. Además, puesto que los ácidos grasos son precursores de la ceramida, la inhibición de la CPT-I puede conducir a la acumulación de ácidos grasos, generación de ceramida y muerte celular por apoptosis. En vista de ello, estudiamos si la síntesis *de novo* de ceramida era capaz de inducir apoptosis en células neurales y, si esto era así, cuál sería el mecanismo implicado y el posible papel de la CPT-I y la cetogénesis en este proceso. Además, se analizó el posible efecto antiapoptótico de la AMPK en células neurales.

Los datos obtenidos muestran que el palmitato induce muerte por apoptosis en astrocitos. Este efecto se acentúa cuando se inhibe farmacológicamente la CPT-I, y no tiene lugar en neuronas. La apoptosis inducida por palmitato se debe a un incremento en la síntesis *de novo* de ceramida, y tiene lugar a través de la activación selectiva de la cascada Raf-1/ERK. Los datos apoyan la hipótesis de que la inhibición de la CPT-I conduciría a la desviación del palmitato a la síntesis de ceramida que, en último término, produciría apoptosis en los astrocitos. Además, la activación farmacológica de la AMPK previno la inducción de la SPT, la acumulación de ceramida, la activación de la cascada Raf-1/ERK y la apoptosis inducida por palmitato.

4. DISCUSIÓN GENERAL Y CONCLUSIONES

Is there an astrocyte–neuron ketone body shuttle?

Manuel Guzmán and Cristina Blázquez

Ketone bodies can replace glucose as the major source of brain energy when glucose becomes scarce. Although it is generally assumed that the liver supplies extrahepatic tissues with ketone bodies, recent evidence shows that astrocytes are also ketogenic cells. Moreover, the partitioning of fatty acids between ketogenesis and ceramide synthesis *de novo* might control the survival/death decision of neural cells. These findings support the notion that astrocytes might supply neurons with ketone bodies *in situ*, and raise the possibility that astrocyte ketogenesis is a cytoprotective pathway.

The weight of the mature human brain is only 2–3% of the total body weight. However, this organ can consume up to 25% of the total body glucose supply. Only a small fraction of this glucose is used for biosynthetic processes; the major part is consumed in energy metabolism, mostly for ion transport and the maintenance of ion gradients[1]. It is generally assumed that, because the brain stores very little fuel, this organ depends on the continuous exogenous supply of metabolic substrates. Hence, pathological conditions that diminish or arrest energy production, such as hypoglycemia or hypoxia/ischemia, lead to a rapid breakdown of transmembrane ion gradients[1]. Unraveling the mechanisms of brain energy metabolism is therefore essential to understanding brain function both in a physiological setting and in pathological situations in which energy production is impaired and neuronal death ensues.

Astrocytes as glucose-metabolizing cells

Evidence accumulated during the past decade supports the notion that astrocytes, the major class of glial cells in the mammalian brain, play a pivotal role in the regulation of brain-glucose metabolism. Owing to their strategic location surrounding intraparenchymal blood capillaries, astrocytes form the first cellular barrier encountered by glucose entering the brain tissue, which makes them a prevalent site of glucose uptake. One of the major products of astroglial glucose metabolism is lactate, which, in turn, constitutes a good substrate for neuronal oxidative metabolism, especially during enhanced neuronal activity. The differential distribution of monocarboxylate transporters and lactate dehydrogenase isoforms between brain cells also supports the notion that astrocytes are lactate 'sources' whereas neurons are lactate 'sinks'. Moreover, brain glycogen is found mainly in astrocytes, and its breakdown, which also results in lactate output, is extremely rapid and finely coordinated with synaptic activity. These and other observations point to the existence of an astrocyte–neuron lactate shuttle for sustaining neuronal energy metabolism[2–4].

