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ESTUDIO DE LOS EFECTOS DE LA DEFICIENCIA DE BIOTINA SOBRE LA ESTRUCTURA Y FUNCIÓN MITOCONDRIAL

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PRESENTA:

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RESUMEN / ABSTRACT

La deficiencia de biotina lleva a la disminución de ATP, el incremento de ADP y AMP y la subsecuente activación de AMPK. Por lo cual se propuso que en esta condición, la función mitocondrial podría estar alterada. Esto fue apoyado por reportes previos de que la deficiencia de biotina causa la disminución de componentes de la cadena respiratoria junto con cambios en la ultra-estructura mitocondrial. Así pues se propuso el objetivo de evaluar el efecto de la deficiencia de biotina sobre la fosforilación oxidativa, así como a los mecanismos implicados. Se encontró que en mitocondrias aisladas de hígado de rata la deficiencia de biotina disminuye la velocidad de consumo de oxígeno, la actividad del Complejo IV y las concentraciones de los citocromos *cc1, b y aa3.* En diversos tejidos se encontró una disminución en la masa mitocondrial, la cual correlaciona con el incremento de dos proteínas asociadas a la mitofagia: BNIP3 y PINK, así como las proteínas HIF-1alfa y STAT3 que disminuyen la función mitocondrial. Debido a lo anterior se evaluó la posibilidad de que STAT3 fuera el responsable de los efectos observados en la deficiencia de biotina. Para probar este planteamiento se inhibió a STAT3 con el inhibidor CAS 1041438-68-9 (Inhibidor de STAT3 VII) en células deficientes de biotina y se observó que las alteraciones sobre la masa mitocondrial y la expresión de BNIP3 y PINK vuelve al nivel del control. De manera contraria, la activación de STAT3 en células controles usando IL-6 causa efectos muy similares a la deficiencia de biotina. En conclusión, STAT3 es al menos en parte responsable de los efectos negativos de la deficiencia de biotina sobre la función mitocondrial, probablemente a través de activar a HIF-1alfa.

ABSTRACT

Biotin deficiency leads to the decrease of ATP and increase of ADP and AMP and the subsequent activation of AMPK. Hence it is proposed that this condition is the cause of impaired mitochondrial function. The latter is supported by previous reports which indicate that in biotin deficiency some respiratory chain components are decreased along with alterations in mitochondrial ultrastructure. Thus the objective of this work is to evaluate the effect of biotin deficiency on oxidative phosphorylation, and the mechanisms implicated. It was found that in rat liver isolated mitochondria that biotin deficiency reduces the consumption of oxygen, the activity of complex IV and cytochrome cc1, b and aa3 concentrations. In various tissues it was found a decrease in mitochondrial mass, which correlates with the increase of two proteins associated with mitophagy: BNIP3 and PINK. The proteins STAT3 and HIF-1α that decrease mitochondrial function were also over expressed. The possibility that STAT3 was responsible for the effects in mitochondrial function observed in biotin deficiency was evaluated. To test this, the STAT3 inhibitor CAS 1041438-68-9 (Inhibitor de STAT3 VII) was used in biotin-deficient cells. Mitochondrial mass and the expression of BNIP3 and PINK in biotin-deficient cells with STAT3 inhibitor were similar to controls. Conversely, activation of STAT3 in control cells using IL-6 caused the increase of PINK, BNIP3 and HIF-1alpha similar to biotin deficient cells. In conclusion, STAT3 is at least partly responsible for the negative effects of biotin deficiency on mitochondrial function, probably through activating HIF-1alpha.

1. INTRODUCCION

La biotina, también conocida como vitamina H o B7, fue identificada en 1936 por Kögl y Tönis cuando estudiaban los requerimientos esenciales para el crecimiento de levaduras (1). Sin embargo, fueron varios años después cuando se empezaron a elucidar las funciones de esta vitamina. En 1950 se demostró que la biotina es un componente indispensable para las reacciones de carboxilación, más tarde en 1958 se encontró una asociación entre la biotina y la enzima acetil CoA carboxilasa (2). Estudios posteriores demostraron que la biotina es cofactor de esta enzima además de otras enzimas que participan en reacciones de carboxilación importantes para el metabolismo intermedio (3).

1.1. Estructura de la biotina y su función como grupo prostético

La estructura de la biotina consiste en dos anillos fusionados, uno llamado imidazol y otro de tetrahidrohidrotiofeno, que contiene azufre, este anillo tiene unida una cadena lateral compuesta por cinco carbonos denominada ácido valérico y termina con un grupo carboxílico (Figura 1) (4).

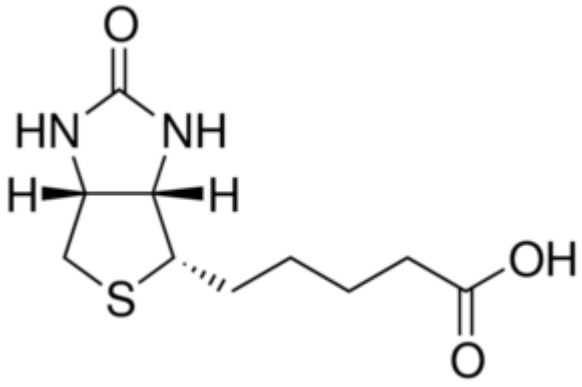

Figura 1. Estructura de la biotina

La función de la biotina como grupo prostético se identificó por la unión covalente entre la biotina y las carboxilasas. En mamíferos existen cinco carboxilasas: la piruvato carboxilasa (PC), la acetil CoA carboxilasa 1 y 2 (ACC 1/2), la propionil CoA carboxilasa (PCC) y la metilcrotonil CoA carboxilasa (MCC) (Figura 2).

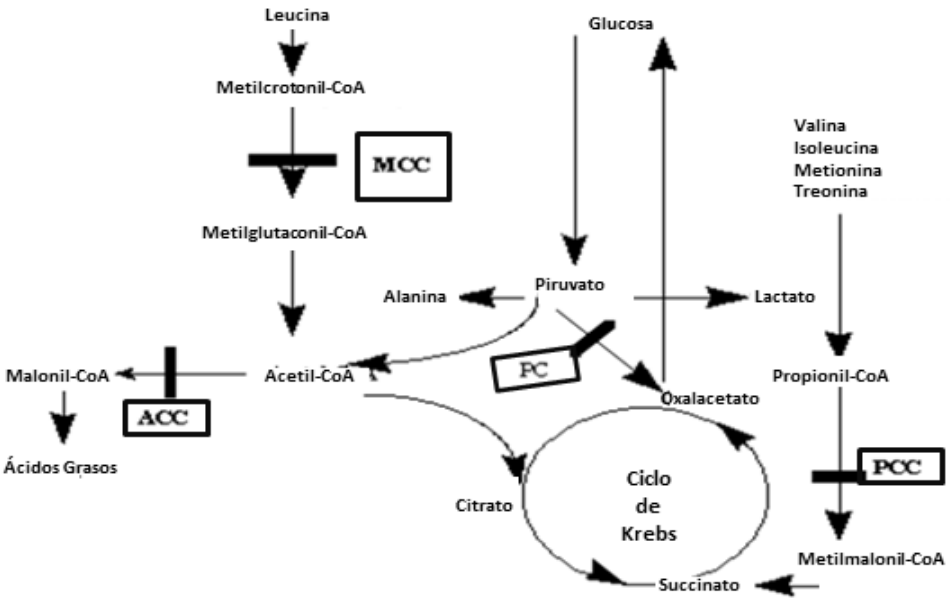

Figura 2. Interrelación entre las rutas metabolicas catalizadas por las enzimas dependientes de Biotina. MCC: metil crotonil CoA carboxilasa**,** ACC: acetil CoA carboxilasa, PC: piruvato carboxilasa, PCC: propionil CoA carboxilasas. (Imagen tomada y modificada de: http://biochem4.okstate.edu/~firefly/Bioch5853/Minireviews/MR2.98%20folder/XW.MR.298/XW.MR.2.98.htm)

Éstas, son sintetizadas como apoenzimas, por lo cual requieren de unirse a la biotina para tener actividad. Esta unión es catalizada por una enzima específica: la proteína ligasa de biotina (BPL) u holocarboxilasa sintetasa (HCS), por medio de una reacción que depende de ATP y ocurre en dos pasos (5-6):

ATP+ Biotina + HCS → Biotina- AMP-HCS + PPi

biotina- AMP-HCS + apocarboxilasa → holocarboxilasa + AMP + PPi + HCS

Las carboxilasas dependientes de biotina catalizan la unión covalente del bicarbonato a ácidos orgánicos y se produce mediante una secuencia de reacciones (Figura 3).

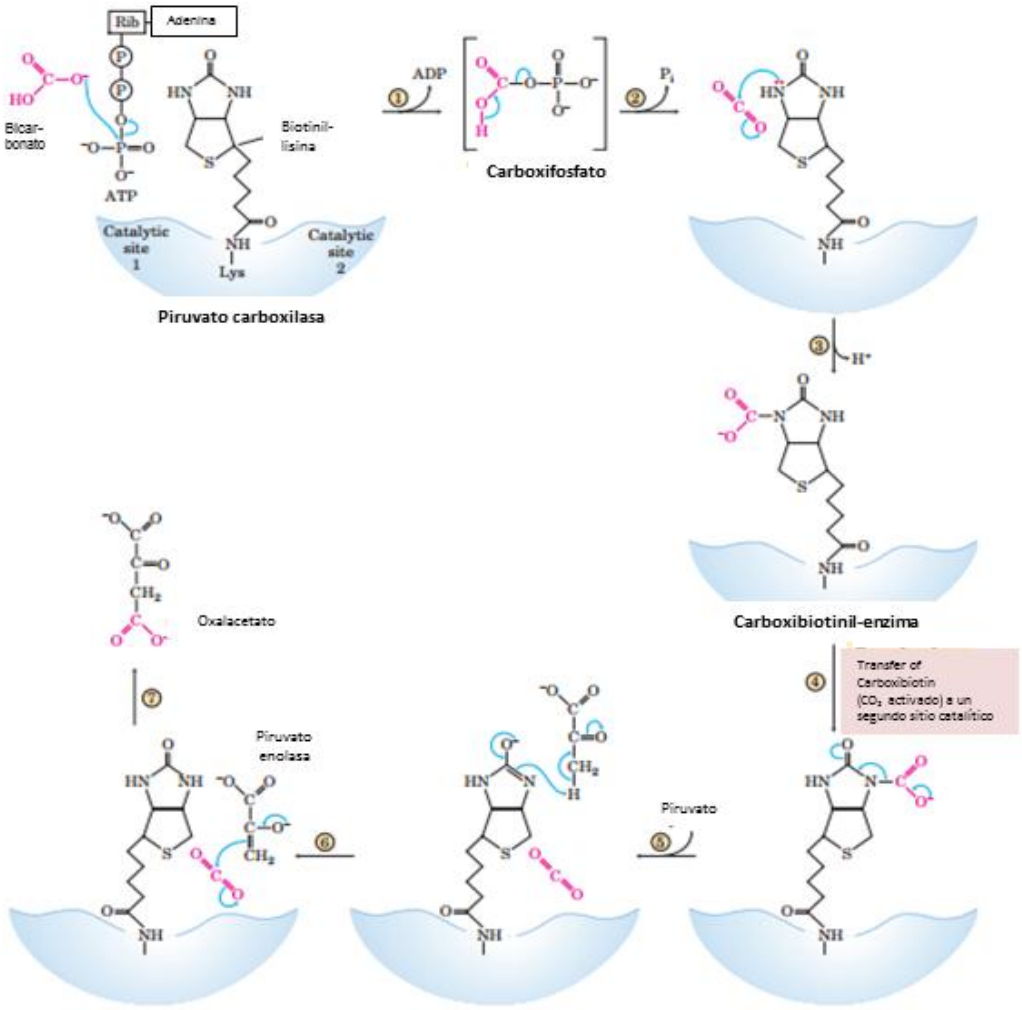

Figura 3. Reacciones de las carboxilasas dependientes de biotina. Las carboxilasas dependientes de biotina catalizan la unión covalente del bicarbonato a ácidos orgánicos mediante la siguiente secuencia: primero, el bicarbonato y el ATP forman un intermediario llamado carboxifosfato (paso 1-3). Posteriormente, el CO_2 formado puede reaccionar con la biotina para formar carboxibiotina (paso 3). En la segunda fase de la reacción (pasos 5-7), el CO_2 reacciona con el piruvato para forma oxalacetato (ejemplo de la reacción mediada por la piruvato carboxilasa) (Imagen tomada y modificada del libro Principios de Bioquímica 4ª

En la primera fase, el bicarbonato es activado al reaccionar con el ATP produciendo un intermediario llamado carboxifosfato, el cual se descompone en bióxido de carbono. El

CO2 reacciona con el nitrógeno 1 del motivo biotinil en la holocarboxilasa para formar 1´-N-carboxibiotinil carboxilasa con la liberación de fosfato inorgánico. Subsecuentemente, la biotina actúa como acarreador para transportar el CO_2, desde un sitio activo a otro en la misma enzima. En una segunda fase, el 1´-N-carboxibiotinil carboxilasa incorpora el CO_2 a un aceptor específico para cada carboxilasa que se libera en el segundo sitio activo (7). Finalmente las holocarboxilasas son degradadas proteolíticamente generando péptidos de biotinil, éstos son degradados por la biotinidasa para liberar la biotina, la cual puede ser reutilizada por otras carboxilasas (8).

1.2. Función de la biotina en la regulación de la expresión de genes

Además de su papel como grupo prostético, la biotina tiene otras funciones biológicas, tales como la regulación de genes a nivel transcripcional y/o postranscripcional; dichos genes participan en diversos procesos, por ejemplo: el metabolismo de la glucosa (9-11).

En procariontes, específicamente en *E. coli*, la biotina puede regular su propia síntesis a través del operón Bio. Debido a la función reguladora que tiene la biotina en procariontes, se comenzó a estudiar esta función en eucariontes. Las levaduras no son capaces de sintetizar biotina de novo, al igual que otros eucariontes, no obstante, estos organismos aún conservan genes que le permiten sintetizarla a partir de precursores como el pimeloil- CoA o el ácido 7-ceto-8-aminopelargónico (KAPA). Estos son introducidos a la célula por medio de los transportadores Bio5p y la proteína permeasa

de biotina (vht1) y posteriormente son metabolizadas para generar biotina por los genes Bio1 (12-14).

En mamíferos se ha observado que la biotina puede modificar la expresión de diversos genes (15). En el laboratorio del Dr. Velázquez se estudió el efecto de la deficiencia de biotina sobre la expresión de genes asociados a su propia función. Se observó que cuando se produce deficiencia de biotina en ratas, disminuye la expresión de la holocarboxilasa sintetasa (HCS) y las carboxilasas PC y PCC en varios tejidos, como son el hígado, el riñón y el musculo esquelético. Esta disminución se revierte con la administración intravenosa de biotina en un lapso de 24 horas. Resultados similares se observaron en la línea celular HepG2 crecidas en un medio de cultivo libre de biotina durante 15 días (16 y 17).

De la misma forma, la biotina tiene efectos en la expresión de otros genes que no están asociados a su función. Dakshinamurti demostró que las concentraciones del RNA mensajero de glucocinasa (GK) disminuyen en la deficiencia de biotina. Además estudios realizados en ratas diabéticas demostraron que la expresión génica de la fosfoenol piruvato carboxicinasa (PEPCK) esta aumentada con respecto al control, y que la administración de biotina disminuye su expresión (18-24).

La biotina también afecta la expresión de algunos oncogenes como son: N-myc, C-myb y raf. La expresión de estos genes disminuye en un 47% cuando se incrementa las concentraciones de biotina a 25 pM. De manera similar, disminuye la expresión de varios factores de transcripción como NF-kB, SP1 y SP3 así como el receptor de la interleucina 2 (IL-2) y la interleucina 4 (IL-4) en células Jurkat (25-27).

1.3. Fuentes de biotina en la dieta

La mayor parte de la biotina ingerida en la dieta se encuentra unida a residuos de lisina en las proteínas y una vez en el intestino la unión es degradada por proteasas para generar biocitina (biotinil-ε-lisina) y péptidos unidos a biotina. Posteriormente la enzima biotinidasa libera a la biotina de la biocitina y de los péptidos para así poder ser absorbida en el intestino (28).

El transporte de la biotina al interior de la célula se realiza a través de transporte pasivo y activo. La difusión pasiva se lleva a cabo cuando la concentración de biotina extracelular excede 25 μMol/L. Por el contrario, cuando la concentración es menor a 5 μMol/L, predomina el transporte activo en donde la captación esta mediada por dos trasportadores: uno conocido como transportador multivitamínico de sodio (SMVT) y en menor medida otro conocido como transportador de monocarboxilatos (MCT1) (29 y 30).

La síntesis de biotina se lleva a cabo por las plantas, la mayoría de las bacterias y algunos hongos. Los mamíferos deben obtener la biotina de fuentes externas como son la dieta o la flora microbiana intestinal. El requerimiento diario de biotina no se conoce con exactitud y por tanto, las recomendaciones para el consumo adecuado se basan en el consumo promedio de poblaciones aparentemente sanas. La tabla 1 muestra las recomendaciones de consumo de biotina para las distintas etapas de la vida.

Se debe tomar en cuenta que estas recomendaciones son inciertas porque el contenido de biotina en la mayor parte de los alimentos no ha sido estudiado por ensayos químicos específicos. Sin embargo; se sabe que la biotina está ampliamente distribuida

en los alimentos: la yema de huevo, la carne, cereales y algunos vegetales son los alimentos con mayor contenido de biotina (31-32).

Tabla 1. Consumo adecuado de Biotina

Edad/etapa de la vida	Requerimientos (mg/día)
Adulto	30
Mujer en lactancia	35
Lactante (0-6 meses)	5
Lactante (7-12 meses)	6
Niños (1-3 años)	8
Niños (4-9 años)	12
Pubertad (9-13 años)	20
Adolescencia(14-18 años)	25

(Tomada y modificada del libro Principios de Bioquímica 4ª Edición, Leninger 2007)

1.4. Deficiencia de Biotina

La primera prueba de la necesidad de biotina en la dieta humana viene de Sydenstricker, 1942 (33). En este estudio se sometieron a adultos voluntarios a una dieta completa en calorías, proteínas y vitaminas, excepto en biotina. Lo anterior se logró suplementando diariamente la dieta con 200 g de huevo crudo. Después de 3 a 4 semanas los voluntarios desarrollaron una dermatitis escamosa, después de 7 semanas mostraron una llamativa palidez grisácea la cual fue atribuida en parte a cambios en la hemoglobina, lo cual se acompañó de anemia microcítica a pesar de un adecuado consumo de hierro. Además de prominentes síntomas de cambios en el estado mental,

mialgias, parestesia localizada y anorexia acompañada con náuseas. Algunos individuos se quejaron de dolor en el pecho y sus electrocardiografías mostraron evidencia de isquemia coronaria. Así mismo se presentaron incrementos en los pigmentos biliares y colesterol en el flujo sanguíneo. Todos los síntomas fueron revertidos dentro de los 5 días consecutivos a una inyección diaria de 150 μg de biotina. Otras manifestaciones clínicas comunes en la deficiencia de biotina son: alopecia. ataxia, hipotonía, acidosis cetogénica y/o orgánica, convulsiones, infecciones e irritación en la piel especialmente alrededor de los ojos, boca y nariz, alteraciones en el sistema inmune, y por último, retraso en el desarrollo en niños (34).

El consumo de huevo crudo es la principal causa de deficiencia de biotina, ya que la avidina contenida en la clara atrapa a la biotina y no permite su absorción (35). Otras causas de deficiencia de biotina son:

- Nutrición parenteral total sin suplementación de biotina (36).
- El uso prolongado de algunas drogas anti convulsionantes como fenitoina, primidona, y carbamazepina puede llevar a deficiencia de biotina, ya sea porque inhiben el transporte de biotina en la mucosa intestinal o porque aceleran su catabolismo (37).
- Malnutrición severa (38).
- Embarazo. Se ha observado que la deficiencia de biotina es muy común en mujeres embarazadas y tiene implicaciones muy importantes en el desarrollo del feto ya que es teratogénica (39).

1.4.1. Efectos de la deficiencia de biotina sobre el metabolismo celular y vías de señalización

Ortega-Cuellar y col (40) demostraron a través de microarreglos que en realidad la deficiencia de biotina no solo afecta la expresión de glucocinasa sino que cambia la expresión de una gran variedad de genes asociados al metabolismo del carbono. Le deficiencia de biotina lleva a una disminución considerable de la expresión de genes que codifican para enzimas que actúan en la glucólisis, tales como: la glucocinasa, la piruvato cinasa, y la lactato deshidrogenasa, entre otras. Mientras que la expresión de fosfoenol piruvato carboxicinasa, enzima que participa en la gluconeogénesis esta aumentada. Por otro lado, los genes involucrados en el metabolismo de lípidos también cambiaron su expresión en la deficiencia de biotina. La ATP citrato liasa, la sintasa de ácidos grasos y la acetil coenzima A carboxilasa son enzimas que participan en la síntesis de ácidos grasos, y cuya expresión disminuye en la deficiencia de biotina. Por su parte, la expresión genica de las enzimas Carnitin Palmitoil Transferasa 1α y la Acil Coenzima A Deshidrogenasa, que corresponden a la vía de β-oxidación de ácidos grasos, están considerablemente aumentadas (40).

Los cambios en la expresión génica estaban acompañados de adaptaciones en diversas vías de señalización como es la cinasa activada por AMP (AMPK) proteína que es conocida como el sensor energético celular. Esta proteína se encontró más fosforilada (en el sitio de activación) en el hígado de ratas deficientes de biotina. Como lo indica su nombre, el principal activador de AMPK es el aumento de las concentraciones de AMP. Cuando la relación ATP/ADP cae, ya sea por algún estrés que inhiba la síntesis de ATP o que los requerimientos celulares incrementen su consumo, la enzima adenilato cinasa

amplifica la señal, ya que cataliza la reacción (2ADP ↔ATP + AMP), de esta manera aumenta las concentraciones de AMP y por tanto la activación de AMPK. De hecho, estudios previos realizados tanto por Velázquez-Arellano et al. (41) y Hernández-Vázquez et al. (42) demostraron que la deficiencia de biotina lleva a una disminución en los niveles de ATP, al mismo tiempo que incrementa las concentraciones de ADP y AMP.

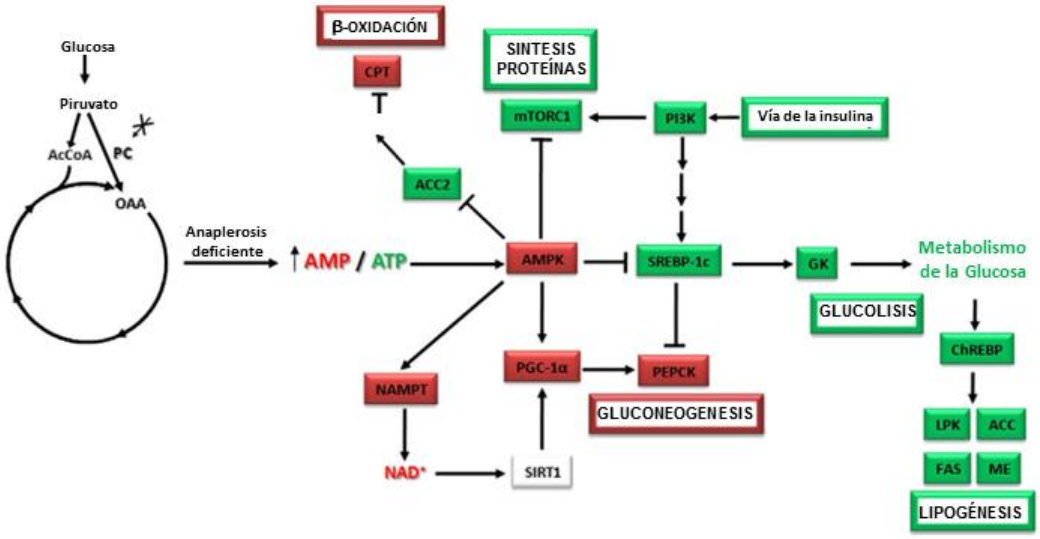

Figura 4. Modelo propuesto para explicar los cambios sobre vías de señalización y la expresión de genes asociados al metabolismo celular causados por la deficiencia de biotina. La reducción de la anaplerosis causada por la deficiencia de biotina aumenta la relación AMP/ATP, este cambio activa a AMPK provocando cambios concertados en las vías de señalización y expresión de genes del metabolismo del carbono. El color rojo indica el aumento de la transcripción de dichos genes, el verde indica disminución, mientras el blanco indica que no hubo cambios (Imagen tomada y modificada de Velázquez- Arellano A, 2011 (41)).

Con el fin de explicar tanto los cambios en la expresión de los genes del metabolismo como la disminución de ATP y la subsecuente activación de AMPK, se propuso un modelo donde se tomaron en cuenta estos hallazgos y lo que se había sido reportado en la literatura hasta ese entonces. En este modelo se propuso que, dado que la

actividad de la piruvato carboxilasa esta disminuida en la deficiencia de biotina, la anaplerosis del ciclo de Krebs está restringida, y por tanto, el flujo por ésta vía se vería afectado, llevando a la disminución de las formas reducidas de NAD y FAD, y esto tendría como consecuencia una menor síntesis de ATP.

Como consecuencia, se activaría AMPK, la cual a su vez tiene una diversidad de proteínas blanco, entre las que se encuentran acetil CoA carboxilasa y SRBP, éstas al ser fosforiladas pueden ser activadas o inhibidas por AMPK, de esta manera se incrementa el flujo por las vías metabólicas que producen ATP como es la β-oxidación de ácidos grasos, mientras que vías que consumen ATP son inhibidas. Así AMPK logra restablecer los niveles de ATP en las células (Figura 4) (41).