Astrocytes as fatty acid-metabolizing cells

Although studies on metabolic regulation in the brain have focused mostly on carbohydrate and amino acid metabolism, the ketone bodies 3-hydroxybutyrate and acetoacetate can replace glucose as the major source of brain energy in situations such as starvation and development[5,6]. The liver is generally believed to be the major organ that supplies the brain with ketone bodies. However, it has been reported that astrocytes can also produce ketone bodies from fatty acids[7] and Leu[8]. Large numbers of studies have established the pathways and regulatory mechanisms of fatty acid oxidation and ketone body production in the liver[9–11] (Fig. 1). In addition, the properties of the ketogenic system in cultured astrocytes have been examined recently. The general conclusion from these studies is that ketone body production by astrocytes and hepatocytes has similar properties. This assumption is based mainly on the following observations:

(1) Ketogenic capacity. Hepatocytes[12] and astrocytes[13,14] show a greater preference for fatty acids (rather than glucose) as their primary metabolic fuel, and produce large amounts of ketone bodies, which in turn constitute the bulk of the fatty acid-oxidation products in the two types of cells[12–14].

(2) Regulatory sites of ketogenesis. Analyses of metabolic control have shown that carnitine palmitoyltransferase I (CPT-I), the mitochondrial outer membrane CPT (Fig. 1), catalyzes the pace-setting step of ketogenesis in hepatocytes[15,16] and astrocytes[14]. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (Fig. 1) might also have a regulatory role in hepatocytes[17] and astrocytes[18,19].

(3) Properties of CPT-I. CPT-I preparations from hepatocytes[20] and astrocytes[14] are similar with respect to electrophoretic mobility, enzyme kinetics, sensitivity to inhibitors, and recognition by antibodies. Therefore, they are assumed to be the same CPT-I isoenzyme: the so-called liver CPT-I (L-CPT-I). In addition, the control of CPT-I activity by interactions between mitochondria and cytoskeletal components seems to operate in both hepatocytes[21] and astrocytes[14].

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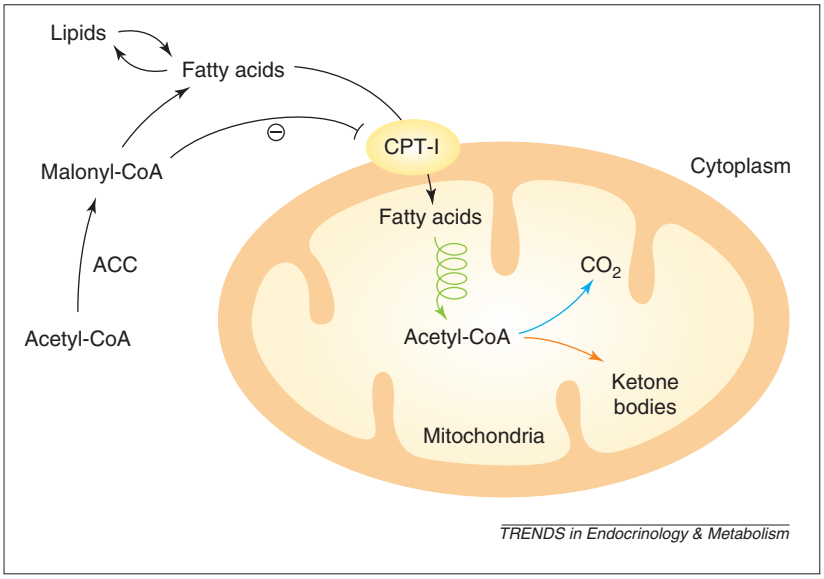

Fig. 1. Overview of fatty acid oxidation and ketogenesis. Fatty acids can undergo β-oxidation (green, coiled arrow) upon translocation into the mitochondrial matrix. CPT-I, which catalyzes the rate-limiting step of this process, is inhibited by malonyl-CoA, the product of the reaction catalyzed by ACC. Inside mitochondria, the acetyl moieties produced by β-oxidation might condense to generate ketone bodies (acetoacetate and 3-hydroxybutyrate) via a pathway in which mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase constitutes the pace-setting step (orange arrow). Alternatively, acetyl-CoA might be further oxidized to CO_2 by the action of the tricarboxylic acid cycle (blue arrow). Abbreviations: ACC, acetyl-CoA carboxylase; CPT-1, carnitine palmitoyltransferase I.