2. PLANTEAMIENTO DEL PROBLEMA

A pesar de que el modelo propuesto por Ortega et al. 2011 (41) para explicar el déficit de ATP es factible, se debe recordar que el principal sitio de síntesis de ATP es la mitocondria, a través de la fosforilación oxidativa. Por lo cual, un cambio en la función mitocondrial podría llevar a modificaciones en la concentración de ATP muy significativas.

La función mitocondrial está estrechamente regulada ya que debe de adaptarse a las condiciones de las células. El flujo por la cadena respiratorio y la masa mitocondrial son los principales determinantes de la función mitocondrial. El primero depende de las concentraciones de ADP y ATP, el potencial de membrana y de los complejos mitocondriales (43-44), factores que podrían estar alterados en la deficiencia de biotina. Por un lado, Atanma et al. (45), demostraron que en fibroblastos deficientes de biotina disminuye el nivel del grupo prostético hemo (componente esencial de los citocromos b, c y a en los complejos respiratorios III y IV), así como la expresión de la subunidad COX II del complejo IV de la cadena respiratoria. Mientras que en otros estudios se ha reportado que la deficiencia de biotina causa la disminución del consumo de oxígeno en mitocondrias aisladas junto con cambios en la ultra-estructura mitocondrial, en particular se han observado cambios en los fosfolípidos que conforman la membrana mitocondrial (46-47). Con respecto a la masa mitocondrial, ésta depende tanto de la síntesis o biogénesis mitocondrial como de la degradación de las mitocondrias (mitofagia). Ambas son reguladas por diversos mecanismos entre los que se encuentran factores nucleares como PGC-1α, HIF-1α y la proteína AMPK, entre otras. Se ha reportado que la

deficiencia de biotina, así como otros micronutrientes como la tiamina o de algunos aminoácidos como la leucina, tiene efectos en HIF-1α y AMPK (48 y 49). Con respecto a HIF-α se ha observado que uno de sus principales activadores es la vía de señalización de STAT3, ésta es activada por citocinas como IL-6, la cual se ha encontrado sobre expresada en deficiencia de biotina (50 y 51). A partir de lo anterior se plantearon las siguientes preguntas:

¿La deficiencia de biotina causa alteraciones en la fosforilación oxidativa? Y de ser así ¿Cuáles son los mecanismos responsables?

3. HIPOTESIS

La deficiencia de biotina lleva a una disminución de la fosforilación oxidativa que es causada por modificaciones en la vía de señalización de STAT3.

4. OBJETIVO GENERAL

Evaluar el efecto deletéreo de la deficiencia de biotina sobre la fosforilación oxidativa, así como los mecanismos moleculares responsables.

5. OBJETIVOS PARTICULARES

1. Caracterizar la alteración de la fosforilación oxidativa inducida por la deficiencia de biotina.

2. Estudiar las modificaciones en la masa y ultra-estructura mitocondrial ocasionadas por la deficiencia de biotina.

3. Analizar las alteraciones en vías de señalización como STAT3 que regulan la fosforilación oxidativa durante la deficiencia de biotina.

6. METODOLOGÍA

6.1 Modelos experimentales

6.1.1 Modelo animal

Se usaron ratas Wistar macho recién destetadas, las cuales fueron divididas en dos grupos con base en el tipo de dieta.

-Dieta deficiente de biotina que contenía 30% de avidina que se une con alta afinidad a la biotina libre en el alimento y no permite que sea absorbida en el intestino (TD. 87126, Harlan Teklad, Madison WI, USA).

-Dieta control que contenía 30% de avidina pero está suplementada con 0.03 g/Kg de biotina (TD. 81079, Harlan Teklad, Madison WI, USA).

De acuerdo a una investigación previa, después de 4 semanas de ser alimentados con esta dieta los animales desarrollan los efectos de la deficiencia de biotina y fueron utilizados para los experimentos que se describirán a continuación (42).

6.1.2 Cultivo celular

- Cultivo primario de hepatocitos: Se usaron ratas con las características descritas en la sección de modelo animal. Los animales fueron anestesiados con ketamina / xilazina 50 mg / kg. El hígado se diseccionó y los hepatocitos se aislaron usando el método de Berry y Friend (48). Las células se suspendieron en Ringer-Krebs-fosfato: 5.7 mM KCl, NaCl 120 mM, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 1.2 mM $CaCl_2$, pH 7.4. El medio se calentó a 37 ° C y se burbujeó con O_2 / CO_2 (95/5%) antes de su uso. La viabilidad

celular se comprobó por el método de exclusión tripano; todos los experimentos se llevaron a cabo solamente cuando se observó viabilidad mayor al 95%.

- Los hepatocitos aislados y las líneas celulares U373 de Glioblastoma y Fibroblastos Embrionarios de Ratón (MEF) Wild Type y Doble Knock Out de AMPK (ATCC: HB-8065, Manassas, VA EE.UU.) se cultivaron utilizando medio DMEM (Dubelcco Modified Eagle´s Medium) suplementado con Suero Fetal Bovino al 10% y penicilina 100 UI/ml, estreptomicina 100 μg/ml y gentamicina 50 μg. (Gibco, Life Technologies, Grand Island, NY. EE.UU.) y fueron mantenidas a 37 °C y 5% de CO2. El medio deficiente en biotina fue preparado por diálisis de suero fetal bovino usando un protocolo descrito anteriormente (50). La deficiencia de biotina se demostró por Western blots con estreptavidina. Cuando se indujo la deficiencia de biotina, las células se sembraron en medio completo, que fue sustituido después de 24 h con un medio deficiente en biotina y se incubó durante los tiempos que requirieron los experimentos.

6.2 Aislamiento de mitocondrias

Las mitocondrias se obtuvieron de acuerdo con un método descrito previamente (49). Se utilizaron ratas Wistar macho que fueron sacrificadas y se obtuvo el hígado, el cual fue colocado en el buffer A que contiene sacarosa 40 mM, 220 Manitol, 1m M EGTA, 5 mM Tris, pH 7.4 y se homogeneizó usando una mano de mortero de teflón. Las mitocondrias se aislaron por centrifugación diferencial y todo el tiempo se mantuvieron en una temperatura cercana a 4 °C. La cantidad de proteína se determinó utilizando el

método de Biuret. La calidad de las preparaciones fue evaluada por el consumo de oxígeno como se describe a continuación.

6.3. Evaluación de consumo de oxígeno

Se usaron ya sea mitocondrias aisladas o células intactas (cultivo primario de hepatocitos), las que se colocaron en una cámara de temperatura controlada con agitación constante. La concentración de oxígeno se controló usando un oxímetro equipado con un electrodo específico Clark (Strathkelvin Instruments, Escocia). Las mitocondrias (0,5 mg de proteína) se suspendieron en Buffer A en presencia de 10 de mM fosfato, 10 mM de glutamato y 10 mM de malato. La respiración se monitorizó en las condiciones anteriores (estado 2) y después de la adición de ADP (estado 3).

Los hepatocitos se tripsinizaron y se resuspendieron en los respectivos medios de cultivo (completo o deficiente de biotina) a una densidad conocida ($2x10^5$ células/ml) y se mantuvieron a agitación constante. El consumo de oxígeno se midió antes y después de la adición de oligomicina (10 mM), un inhibidor especifico de la ATP sintasa mitocondrial. La sensibilidad hacia la oligomicina fue interpretada como la tasa de fosforilación oxidativa como se informó anteriormente (52).

En todos los casos, se calculó la tasa respiratoria basándose en la concentración de oxígeno en función del tiempo teniendo en cuenta la concentración de proteína o la densidad celular, según sea el caso.

6.4 Determinación de la actividad del complejo IV mitocondrial

La actividad del citocromo c oxidasa (COX) o Complejo IV mitocondrial se evaluó a través del consumo de oxígeno. Las mitocondrias (0,5 mg de proteína) se preincubaron con antimicina A (10 mM) y ascorbato 5 mM (1 mM). La actividad de COX fue estimulada por la adición de 10 mM TMPD (N, N, N ', N'-tetrafenilenediamine). La actividad se reportó como la cantidad de oxígeno consumido por minuto.

6.5 Cuantificación de los citocromos

Para la cuantificación de los citocromos *b, cc1 y aa3* se usaron mitocondrias aisladas de hígados de rata (1 mg de proteína). Las mitocondrias fueron tratadas con ferricianuro de Sodio, con el fin de medir los espectros de absorción en el estado oxidado a distintas longitudes de onda (500-650 nm) a través de un espectrofotómetro de doble haz (Aminco DW 2, Olis Inc.). Posteriormente se agregó hidrosulfito de sodio (ditionita), el cual, reduce a los citocromos y se repitió la medición con el espectrofotómetro. Se graficó la diferencia entre ambos espectros, con esto se obtuvo un trazo en el cual aparecieron los picos característicos de los citocromos. El citocromo c tiene un máximo de absorción a 550 nm, mientras que los citocromos b y aa3 absorben a 560 y 603 nm, respectivamente (Figura 5). Los citocromos se cuantificaron espectrofométricamente utilizando los valores reportados previamente de los coeficientes de extinción molar (ε) (aa3: 24 M-1 cm-1, b: 28,5 M-1 cm-1, cc1: 21 M-1 cm-1) (53).

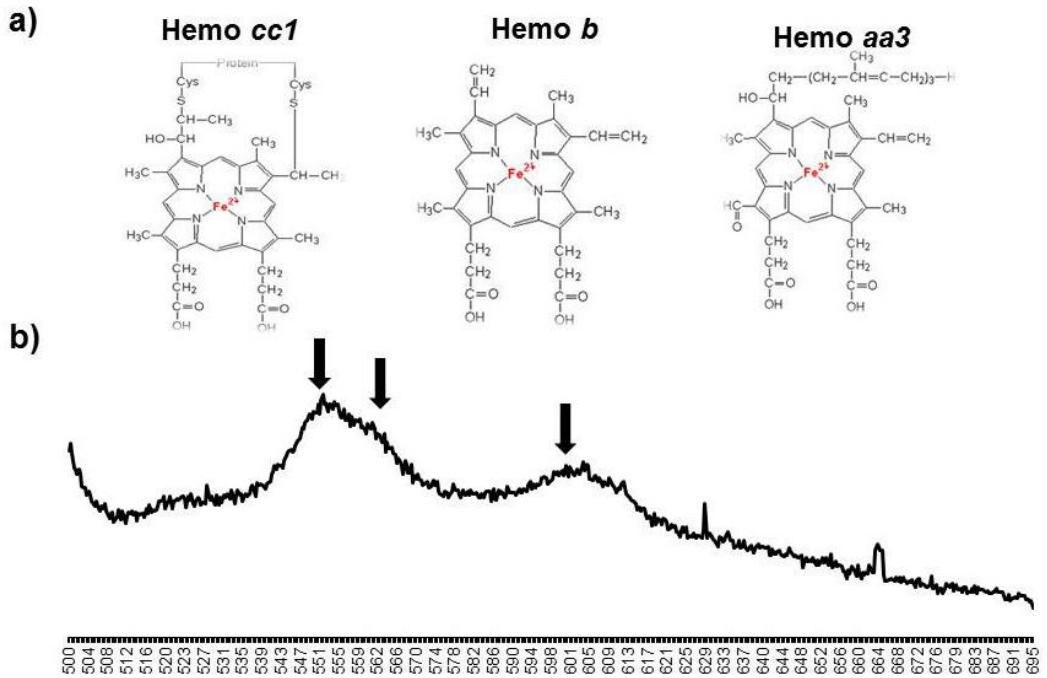

Figura 5. Cuantificación de los citocromos por espectrometría diferencial. . Esta técnica se basa en en un barrido a distintas longitudes de onda (500-650 nm). Las flechas indican la longitud de onda en la que se observa el máximo de absorción de cada citocromo: el citocromo c tiene un máximo de absorción a 550 nm, mientras que los citocromos b y aa3 absorben a 560 y 603 nm, respectivamente.

6.6 Actividad de citrato sintasa

La actividad de citrato sintasa se midió en extractos de proteína obtenidos al homogenizar el tejido o cultivo celular con un buffer de lisis que contenía Triton X-100 0,1%, 50 mM de KCl, 1 mM de EDTA, 1 mM de EGTA, 5 mM de fosfato, 50 mM HEPES, pH 7,0) suplementado con un cóctel inhibidor de la proteasa (Complete Mini, Roche). La proteína se cuantificó por el método de Bradford. Para cada medición se usaron 30 µg de proteína, los cuales se incubaron con 30 mM de acetil-CoA, 10 mM de oxalacetato y 10 mM de DTNB. Se monitorizó la absorbancia a 412 nm en un espectrofotómetro Genova-Nano (Jenway).

6.7 Tinción de las mitocondrias con Mitotracker

Los hepatocitos primarios se sembraron en cubreobjetos y se trataron con Mitotracker CMXRos 25 nM (Invitrogen) durante 15 minutos, se lavaron dos veces con PBS y se fijaron con paraformaldehído al 4%. Los cubreobjetos fueron montados en placas de vidrio y se visualizaron mediante microscopía confocal (Zeiss LSM 5 PASCAL).

6.8 Estudios de la estructura mitocondrial con microscopia electrónica

Ratas control y ratas deficientes de biotina fueron perfundidas a través del ventrículo cardíaco izquierdo con 4% de paraformaldehído en Buffer de fosfato al 0.1 M de solución salina (pH 7,4). Se retiró una muestra de tejido muscular de los glúteos, se seccionó, y se transfirió al mismo fijador durante 1 h. Posteriormente, las muestras se trataron con 0,5% de tetróxido de osmio en ácido cacodílico a pH 7.4, y se deshidrataron en alcohol extremo embebido en resina EPON. Secciones de una micra de grosor en el área gris/plata de los colores de interferencia en la plata/zona gris de la gama de colores de interferencia se tiñeron con acetato de uranilo y citrato de plomo y se examinan bajo un microscopio electrónico de transmisión JEOL JEM-1400. Estos experimentos se realizaron en con el laboratorio de neuropoatologia experimental del Instituto Nacional de Neurología.

6.9 Western Blots

Las muestras se congelaron en nitrógeno líquido y se homogeneizaron con un Buffer que contiene HEPES 50 mM, KCl 50, EGTA 1 mM,-β glicerol fosfato 5 mM, Triton X-100 0,1% (v / v), inhibidores de la proteasa mM, EDTA 1 mM mini Complet (Roche Diagnostics, Mannheim, Alemania), 50 mM de fluoruro de sodio, 1 mM de ortovanadato

de sodio, pirofosfato de sodio al 5 mM y PMSF al 0,2 mM (todos de Sigma-Aldrich). Los homogeneizados se centrifugaron a 12.000 xg durante 15 minutos a 4 ºC y se utilizaron los sobrenadantes. Los extractos de proteína se separaron mediante electroforesis SDS-PAGE en un gel de poliacrilamida 10%. Las proteínas se transfirieron sobre membranas de nitrocelulosa (Bio-Rad Laboratories, Inc. Alemania) y después se bloquearon con leche sin grasa 1% y se incubaron con diferentes anticuerpos primarios: fosfo-AMPKα (Thr172) # 2 531, AMPKα(23A3) # 2.603 (Cell Signaling Technology, Inc. Danvers, MA. EE.UU.), fosfo-STAT3 (Tyr 705) # 7993, STAT3 (C-20) # 482, PINK1 (H-300) # 33796, BNIP-3 (Ana40) # 56167), mtTFA (E-16) # 30963, HIF-1α (Y-15) # 12542, y α-tubulina (TU-02) # 8035 (Santa Cruz Biotechnology, Dallas, Texas. EE.UU.). Las membranas se lavaron tres veces con PBS y se incubaron con los respectivos anticuerpos secundarios conjugados con HRP. Las transferencias se revelaron utilizando un sustrato quimioluminiscente HRP Immobilion Occidental (Millipore Corporation, Billerica, MA.USA). Las imágenes fueron adquiridas en un Sistema FUSION FX (VILBER Lourmat, Francia) y analizadas en ChemiDoc Sistema XRS (Bio-Rad Laboratories, Inc. Alemania).

6.10 Análisis estadísticos

Todos los experimentos se realizaron por triplicado y en tres ocasiones diferentes. Los resultados se presentan como la media $\pm$ desviación estándar, la significancia estadística se evaluó con ANOVA y la prueba t de Student. Las diferencias se consideraron estadísticamente significativas con un valor de $p < 0.05$.

7. RESULTADOS

7.1. Efecto de la deficiencia de biotina sobre la función mitocondrial

Como se mencionó en la metodología, los experimentos se realizaron empleando ratas que fueron alimentadas durante 4 semanas con una dieta diseñada para inducir deficiencia de biotina. Un método para comprobar que los organismos desarrollaron la deficiencia de esta vitamina es por medio de blots de estreptavidina, ésta se une covalentemente a la biotina, de modo que la intensidad de la señal depende de la concentración de las carboxilasas que están biotiniladas. En la figura 6 se muestra un blot representativo usando extractos de hígado de ratas control y ratas deficientes de biotina. En los carriles correspondientes a las muestras de ratas deficientes de biotina se observa que la intensidad de estas bandas es considerablemente menor con respecto a los controles, lo cual demuestra que hay menos biotina unida a estas proteínas. Con este análisis se comprueba que los animales que se usaron para experimentar presentan efectivamente una deficiencia de biotina.

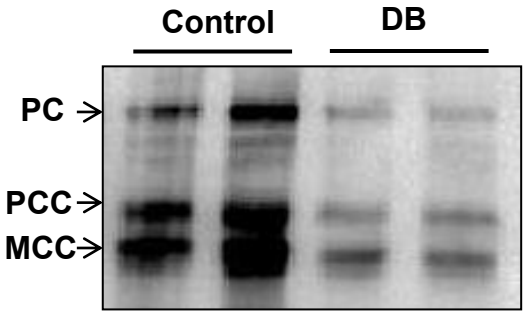

Figura 6. Blot de carboxilasas biotiniladas. Extractos de proteína de hígado de ratas control y deficientes de biotina (DB). En cada pozo se cargaron 30 μg de proteína. Se incubaron con streptavidina. PC: piruvato carboxilasa, PCC: propionil CoA carboxilasa y MCC: metil crotonil

Una vez comprobada la deficiencia de biotina se procedió a evaluar el consumo de oxígeno en mitocondrias aisladas de hígado de ratas deficientes y controles. El trazo se

empezó usando Glutamato/Malato como sustratos (Estado 2 respiratorio), posteriormente se agregó ADP (Estado 3 respiratorio) (Figura 7a). Si las mitocondrias están acopladas, el ADP debe de acelerar el consumo de oxígeno, por lo tanto, una relación estado 3/estado 4 (coeficiente respiratorio) mayor a 2, indica que las mitocondrias están acopladas. En las mitocondrias de hígado de las ratas control, el coeficiente respiratorio fue de 4.3 mientras que en las deficientes de biotina fue de 3. Así mismo, el consumo de oxígeno en el estado 3 fue mayor en las mitocondrias de ratas controles (50 nmoles O2/min/mg de proteína) en comparación con las mitocondrias de ratas deficientes (30 nmoles O2/min/mg de proteína) (Figura 7b), es decir, la velocidad de consumo de oxígeno disminuyó en un 40% en las mitocondrias aisladas de hígados de ratas deficientes de biotina. Con lo anterior se demuestra que la actividad de la cadena respiratoria está afectada en la deficiencia de biotina.

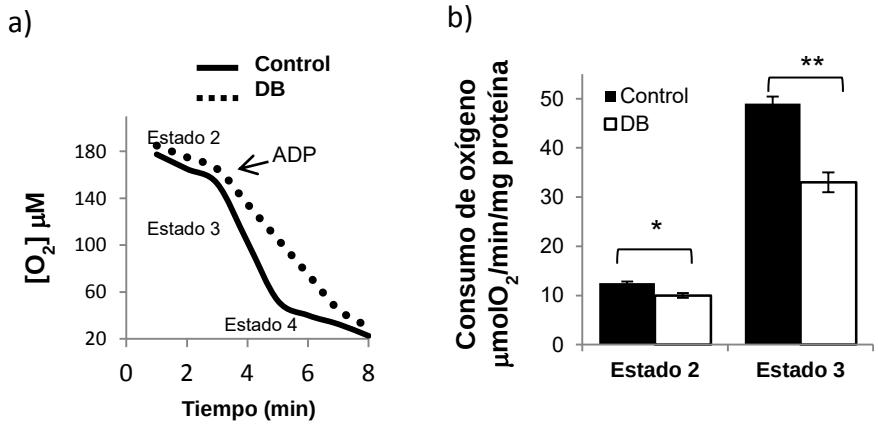

Figura 7. Consumo de oxígeno en mitocondrias aisladas de hígado. a) El consumo de oxígeno se midió usando como sustratos glutamato/malato (estado 2), la adición de ADP acelera el consumo de oxígeno (Estado 3), cuando el ADP se agota, disminuye la velocidad de consumo de oxígeno (Estado 4). b) Velocidad del consumo de oxígeno en el estado 2 y en el estado 3. * p< 0.01, ** p<0.001 (Prueba t de Student).

Evidencia previa a este trabajo indica que la deficiencia de biotina podría afectar a la actividad de complejo IV mitocondrial, ya que por medio de western blot se observó que

la expresión de la subunidad COXII del complejo IV está disminuida en la deficiencia de biotina (45). Por lo cual, se evaluó la actividad del complejo IV usando mitocondrias aisladas. Se inhibió al complejo III con antimicina y posteriormente se agregaron TMPD y ácido ascórbico como sustrato de citocromo c, éstos impulsan el consumo de oxígeno y la actividad del complejo IV se toma como la velocidad del consumo de oxígeno (μmol O_2/min/mg de proteína). Las mitocondrias de hígado de ratas control presentaron una actividad de 0.97 μmol O_2/min/mg de proteína, mientras que las deficientes de biotina tuvieron 0.57 μmol O_2/min/mg de proteína de actividad, es decir, la deficiencia de biotina disminuyó la actividad de éste complejo en un 40% (figura 8). La disminución del complejo IV puede explicar en gran parte las alteraciones en el flujo de electrones que se mostraron previamente ya que, éste complejo controla gran parte del flujo respiratorio (56).

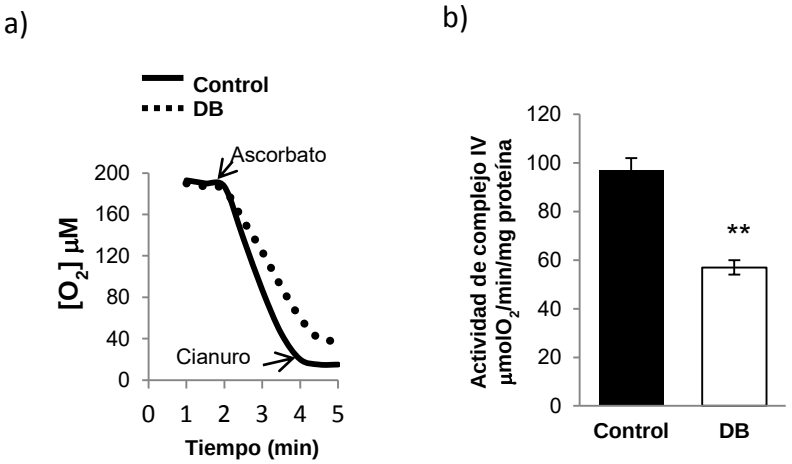

Figura 8. Actividad de complejo IV en mitocondrias aisladas de hígado. a) Se inhibe al complejo III con antimicina, se comienza el trazo con TMPD, el consumo de oxígeno se acelera cuando se añade ascorbato, el consumo de oxígeno es completamente sensible al cianuro. b) La actividad del complejo IV se determina como la velocidad del consumo oxígeno cuando se añadió el ascorbato. ** $p<0.001$ (Prueba t de Student).

Una posible explicación para la disminución de la actividad del complejo IV mitocondrial es la disminución en la síntesis del grupo hemo como había sido reportado previamente (45). El grupo hemo es indispensable para la síntesis de los citocromos *cc1, b y aa3* que forman parte de los complejos III y IV. Con base en lo anterior se decidió medir la cantidad de citocromos en mitocondrias aisladas. En la figura (Figura 9a) se muestran trazos representativos de los espectros diferenciales empleados para cuantificar los citocromos. En el trazo correspondiente a mitocondrias de ratas control se pueden distinguir claramente cada uno de los picos correspondientes a los citocromos *b* (560 nm), *cc1* (550 nm) y *aa3* (603 nm), mientras que en el trazo de mitocondrias deficientes de biotina los picos están menos definidos y las absorbancias a cada longitud de onda son considerablemente menores. Posteriormente se cuantificaron los citocromos usando sus respectivos coeficientes de extinción, la deficiencia de biotina causa la disminución de los tres tipos de citocromos en alrededor del 50% (Figura 9b).

a) b)

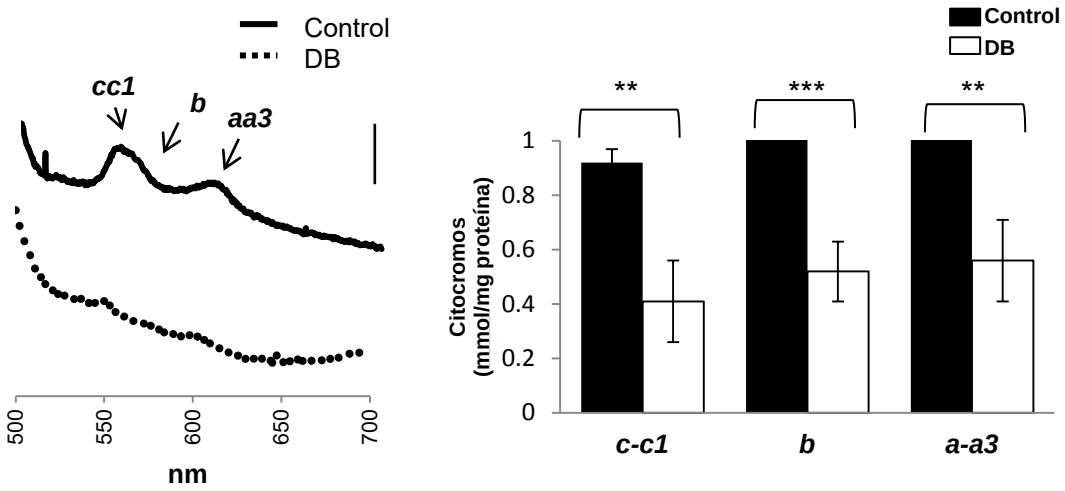

Figura 9. Concentraciones de citocromos en mitocondrias aisladas de hígado. a) Trazo representativo de espectrometría diferencial en mitocondrias de controles y deficientes de biotina (DB). b) Cuantificación usando los coeficientes de extinción para cada citocromo. ** p<0.01, *** p< 0.001 (Prueba t de Student).

Con esto resultados se explica la disminución de la actividad del complejo IV mitocondrial y brinda evidencias de que el complejo III también podría estar disminuido

Los resultados anteriores demuestran que la deficiencia de biotina causa alteraciones en la fosforilación oxidativa en mitocondrias aisladas. Sin embargo, las células pueden tener muchas adaptaciones para compensar estas alteraciones, ya sea incrementando la cantidad de mitocondrias o activando vías metabólicas como la β-oxidación de ácidos grasos para generar más sustratos para la cadena respiratoria. Por lo que se estudió la fosforilación oxidativa en células completas con el fin de evaluar si las alteraciones observadas en las mitocondrias aisladas se mantenían en el contexto de una célula completa. Se usaron cultivos primarios de hepatocitos obtenidos de ratas controles y deficientes de biotina. Los hepatocitos se tripsinizaron y se suspendieron en un medio que contenía 5 mM de glucosa en una concentración de $2x10^5$ células por mililitro, se colocaron en el oximétro y se empezó el trazo. El consumo de oxígeno fue considerablemente menor en los hepatocitos de ratas deficientes de biotina, empero, el consumo de oxígeno por sí mismo no refleja el estado de la fosforilación oxidativa debido a que existen procesos fisiológicos que disipan los protones y por tanto desacoplan la respiración de la síntesis de ATP. Por esta razón se usó oligomicina, la cual es un inhibidor específico del complejo V o ATP sintasa. La sensibilidad a este inhibidor puede dar un estimado del flujo respiratorio que está asociado a la fosforilación oxidativa. La respiración sensible a la oligomicina disminuyó un 50% en los hepatocitos de ratas deficientes de biotina en comparación con los controles (Figura 10). Esto indica

que la producción de energía es menos eficiente como consecuencia de la deficiencia de biotina como se sugería anteriormente.

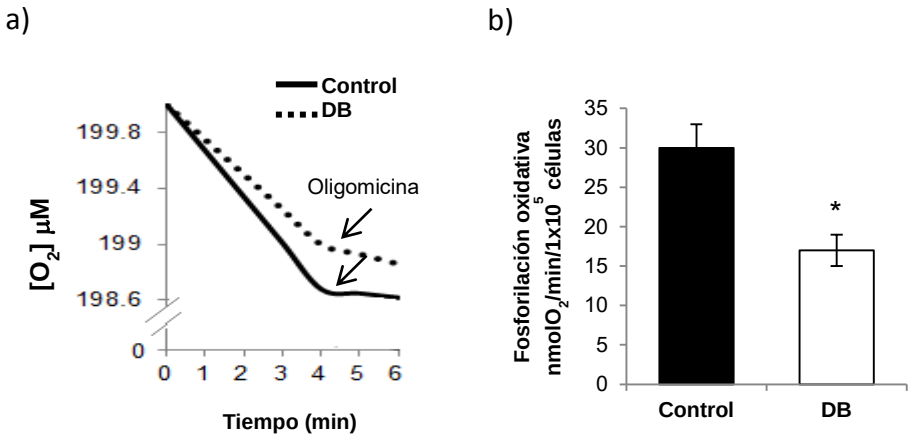

Figura 10. Fosforilación oxidativa en cultivo primario de hepatocitos. a) Trazo representativo donde se muestra las diferencias en el consumo de oxígeno de los hepatocitos Controles y Deficientes de Biotina (DB) antes y después de la adición de oligomicina b) El consumo de oxígeno posterior a la adición de la oligomicina se restó al previo, el resultado se tomó como las fosforilación oxidativa. *p< 0.001 (Prueba t de Student).

7.2. Las alteraciones en la fosforilación oxidativa están acompañadas con la disminución en la masa y ultra-estructura mitocondrial

Los resultados anteriores nos llevaron a pensar que la deficiencia de biotina podría causar la disminución en la masa mitocondrial, ya que se ha observado que tanto las concentraciones de citocromo c como la enzima citrato sintasa correlacionan con la masa mitocondrial (57). De hecho la actividad de citrato sintasa es un marcador muy utilizado para estimar la cantidad de mitocondrias. Se midió la actividad de esta enzima en los hepatocitos aislados, tanto controles como deficientes de biotina y se encontró

que la deficiencia de esta vitamina lleva a una reducción del 30% en la actividad de citrato sintasa (figura 11a).

Los hepatocitos de ratas deficientes de biotina también presentaron una disminución del 30% en la expresión de la proteína del factor de transcripción mitocondrial A (TFAM) (Figura 11b). Esta es una proteína que se transcribe en el núcleo y posteriormente es transportada hacia la mitocondria. Tiene dos funciones: por un lado regula la replicación del DNA mitocondrial, y por otro se une a él protegiéndolo de la degradación. Se ha observado que las concentraciones de TFAM correlacionan con las del DNA mitocondrial (58 y 59). Este hallazgo apoya al resultado de citrato sintasa como evidencia de que la deficiencia de biotina disminuye la masa mitocondrial.

En ese mismo sentido, se empleó la tinción con mitotracker como otro método de evaluación de la masa mitocondrial. La Figura 11c muestra dos fotografías representativas (control y deficiente de biotina) de hepatocitos primarios teñidos con mitotracker. Se puede observar que los hepatocitos de ratas deficientes de biotina presentan menos tinción en comparación con el control.

Después de lo anterior expuesto, se pensó que la ultra-estructura mitocondrial podría estar afectada, ya que diversos estudios realizados en deficiencia de biotina o en deficiencia génica de algunas de las carboxilasas se encontró hinchamiento mitocondrial e incluso alteraciones en la membrana mitocondrial. Así que se decidió realizar estudios con microscopia electrónica con el fin de evaluar estos parámetros. Dado que la mayor parte de los estudios de este tipo se realizan en músculo, se usó este mismo tejido para que los resultados puedan ser comparables. En la Figura 11d se

muestran fotografías de microscopía electrónica de músculo de ratas controles y ratas alimentadas con dieta para inducir deficiencia de biotina durante 4, 6 y 10 semanas.

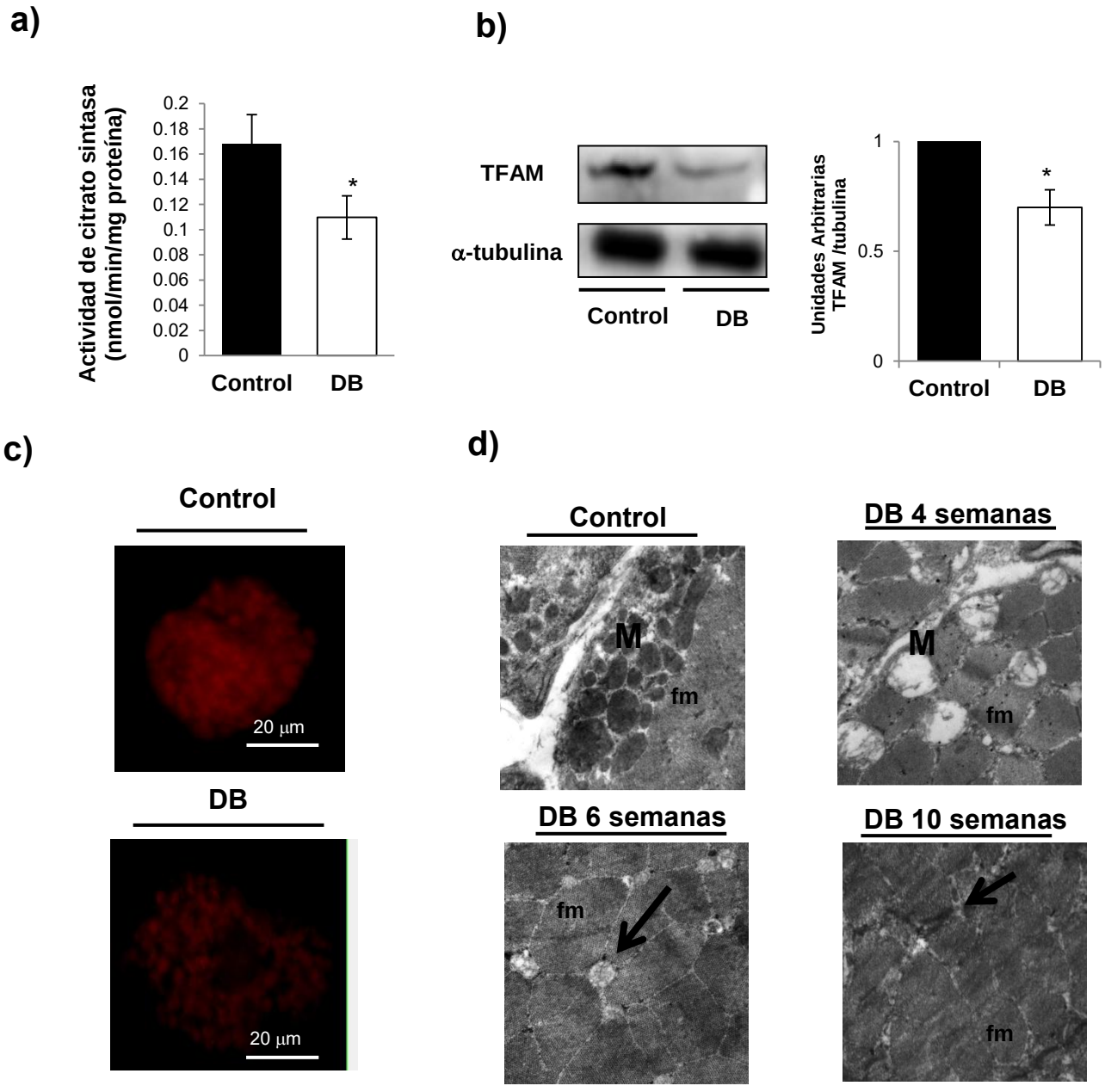

Figura 11. Efecto de la deficiencia de biotina sobre la masa y ultra-estructura mitocondrial. a) Actividad de citrato sintasa en hepatocitos controles y deficientes de biotina (DB). b) La expresión de TFAM se determinó por medio de WesternBlot en extractos de cultivo primario de hepatocitos. c) Tinción con Mitotracker Red CMXRos En cultivo primario de hepatocitos Controles y Deficientes de Biotina (DB). d) Microscopía electrónica en cortes de musculo de ratas Control y ratas con 4, 6 y 10 semanas de Deficiencia de Biotina (DB). *p< 0.05 (Prueba t de Student).

En la imagen representativa del músculo control se observa un cúmulo de mitocondrias (M) y las fibras musculares (fm) conservadas. En el músculo de ratas alimentadas con la dieta para inducir deficiencia de biotina por 4 semanas, se observa menor número de mitocondrias (M), además de que presentan una ultra-estructura alterada debido a edema.

En las muestras de músculo de ratas con 6 semanas de deficiencia de biotina, presentan además de la reducción en el número de mitocondrias, una disminución de tamaño (flecha). En el músculo de ratas tratadas por 10 semanas se observan fibras musculares con pérdida de su arquitectura, con escasas mitocondrias (flecha) las cuales están disminuidas en su tamañ

7.3. La deficiencia de biotina incrementa la expresión de proteínas que participan en vías de mitofagia

En el marco de las observaciones anteriores, el siguiente paso fue investigar los mecanismos que llevan a la disminución en la masa mitocondrial. Lo procesos de síntesis y degradación de las mitocondrias son finamente regulados por la célula, ya que a pesar de que las mitocondrias son el principal sitio de síntesis de ATP, estos organelos son también generadores de especies reactivas de oxígeno, y bajo ciertas circunstancias pueden desatar la muerte celular (60 y 61).

Se conoce como mitofagia a un tipo específico de autofagia que tiene como objetivo la remoción de mitocondrias dañadas, decadentes o superfluas. Las vías de señalización que controlan la mitofagia, así como las proteínas que participan en la progresión de la misma siguen siendo estudiadas y son complejas. Sin embargo, existen dos proteínas

que se pueden usar como marcadores de una vía de mitofagia activa, la PTEN-induced putative kinase 1 (PINK1) y la BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3). Se propone que la primera participa en la degradación de mitocondrias dañadas o despolarizadas, mientras que la segunda es activada en condiciones de privación de nutrientes o ATP (62-65). Como ya se ha mencionado, ambas condiciones (daño mitocondrial y privación de nutrientes) están presentes en la deficiencia de biotina. Por este motivo se evaluó la expresión de estas proteínas en hígados de ratas controles y deficientes de biotina (Figura 12a), se observó que tanto PINK1 como BNIP3 están sobre-expresadas en la deficiencia de biotina. Esta sobre-expresión correlaciona con la disminución de TFAM, y la activación de AMPK. Estos resultados sugieren fuertemente que la disminución en la masa mitocondrial observada en la deficiencia de biotina se debe a la activación de la mitofagia.

Si bien es cierto que las vías de mitofagia pueden ser activadas por los mecanismos que se explicaron arriba, existen evidencias de que en la deficiencia de biotina, tanto PINK como BNIP3 pueden ser activadas por proteínas señalizadoras, una de las cuales es el factor de transcripción inducido por hipoxia 1α (HIF-1α) (66), el cual como lo indica su nombre, es activado cuando las concentraciones de oxígeno disminuyen considerablemente. También se sabe que también puede ser activado sin que cambien las concentraciones de oxígeno, condición conocida como pseudo-hipoxia. Existen reportes de que deficiencias de otras vitaminas como es la tiamina, activan a HIF-1α (66). De manera semejante la deficiencia de biotina incrementa la expresión de HIF-1α hasta dos veces en comparación con el control (Figura 12b).

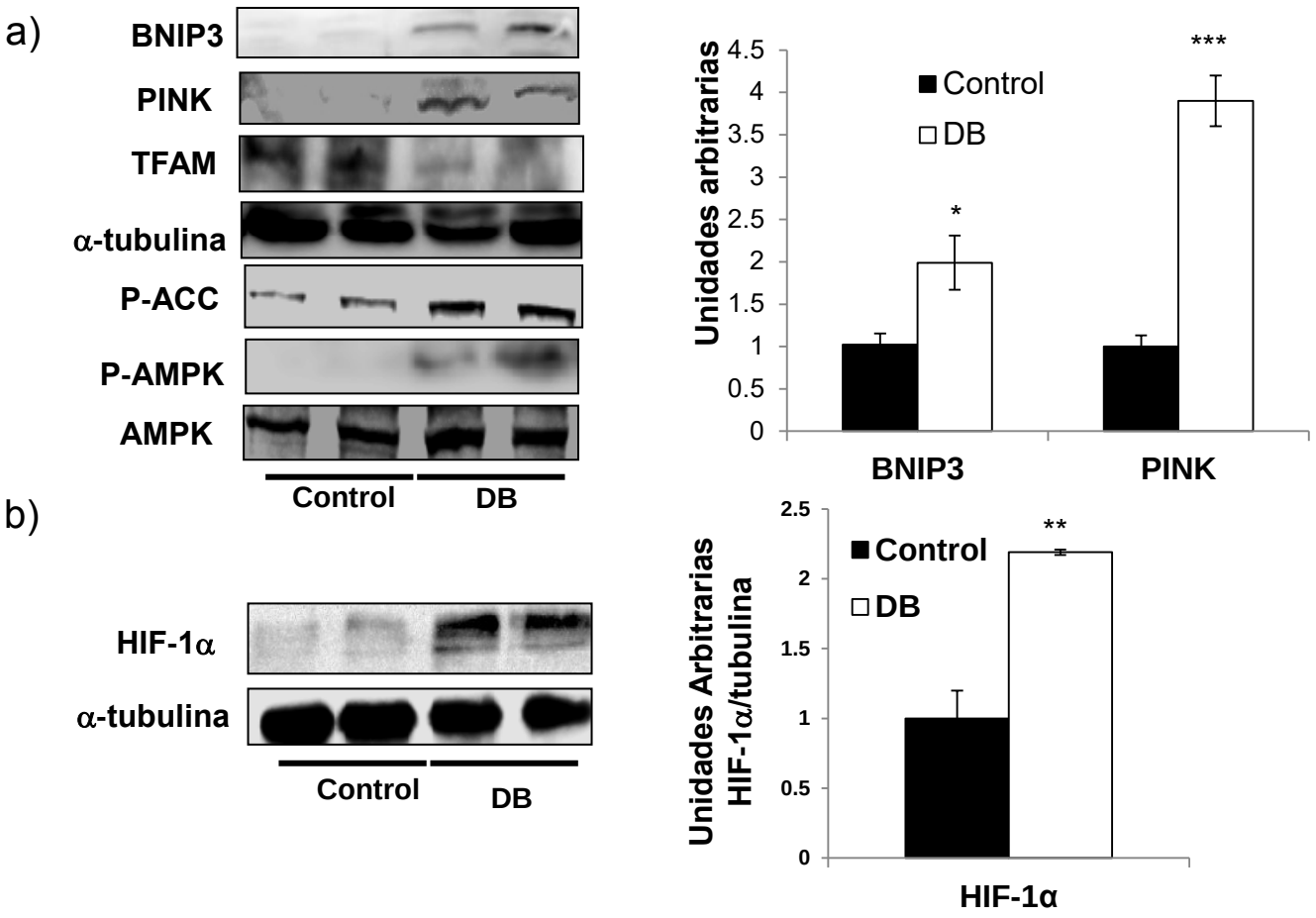

Figura 12. La deficiencia de biotina lleva a la activación de vías para la degradación de mitocondrias. a) Expresión de BNIP3, PINK, TFAM, p- AMPK y p-ACC en hígado de ratas control y deficientes de biotina (DB). b) Expresión de HIF-1α en hígados de ratas control y deficientes de biotina (DB). *p< 0.05, **p<0.001, ***p<0.0001 (Prueba t de Student).

7.4. La deficiencia de biotina tiene un efecto similar sobre la disminución de lamasa mitocondrial y la sobre expresión de proteínas de mitofagia en el cerebro.

Dada la importancia que tienen tanto la fosforilación oxidativa como los procesos de mitofagia en el cerebro (67), se decidió evaluar los efectos de la deficiencia de biotina en este órgano. Se observó que en el cerebro la deficiencia de biotina causa la disminución de la actividad de citrato sintasa hasta en un 50%. Conjuntamente disminuyó la expresión de TFAM al mismo tiempo que incremento BNIP3 y HIF-1α (Figura 13a y

13b). Cabe resaltar que se ha observado que tanto la deficiencia de biotina como la deficiencia genética de biotinidasa pueden causar alteraciones neurológicas (34,19, 68), lo cual es notable, ya que siempre se ha supuesto que el cerebro es un órgano privilegiado en que la biotinilación de las carboxilasas no se altera a pesar de que se induzca una deficiencia de biotina (69).

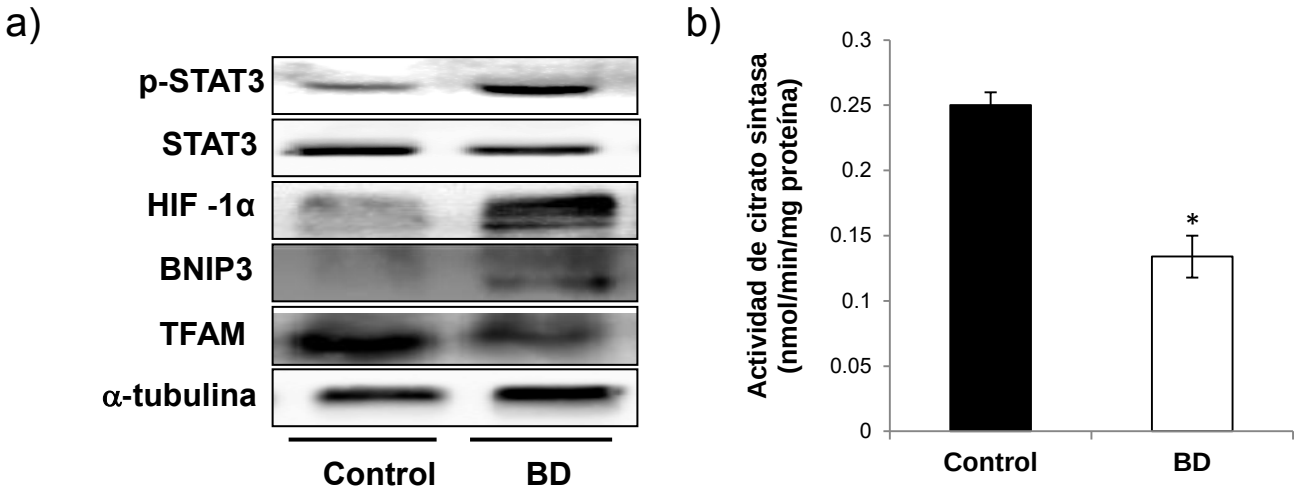

Figura 13. La deficiencia de biotina tiene efectos en el cerebro similares a los encontrados en hígado. a) Expresión de HIF-1α, BNIP3, PINK, TFAM, p- STAT3 en cerebros de ratas control y deficientes de biotina (DB). b) Actividad de citrato sintasa en cerebros de ratas control y deficientes de biotina (DB). *p<0.001 (Prueba t de Student).

Fue evidente entonces, que se requeriría una explicación que no incluyera por si misma a la función de la biotina como cofactor de las carboxilasas para que fuera capaz de explicar por qué existen alteraciones similares tanto en el hígado como en el cerebro. Con respecto a lo anterior, se ha visto que la deficiencia de biotina tiene efectos a nivel sistémico, ya que cuando se presenta dicha condición, se incrementa la concentración de diversas moléculas en sangre, como es el lactato y otros ácidos orgánicos (66). También, como se ha reportado por otros grupos de trabajo, se incrementan las

concentraciones séricas de citocinas como son el factor de necrosis tumoral α (TNFα), la interleucina 1β (IL-1β) y la interleucina 6 (IL-6) (50, 70-71). Estas proteínas son producidas por el sistema inmunológico o por órganos como el hígado y se sabe que tienen receptores en diferentes tipos celulares, como la glía del cerebro.

IL-6 es de interés particular puesto que se sabe que es un activador de STAT3 (Signal Transducer and Activator of Transcription 3) a través de su receptor de membrana JAK2 (Janus Kinase 2). Se ha observado que la activación de STAT3 tiene efectos en el metabolismo energético, incrementando el flujo por la glucolisis mientras que inhibe la función mitocondrial. Se ha propuesto que esto ocurre al incrementarse la expresión y estabilidad de HIF-1α (72 y 73). Por esta razón se evaluó la fosforilación de STAT3 en la serina 705. Se encontró más fosforilada a STAT3 en cerebros de ratas deficientes de biotina que en los controles (Figura 13a). Todo lo anterior, nos llevó a pensar que la activación de STAT3 podría ser la causa de la disminución de la masa mitocondrial en la deficiencia de biotina a través de desencadenar la mitofagia, por lo cual no propusimos a poner a prueba este planteamiento.

7.5. La activación de STAT3 precede a la disminución de la masa mitocondrial

Se trasladaron los experimentos a una línea celular de glioblastoma, este tipo de células han sido ampliamente usada para el estudio de las vías de señalización de interés para este trabajo (72 y 73). Para que el planteamiento de que STAT3 es la causa de la disminución de la masa mitocondrial sea viable, la activación de esta proteína tiene que preceder a las otras alteraciones ocasionadas por la deficiencia de biotina. Para estudiar esto, las células se sembraron en un medio que tenía 10% de SFB dializado.

Se tomaron muestras a los días 1, 2, 3, 4 y 5 después de iniciado el tratamiento con el suero libre de biotina con el fin de evaluar el curso temporal en el que aparecen los efectos de la deficiencia de biotina. La fosforilación de STAT3 se observó desde el día 2 de tratamiento, mientras que HIF-1α, BNIP3 y PINK se sobre-expresaron hasta el día 3 (Figura 14a).

Estos hallazgos tienen una correlación inversa con la expresión de TFAM y la actividad de citrato sintasa los cuales comenzaron a disminuir a partir del día 3. Con esto queda comprobado que el primer evento en la deficiencia de biotina es la activación de STAT3; Sin embargo, esto no demuestra una relación causa-efecto, por lo cual el siguiente paso fue hacer experimentos donde se evaluara el efecto de activar o inhibir a STAT3 sobre HIF-1α, BNIP3, PINK, TFAM y la actividad de citrato sintasa (Figura 14 a y b).