(4) Properties of acetyl-CoA carboxylase. The levels of malonyl-CoA, the main physiological inhibitor of CPT-I, and the kinetic and regulatory properties of acetyl-CoA carboxylase, the malonyl-CoA-synthesizing enzyme (Fig. 1), are similar in hepatocytes[22] and astrocytes[14].

(5) Flexibility of ketogenesis. As in hepatocytes[10,11], CPT-I and ketogenesis in astrocytes are very flexible, and become finely modulated by stimuli coupled to activation of, for example, cAMP-dependent protein kinase[14] and AMP-activated protein kinase (AMPK)[23].

The notion that astrocytes display a high rate of fatty acid utilization and ketogenesis is compatible with their important ability to carry out other pathways of lipid metabolism, such as lipid synthesis[24] and lipoprotein secretion[25]. Moreover, astrocytes have recently been shown to express peroxisome proliferator-activated receptors, a family of transcription factors that are believed to mediate fatty acid-dependent gene induction[26]. Therefore, similar to hepatocytes, astrocytes can be considered versatile lipid-metabolizing cells.

Astrocyte ketogenesis during synaptic activity

Astrocytes are associated intimately with neurons and also surround synapses. Owing to their close proximity to synaptic clefts, astrocytes are in a prime location to receive synaptic information from released neurotransmitters. In fact, astrocytes express a wide range of neurotransmitter receptors and transporters[27]. In general, it is assumed that the supply of lactate from astrocytes for neuronal oxidative metabolism is enhanced in situations of high synaptic activity[2–4,28]. For example, adenylyl cyclase-activating receptors such as β-adrenoceptors and vasoactive intestinal peptide receptors are present in astrocytes. An increase in cAMP concentration in cultured astrocytes has been shown to stimulate glycogen mobilization, glycolysis and lactate output[28,29]. Similarly, cAMP activates ketogenesis in astrocytes via phosphorylation and inactivation of acetyl-CoA carboxylase by cAMP-dependent protein kinase. This leads to a decrease in the malonyl-CoA concentration and release of the inhibition of CPT-I (Ref. 14). In addition, glutamate – the main excitatory neurotransmitter in the mammalian brain – is released from neurons and taken up avidly by astrocytes. This process triggers glucose utilization and lactate production in the astrocytes[28,29].

Similarly, it has been found that glutamate enhances ketogenesis in cultured astrocytes by a process that seems to be independent of glutamate receptors but dependent on glutamate transporters (C. Blázquez and M. Guzmán, unpublished). Moreover, endocannabinoids, a family of endogenous neuromodulatory ligands of cannabinoid receptors, activate glycolysis[30], as well as CPT-I and ketogenesis[31], in astrocytes via the lipid second messenger ceramide. These observations indicate that neurotransmitters released by neurons during enhanced synaptic activity might interact with astrocytes and induce the production of lactate, as well as ketone bodies, in these cells, as substrates for neuronal oxidative metabolism. In this respect, it has been reported that not only lactate[32], but also 3-hydroxybutyrate[33], can replace glucose as fuel and preserve neuronal synaptic function and structural stability. In particular, the effect of 3-hydroxybutyrate was more remarkable during early postnatal development[33], a situation in which ketone bodies are known to constitute relevant substrates for neuronal oxidative and biosynthetic processes[5,6].

Astrocyte ketogenesis during hypoxia

Glucose utilization by the brain in a physiological setting is nearly matched by carbon dioxide production. However, when the local glycolytic rate exceeds the local oxidative metabolic rate, lactate can accumulate in the brain[34,35]. This is especially notable during ischemia/hypoxia, conditions in which the anerobic metabolism of glucose in astrocytes is stimulated, and which therefore lead to an enhanced output of lactate that might be available for neuronal metabolism[34,35]. However, the remarkable feedback inhibition of astroglial glycolysis at low pH upon lactate accumulation[34], as well as the blockade of lactate utilization by neurons during hypoxia[23,34,35], to a certain extent prevents the use of lactate as neuronal fuel. By contrast, no significant depression of ketone body production by astrocytes occurs when the pH of the medium is reduced to 6.6 (Ref. 23). In addition, 3-hydroxybutyrate is preferred over lactate as a substrate for neuronal oxidative metabolism, and oxidation of this compound by neurons is not significantly impaired during hypoxia[23]. These observations suggest that ketone bodies might be used, together with lactate, as substrates for neuronal oxidative metabolism during hypoxia.

A role for AMPK in the maintenance of astroglial ketogenesis during hypoxia has been recently

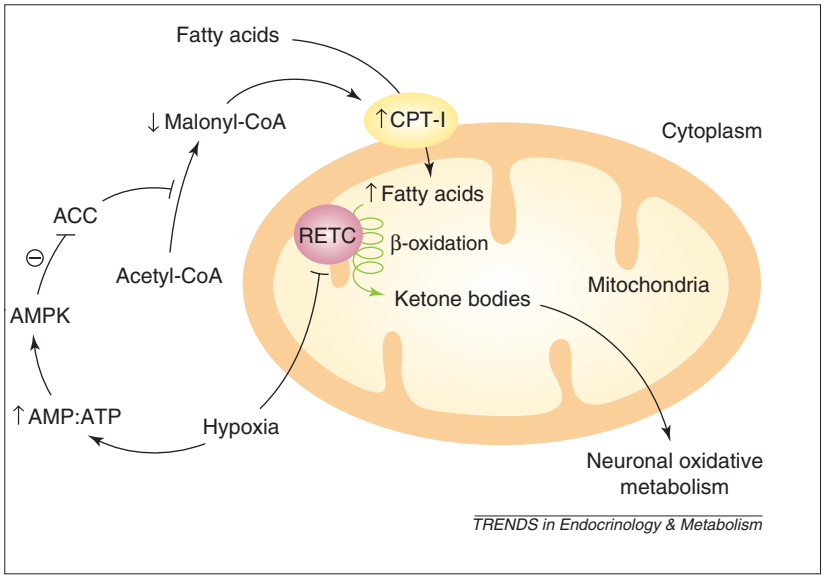

Fig. 2. Proposed model for the role of AMPK in sustaining ketone body production by astrocytes during hypoxia. An increase in the cellular AMP:ATP ratio occurs in hypoxia owing to the inhibition of the RETC. This leads to the stimulation of AMPK, which in turn phosphorylates and inactivates ACC, thereby decreasing the malonyl-CoA concentration. Therefore, CPT-I is released from inhibition, and can therefore increase the supply of fatty acid substrates, which might compensate for the depression of intramitochondrial fatty acid oxidative metabolism, thereby allowing astroglial ketogenesis to be maintained at a high rate. Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyltransferase I; RETC, respiratory electron transporting chain.

suggested[23]. This kinase plays a major role in cell responses to metabolic stress. AMPK is activated by AMP both directly, and via activation of an upstream AMPK kinase. Once activated, AMPK phosphorylates and inactivates several regulatory enzymes involved in biosynthetic pathways. Hence, the AMPK cascade seems to have evolved to monitor the energy status of the cell, and to initiate appropriate energy-conserving mechanisms in response to ATP depletion during metabolic stress[36]. AMPK activation during hypoxia occurs in several cells, including astrocytes[23,36]. On the basis of cell culture and microdialysis experiments, AMPK activation has been proposed to compensate for the depression of fatty acid β-oxidation during hypoxia, thereby sustaining the supply of ketone bodies from astrocytes to neurons via the cascade depicted in Fig. 2. It is also worth noting that non-esterified fatty acids accumulate in the brain during hypoxia, and that this might provide more substrates for astrocyte ketogenesis.