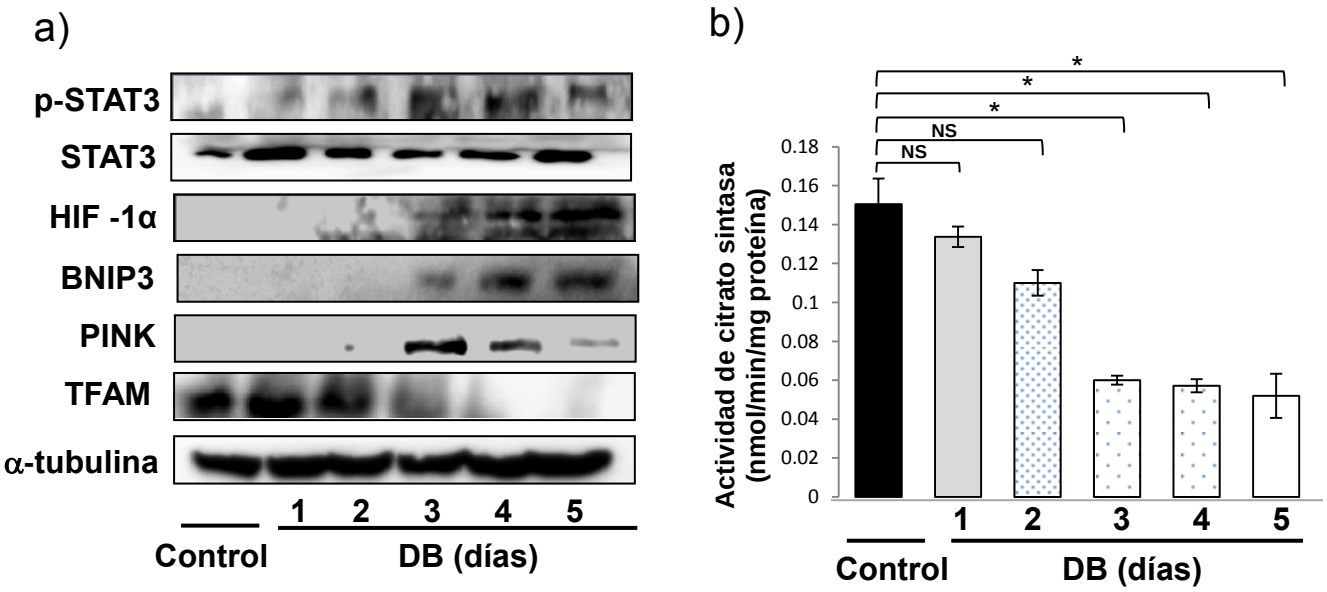

Figura 14. Evaluación del curso temporal de los efectos de la deficiencia de biotina. a) Expresión de HIF-1α, BNIP3, PINK, TFAM y p- STAT3 en cultivo de U373 con medio Control o cultivadas en medio con SFB dializado (DB) durante 1, 2, 3, 4, 5 días. b) Actividad de Citrato Sintasa en células cultivadas en las mismas condiciones. *p<0.001 (Prueba t de Student).

7.6. Efecto de la activación y la inhibición de STAT3 sobre las vías de mitofagia y la masa mitocondrial en la deficiencia de biotina

La activación de STAT3 se logró agregando IL-6 al medio de cultivo tanto en células U373 controles como deficientes de biotina durante 24 horas. Se puede observar en la figura 15a que agregar IL-6 al medio de cultivo fue suficiente para sobre-expresar HIF-1α, BNIP3 y PINK en las células controles, mientras que en las células deficientes de biotina IL-6 no tuvo mayor efecto.

Además, la adición de IL-6 en el control causó la disminución de los niveles de TFAM y de la actividad de citrato sintasa, mientras que en las deficientes de biotina estos dos parámetros ya estaban considerablemente disminuidos y la adición de IL-6 ya no tuvo un efecto adicional (Figura 15 a y b).

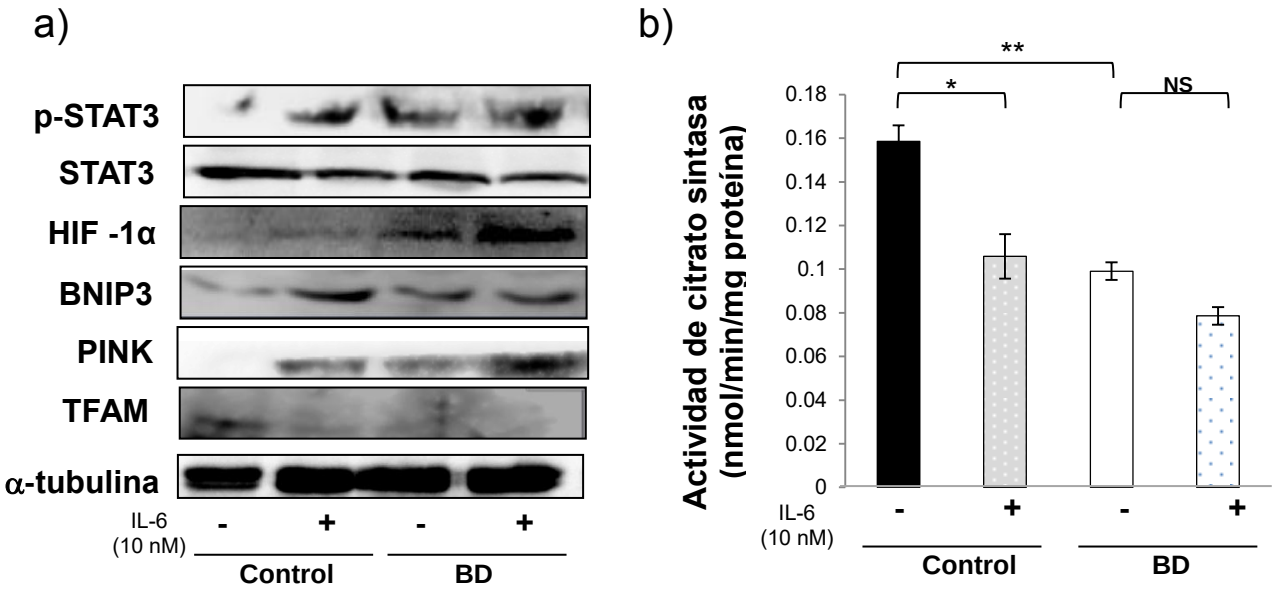

Figura 15. Efecto de la acivación de STAT3 con IL-6 sobre los marcadores de mitofagia y la masa mitocondrial. a) Expresión de HIF-1α, BNIP3, PINK, TFAM y p- STAT3 en cultivo de U373 con medio Control o cultivadas en medio con SFB dializado (DB). b) Actividad de Citrato Sintasa en células cultivadas en las mismas condiciones. *p<0.001 (Prueba t de Student).

Cuando se inactivó a STAT3 con el inhibidor III WP1066 se pudo observar que disminuyó la expresión tanto de HIF-1α como de BNIP3 y PINK en los controles, mientras que la actividad de citrato sintasa aumentó.

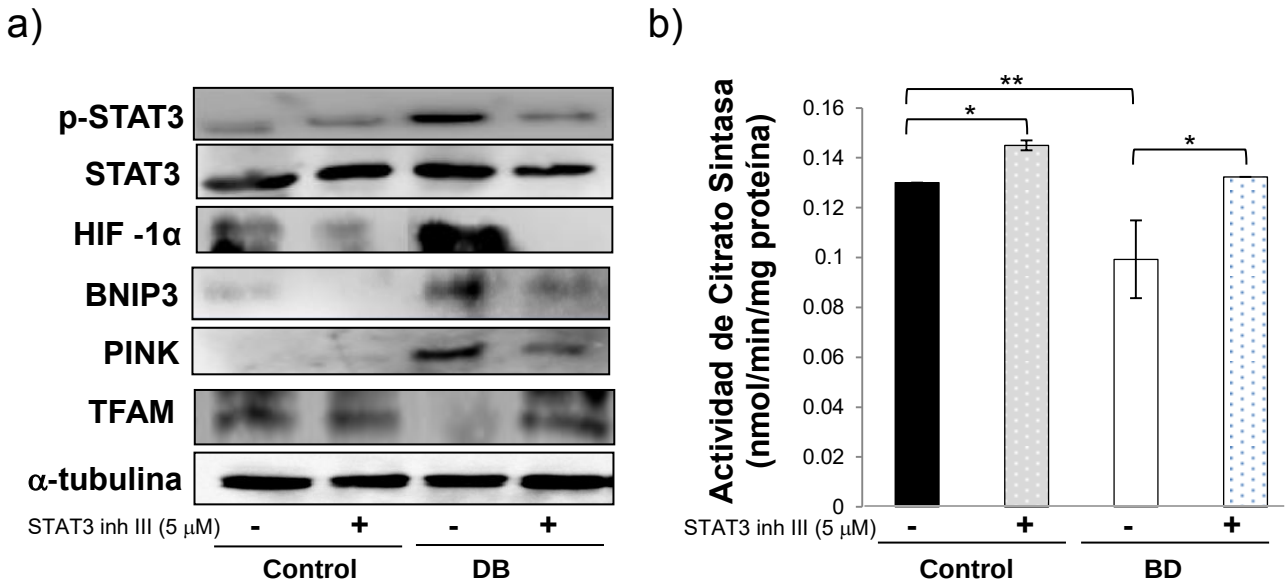

Figura 16. La inhibición de STAT3 revierte los efectos de la deficiencia de biotina sobre las vías de mitofagia y la masa mitocondrial. a) Expresión de HIF-1α, BNIP3, PINK, TFAM y p-STAT3 en cultivo de U373 con medio Control o cultivadas en medio con SFB dializado (DB) durante 5 días en la presencia o ausencia de 5 mM del Inhibidor de STAT3 WP1066. b) Actividad de Citrato Sintasa en células cultivadas en las mismas condiciones. *p<.05, **p<0.01 (Prueba t de Student).

En el caso de las células deficientes de biotina el inhibidor VII de STAT3 restauró la expresión de estas proteínas a un nivel muy similar al control. Así mismo, la adición del inhibidor logro regresar la actividad de citrato sintasa a los niveles del control (Figura 16 a y b).

7.7. El efecto de la activación de AMPK en la deficiencia de biotina sobre la masa mitocondrial

Como ya se ha aclarado, la deficiencia de biotina lleva a la activación de AMPK, la cual ha sido reportado que incrementa la biogénesis mitocondrial, por tanto, incrementa la masa mitocondrial (74).

Lo anterior parece contradictorio con los hallazgos descritos en este trabajo, por lo cual nos propusimos evaluar la contribución que tiene la activación de AMPK sobre la masa mitocondrial en la deficiencia de biotina. Se usó una línea celular de fibroblastos embrionarios de ratón Wild Type (MEF WT) y doble Knock Out de AMPK (MEF DKO). Se evaluó la actividad de citrato sintasa y la expresión de TFAM como indicadores de masa mitocondrial (FIG 17a y b) tanto en células cultivadas en medio control como en medio libre de biotina y se observó que comparadas con las células MEF WT control, la actividad de citrato sintasa disminuyó un 20% en las MEF DKO, es decir, con tan solo eliminar a AMPK se ve disminuida la actividad de citrato sintasa, un efecto similar se observó en TFAM.

En las MEF WT deficientes de biotina la actividad de citrato sintasa disminuyó un 40%, lo cual fue muy similar a lo encontrado en hígado y células de cerebro, en cambio la actividad de ésta enzima se redujo hasta en un 60% en las células MEF DKO. De igual forma, TFAM disminuyó aún más su expresión en las células MEF DKO. Además, se evaluó la expresión de HIF-1α, BNIP3 y PINK en estas mismas condiciones. La expresión de estas proteínas fue considerablemente mayor en las MEF DKO deficientes de biotina en comparación con las WT también deficientes, de hecho la eliminación de

AMPK fue suficiente para que estas proteínas se sobre expresaran en los controles. Estos resultados indican que en efecto, AMPK podría tener un papel al incrementar la masa mitocondrial; Sin embargo, su efecto no logra contrarrestar a las otras vías que activan a la mitofagia.

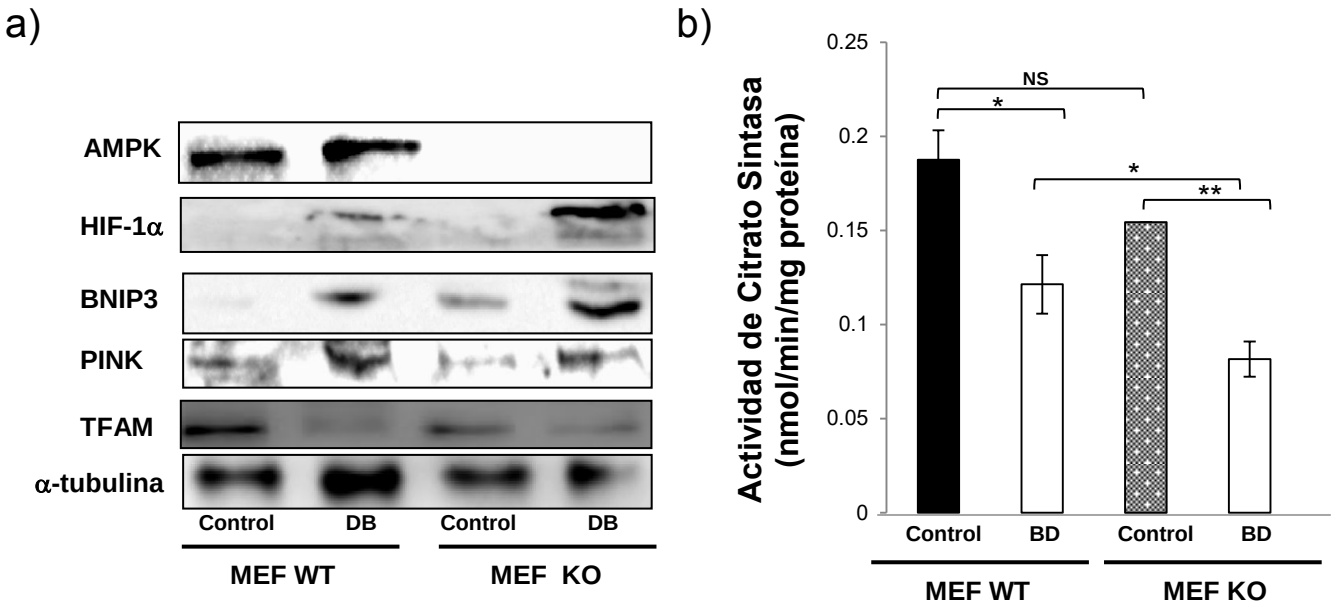

Figura 17. Los efectos de la deficiencia de biotina sobre las vías de mitofagia se acrecientan cuando AMPK es eliminado. a) Expresión de AMPK, HIF-1α, BNIP3, PINK y TFAM en Fibroblastos Embrionarios de Ratón (MEF) Wild Type (WT) y Knock Out (KO) con medio Control o cultivadas en medio con SFB dializado (DB) durante 7 días. b) Actividad de Citrato Sintasa en células cultivadas en las mismas condiciones. NS: No significativo, *p<.05, **p<0.01 (Prueba t de Student).

8. DISCUSIÓN

En este trabajo se estudió el efecto que tiene la deficiencia de biotina sobre la función y la masa mitocondrial y se observó que esta condición lleva a la disminución de la fosforilación oxidativa y la masa mitocondrial, los datos indican que esto se debe a la expresión de ciertas proteínas como HIF-1α, la cual es un activador de PINK y BNIP3 que causan la degradación especifica de las mitocondrias, proceso conocido como mitofagia. Se encontró que la activación de STAT3 aumenta la expresión de HIF-1α, PINK y BNIP3 y causa la disminución en la masa mitocondrial (Figura 18).

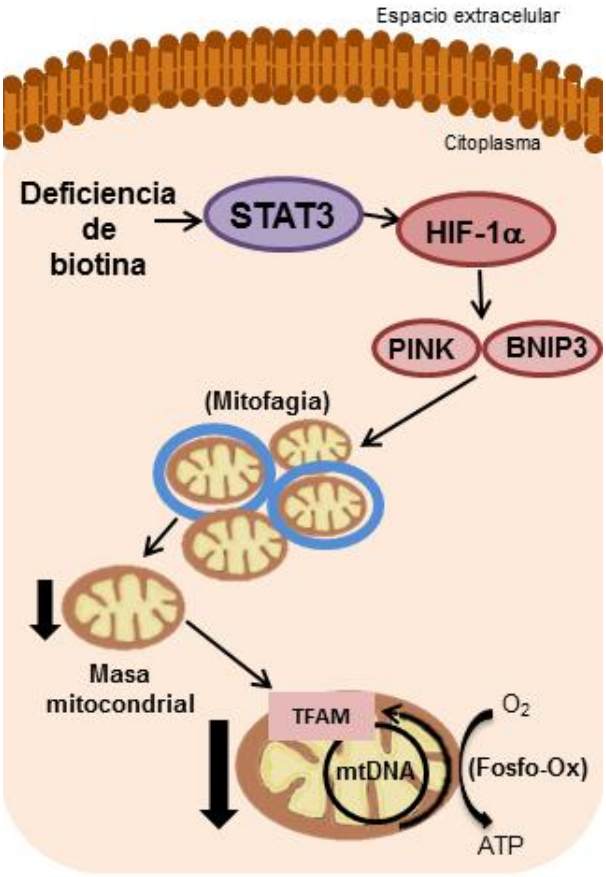

Figura 18. Mecanismo propuesto por el cual la deficiencia de biotina lleva a la disminución e la masa mitocondrial y la fosforilación oxidativa. En la deficiencia de biotina la proteína STAT3 esta mas activa, ésta aumenta la expresión de HIF-1α, la cual a su vez, activa a BNIP3 y PINK que participan en la degradación especifica de las mitocondrias (Mitofagia). Lo anterior causa la disminución en la masa mitocondrial y la fosforilación oxidativa.

Sin embargo, la razón por el cual se activa STAT3 no está clara. Se sabe que se incrementan las concentraciones séricas de ciertas citocinas como IL-6, los mecanismos involucrados no se conocen bien, se ha propuesto que la deficiencia de biotina lleva a cambios epigenéticos que tienen como consecuencia mayor actividad de NFκB, el cual es el factor de transcripción de citocinas como IL-6.

Por otro lado, en diversos trabajos se ha demostrado que en los organismos deficientes de biotina, se acumulan metabolitos tóxicos como son ciertos ácidos orgánicos, uno de los cuales es la propionil CoA, la cual tiene diversos efectos en el metabolismo oxidativo. En particular, inhibe al complejo de la piruvato deshidrogenasa, a la α-Cetoglutarato deshidrogenasa y al complejo mitocondrial III. De hecho en los casos clínicos de enfermedades congénitas como la deficiencia de biotinidasa, la administración de L- carnitina es usada como un coadyuvante del tratamiento, debido a que promueve la eliminación de éstos ácidos. En estudios in vitro se ha observado que la adición de carnitina puede revertir los efectos adversos que tiene la propionil CoA sobre la función mitocondrial. Sin embargo, nunca se ha hecho un estudio riguroso sobre sus efectos *in vivo* para comprobar si estas observaciones son reproducibles y si tienen relevancia fisiológica (75).

Basándose en las consideraciones anteriores, es posible que existan dos mecanismos distintos que tienen efectos negativos sobre la función mitocondrial en la deficiencia de biotina, pero también surge la posibilidad de que la activación de STAT3 y sus consecuencias sobre la masa mitocondrial este asociada a la acumulación de dichos metabolitos tóxicos.

Existen pocos o nulos estudios sobre la prevalencia de deficiencia de biotina, se considera que existen pocos casos, ya que rara vez se presentan las manifestaciones clínicas de la deficiencia de esta vitamina en la población. No obstante existe la posibilidad de que algunas personas padezcan de deficiencia de biotina marginal, es decir, el consumo de la vitamina no cubra sus requerimientos pero la deficiencia no se vuelve evidente a nivel clínico. Esta situación es de particular interés durante el embarazo, ya que la deficiencia de biotina es teratogénica, y se ha observado que alrededor del 50% de las mujeres embarazadas presentan deficiencia de biotina marginal (76). No existen estudios sobre la deficiencia de biotina marginal en otros grupos etarios.

Por esta razón, la relevancia de este trabajo no consiste en que la deficiencia de biotina sea un problema de salud pública. Sino que recae en la importancia que tienen las proteínas que se estudiaron con el metabolismo mitocondrial. En particular, se encontró nueva evidencia que apoya la interrelación entre proteínas STAT3, HIF-1α con la función y la masa mitocondrial. Dichos efectos son bastante similares a algunas situaciones patológicas, por lo que los resultados encontrados en la deficiencia de biotina podrían contribuir a su entendimiento.

Por un lado, se ha observado que la activación de STAT3 está relacionada con las primeras etapas del cáncer y es en parte responsable de las anormalidades metabólicas de las células cancerosas. A HIF-1α por su parte, se le atribuye el efecto Warburg, en el cual se incrementa la utilización de glucosa y se inhibe la fosforilación oxidativa (77).

Por otro lado, se sabe que la disfunción mitocondrial está fuertemente ligada a enfermedades neurodegenerativas como son el Parkinson, Alzheimer entre otras. Se ha observado que cuando se hace un Knockdown de HIF-1α en modelos animales, se pueden revertir las alteraciones mitocondriales y las anormalidades neuronales (78).

Las perspectivas de este trabajo van dirigidas a: 1) Entender mejor las causas de la activación de STAT3 en deficiencia de biotina. 2) Estudiar que zonas específicas del cerebro están siendo afectadas por la deficiencia de biotina y correlacionar esto con signos neurológicos que se presentan en esta condición. 3) En el cerebro no hay cambios en la biotinilación de las carboxilasas, por lo cual siempre ha sido aceptado que es un órgano protegido, y a pesar de esto, en este trabajo se observaron alteraciones importantes en este órgano. Debido a esto se propone estudiar si la biotina regula a un nivel epigenético la expresión de STAT3, de manera independiente a su función como cofactor de carboxilasas.

9. CONCLUSIÓN

La deficiencia de biotina lleva a la disminución de la función y de la masa mitocondrial al menos en tres tejidos distintos hígado, músculo y cerebro. Este efecto fue debido al menos en parte a la activación de dos proteínas de mitofagia: BNIP3 y PINK. En este trabajo se demostró que la activación de STAT3 lleva a la activación de estas proteínas presuntamente a través de incrementar la expresión de HIF-1α.

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APENDICE 1

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Biotin deprivation impairs mitochondrial structure and function and has implications for inherited metabolic disorders☆

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abstract

Certain inborn errors of metabolism result from deficiencies in biotin containing enzymes. These disorders are mimicked by dietary absence or insufficiency of biotin, ATP deficit being a major effect, whose responsible mechanisms have not been thoroughly studied. Here we show that in rats and cultured cells it is the result of reduced TCA cycle flow, partly due to deficient anaplerotic biotin-dependent pyruvate carboxylase. This is accompanied by diminished flow through the electron transport chain, augmented by deficient cytochrome c oxidase (complex IV) activity with decreased cytochromes and reduced oxidative phosphorylation. There was also severe mitochondrial damage accompanied by decrease of mitochondria, associated with toxic levels of propionyl CoA as shown by carnitine supplementation studies, which explains the apparently paradoxical mitochondrial diminution in the face of the energy sensor AMPK activation, known to induce mitochondria biogenesis. This idea was supported by experiments on AMPK knockout mouse embryonic fibroblasts (MEFs). The multifactorial ATP deficit also provides a plausible basis for the cardiomyopathy in patients with propionic acidemia, and other diseases. Additionally, systemic inflammation concomitant to the toxic state might explain our findings of enhanced IL-6, STAT3 and HIF-1α, associated with an increase of mitophagic BNIP3 and PINK proteins, which may further increase mitophagy. Together our results imply core mechanisms of energy deficit in several inherited metabolic disorders.

1. Introduction

To understand better the metabolic and genomic functions of indispensable nutrients that participate as cofactors of intermediary metabolism and their repercussions for disease, we have used biotin deprivation (BtDEF) as an experimental model [1]. Biotin (Bt) is the prosthetic group of carboxylases (named according to their substrates: pyruvate -PC, propionyl CoA - PCC, 3-methyl crotonyl CoA - 3MCC and acetyl CoA - ACC) [2], whose activities are severely reduced in this vitamin deficiency. There is also a genetic syndrome, Multiple Carboxylase Deficiency [2], in which there is a combined deficiency of the four carboxylases due to deficient activities of either holocarboxylase synthetase or biotinidase. In particular, PC is a very important anaplerotic enzyme [3–6]. PCC also has an anaplerotic role and catalyzes a crucial step in the catabolism of isoleucine, valine, threonine, odd-chain fatty acids and cholesterol; its genetic deficiency causes propionic acidemia, a severe inherited metabolic disorder [7] that can cause cardiomyopathy [8,9]. Although the mechanism involves mitochondrial dysfunction [10], the detailed metabolic basis has not been well elucidated.

Biotin dietary requirements are not accurately known, but a normal diet may not satisfy the requirement for some individuals in particular circumstances [2,11]. Nutritional BtDEF is found in pregnancy [12], protein-energy undernutrition [13–15] and in rare cases of high avidin consumption as in raw egg diets [11]. Part of the required Bt is provided endogenously by hydrolysis of biotin-lysine (biocytin) and biotin-bound peptides catalyzed by the enzyme biotinidase (BTD) during carboxylases degradation, thus allowing the released Bt to be re-utilized in the biotin cycle [2]. Children with genetic deficiency of BTD become

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severely ill with many of their symptoms resembling those of dietary BtDEF [16]. Wolf et al. produced a BTD-KO mouse [17], in which we found metabolic and genomic changes similar to those found in rat BtDEF [18], (see below).

We have previously reported that in livers of biotin deprived rats there are changed levels of mRNAs of several genes of carbon metabolism and an ATP deficit, increased AMP/ATP ratio and increased activity of AMP kinase (AMPK) [1,19]. ATP generation is a complex phenomenon to which several factors contribute [20], such as TCA cycle anaplerosis, the electron transfer chain (ETC) enzymes, and functional mitochondria. AMP kinase (AMPK) is the main energy sensor in eukaryotes [21]. AMPK is activated by decreased ATP; AMPK activation promotes ATP generating processes, including augmentation of mitochondria [22], and diminishes ATP consumption. Among its effects there is an augmentation of mitochondria. The mechanisms underlying these findings are not completely understood. This article addresses these queries.