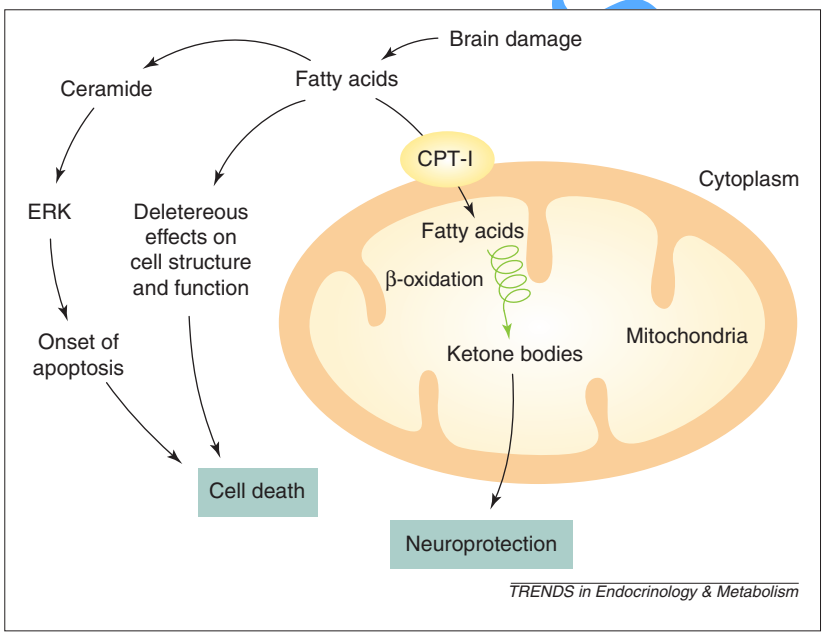

Fig. 3. Proposed model for the role of astrocyte CPT-I in the control of the cell survival/death decision. Non-esterified fatty acids might accumulate upon brain damage and exert several deleterious actions, both directly and by acting as biosynthetic precursors of the proapoptotic lipid ceramide, which induces apoptosis by activating ERK. By favoring the mitochondrial metabolism of fatty acids, CPT-I might be involved in preventing these negative effects. In addition, ketone bodies supplied by astrocytes to neurons might sustain neuronal activity and be cytoprotective. Abbreviations: CPT-1, carnitine palmitoyltransferase I; ERK, extracellular signal-regulated kinase.

Astrocyte ketogenesis and apoptosis

The hypothesis that ceramide acts as a second messenger in the induction of apoptosis has attracted considerable attention during the past few years. In general, it is thought that, in ceramide signaling pathways leading to apoptosis, ceramide is generated by the hydrolysis of sphingomyelin catalyzed by sphingomyelinases[37]. However, the *de novo* synthesis pathway has been gaining acceptance as an alternative means of generating an apoptotic pool of ceramide[38]. Indeed, a significant contribution of the *de novo* pathway to ceramide generation and apoptosis has been reported recently in several systems using cultured neural cells[39–42]. Because fatty acids are biosynthetic precursors of the proapoptotic lipid ceramide, it is conceivable that inhibition of CPT-I and ketogenesis might result in an accumulation of fatty acids in the cytoplasm, and an increase of ceramide synthesis and apoptosis[43]. In fact, this is what occurs in astrocytes[40]. It has also been shown that the extracellular signal-regulated kinase cascade is the downstream target of *de novo*-synthesized ceramide that is involved in the apoptosis of astrocytes[40] and glioma cells[41] (Fig. 3).