Here we show that in BtDEF all the above-mentioned factors for ATP generation converge, but, in addition and unexpectedly, there is also a severe decrease of mitochondria, in spite of the AMPK activation that would be expected to increase their number. We observed severe mitochondria damage, likely due to accumulation of toxic acyl CoA compounds caused by blocks at steps catalyzed by biotin-dependent carboxylases, particularly propionyl CoA carboxylase [2]. In addition, the harmed mitochondria and concomitant ATP deficit are the likely basis of cardiomyopathy in propionic and methylmalonic acidemias [8,9]. Additionally, we have also found changes that suggest a signaling pathway that increases mitophagy when there is a toxic state and inflammation.

2. Results

2.1. Impaired TCA cycle anaplerosis

In isolated hepatocytes from control and BtDEF rats, anaplerosis was evaluated by assessing pyruvate and TCA metabolism by quantitating changes in metabolite abundance relative to total protein and by quantitating ^{13}C enrichment from pyruvate-3-^{13}C and from lactate-3-^{13}C into several key metabolites. As depicted in Fig. 1A, pyruvate abundance decreased in the deficient cells relative to controls; lactate and alanine abundance increased. Abundance of the TCA cycle metabolites citrate and, to a lesser extent, α-ketoglutarate was decreased; there was a moderate increase of glutamate and glutamine. On the other hand, the branched-chain amino acids leucine, isoleucine, and valine increased in the deficient hepatocytes relative to controls.

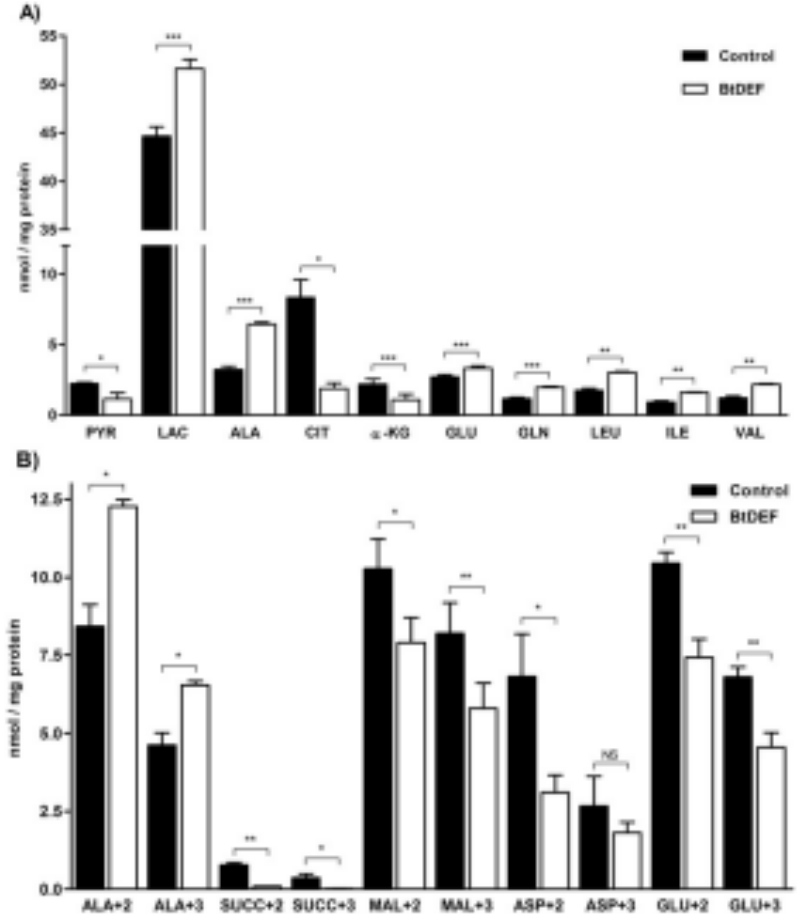

Fig. 1. Reduced flow through the TCA cycle is related to deficient pyruvate carboxylase-driven anaplerosis, as shown by ^{13}C carbon enrichment experiments. (A) Selected metabolite concentrations (nmoles/mg protein) in acid extracts from freshly isolated hepatocytes after 10 min incubation in Ringer–Krebs-phosphate (RKP) buffer containing 0.3 mM sodium pyruvate-3-^{13}C and 3 mM sodium L-lactate-3-^{13}C. Pyr: pyruvate, lac: lactate, ala: alanine, cit: citrate, α-kg: α-ketoglutarate, glu: glutamate, gln: glutamine, leu: leucine, ile: isoleucine, val: valine. (B) Degree of isotopic enrichment in the same acid extracts in +1, and +2 species in intermediary metabolites estimating the flow through the pyruvate metabolism and the TCA cycle pathways. Ala: alanine, succ: succinate, mal: malate, asp: aspartate, glu: glutamate. Corrected atoms per excess (APE) was determined in control (white bar) and BtDEF hepatocytes incubated as in (A) but for 30 min. n = 4 independent experiments. The error bars represent SEM. * $P \le 0.05$, ** $P \le 0.01$, *** $P \le 0.001$, Student's t test.

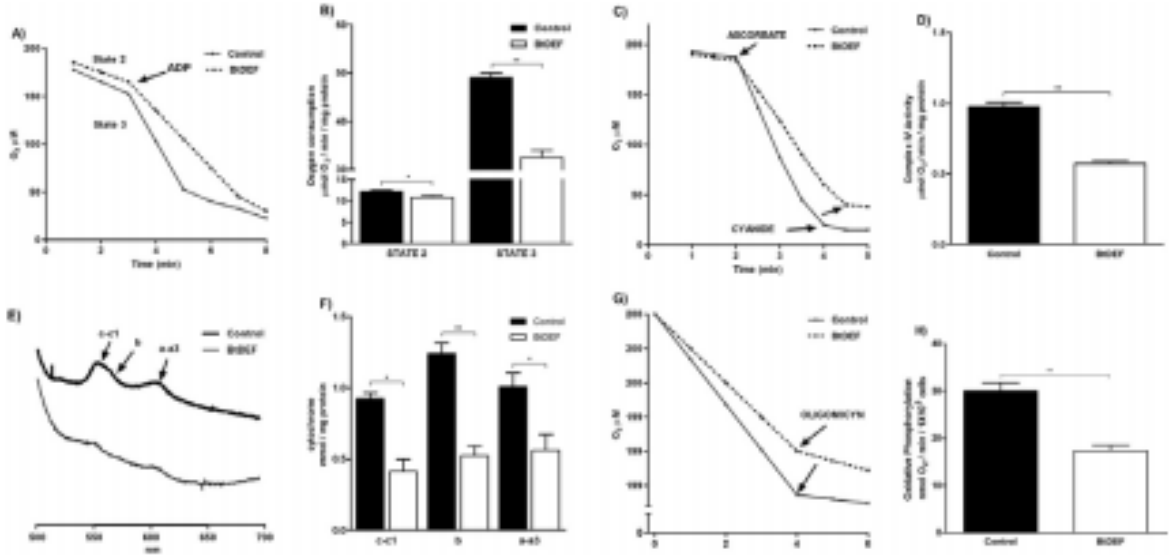

Fig. 2. Respiration and oxidative phosphorylation after biotin depletion. (A) Oxygen consumption of isolated mitochondria (0.5 mg protein/mL) from control and BIDEF livers. 10 mM Glutamate and 10 mM malate were used as substrates and respiration was registered by a Clarke electrode before and after adding 0.80 μM ADP (states 3 and 4, respectively). (B) Quantification of both state 3 and 4 respiratory rates expressed by the arrows under the curves. (C) Representative traces of complex IV activity of isolated mitochondria from BIDEF and control livers. Mitochondria (0.5 mg protein/mL) were incubated with 10 μM antimycin A and 10 μM TMPD. Oxygen uptake was initiated by the addition of 5 mM ascorbate, and the experiment was stopped by the addition of 1 mM cyanide. (D) Quantification of complex IV activity expressed by the arrows under the curves. (E) Comparison of control and BIDEF cytochromes c + c1, b and a + a3 identified by their characteristic peaks at 548–550, 562–570 and 603–630 nm (indicated by arrows) using differential scanning spectrometry of their oxidized and reduced forms. The total cytochrome content in mitochondria was 1 mg protein, isolated from rats fed with either a normal (control) or biotin-deficient (BIDEF) diets. Bar indicates 0.05 absorbance units. (F) Quantification of cytochrome content as obtained using the respective maximum absorbance values and the appropriate molar extinction coefficients. (G) Oxidative phosphorylation studied by oxygen consumption of both control and BIDEF isolated rat hepatocytes after the addition of 10 μM oligomycin. (H) Quantification of oxidative phosphorylation rate calculated as the difference between the respiratory rates before and after oligomycin additions as in 3 independent experiments. The error bars represent SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 (Student's t test).

To further characterize pyruvate metabolism in BtDEF, control and deficient primary hepatocyte cultures were incubated with pyruvate-3-^{13}C and lactate-3-^{13}C, and their conversion into other metabolites was followed by their ^{13}C enrichment. The excess percentage of ^{13}C atoms after 10 min incubation was substantially reduced in succinate, malate, glutamate and aspartate in the deficient cells (Fig. 1B), indicating reduced anaplerosis and a reduced TCA cycle flow. As expected there was an increased enrichment of ^{13}C in alanine.

2.2. Impaired oxidative phosphorylation (OXPHOS) in BtDEF

Decreased flow through the TCA cycle diminishes the electron flow through the mitochondrial respiratory chain [23], with consequent reduction of oxidative phosphorylation (OXPHOS). We evaluated this relationship in livers of control and BtDEF rats. Oxygen consumption rate was measured in isolated liver mitochondria by means of a Clarke electrode. As shown in Fig. 2A and B, respiratory states 2 (before the addition of ADP) and 3 (after the addition of ADP; phosphorylation state) were significantly reduced in BtDEF. There was a decreased oxygen consumption of about 30% in state 3 in BtDEF indicating a substantial impairment of the respiratory chain flow.

Because cytochrome c oxidase activity (complex IV) has an important role in the regulation of the flux through the respiratory chain, we assessed cytochrome c oxidase activity by measuring the mitochondrial oxygen consumption after providing ascorbate as substrate and then by looking at the effect of cyanide (Fig. 2C). Cytochrome c oxidase activity was 40% lower in hepatic biotin deficient mitochondria relative to controls (Fig. 2D), a result likely due to the decreased cytochrome content in BtDEF that we observed (Fig. 2E and F).

Finally, we explored oxidative phosphorylation using primary cultured hepatocytes. The rate of oxygen consumption before and after the addition of oligomycin (a complex V specific inhibitor), as an indicator of ATP synthesis, was reduced by approximately 35% (Fig. 2G and H).

2.3. Diminished mitochondrial mass

We additionally found a reduction of mitochondria mass, evaluated by decreased amount of the mitochondrial transcription factor TFAM [24], diminished activity of the constitutive mitochondrial enzyme citrate synthase [25] and reduced membrane potential shown by diminished staining of mitochondria with mitotracker [26] (Fig. 3A–D, respectively). We supposed that these results are caused by accumulation of acyl CoA compounds, mainly propionyl CoA. [27,28], caused by decreased activity of biotin-dependent PCC [29]. We studied these organelles by transmission electron microscopy of control and BtDEF rat gluteal muscle. The muscle of the control rats showed well-preserved structures, with mitochondria arranged in clusters, and myofibrils with normal architecture. At 4 weeks of treatment, mitochondria were observed with broken internal membranes, denoting edema. At 6 weeks of biotin depletion, preserved myofibrils were observed; however a reduction in size and altered ultrastructure of mitochondria were present. Finally, at 10 weeks of biotin depletion, damaged myofibrils were observed, along with a severe decrease in mitochondria population (Fig. 4).

To have a better estimate of the accumulation of acyl CoA compounds that are difficult to measure directly because of their inherent instability, we administered large amounts of carnitine to rats at the same time that we made them BtDEF. Since carnitine is exchanged with coenzyme A from acyl CoA compounds, producing acyl carnitines [30], we reasoned that if there is an important accumulation of propionyl and other acyl CoA compounds, exceeding the carnitine body pool, administration of a large supplement would further raise the acyl carnitine amount, which is known to be already elevated in BtDEF. As shown in Fig. 5A, propionyl carnitine was indeed elevated over controls in BtDEF rat blood samples and was further increased in animals to which a large amount of carnitine was administered, indicating that they had elevated levels of the acyl CoA compound that was then conjugated with carnitine. Correspondingly, blood free carnitine levels were lower in BtDEF compared to controls (Fig. 5B), likely due to its use for conjugation of the increased acyl compounds, and increased to control levels when there was carnitine supplementation. Recent findings in BtDEF HepG2 cultured cells (originated from a human hepatoma) strengthen our results [31].

These findings may seem paradoxical because it is well known that activation of the energy sensor AMPK promotes mitochondria biogenesis [22]. However the mitochondrial damage likely augments mitophagy at a higher proportion than the rate of increase in mitochondrial mass induced by AMPK activation. Thus the paradox is resolved. Furthermore, in BtDEF the amount of mitochondria, as estimated by Tfam and by citrate synthase (CS) activity, was more reduced in mouse embryonic fibroblasts (MEFs) whose genes for the AMPK catalytic α1 and α2 subunits had been eliminated (KO), than in wild type MEFs with their AMPK genes intact (Fig. 6A and B), supporting the idea that although the activation of AMPK may increase the mitochondria mass, this effect is overwhelmed by their increased destruction in BtDEF.

2.4. Mechanistic studies on mitophagy

We considered the development of systemic inflammation as a result of the toxic state in BtDEF [32]. In inflammation, there is an increase of NF-kβ, which has previously been reported in this vitamin deficiency [33] and that enhances interleukin transcription, such as interleukin 6 (IL-6). IL-6 binds to its JAK receptor, and phosphorylates and activates STAT3, presumably promoting its nuclear translocation. This is a transcription factor for HIF-1α [34], which may induce mitophagy via activation of proteins like BNIP3 and PINK [35].

To probe this mechanism, we followed the temporal course in glioblastoma cells, of changes of p-STAT3 and HIF-1α, proteins that are part of the proposed pathway that transduces a toxic/inflammatory signal to the mitophagic PINK and BNIP3 proteins, promoting mitophagy. As can be seen in Fig. 7, STAT3 changed first, then HIF-1α, and PINK and BNIP changed last, supporting the proposed pathway.

STAT3 Inhibitor III was then added to the BtDEF glioblastoma cultures. The mitochondrial markers CS and TFAM were increased, pointing towards augmented mitochondria mass, with concomitant reduction of p-STAT3, HIF-1α, BNIP3 and PINK (Fig. 8).

Finally we tested the hypothesis by adding IL-6, as a physiological activator of STAT3, to control (biotin-containing) and BtDEF glioblastoma cells. There was an increase of HIF-1α, BNIP3 and PINK, with reduction of TFAM levels and CS activity, and these changes were further augmented in BtDEF cultures (Fig. 9 left). These changes were reversed when the STAT3 inhibitor was added (Fig. 9 right). These results provide strong mechanistic support for the pathway we postulate to be involved in triggering aspects of the mitochondrial response to BtDEF.

3. Discussion

We studied different mechanisms that impinge on the generation of ATP in starvation for biotin (BtDEF), a vitamin that is the cofactor of carboxylases, enzymes that participate in the metabolism of carbohydrates, lipids and amino acids [2]. Previously we discovered a severe energy shortage in such a condition [19]. In this paper we limited these investigations to aspects of mitochondria structure and function. We found that part of this shortage is caused by reduced anaplerosis due to deficient activity of biotin-dependent pyruvate carboxylase [3,5], and the associated flux reduction through the TCA cycle, with concomitant decreased mitochondria respiration (Figs. 1 and 2A). These findings were expected from the known anaplerotic function of biotin-dependent pyruvate carboxylase [6]. Additionally, we found diminished activity of cytochrome c oxidase (respiratory chain complex IV) (Figs. 2C and 2D) associated with diminished cytochromes

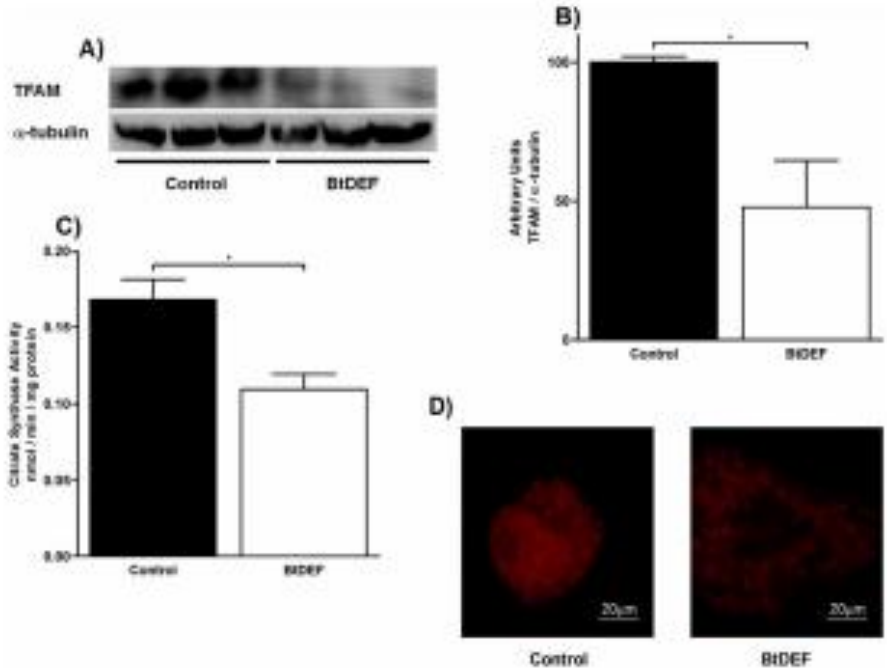

Fig. 1. Assessment of mitochondrial content in normal and biotin-deficient rat livers. (A and B) Mitochondrial transcription factor TFAM expression was assessed by Western blots and quantified by densitometry. (C) Citrate-synthase activity was determined in cultured hepatocytes isolated from the livers of rats fed with a normal (control) or a biotin-deficient diet. (D) Mitochondrial membrane potential shown by staining with mitotracker red CMXRos staining of control and BtDEF rat hepatocytes. n = 3 independent experiments. Results of citrate synthase activity are expressed as mean values ± SEM. * $P \leq 0.05$, (Student's *t* test).

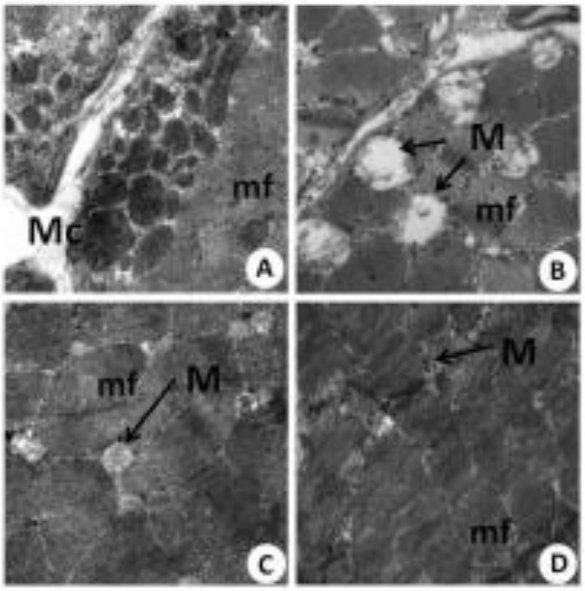

Fig. 4. Reduction of muscle mitochondria mass in BtDEF. The ultrastructure of gluteal-muscle mitochondria of control and rats fed with BtDEF diet during 2, 4 and 6 weeks was analyzed by electron microscopy. (A) Control muscle showing cluster of mitochondria (Mc) and myofibrils (mf) with preserved ultrastructure appearance. (B) Muscle of 4 week treated group where mitochondria (M) with edema was observed (arrows). (C) In the group treated for six weeks, mitochondria (M, arrow) with reduced size were found. (D) At 10 weeks of BtDEF, reduced population of mitochondria (M) and damaged myofibrils (mf) were observed. (Uranyl acetate/lead citrate staining, 30,000×).

(Figs. 2E and 2F), whose functional group is heme, the prosthetic group of cytochromes, one of whose precursors is succinyl CoA, an intermediate of the TCA cycle. Heme and concomitant complex IV shortages have been reported to be deficient in biotin deprivation [36]. All these findings explain the reduced oxidative phosphorylation in BtDEF, as shown by a more pronounced decreased oxygen consumption by biotin-deprived, oligomycin-treated mitochondria than in the controls (Figs. 2G and 2F).

Furthermore, there was a severe damage and reduction in the amount of mitochondria (Fig. 3) in accordance with a previous preliminary report [37]. This finding is explained by an accumulation of compounds toxic for mitochondria like propionyl CoA, derived from the catabolism of branched chain amino acids, methionine, threonine, odd-chain fatty acids and cholesterol, and due to carboxylases like PCC in BtDEF. These toxic acyl CoA compounds are known to damage mitochondria in the inherited metabolic disorders propionic and methylmalonic acidemias [7].

The inferred accumulation of toxic propionyl CoA was evidenced by a further increase of propionyl carnitine, over an already elevated level in blood from BtDEF rats, when they were given extra carnitine (Fig. 6), indicating that they had elevated levels of acyl CoA compounds that were then conjugated with carnitine. Carnitine is exchanged with coenzyme A from acyl CoA compounds in the mitochondria matrix, producing acyl carnitines that are not toxic and are readily excreted in the urine or at the culture media [30]. Indeed, carnitine is a very useful therapeutic adjuvant in propionic and methylmalonic acidemias, severe inborn errors of metabolism in which propionyl CoA readily accumulates [7]. Dysfunctional mitochondria with impairment of oxidative phosphorylation in PA have previously been reported [10]. Therefore the ATP shortage in BtDEF is of multifactorial nature, particularly with increased mitochondria damage. It is also very probably the basis of the cardiomyopathy that has been observed in several cases of propionic acidemia (PA) [8,9].

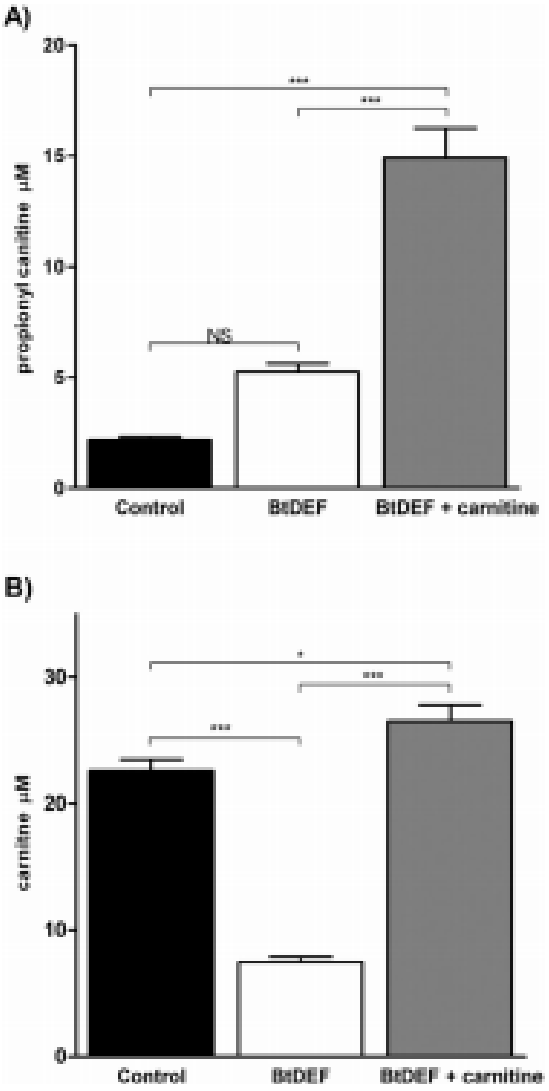

Fig. 5. Propionyl CoA accumulates to a large degree in BtDEF rats as revealed by L-carnitine supplementation studies, in which L-carnitine 200 mg/kg was injected intraperitoneally daily in 4 weeks. (A) Blood free carnitine was lower in BtDEF and increased to control levels with L-carnitine supplementation. (B) Blood levels of propionyl carnitine determined by tandem mass spectrometry were increased in BtDEF compared to control rats and were further elevated when they received an L-carnitine supplement. n = 3 independent experiments. Results are expressed as mean values ± SEM. *P b 0.05, ***P b 0.001 (ANOVA Bonferroni's multiple comparison test).

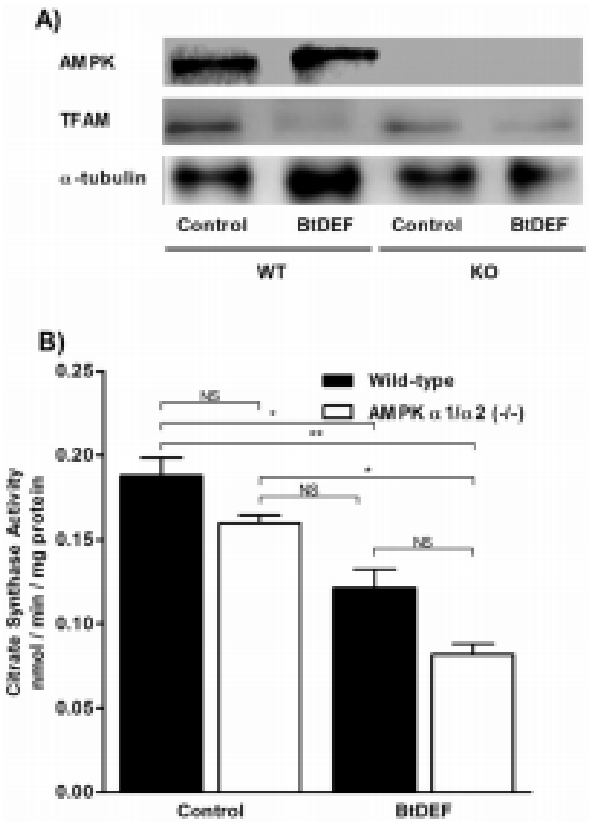

Fig. 6. Mitochondria mass is further decreased in double knockout (dKO) mouse embryonic fibroblasts (MEFs) for the catalytic α1 and 2 subunits of AMPK. (A) Activity (degree of phosphorylation at α1 subunit Thr172) AMPK and amount of TFAM assessed by Western blots in control and BtDEF wild type (WT) and dKO MEFs. (B) Citrate synthase activity. n = 3 independent experiments. Results of citrate synthase activity are expressed as mean values ± SEM. *P b 0.05, **P b 0.01, ***P b 0.001 (ANOVA Bonferroni's multiple comparison test).

In turn, the extensive mitochondria damage explains the apparent contradiction between our observed reduction of mitochondrial amount and a predicted increased mitochondria biogenesis [38] in the face of activation of the eukaryotic energy sensor AMP kinase (AMPK) due to cellular energy reduction in BtDEF. The increase of these organelles due to AMPK activation is very likely thwarted by a higher rate of destruction by mitophagy. Further evidence comes from reduced activity of the mitochondria marker citrate synthase (i.e., mitochondria amount) in BtDEF wild type (WT) mouse embryonic fibroblasts (MEFs), that was further decreased in double knockout (dKO) MEFs for the catalytic α1 and α2 subunits of AMPK (Fig. 5).

Additionally, we propose that in conditions of toxicity and inflammation as in BtDEF, mitophagy is further driven by a new signal transduction pathway that we are herewith proposing, active in inflammatory circumstances like the accumulation of toxic acyl CoA compounds in conditions such as BtDEF, involving increased NF-kβ, IL-6 and STAT3, HIF-1α and eventually mitophagosome proteins BNIP3 and PINK. Very recently, it has been reported that the ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy [39]. Supporting this hypothesis is the sequence of temporal changes of these proteins after BtDEF (Fig. 7), the decrease in the signaling proteins and augmentation of mitochondria (as assessed by Tfam and citrate synthase) by an STAT3 inhibitor (Fig. 8), and the exacerbation of the BtDEF effects after IL-6 addition to cell cultures (Fig. 9).

There are other medical implications to these findings, particularly the toxic damage of mitochondria as an important causal factor of respiratory energy shortage in the inherited metabolic disorders PA, MMA and other organic acidemias like Leigh disease, maple syrup urine disease, glutaric acidemia, some of the mitochondrial fatty acid disorders, among others [40], and possibly some non-Mendelian neurodegenerative diseases. With respect to nutritional biotin deprivation, we previously showed that nearly a third of Mexican children with protein-energy undernutrition are biotin deficient [13,14] with important metabolic outcomes [15]. Furthermore, marginal BtDEF has been reported in about a third of Caucasian middle class American pregnant women [12], which may have consequences in their children, such as diabetes and metabolic syndrome, when they become adults [41,42].

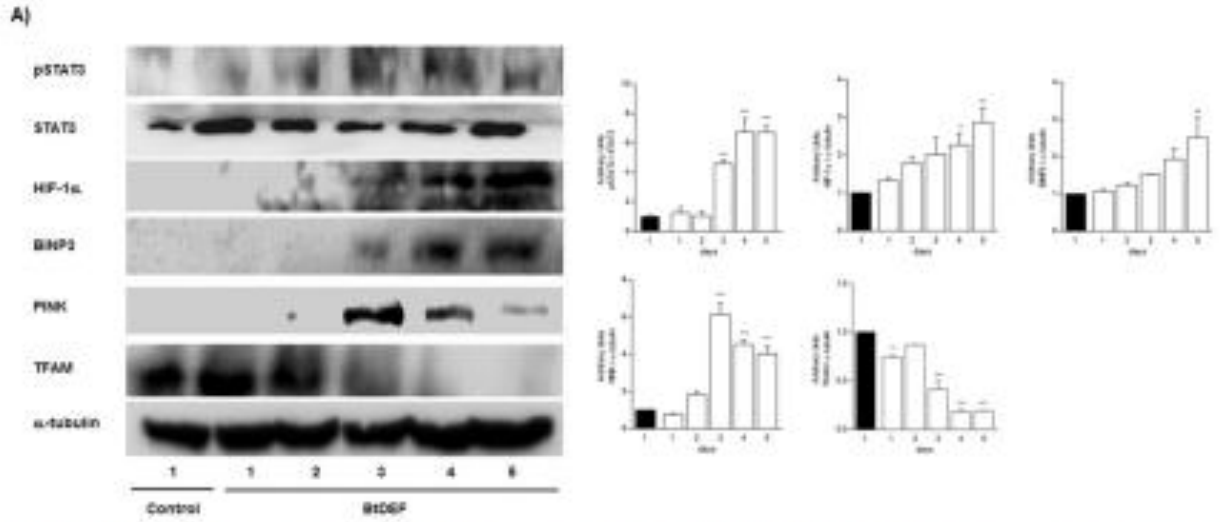

Fig. 7. Temporal course of changes in BtDEF glioblastoma cells, of proteins that may transduce a toxic/inflammatory signal to the mitophagic PINK and BNIP3 proteins. A) Western blot analysis of STAT3, HIF-1α, of the mitophagic proteins PINK and BNIP3 and the mitochondrial marker TFAM, and B) citrate synthase activity, in consecutive days after BtDEF was started. Western blot bands were quantified by densitometry. Tubulin is used as a load control. n = 3 independent experiments. Results of citrate synthase activity are expressed as mean values ± SEM. *P b 0.05, **P b 0.01, ***P b 0.001 (ANOVA Dunnett's multiple comparison test).

In conclusion, the findings reported in this article establish that the deficiency of biotin, a vitamin essential for energy metabolism and for catabolism of several amino acids and fatty acids, results in multifactorial derangements of ATP generation, with outstanding mitochondrial damage. It is likely that scarcity of similar nutrients, for example thiamine, lead to analogous disturbances. They represent phenocopies of several inherited metabolic diseases and of some energy defective neurologic and cardiac illnesses, and thus their thorough understanding will probably help to their better diagnosis and treatment.

4. Materials and methods

4.1. Biotin deficiency in rats

Biotin deficiency was induced in rats as previously reported [43]. Briefly, Wistar male rats (aged 21 days and 80–90 g of weight) were fed a commercial diet (TD. 81,079 Harlan Teklad. Madison, WI) supplemented with 30% egg white as source of avidin. Control rats were males of similar age and weight, and fed with the same commercial diet, supplemented with 4 mg/kg biotin (TD. 97,126 Harlan Teklad. Madison, WI). Rats were housed individually in air-filtered cages, and were exposed to 12 h light/dark cycles with free access to food and water. After 4 weeks of biotin deprivation, they and their controls were sacrificed by cervical dislocation and their livers were immediately separated and immersed in liquid nitrogen, and were stored at −70 °C for later analysis [44]. Biotin deficiency was proved by streptavidin Western blots, as has been reported in some of our previous articles [1,44].

4.2. Hepatocyte isolation

Male Wistar rats weighing 150–180 g were fed control and biotin-deficiency diet for 4 weeks, respectively. The animals were anesthetized with ketamine/xylazine 50 mg/kg. The liver was dissected and hepatocytes were isolated using the method of Berry and Friend [45]. The cells were suspended in Ringer–Krebs-phosphate: 5.7 mM KCl, 120 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 1.2 mM $CaCl_2$, pH 7.4. The

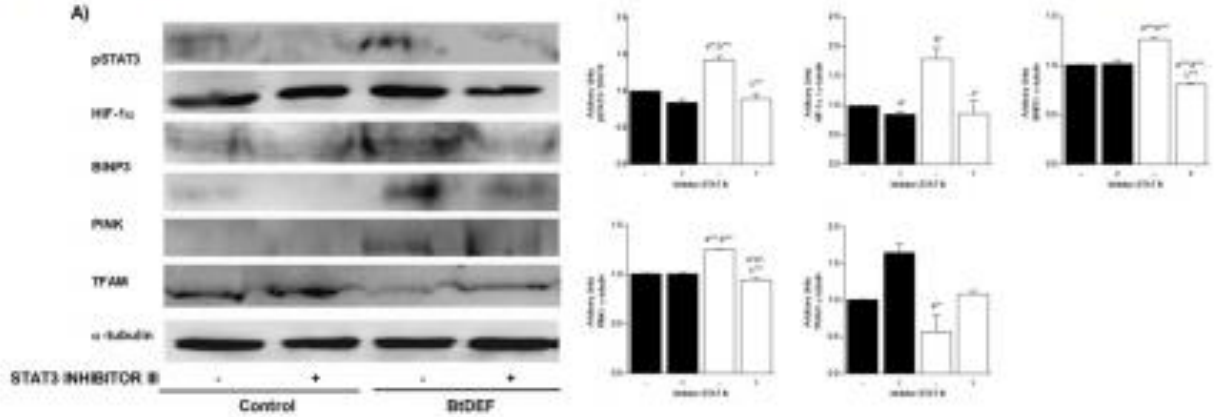

Fig. 8. Decrease of the mitophagic PINK and BNIP3 proteins after STAT3 was inhibited by STAT3 inhibitor III in the BtDEF glioblastoma cultures. Effects of the STAT inhibitor III on of STAT3, HIF-1α, the mitophagic proteins PINK and BNIP3, the mitochondrial marker TFAM, and citrate synthase activity. by Western blot analysis. Bands were quantified by densitometry and compared as follows (a) control without STAT3 inhibitor III, (b) control with STAT3 inhibitor III and (c) BtDEF without STAT3 inhibitor III. Tubulin is used as a load control. n = 3 independent experiments. Results of citrate synthase activity are expressed as mean values ± SEM. *P b 0.05, **P b 0.01, ***P b 0.001 (ANOVA Bonferroni's multiple comparison test).

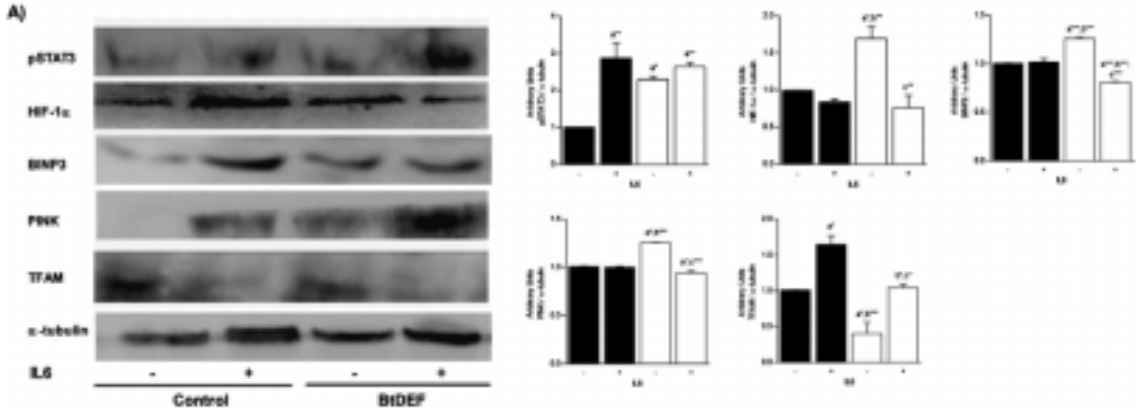

Fig. 9. Effects of interleukin 6 (IL6) as a physiological activator of $GATA_3$ on HIF-1α, BNIP3 and PINK proteins and on the mitochondria markers TFAM and citrate synthase. Bands were quantified by densitometry and compared as follows: (a) control without IL6, (b) control with IL6 and (c) BtDEF without IL6. Tubulin is used as a load control. n = 3 independent experiments. Results of citrate synthase activity are expressed as mean values ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 (ANOVA Bonferroni's multiple comparisons test).

medium was warmed to 37 °C and bubbled with O_2/CO_2 (95/5%) prior to use. Cell viability was assayed by the trypan exclusion method; all the experiments were performed only when N 95% viability was observed.

4.3. Cell culture

Primary culture of hepatocytes, HepG2 cells (ATCC: HB-8065, Manassas, VA, USA) and U373 glioblastome cell line were cultured using DMEM medium (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum and penicillin 100 IU/ml, streptomycin 100 μg/ml and gentamicin 50 μg (Gibco, Life Technologies, Grand Island, NY, USA) at 37 °C and 5% CO_2. Biotin deficient medium was prepared by dialyzing serum using a protocol previously described [46]. Biotin deficiency was proved by streptavidin Western blots (see above). When biotin deficiency was induced, cells were seeded in complete medium, which was replaced after 24 h with a biotin-deficient media and incubated for different times.

4.4. ^{13}C enrichment experiments

Pyruvate and TCA cycle metabolism was studied measuring metabolite concentrations and ^{13}C enrichment, from pyruvate-3-^{13}C and lactate-3-^{13}C, of several key metabolites. Freshly isolated hepatocytes (1×10^6 cells/ml) from control and BtDEF rats were incubated in Erlenmeyer flasks with Ringer–Krebs-phosphate buffer (5 ml final volume) in a shaking water bath at 30 °C for 10 min and 0.3 mM sodium pyruvate-3-^{13}C and 3 mM sodium L-lactate-3-^{13}C, pH 7.4 were added. At the end of the incubation, an 100 μl aliquot was taken for protein determination, and the incubation was stopped with 100–150 μl of $HClO_4$ (60%). The extracts were neutralized with 4 N KOH. Metabolite concentrations were quantified in 50 μl of neutralized samples directly injected into HPLC (Varian, Agilent, Palo Alto) using pre-column derivatization with o-phthalaldehyde and fluorescent detection, as previously described [47].

For measurement of the ^{13}C enrichment in different metabolites, samples were prepared as described previously [48–50]. These studies were carried on at the University of Pennsylvania at Philadelphia Medical School Core Center, using a Hewlett-Packard 5970MSusing Gas chromatography–Mass Spectrometry (GC–MS) D and/or 5971 Mass Selective Detector (MSD), coupled with a 5890 HP-GC, GC–MS Agilent System (6890 GC-5973MSD) or Hewlett-Packard (HP-5970MSD), using electron impact ionization with an ionizing voltage of -70 eV and an electron multiplier set to 2000 V. ^{13}C enrichment in a given mass isotopomer is expressed by molar percent enrichment (MPE), which is the molar fraction (%) of the analyte containing ^{13}C atoms above natural abundance. The MPE was calculated using the peak area from GC-MS ions corrected for natural abundance as described (I. Nissim, personal communication) [51,52].

4.5. Mitochondria isolation

Mitochondria were obtained according to a previously described method [37]. Livers were obtained from male Wistar rats (control and biotin-deficient) placed in ice-cold buffer A (40 mM sucrose, 220 Mannitol, 1 m M EGTA, 5 mM Tris, pH 7.2) and homogenized using a teflon pestle. Mitochondria were isolated by differential centrifugation. Protein amount was determined using the Biuret Method. The quality of preparations was evaluated by oxygen consumption as described below.

4.6. Oxygen consumption experiments

Isolated mitochondria or intact cells (primary culture hepatocytes) were placed in a temperature-controlled chamber with constant stirring. Oxygen concentration was monitored using an oxygraph equipped with a specific Clark electrode (Strathkelvin Instruments, Scotland). Mitochondria (0.5 mg protein) were suspended in buffer A in the presence of 10 mM phosphate, 10 mM glutamate and 10 mM malate. Respiration was monitored in these conditions (State 2) and after ADP addition (State 3). Intact hepatocytes and were tripsinized, resuspended in the respective media (complete or biotin-deficient) at a known density (2×10^6 cells/ml) and maintained at constant stirring. Oxygen consumption was measured before and after the addition of oligomycin (10 μM), a specific mitochondrial ATP synthase inhibitor. The sensitivity towards oligomycin was interpreted as the oxidative phosphorylation rate as previously reported [14]. In all cases, the respiratory rate was calculated based on "oxygen concentration vs. time" plots taking into account the amount either the protein concentration or cell density, respectively.

4.7. Complex IV activity

Cytochrome oxidase (COX) (complex IV) activity was assessed through oxygen consumption [53]. Mitochondria (0.5 mg protein) were preincubated with antimycin A (10 μM) and 5 mM ascorbate (1 mM). COX activity was stimulated by the addition of 10 μM TMPD (*N,N,N',N'*-tetraphenylenediamine). COX activity was reported as the amount of oxygen consumed per minute.

4.8. Cytochrome quantification [54]

Isolated mitochondria (1 mg protein) were treated with dithionite and the absorption spectrum was recorded in a double-beam spectrophotometer (Aminco DW 2, Olis Inc.). Afterwards, saturated ferricyanide was added to mitochondrial suspension in order to record the oxidized state of mitochondrial cytochromes. The final plot is constructed taking into account the difference between the two spectra. Cytochromes were quantified using the previously reported values of the molar extinction coefficients (ε) [38]. Cytochromes *a* + *a3*: 24 $M^{-1}cm^{-1}$, cytochrome *b*: 28.5 $M^{-1}cm^{-1}$, cytochromes *c* + *c1*: 21 $M^{-1}cm^{-1}$.

4.9. Citratesynthase (CS) assay

Protein extracts were extracted by treating cells (either primary rat hepatocytes, HepG2 or mouse hepatocytes) with lysis buffer (Triton X-100 0.1%, KCl 50 mM, EDTA 1 mM, EGTA 1 mM, β-glycerol phosphate 5 mM, HEPES 50 mM, pH 8.0) supplemented with a protease inhibitor cocktail (Complete Mini, Roche). Protein was quantified by the Bradford method. Extracts (30 μg protein) were incubated with Acetyl-CoA 30 mM, Oxaloacetate 10 mM and DTNB 10 mM. Absorbance was monitored at 412 nm in a Genova-Nano spectrophotometer (Jenway).

4.10. Mitotracker staining

Cells (primary hepatocytes or HepG2) were seeded in coverslips and fixated with 4% paraformaldehyde. Samples were treated with Mitotracker CMCRos 25 nM (Invitrogen) for 15 min and washed twice with PBS. Coverslips were mounted on glass plates and they were visualized by confocal microscopy (ZEISS LSM 5 PASCAL).

4.11. Acylcarnitine analysis

L-carnitine (Sigma Aldrich, St. Louis, MO) was supplemented through an intraperitoneal injection 200 mg/kg every 24 h for 4 weeks to control and BtDEF rats to investigate acyl CoA accumulation. Dried blood spots were collected from all rats on Whatman 903 filter paper and sent to the Massachusetts Medical School for analysis. Blood spots were analyzed using electrospray ionization liquid chromatography–mass spectrometry (LC–MS) with a Quattro Micro API (Micromass) tandem mass spectrometer [55]. All procedures for sample preparation and

MS analysis were performed by NeoGram AAAC Spectrometry kit (Perkin Elmer, MA, USA) according to the manufacturer's protocol. Briefly, single disks were punched from each dried blood spot using an automatic 3-mm punch. One disk was added per well. Using a multichannel pipette, 190 μl of working extraction solution (containing a mixture of the respective stable-isotope-labeled internal standards) was added to each well. The plate was covered with aluminum foil, followed by shaking at 650 rpm and incubation for 30 min at 30 °C. The plate was finally placed in the auto-sampler for test.

4.12. Electron microscopy studies

At the end of the treatments, the animals were perfused via the left cardiac ventricle with 4% paraformaldehyde in 0.1 M saline phosphate buffer (pH 7.4). A sample of gluteal muscle tissue was removed, sectioned, and transferred to the same fixative for a further 1 h. Samples were postfixed in a 0.5% osmium tetroxide in pH 7.4 cacodylic acid, dehydrated in alcohol and embedded in EPON resin. One-micron thick sections at the silver/grey area of the spectrum of interference colors were stained with uranyl acetate and lead citrate and examined under a JEOL JEM-1400 transmission electron microscope.

4.13. Western blots

Samples frozen in liquid nitrogen were homogenized with an extraction buffer containing HEPES 50 mM, KCl 50 mM, EDTA 1 mM, EGTA 1 mM, β-glycerol phosphate 5 mM, Triton X-100 0.1% (v/v), protease inhibitors miniComplet (Roche Diagnostics, Mannheim, Germany), sodium fluoride 50 mM, sodium orthovanadate 1 mM, sodium pyrophosphate 5 mM and PMSF 0.2 mM (all from Sigma-Aldrich). The homogenates were centrifuged at 12,000 × *g* for 10 min at 4 °C and supernatants were used. Proteins in extract samples were separated by SDS-PAGE electrophoresis in a 10% polyacrilamide gel. Proteins were blotted on nitrocellulose membranes (Bio-Rad Laboratories, Inc., Germany) and afterwards they were blocked with 1% non-fat milk and incubated with different primary antibodies: phospho-AMPKα (Thr172) #2531, AMPKα (23A3) #2603 (Cell Signaling Technology, Inc. Danvers, MA, USA), phospho-STAT3 (Tyr 705) #7993, STAT3 (C-20) #482, PINK1 (H-300) #33,796, BNIP-3 (Ana40) #56,167), mtTFA (E-16) #30,963, HIF-1α (Y-15) #12,542, and α-tubulin (TU-02) #8035 (Santa Cruz Biotechnology, Dallas, Texas, USA). Membranes were washed three times with PBS and incubated with the respective HRP-conjugated secondary antibodies. Blots were revealed using a chemiluminescent substrate HRP Immobilion Western, (Millipore Corporation, Billerica, MA, USA). Images were acquired in a FUSION FX System (VILBER LOURMAT, France) and analyzed in Chemidoc XRS System (BIO-RAD Laboratories, Inc, Germany).

4.14. Statistical analysis

Date are shown as the mean +/− S.E. Statistical analysis two-tailed Student's t.test, or one way analysis of variance (ANOVA) with the Dunnett's and Bonferroni's multiple comparison test was performed for the groups of interest. The analysis was done with the software Prism 5.0 (GraphPad). Differences were considered significant at * P b 0.05, ** P b 0.01, *** P b 0.001.

4.15. Study approval

The animals were handled according to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (National Academy of Sciences, Washington, DC, USA, 1996) and approval was obtained from the Animal Research Committee (CINVA) of the Instituto de Investigaciones Biomedicas of the Universidad Nacional Autonoma de Mexico, for all the animal procedures performed in this project.

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APENDICE 2

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Temporal development of genetic and metabolic effects of biotin deprivation. A search for the optimum time to study a vitamin deficiency☆

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ABSTRACT

Biotin deficiency (Bt-D) is usually studied at the point at which the animal model exhibits the signs of full-blown deficiency symptoms; in rats, this typically occurs at 6–8 weeks of feeding a deficient diet. To differentiate specific deficiency effects from those of undernutrition, biotin sufficient and deficient rats were studied at 2, 3, 4, and 5 weeks on the deficiency diet, before the onset of weight loss and deficiency signs. The deficiency state was confirmed by biochemical and molecular analyses. Blood and liver metabolites were determined and western blots of signaling proteins, and qRT-PCR gene expression studies. The main effects of Bt-D were already well established by the fourth week on the diet; thus, we consider the fourth week as the optimum time to study the consequences of biotin depletion. Early effects, which were already apparent at week 2, included cellular energy deficit (as assessed by increased AMP/ATP ratio), activation of the AMPK energy sensor, and changes of carbon metabolism gene transcripts (e.g., phosphoenolpyruvate carboxykinase, carnitine palmitoyl transferase 1, liver glucokinase and fatty acid synthetase). Reduced post-prandial blood concentrations of glucose were also observed early; we speculate that these are attributable to augmented sensitivity to insulin and increased glucose utilization, a likely effect of AMPK induction of translocation of glucose transporter GLUT4 to the cell membranes and increased hexokinase expression. Other late-onset changes (week 4) included increased serum concentrations of lactate and free fatty acids and decreased liver glycogen and serum concentrations of triglycerides and total cholesterol. The identification of the early specific molecular and metabolic disturbances of biotin deficiency might be useful in identifying individuals with marginal deficiency of this vitamin, which appears to be common in normal human pregnancy. The study of time-course of other vitamin deficiencies, such as this one, might help to better understand and cope with their effects.

1. Introduction

Biotin, the essential prosthetic group of the five mammalian biotin-dependant carboxylases, is a water-soluble vitamin (for a review see [1]). Biotin is bound covalently and facilitates in the addition of a carbon atom from bicarbonate (in the form of CO_2) the various substrates. Four carboxylases in mammals catalyze critical reactions of intermediary metabolism: (1) Pyruvate carboxylase (PC), the main anaplerotic enzyme whose product is oxaloacetate (OAA); which is also a precursor of glucose in gluconeogenesis, and of fatty acids in lipogenesis. (2) Acetyl CoA carboxylase (ACC I and II; also referred to as ACC<alpha>and<beta>). Both enzymes produce malonyl CoA from acetyl CoA. One is cytosolic and catalyzes production of the compound that is rate limiting in fatty acid synthesis; the other is located on the outer mitochondrial membrane and controls fatty acid oxidation in mitochondria through the inhibition of carnitine palmitoyltransferase I by its production of malonyl CoA, which also acts as inhibitor of β-oxidation. (3) Propionyl CoA carboxylase (PCC) participates in the catabolism of several amino acids (isoleucine, valine, methionine, threonine) and in odd-chain fatty acid oxidation. (4) 3-Methylcrotonyl CoA carboxylase (MCC) catalyzes a step in leucine catabolism; its deficient activity leads to accumulation of the collateral products 3-hydroxyisovaleric acid (3HIA) and 3-HIA carnitine, that are sensitive markers of biotin deficiency [2–4].