Work by Bazan in the early 1970s demonstrated an enhanced breakdown of cellular glycerolipids and a concomitant accumulation of non-esterified fatty acids in several models of brain trauma and/or ischemia[44,45]. The breakdown of membrane phospholipids seems to be the result of Ca^{2+}-induced stimulation of phospholipases and impaired phospholipid resynthesis resulting from energy depletion, and could be involved in irreversible damage of membrane structure and function[46]. Hence, ceramide synthesized *de novo* from non-esterified fatty acids might contribute to cell death in situations of brain damage (Fig. 3). In astrocytes, AMPK is stimulated in hypoxia[23] and AMPK activation prevents fatty acid-induced *de novo* ceramide synthesis and apoptosis[47]; thus, it is possible that the balance between proapoptotic fatty acid accumulation and antiapoptotic AMPK/CPT-I stimulation determines ceramide levels and, therefore, influences the survival/death decision of neural cells. In addition, activation of CPT-I and ketogenesis could be a

mechanism for preventing various direct, detrimental effects exerted by non-esterified fatty acids on brain structure and function; for example, the uncoupling of oxidative phosphorylation, the disruption of plasma membrane and mitochondrial ion fluxes, the inhibition of membrane receptors, enzymes and ion channels, and the elevation of synaptic glutamate concentration[46] (Fig. 3). Moreover, the observation that, similar to lactate[48], ketone bodies might directly protect neurons from death[49], supports a role for astrocyte ketogenesis as a brain-protective pathway (Fig. 3).

Concluding remarks

Recent studies indicate that astrocytes, in addition to their high glycolytic capacity to yield lactate, produce significant amounts of ketone bodies. These observations raise the exciting possibility that ketone bodies produced by astrocytes might be used together with lactate as substrates for neuronal oxidative metabolism in situations such as enhanced synaptic activity and hypoxia. Although the extent to which ketone body formation by astrocytes occurs *in vivo* remains to be determined, it should be kept in mind that astrocytes outnumber neurons nine to one and take up half the volume of the brain[50]. Moreover, the partitioning of fatty acids between oxidation and ceramide synthesis might control the survival/death decision of neural cells. In view of all these observations, we eagerly anticipate that the astrocyte–ketone body connection will prove to be a pivotal factor in brain energy homeostasis, which might have important pathophysiological implications.

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Multiple endocrine neoplasia type 1: new clinical and basic findings

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Multiple endocrine neoplasia type 1 (MEN1) provides a prime example of how a rare disease can advance our understanding of basic cell biology, neoplasia and common endocrine tumors. MEN1 is expressed mainly as parathyroid, enteropancreatic neuroendocrine, anterior pituitary and foregut carcinoid tumors. It is an autosomal dominant disease caused by mutation of the *MEN1* gene. Since its identification, the *MEN1* gene has been implicated in many common endocrine and non-endocrine tumors. This is a brief overview of recent scientific advances relating to MEN1, including newly recognized clinical features that are now better characterized by genetic analysis, insights into the function of the *MEN1*-encoded protein menin, and refined recommendations for mutation testing and tumor screening, which highlight our increasing understanding of this complex syndrome.

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The gene responsible for multiple endocrine neoplasia (*MEN1*) has recently been identified[1]. This disease predisposes to tumor development in many tissues with only partial penetrance in each (Fig. 1). The clinical diagnosis of MEN1 can be made in a patient who has tumors in at least two of the following tissues: parathyroid, gastrin cells and pituitary cells (lactotropes). A simple definition of familial MEN1 is a cluster of a minimum of two first-degree relatives, where one has MEN1 and the other has at least one feature of MEN1. Estimates of the prevalence of MEN1 range from 0.01 to 2.50 per thousand[2–5]. Clinical manifestations of the disease are related to the affected organ and can include hormone hypersecretion, and mass effects from tumor size and malignancy. The clinicopathological features of MEN1-associated tumors are generally similar to those of tumors arising sporadically in the same tissue; however, there are distinguishing features of the disease that are characteristic of this and other hereditary neoplasia syndromes[6]. First, the term 'multiple' describes both the multiplicity of tumors that occur in a single affected organ (such as the parathyroids or the islets) and the occurrence of tumors in many different endocrine organs. Tumor multiplicity can result in tumor recurrence after an apparent cure if the affected tissue was incompletely excised (as with subtotal parathyroidectomy). Second, some MEN1 tumors (i.e. parathyroid and gastrin cell) typically present approximately one or more decades earlier than sporadic tumors of the same tissue type. Third, some, but not all, MEN1 tumors have malignant potential (Fig. 1). Indeed, MEN1-related malignancy is a major cause of death for MEN1 patients[7,8].

Primary hyperparathyroidism is the most common and usually the earliest endocrine expression of MEN1 (Refs 3,9,10), occurring in 80–100% of MEN1 patients by the age of 40 years (Refs 5,11). Gastrin-secreting tumors, the most common functional MEN1-associated enteropancreatic neuroendocrine tumors, are a major cause of morbidity and mortality[8]. Approximately 50% of MEN1 patients develop gastrinomas by the age of 50 years (Ref. 6). These gastrinomas are often multiple[12] and metastatic at diagnosis[13], with 15% progressing to an aggressive malignancy[14,15]. MEN1-related insulinomas occur in 10–35% of cases (G. Tamburrano *et al.*, unpublished)[6,16]. Because hyperinsulinemia is usually caused by one benign islet adenoma, surgery is the best primary therapy for this enteropancreatic tumor[17,18]. Non-functional enteropancreatic tumors, and tumors that produce pancreatic polypeptide, are common and often multiple in MEN1, and can become malignant at a later date[19]. Pituitary tumors, most commonly prolactinomas, are present in 10–50% of symptomatic MEN1 patients[4,20]. A substantial number of MEN1 pituitary tumors are 'nonfunctional'[4,21]. Foregut carcinoid tumors, also

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SUMARIO

Los cuerpos cetónicos pueden reemplazar a la glucosa como principal fuente energética cerebral en situaciones en las que ésta es escasa. Aunque se ha asumido que el hígado es el órgano que aporta cuerpos cetónicos a los tejidos extrahepáticos, existían algunas evidencias en la bibliografía de que los astrocitos también son capaces de producirlos. Así, en este trabajo se ha tratado de caracterizar la ruta cetogénica en astrocitos, estudiar sus mecanismos de regulación, y evaluar su posible papel citoprotector en situaciones patológicas, como la hipoxia y la apoptosis inducida por ácidos grasos. A partir de los resultados obtenidos, y a modo de resumen, pueden extraerse las siguientes conclusiones generales:

1.- Los astrocitos son capaces de producir cantidades significativas de cuerpos cetónicos, que podrían ser cedidos a las neuronas para ser utilizados como fuente energética alternativa a la glucosa. Las propiedades de la cetogénesis en astrocitos son muy semejantes a las de la cetogénesis en hepatocitos, lo cual apoya una posible funcionalidad biológica de aquella.

2.- La cetogénesis en astrocitos es un proceso flexible, y se encuentra finamente regulado por al menos dos proteína quinasas, la PKA y la AMPK. Así, un incremento en la concentración de cAMP (que activa la PKA) o de AMP (que activa la AMPK) estimula la producción de cuerpos cetónicos por inactivación de la ACC, descenso en la concentración de malonil-CoA y desinhibición de la CPT-I. Además, la acción activadora directa de la ceramida sobre la CPT-I estimula la producción de cuerpos cetónicos.

3.- La AMPK astroglial podría ejercer un papel citoprotector en situaciones de hipoxia (aumentando el aporte de cuerpos cetónicos a las neuronas) y apoptosis inducida por ácidos grasos (inhibiendo la síntesis *de novo* de ceramida). La cetogénesis astroglial también podría impedir algunos de los efectos perjudiciales provocados por la acumulación de ácidos grasos en el cerebro. Así, el equilibrio entre la oxidación de ácidos grasos y la síntesis de ceramida desempeñaría un papel importante en el control de la decisión supervivencia/muerte celular.