We discovered a new function of biotin as regulator of the genetic expression of carboxylases and of holocarboxylase synthetase (HCS), the enzyme that covalently binds biotin to the apocarboxylases [5]. More recently, we found that biotin deficiency by means of an avidin-containing diet that contains adequate glucose modifies the

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expression of several genes involved in carbon metabolism in rat liver, in *C. elegans* and in *S. cerevisiae* [6]. We also found that biotin deficiency causes an ATP deficit in the three species, which activates the energy sensor AMPK [7].

Biotin deficiency can be produced experimentally by adding avidin to the animals' diet, and its full blown effects are usually observed at 6–8 weeks of feeding the deficiency producing diet [2,5,6,8–14]. This vitamin deprivation bears several similarities to multiple carboxylase deficiency, a group of inherited metabolic disorders caused by mutations in the genes for intestinal biotin transport [15], holocarboxylase synthetase [16], and biotinidase [17], in that in all of these conditions the activities of the different carboxylases are greatly reduced. In particular biotinidase (the enzyme that allows biotin reuse by freeing it from biocytin in the biotin cycle) deficiency [17] leads to acute biotin depletion. Recently, a biotinidase deficient knockout mouse has been generated [18].

A serious problem of the dietary experimental model as it is generally used [9,12–14] is that when the animals present full-blown deficiency signs, they are also severely undernourished. Therefore, it is not possible to differentiate which effects are specific of the biotin deficiency and which are due to general malnutrition. Similar studies inducing biotin deficiency experimentally by egg white feeding in animals or human volunteers have also clearly demonstrated deficient propionyl CoA carboxylase activity and increased excretion of 3-hydroxyisovaleric acid and 3-hydroxyisovaleryl carnitine prior to the onset of both symptoms and signs of overt biotin deficiency [2,3,14,19,20]. Furthermore, for biotin, the focus of research is currently shifting from the consequences of moderate to severe deficiency to that of the consequences and mechanisms of marginal deficiency [21]. This is the result of both the modern emphasis on optimizing nutritional status and because substantial information is accumulating that a significant proportion of women undergoing normal pregnancy develop marginal biotin deficiency. Here we show that the genetic expression and metabolic effects of biotin deficiency [6] also appear earlier than the phenotype (underweight, deficiency signs), thus being specific to the lack of the vitamin. This knowledge will be critical to optimize the design of the experiment model of biotin deficiency and to use it to probe interactions between metabolic regulation and genetic expression control in nutrigenomics.

2. Material and methods

2.1. Biotin-deficient animals

Biotin deficiency was induced as follows: Male rats (Wistar strain), aged 21 days (their weaning age) and 80–90 g of weight, were made biotin deficient (Bt-D) by feeding commercial diet (TD. 81079 HARLAN Teklad. Madison, WI) containing 30% of white egg which contains negative avidin. The control rats (biotin sufficient Bt-S) were similar in gender, age, and weight and were fed with a similar diet but the diet was supplemented with biotin 4 mg/kg (TD. 97126 HARLAN Teklad. Madison, WI). For convenience considerations, standard biotin deficiency studies are done in young animals. Rats were housed individually in an air-filtered cage under 12-hour light/dark cycles and were allowed free access to water. Bt-S and Bt-D rats were sacrificed at 2, 3, 4, and 5 weeks; at each of these times, none of the rats had developed any features of biotin deficiency as previously described. The animals were sacrificed by decapitation before immediate isolation of the liver. Livers were immediately placed in liquid nitrogen and stored at −70 °C before utilization.

2.2. Acyl carnitine analysis

Dried blood spots were collected from all rats on Whatman 903 filter paper. Blood spots were analyzed using electrospray ionization liquid chromatography-mass spectrometry (LC-MS) with a Quattro Micro API (MicroMass) tandem mass spectrometer. All procedures for sample preparation and MS analysis were performed by NeoGram AAAC Spectrometry kit (Perkin Elmer, MA, USA) according to the manufacturer's protocol. Briefly, single disks were punched from each dried blood spot using an automatic 3-mm punch. One disk was added per well. Using a multichannel pipette, 190 µl of working extraction solution (containing a mixture of the respective stable-isotope-labeled internal standards) was added to each well. The plate was covered with aluminum foil, followed by shaking at 650 rpm and incubation for 30 minutes at 30 °C. The plate was finally placed in the auto-sampler for testing.

2.3. Holocarboxylases estimation

To assess biotin status, we determined the degree of carboxylase biotinylation by streptavidin Western blots [14]. Aliquots containing 30 µg of total protein were subjected to SDS-PAGE. The gel was blotted on a 0.45-µm nitrocellulose membrane, using a semidry transfer cell (Bio-Rad), at 20 V for 25 min. The membrane was incubated with streptavidin-alkaline phosphatase for 2 h [15]. The visualization was made using as chemiluminescent substrate HRP Immobilon™ Western (Millipore Corporation, Billerica, MA), and was analyzed in a ChemiDoc™ XRS system (BIO RAD, Philadelphia, PA). Holoenzyme abundances for PC, PCC and MCC were quantified by densitometry of the bands. Total protein was determined by the Bradford method [22] using BSA as the standard.

2.4. Blood biochemistry

Blood samples were taken from the tail vein of non-fasting Bt-S and Bt-D rats. Glucose concentration was measured using and automatic glucometer (Accuchek, Performa, Roche, Manheim, Germany). Concentrations of triglycerides, cholesterol, and lactate were measured using the corresponding assay strips (Accutrend Plus, Roche, Manheim, Germany). For determination of insulin, serum was collected (25 µl) and insulin was measured using immunoassay (ELISA) based on a commercial kit (ALPCO Diagnostics, Salem, NH).

2.5. Liver glycogen

Ten mg of liver was homogenized in 200 µl of water. The homogenate was boiled for 5 minutes to inactivate the enzymes and then centrifuged at 13,000 rpm for 5 minutes to eliminate all the insoluble material. 10 µl of supernatant was assayed using the EnzyChrom™ Glycogen Assay Kit (BioAssay Systems, Hayward, CA), following the fabricant's instructions.

2.6. Adenine nucleotides

ATP, ADP and AMP levels were determined by HPLC according to the method of Delaney and Geiger [21]. Rats were sacrificed by concussion. A portion of hepatic tissue (0.8–1 g) was removed and immediately immersed in 3 ml of perchloric acid 8%, homogenized, and centrifuged (10,000 rpm for 10 minutes at 4 °C). The supernatant was collected stored at −80°. Upon thawing, the supernatant was neutralized with with K_2CO 3 M (6% final volume) and re-centrifuged. Then the adenine nucleotides content was determined by HPLC. One hundred µl of a 1:10 dilution of each sample was injected on to the HPLC. Nucleotides were separated on an ACE 5 µm C18 (150×4.6 mm) column using an HPLC KNAUER system (Smartline System Isocratic HPLC with UV detection). The mobile phase consisted of KH_2PO_4 1 M, 20% of methanol and ION PAIR COCKTAIL Q6 4 mM (Hexyltriethylammonium Phosphate) with a flux of 1.5 ml/min. The external standard was treated identically to the hepatic homogenates; the ATP, ADP and AMP spikes were

identified based on their retention times. The data are expressed in μmol/g of wet tissue.

2.7. Western blot analyses

Aliquots of liver samples frozen in liquid nitrogen were homogenized with an extraction buffer containing HEPES 50 mM, KCl 50 mM, EDTA 1 mM, EGTA 1 mM, β-glycerol phosphate 5 mM, Triton X-100 0.1% (v/v), protease inhibitors (miniComplet, Roche), sodium fluoride 50 mM, sodium orthovanadate 1 mM, sodium pyrophosphate 5 mM and PMSF 0.2 mM (all from Sigma-Aldrich). The homogenates were centrifuged at 12,000 x g for 30 minutes at 4 °C and supernatants were used. Protein concentration was determined by the Bradford method [22] using BSA as the standard. Aliquots containing 50 μg of total protein were subjected to SDS-PAGE. The gel was blotted on a 0.45 μm nitrocellulose membrane, using a semi-dry transfer cell (Bio-Rad), at 20 V for 25 min. Probing of the membranes was carried out with various antibodies: 1:1000 AMPKα (23A3) #2603, p-AMPKα (Thr172) #2531, p-ACCα (Ser79) #3661 and as secondary 1:2000 anti-rabbit IgG #7074S (Cell Signaling Technology, Inc. Danvers, MA).

2.8. Gene expression analyses

RNA was isolated from liver samples that had been frozen in liquid nitrogen. Complementary DNA was generated by M-MLV reverse transcriptase enzyme (Invitrogen) and random hexamers (deoxy-NTPs; Amersham science). The analysis was performed by quantitative reverse-transcriptase PCR (qRT-PCR) on ABI PRISM 7700 Real Time PCR machine (Applied Biosystems) using Taqman probes. The level of each target mRNA was examined and normalized to 18S rRNA using the $2^{-\Delta\Delta CT}$ method [23].

2.9. Statistical analyses

Data are shown as the mean ± S.E. Statistical analysis (two-tailed Student's t-test, or one way ANOVA with the Tukey posthoc test) was performed for the groups of interest. The analysis was done with the software Prism 5.0 (GraphPad). Differences were considered significant at * $P<0.05$.

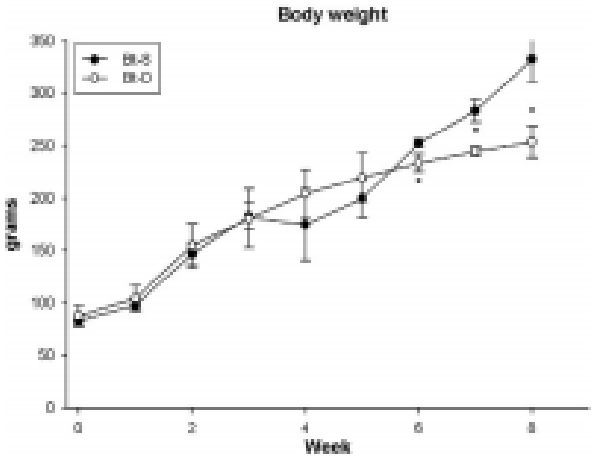

Fig. 1. Body weight does not vary between the biotin-starved rats (clear circles) and the controls (solid circles) before the 6th week of the biotin deficiency diet. Values are the mean ± S.D. n = 5 animals. * $P<0.05$.

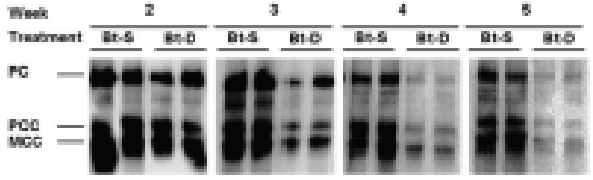

Fig. 2. Holocarboxylases diminish from week 2 of deficient diet and declines with time. Liver streptavidin western blots. PC (pyruvate holocarboxylase; PCC (propionyl-CoA holocarboxylase); MCC (3-methylcrotonyl-CoA holocarboxylase). Bt-S: biotin sufficient (control); Bt-D: biotin deficient. The different duplicate lanes are samples from two different animals treated in the same ways.

3. Results

3.1. Clinical and biochemical indicators of biotin deficiency

Body weight of biotin-starved rats did not differ significantly from the controls until the 6th week of the deficient diet, (Fig. 1). Before then, no classical deficiency signs were observed. Food intake did not differ between the two groups until week 6 (data not shown). However, biochemical markers clearly showed the animals were developing multiple consequences of biotin deficiency by week 2.

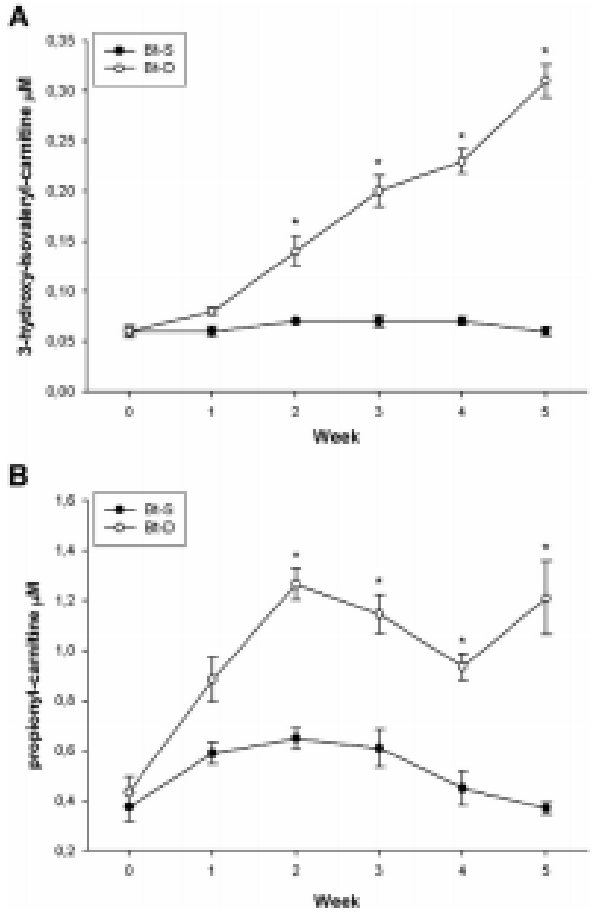

Fig. 3. Blood 3-hydroxy-isovaleryl (A) and propionyl (B) carnitine levels increased very early after the start of biotin-deficient diet. Values show the blood concentration of the acylcarnitines in non-fasted animals. Bt-S (solid circles) and Bt-D (clear circles). Data are means ± SEM; n = 5 animals per group. * $P<0.05$.

To evaluate biotin deficiency, we studied three liver holocarboxylases by means of streptavidin western blots: PC, PCC and MCC; ACC could not be visualized because of its much higher molecular weight [6]. There were differences between them and the differences were already apparent at week 2 and became more pronounced in the following weeks (Fig. 2). At week 5 the three holocarboxylases were much fainter in the deficient than in the control homogenates. Additionally, beginning at week 2 the blood levels of 3-hydroxyisovaleryl (3-HIA) carnitine were increased relative to controls (Fig. 3). We also observed a similar increase in blood levels of propionyl carnitine, a compound directly derived from the PCC substrate propionyl CoA [24]. The increase in these acyl carnitines is strong evidence of biotin depletion (Fig. 3).

3.2. Time course of biochemical effects of biotin depletion

Biotin-starved non-fasting rats had significant reductions in blood glucose from the second week of the diet (Fig. 4A). Later on during the deficiency diet, lactate and free fatty acids levels increased (Fig. 4B and C), and cholesterol and triglycerides levels decreased (Fig. 4D and E). Blood insulin increased with age in both Bt-D and Bt-S rats, but the increase was less pronounced in the Bt-D group by the fourth week (for numerical values see supplementary Table 1). Liver glycogen increased from the second week on in both Bt-S and Bt-D rats, but in Bt-D livers, there was a sharp decrease by the fourth weeks (Fig. 5).

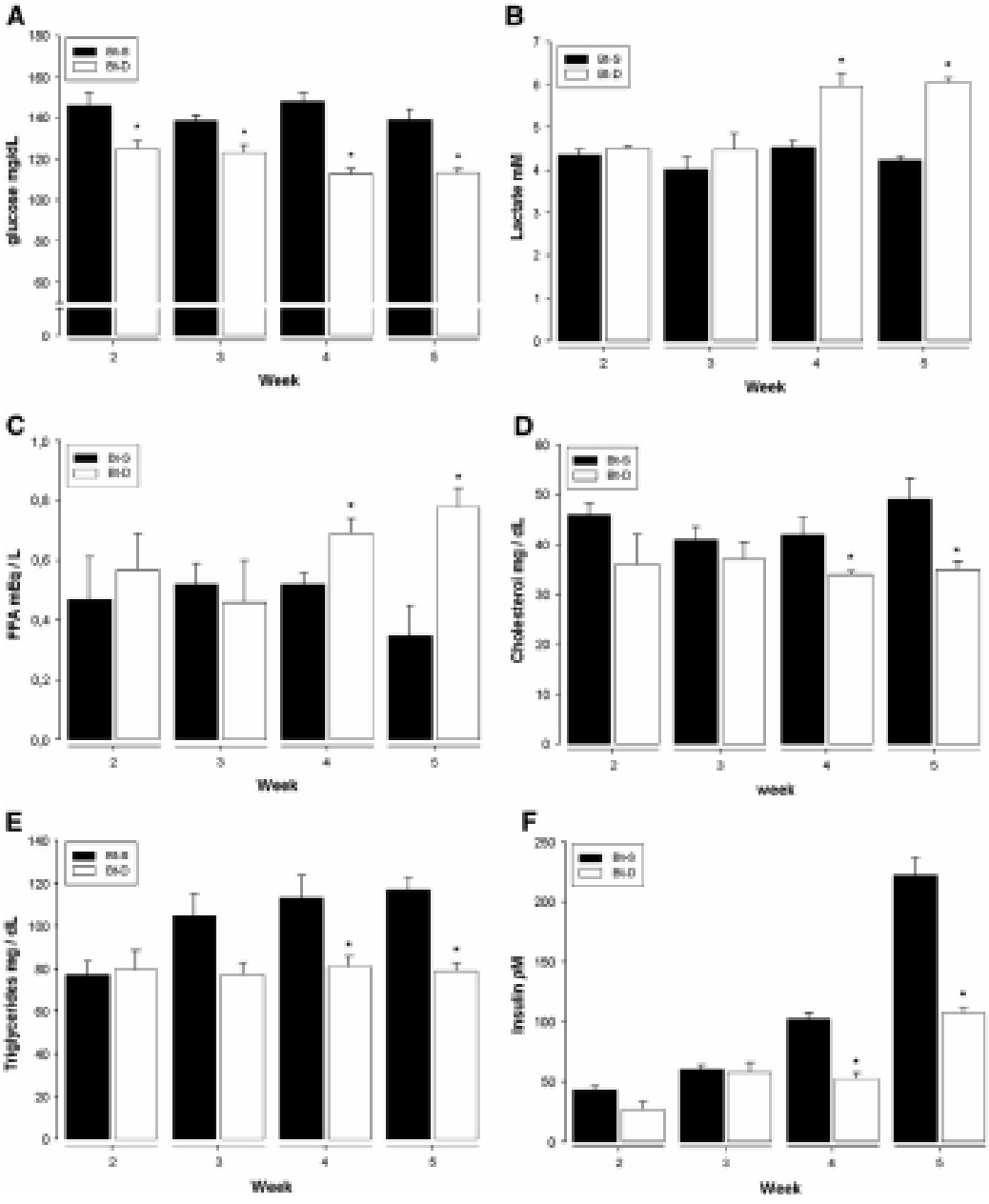

Fig. 4. Temporal changes in non-fasting plasma concentrations of glucose mg/dL, lactate mM, FFA mEq/L, cholesterol (mg/dL), triglycerides (mg/dL) and insulin pM during deprivation of biotin. Bt-S: filled bars and Bt-D: clear bars. Values are the mean ± SEM; n = 5 animals per group. * P < 0.05.

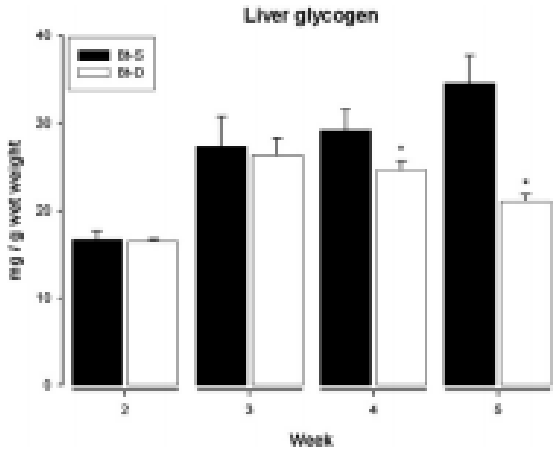

Fig. 5. Liver glycogen diminishes in Bt-D (open bars) compared to Bt-S (filled bars) from week 4 of the biotin deficient diet. Values are the mean ± SEM; n = 5 animals per group. * P<0.05.

3.3. Liver adenine nucleotides and AMPK activation

We had previously reported reported decreased ATP in biotin depleted rat liver [7]. Fig. 6 shows that the hepatic AMP/ATP ratio increased steadily beginning in the second week of the biotin deficiency diet. The content of both nucleotides changed markedly. AMP content had risen almost twice the initial value by the fourth and fifth weeks of Bt-D diet (0.07 nmol/g to 0.13 nmol/g or 0.11 nmol/g to 0.22 nmol respectively), whereas ATP content decreased at significant levels for the same period (1.55 ± 0.09 to 1.27 ± 0.04 and 1.57 ± 0.06 to 1.19 ± 0.06) supplementary material (Table 2). A similar time course was followed by the activated (phosphorylated) form of AMP-dependent protein kinase (AMPK), the main cellular energy sensor [25], and by one of its targets, the phosphorylated form of acetyl-CoA carboxylase (Fig. 7).

3.4. Gene expression for enzymes involved in carbon metabolism

We also studied the time course of changes in the expression of four genes, selected because they catalyze essential steps in intermediary metabolism: glucokinase (GK) for glycolysis; phosphoenolpyruvate carboxykinase (PEPCK) for gluconeogenesis, carnitine palmitoyl transferase 1 (CPT-1) for fatty acid oxidation and fatty acid synthase (FAS)

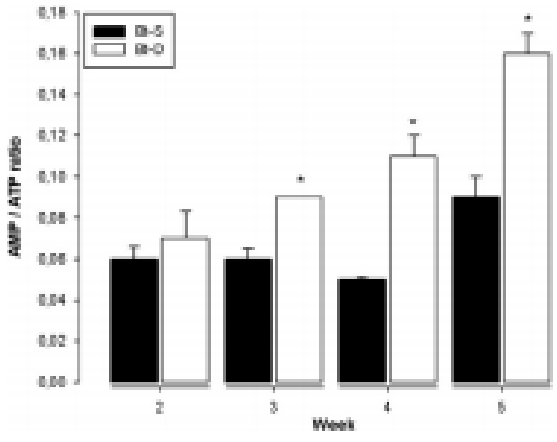

Fig. 6. The AMP/ATP increase starts very early during biotin starvation. Bt-S: filled bars, Bt-D: clear bars. Values are the mean ± SEM; n = 5 animals per group. * P<0.05.

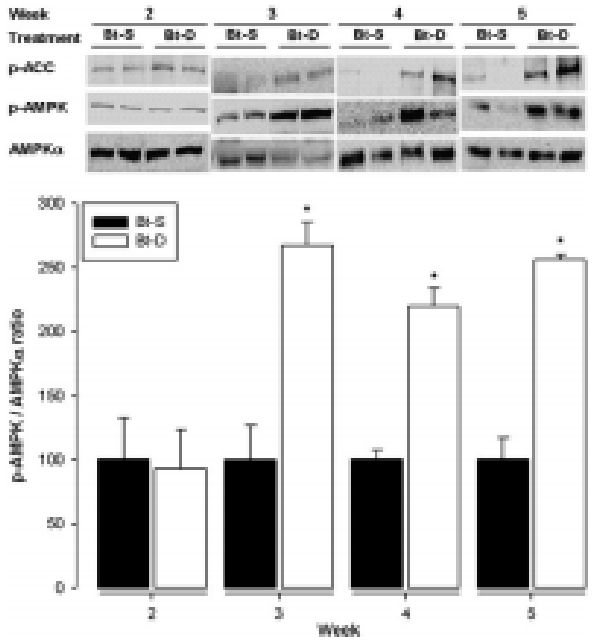

Fig. 7. Early increase of liver AMPK activation and of ACC phosphorylation during biotin starvation. Bt-S: filled bars; Bt-D: clear bars. Values are the mean ± SEM; n = 5 animals per group. * P<0.05. The different duplicate lanes are samples from two different animals treated in the same ways.

for fatty acid synthesis. Although the mRNAs of the genes studied changed only gradually since the second week in the Bt-D group (Fig. 8) as previously indicated [6], the differences in the Bt-D group were striking and highly significant. Glucokinase and fatty acid synthase were substantially down regulated, and phosphoenolpyruvate carboxykinase and carnitine palmitoyltransferase I were substantially up regulated in the same fashion that we had found earlier [6].

4. Discussion

We studied the time-course of biochemical and genetic changes in biotin deficient diet, prior to weight loss (Fig. 1), diminished food intake, or the onset of the signs of biotin depletion. The present results show that the main metabolic and genetic expression effects of biotin deficiency are already well established by fourth week of the diet; thus we consider four weeks as an optimum time to study the consequences of biotin starvation, without possible confounding effects of general malnutrition. Almost all published studies on Bt-D, including our own, have been performed at 8 weeks of the experimental diet [8,11–13], when the animals present vitamin deficiency sign and several ill, also protein and energy malnutrition. Our studies offer novel insight into the consequences of biotin deficiency in the most common animal model.

Carboxylase activities are substantially decreased by the second week of diet, as evidenced by the heightened 3-hydroxyisovaleryl carnitine and propionyl carnitine blood concentrations (Fig. 2). These compounds are derived from the accumulation of the substrates of 3-methylcrotonyl CoA carboxylase (MCC) [25] and of propionyl CoA carboxylase (PCC) [23] revealing metabolic blocks at these steps. These observations confirm our previous work [13,18], and are similar to the urine acylcarnitine levels and related substrate to product ratios recently reported by Boguslewicz, et al. [26], which likely increase human relevance of our observations. Furthermore, the urinary excretion of 3-HIV is consistent with a decrease in the lymphocytes PCC activity [27]. Moreover, the amounts of three of the four hepatic holocarboxylases, (PC, PCC, and MCC), progressively

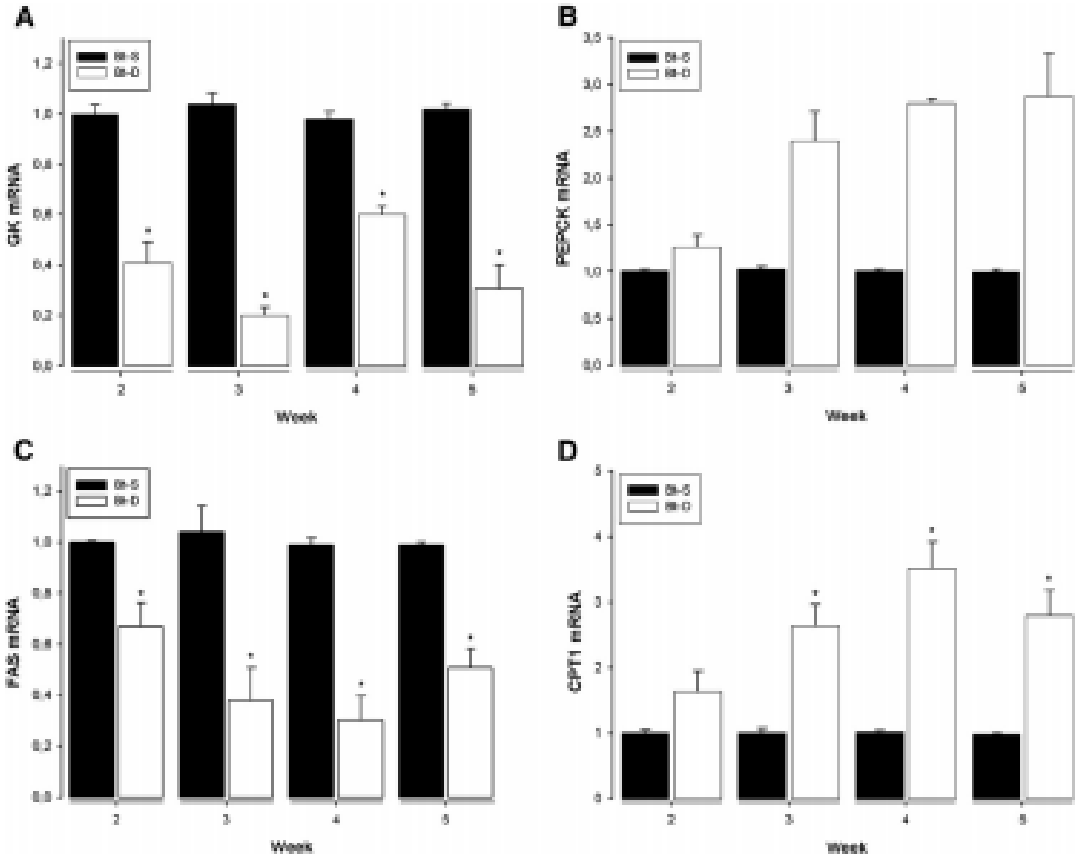

Fig. 8. Expression of representative genes of main liver carbon metabolism pathways changed very early during biotin starvation. A) Glucokinase (GK), B) phosphoenolpyruvate carboxykinase (PEPCK), C) fatty acid synthetase (FAS), D) carnitine palmitoyl transferase 1 (CPT1). Bt-S: filled bars, Bt-D: clear bars. Values are the mean ± SEM, n = 5 animals per group. * P<0.05.

decreased beginning in week 2 of the deficiency diet (Fig. 3). In contrast, free carnitine significantly decreased from week 4 in the biotin-deprived rats compared to the controls (data not shown), most likely as a consequence of the production of the acyl carnitines.

Although all these effects were clearly evident by week 4, some of them occurred as early as week 2; others occurred only by week 4. Among the former are reduced (post-prandial) glycemic levels, cellular energy deficit (as assessed by an increased AMP/ATP ratio), activation of the AMPK energy sensor with phosphorylation of its ACC target, and changes of carbon metabolism gene transcripts (increase of those for PEPCK and CPT-1 and decrease of those for GK and FAS). We hypothesize that these changes are an adaptation to the energy shortage; AMPK is activated leading to induction of ATP producing processes and reduction of those consuming ATP [25].

In contrast, only at the fourth week did we observe decreased blood insulin and liver glycogen, increased serum lactate, and decreased free fatty acids, triglycerides, and total cholesterol. It is noteworthy that blood glucose levels are already lower in the biotin-starved rats than in the controls by the second week while blood insulin is similar in the two groups until week 4; at that time, insulin is lower in the deficient animals relative to biotin sufficient controls. A possible explanation for these observations is that biotin deprivation might have first augmented the sensitivity to insulin, leading to increased glucose uptake by tissues such as adipose and muscle. AMPK activation may have contributed to increased insulin sensitivity [28]. Several studies report that AMPK elevation promotes the translocation of the glucose transporter GLUT4 to the cell membrane of adipose tissue and muscle [29,30], as well as increasing muscle and heart hexokinase [28] expression, thus augmenting glucose utilization. Furthermore, we have found that the insulin pathway is abnormally activated in biotin starvation (*submitted manuscript*). Although, in the present work, we confirmed previous reports of liver glucokinase repression [6,8,31], the contribution of this repression to overall glucose homeostasis is likely marginal. We are currently studying metabolic flux through the main carbon metabolism pathways in an attempt to gain a more complete understanding of the metabolic effects of biotin starvation. The delayed diminution of blood insulin may reflect a delayed perturbation of insulin production/secretion by the pancreatic beta cells; such a delay has shown to be associated with chronic AMPK activation [29,30]. Although putative inhibition of gluconeogenesis in biotin starvation might also be considered as a mechanism for the blood glucose reduction, we contend this is unlikely to have played a substantial role in our results because samples were obtained from deficient animals in the post-prandial state.

The high serum lactate can reasonably be attributable to the reduced activity of pyruvate carboxylase. A contribution from an increase of glycolysis from glycogen is also conceivable and could help explain the decreased liver glycogen content observed in the biotin deficient rats, besides the well-known inhibition of glycogen synthesis by AMPK [32].

Both the increase of CPT-1 mRNA and of serum free fatty acids suggest an increased fatty acid release from adipose tissue and their

oxidation in peripheral tissues in the biotin-starved animals, caused by lower insulin levels. This would also explain the decrease of serum triglycerides and cholesterol. Although this would lead to increased ATP production at the mitochondria, we have recently discovered a severe mitochondrial dysfunction in biotin deficiency ([33], manuscript in preparation) that explains these apparently paradoxical findings.

A decrease in ACC activity due to biotin deficiency would lead to a decrease in malonyl CoA activity in the microenvironment of the outer mitochondrial membrane. That would lead to increased CPT-1 activity, increased fatty acid transport, and increased fatty acid oxidation, which would increase ATP. However, we have found damaged and dysfunctional mitochondria in biotin-deficient rat livers, that explain the ATP deficit in the face of increased beta-oxidation (manuscript in preparation).

Overall, all these effects point towards a pattern of sequential changes resulting from the interference with the enzymatic functions of biotin-containing carboxylases. In the initial stage, very early after the vitamin deficit and the enzyme deficiencies, there is a cellular energy shortage with the resulting AMPK activation. This leads, presumably through a network of signal transduction pathways [7], to carbon metabolism gene expression changes and increased insulin sensitivity. Later on, diminished blood insulin would cause lipid metabolism alterations and the utilization of fatty acids as the main metabolic fuel in the biotin-starved animals.

In summary, biotin deficiency has wide ranging effects on intermediary metabolism and gene expression that become evident very early during the biotin deficiency, well before weight loss and undernutrition. Therefore, we propose that specific deficiency effects be studied at weeks 4–5 of the experimental diet. This type of approach might well apply to the lack of other indispensable nutrients.

It is interesting, and enhances the potential importance of this study, the recent findings inducing biotin deficiency experimentally in human volunteers [21]. Furthermore, several of the metabolic and genomic consequences of biotin starvation that we have uncovered might not be unique to biotin but might be more general adaptations that mitigate the shortage of essential nutrients, an issue that we are currently pursuing.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgme.2012.09.005.

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APENDICE 3

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Anaplerosis in cancer: Another step beyond the Warburg effect

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ABSTRACT

Biosynthesis is up-regulated in tumors and thus the demand for anabolic intermediates is increased. The metabolic routes providing the building blocks for macromolecules are thus a very attractive target as they are not normally up-regulated in a normal quiescent cell. Some routes for glycolysis-derived intermediates production have been identified, but these do not constitute the whole pool of biosynthetic molecules in the cell, as many of these derive from mitochondria in the Krebs cycle. Indeed, this metabolic pathway is considered a "biosynthetic hub" from which anabolism is fed. If a metabolite efflux is indeed occurring, anaplerotic reactions must keep a steady supply of substrates. In spite of this obvious relevance of anaplerosis, it has been poorly characterized in the malignant cell context. Glutaminolysis and and pyruvate carboxylation are two pathways that function in an anaplerotic fashion. In spite of the increasing evidence implicating these two processes in cancer metabolism their role as intermediate providers is overlooked. In this review we analyze the implications of an active anaplerosis in cancer and we discuss experimental evidence showing the relevance of these metabolic routes in tumor physiology.

Keywords: Anaplerosis; Biosynthesis; Cancer; Glutaminase; Krebs Cycle; Metabolism; Mitochondria; Pyruvate Carboxylase; Warburg Effect

1. INTRODUCTION

Cancer is a group of diseases considered as a public health issue because of their increasing incidence, the lack of effective treatments for the vast majority of the diagnosed cases, and the high mortality rate of cancer patients. These diseases can affect virtually every organ of the human body and they are characterized by the formation of malignant tumors, which in some cases can spread out to distant sites in the body (metastasis). During the last decades, the research on cancer has intensified and some important discoveries have been made in the field, such as the identification of oncogenes, tumor suppressor genes and the development of targeted antitumor drugs. In spite of these advances and some paradigm-shifting breakthroughs, the clinical progress is minimal and it is possible that additional and more specific therapeutic targets remain to be discovered. Still, one of the main challenges for cancer therapy is to discriminate between malignant and normal cells.

Almost 80 years ago Otto Warburg identified that cancer cells display a different metabolic program regarding their normal counterparts. He observed that tumors have a decreased respiration and an enhanced lactate production in presence of oxygen [Reviewed in 1]. The term coined for this phenomenon was "aerobic glycolysis", nowadays known as "the Warburg effect". Notwithstanding the relevance of these findings, the study of cancer metabolism did not receive much attention in the next decades after its discovery, as most of the work done in the cancer field was focused on genetics and the identification of deregulated signal transduction pathways. Nonetheless, the importance of aerobic glycolysis was demonstrated through the implementation of ^{18}fluoro-deoxyglucose-based positron emission tomography (FDG-PET) [2]. Based on the high glycolytic capacity of tumors and their enhanced glucose uptake, this technique allowed the visualization of tumors *in vivo* resulting from the accumulation of the non-metabolizable probe by malignant cells.

However, a decade ago, the aerobic glycolysis phenomenon was rediscovered. Molecular evidence was gathered linking *bona fide* oncogenes and tumor suppressors with the metabolic alterations previously identified by Warburg (**Table 1**). Some of these findings were in accordance to Warburg's original hypothesis: "in cancer cells an irreversible damage to mitochondria must

Table 1. Metabolic enzymes evaluated as essential for the maintenance of the glycolytic phenotype in tumors (see text for details and references).

Enzyme	Status in tumors	Normal metabolic role	Additional role in cancer
Hexokinase 2 (HXK2)	Over-expressed	Glucose phosphorylation. Flux-controlling step of glycolysis.	Inhibition of apoptosis
Phosphofructokinase-2 (PFKFB3)	Over-expressed	Activation of phosphofructokinase-1. Glycolytic flux enhancement.	Not reported.
Pyruvate kinase M2 (PKM2)	Over-expressed	Production of pyruvate from phosphoenolpyruvate and ATP generation in glycolysis.	Accumulation of glycolytic intermediates for biosynthesis. Promotion of tumor cell proliferation
Glutaminase (GLS)	Overactive	Deamination of glutamine to glutamate.	Stimulation of lipid biosynthesis
Isocitrate dehydrogenase 1 and 2 (IDH)	Mutated	NADP-dependent decarboxylation of isocitrate to α-ketoglutarate.	Production of the oncometabolite 2-hydroxyglutarate
Succinate dehydrogenase (SDH)	Inactive	Succinate oxidation in Krebs cycle producing fumarate	Tumor suppressor
Fumarase (FH)	Inactive	Conversion of fumarate to malate in the Krebs cycle.	Tumor suppressor

exist concomitantly with the enhancement of the glycolytic flux" (see **Tables 1** and **2**) [3]. For instance, a mitochondrial damage was identified in pheochromocytomas and paragangliomas. These tumors carry a mutation in different subunits of succinate dehydrogenase (the mitochondrial complex II), encoded by the genes SDHB, SDHC and SDHD [4]. Indeed, it has been demonstrated that these tumors display the Warburg effect [5]. Similarly, a biallelic inactivation of the FH gene, coding for mitochondrial fumarase, is inactivated in leyomiomas [4]. More evidence came from studies with the tumor suppressor p53, which is commonly mutated in most cancer types. The absence of p53 impairs the induction of SCO2 which is essential for the assembly of cytochrome oxidase subunit II (COX II), thus rendering the cells less dependent on oxidative phosphorylation [6].

The enhancement of glycolysis was explained by the overexpression of all the glycolytic enzymes, especially those ones considered as flux-limiting in the metabolic pathway: hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) [Reviewed in 7]. The embryonic isoforms, are preferentially expressed in tumors, as they allow a higher metabolic flux compared to those expressed in differentiated tissues [7]. Moreover, some of these enzymes are presumably involved in non-conventional roles by endowing tumor cells with the ability to proliferate and overcome programmed cell death mechanisms. For instance, HK seem to dampen the apoptosis-induction mechanism of Bax by interacting with mitochondria [8]; The M2 isoform of PK appears to be essential for sustaining proliferation in conjunction with the epigenetic machinery and its activity may itself constitute a proliferation signal [9,10]. **Table 1** lists some of the enzymes evaluated as essential for the maintenance of the glycolytic phenotype in tumors.

Furthermore, some oncogenes commonly activated in a number of tumors, such as Akt and MYC, are directly involved in the metabolic switch to aerobic glycolysis (**Table 2**) [11]. It was discovered that one of the main drivers of the Warburg effect is hypoxia-induced factor 1-α (HIF-1α), which is a transcription factor that activates the expression of all glycolysis enzymes, it triggers the translocation of glucose transporters to the plasma membrane, and it limits the substrate influx into the Krebs cycle by allowing the expression of pyruvate dehydrogenase kinase (PDK1) [12,13]. HIF-1α is stable and active in hypoxic conditions allowing the cells to switch to an anaerobic metabolism. However, in several tumors this transcription factor is active even in normoxic conditions by diverse mechanisms.

The relationship between metabolism and proliferation signals in cancer is being unveiled as other signaling molecules which are involved in the regulation of proliferation and differentiation (e.g. LKB1, Cyclin D1 and Notch) [14-16] are inextricably linked to energy metabolism as well. **Table 2** lists some of these, along with their reported function for the maintenance of the glycolytic phenotype. The detailed discussion about oncogenes and tumor suppressors and their specific role in tumor metabolism is out of the scope of this review and the reader is referred to excellent reviews in the literature [11,17].

Based on these discoveries, it seemed that cancer cell metabolism could be regarded as a promising specific target. Several therapeutic approaches have been proposed, such as the inhibition of specific glycolysis enzymes (hexokinase, pyruvate kinase and lactate dehydrogenase) [18-20], the reactivation of mitochondrial metabolism by stimulating pyruvate oxidation [20], re-feeding oxidative substrates, and the inhibition of the hypoxic metabolic program by interrupting the expression and/or activity of HIF-1α [21]. Encouraging results have been obtained *in vitro* for some of these strategies, but their effectiveness in cancer patients is still unknown.

Table 2. Signals commonly deregulated in cancer and their possible role for the aerobic glycolysis maintenance.

Protein	Identified status in cancer	Effect on metabolism
PI3K/Akt	Overactive	Glucose uptake enhancement. Increase in glycolytic flux.
MYC	Overactive	Increased lactate production and glutaminolysis.
Cyclin D1	Overactive	Inhibition of mitochondrial function.
HIF-1α	Up-regulated	Stimulates glucose uptake and glycolysis. Down-regulation of pyruvate supply for mitochondrial consumption.
p53	Inactive	Increased glycolytic flux. Stimulation of the pentose shunt pathway. Down-regulation of mitochondrial metabolism.
LKB1	Inactive	Inhibition of protein biosynthesis. Stimulation of mitochondrial biogenesis.
Notch	Overactive	Glycolytic flux enhancement.

During the last five years it has been also demonstrated that cancer cell metabolism goes beyond a simple interplay between glycolysis and mitochondria, in contrast with Otto Warburg's original proposal. A considerable body of evidence shows that some specific tumors rely mainly on oxidative phosphorylation [Reviewed in 22]. Other previously overlooked metabolic pathways are shown to be crucial for tumor survival: glutamine metabolism is highly important for energy obtaining and as a biosynthetic pathway [23], $NADP^+$-dependent isocitrate dehydrogenases are directly implicated in tumorigenesis through the production of a putative oncometabolite (2-oxoglutarate) [24], and *de novo* fatty acid synthesis is overactive in malignant cells [25]. These findings highlight the need to evaluate cancer cell metabolism in a more exhaustive fashion as many other metabolic pathways might contribute to cancer cell survival and be susceptible of pharmacological inhibition for cancer therapy.

Aerobic glycolysis probably endows cancer cells with the ability to generate biosynthetic intermediates from the glycolytic pathway, thus enabling tumors with the ability to proliferate faster and outnumber their normal counterparts [26]. This current "biosynthesis model" envisions an overall predominance of anabolic metabolism, i.e. intermediates for protein, fatty acids and nucleic acid biosynthesis must be supplied at sufficiently high rates. Some of these can be supplied by glycolysis and the pentose phosphate shunt, and in theory, the rest of them should be obtained from the Krebs cycle. The latter constitutes one of the inconsistencies of this model, as Krebs cycle functioning is driven by the mitochondrial respiratory chain activity, which is assumed to be impaired or down-regulated in cancer. However, recent findings evidence the presence of functional mitochondria in tumor cells and show their significant contribution for anabolic flux in malignant cells (see Section 3.1). These discoveries challenge the "Warburg effect" hypothesis and unveil a complex scenario where energy metabolism in cancer goes beyond a simple interplay between glycolysis and mitochondria. Hence, if the Krebs cycle is indeed functioning as a provider for anabolism, the net metabolite efflux must be considerable, given the high demand for macromolecule building blocks during fast-rate proliferation. In order to keep the functioning of the pathway, the efflux rate must be equal to the influx rate. Thus, the substrate provision rate for Krebs cycle must be consequently increased. A constant pyruvate supply to mitochondria (provided by an accelerated glycolysis) is probably not sufficient to compensate for metabolite efflux, and hence some mechanism assuring a steady intermediate replenishment must be operating. Anaplerotic reactions fulfill such function, as in normal cells their physiological role is to ensure a constant supply of intermediates for the continuous metabolic activity of the Krebs cycle. In the context of a tumor cell with an increased demand for biosynthesis intermediates, anaplerosis reactions might be crucial. Although these metabolic pathways are overlooked in cancer metabolism studies, in this review we highlight their possible role in tumor physiology. We also discuss their potential as therapeutic targets in cancer.

2. ANAPLEROSIS

The Krebs cycle is a central pathway in energy metabolism. This metabolic route provides reducing equivalents that serve as substrate for the mitochondrial respiratory chain, thus contributing to energy (ATP) production in the cell. In addition, it is the source of anabolic precursors, such as aminoacids for protein biosynthesis, of citrate used for *de novo* fatty acid synthesis, of succinyl-CoA required for heme group synthesis, and oxaloacetate, which is an essential intermediate for gluconeogenesis [27]. Thus, there is a constant efflux of intermediates from the Krebs cycle, which must be replenished in order to maintain the carbon flux throughout the oxidative metabolism pathway (**Figure 1**). The anaplerotic reactions are those who regenerate these intermediates used for biosynthetic metabolism. Although there are several enzymes that might fulfill this role (see below), two of them are well characterized: pyruvate carboxylase (PC) and glutaminase (GLS) (**Figure 1**) [28].

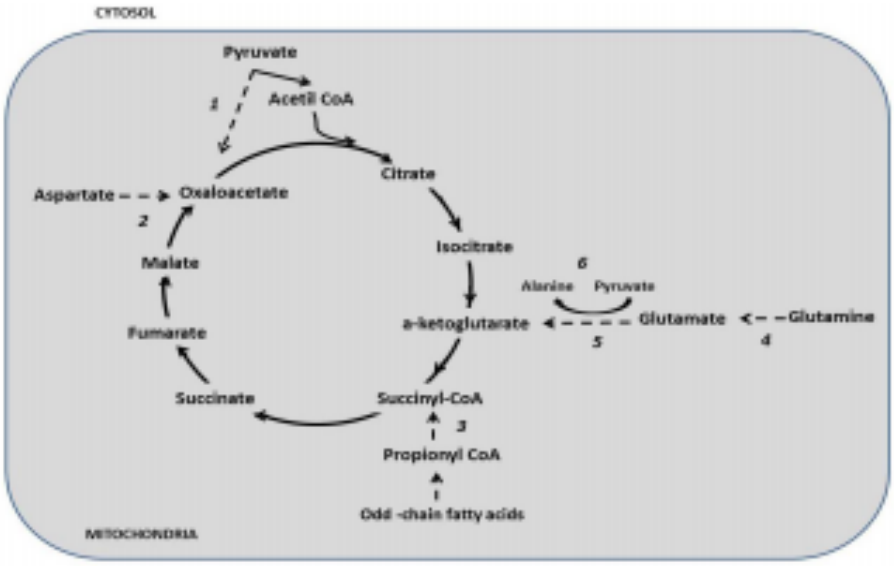

Figure 1. Overview of anaplerotic reactions occurring in mitochondria. Dotted lines indicate the reactions participating in Krebs cycle intermediate replenishment. Pyruvate carboxylase (1) regenerates the oxaloacetate used for citrate synthesis; Aspartate aminotranferase can also produce oxaloacetate, which is the result from the transamination of aspartate (2); Propionyl-CoA carboxylase in the odd-chain fatty acid degradation pathway yield succinyl-CoA (3); Glutaminase deaminates glutamine producing glutamate (4); and the latter serve as the substrate of Glutamate dehydrogenase producing α-ketoglutarate. Alanine aminotransferase also produces α-ketoglutarate from the transamination of glutamate with pyruvate.

Pyruvate carboxylase (PC) is considered the main anaplerotic enzyme and it catalyzes the ATP-dependent conversion of pyruvate to oxaloacetate (**Figure 1**) in a process dependent on biotin as a coenzyme. PC is expressed in a wide variety if tissues, but it is of special relevance in liver, brain, adipose tissue, kidney and pancreatic islets. This enzyme has a relevant role for gluconeogenesis, glycerol synthesis and the down-regulation of fatty acid synthesis [29] (See below). Although there is only one gene coding for PC, there are several variants resulting from alternative splicing. These different forms might be expressed depending on the physiological state of the cell and their specific functionality remains unclear [30].

PC anaplerotic function is essential for insulin secretion since it maintains the pool of Krebs cycle intermediates in order to allow the complete oxidation of glucose to CO_2 and H_2O. This is important, as it is widely known that β-pancreatic cells must oxidize glucose from the blood stream in order to increase the ATP/ADP ratio that triggers insulin secretion [31]. Besides its direct anaplerotic role, PC is also important for the generation of a NADPH pool that might serve as substrate for anabolic pathways [30].

De novo glucose synthesis (gluconeogenesis) is the most studied function of PC in liver. This biosynthetic process is stimulated by glucagon, which enhances pyruvate conversion to oxaloacetate. In a second step, phosphoenol pyruvate carboxykinase (PEPCK) uses the latter to produce phosphoenolpyruvate, which is required for the gluconeogenic pathway [32]. Nonetheless, PC possibly has an anaplerotic role in liver as demonstrated in a NMR analysis in which it was shown that pyruvate carboxylase is the major pathway for pyruvate influx into the Krebs cycle. This study measured an anaplerotic flux seven-fold higher in comparison with pyruvate oxidation [33].

In central nervous system, anaplerotic reactions are essential for neurotransmitter synthesis, such as glutamate and γ-aminobutyric acid (GABA). Both of them can be produced from α-ketoglutarate, which is normally consumed during Krebs cycle functioning. PC role is predominant in astrocytes, where it compensates the constant efflux of intermediates because of glutamate and GABA synthesis [34]. The glutamate synthesized by astrocytes must reach the neurons and it is released in the form of glutamine, which is reconverted to glutamate by the latter in a global process called "the glutamate/glutamine cycle". It has been demonstrated that the glutamine flux is dependent on the activity of astrocyte PC [35]. Thus, normal brain functioning rely on PC activity.

Glutaminase (GLS) is a mitochondrial enzyme that catalyzes the deamination of glutamine to produce ammonium ions and glutamate (**Figure 1**). There are two

glutaminase isoforms: the K-type, the main isoform detected in kidney, and the L-type, which is predominant in liver. The former is the ubiquitous and detected in non-hepatic tissues. In spite of the long-time consideration that the K-form was the only one present in brain, both isoforms can be found in this organ and they might play a differential metabolic role [36]. In liver, this enzyme can be part of an anaplerotic route to produce α-ketoglutarate in conjunction with aspartate-alanine aminotransferase (ALT), which catalyzes its transamination with pyruvate to yield α-ketoglutarate and alanine. In brain, a similar pathway has been identified as part of the catabolism of the neurotransmitter glutamate, which can also be transaminated in a similar manner by astrocytes [37].

Some other metabolic pathways can provide for Krebs cycle intermediates and thus could be classified as anaplerotic. Such is the case for the reaction catalyzed by adenylosuccinate synthetase in skeletal muscle that synthesizes fumarate and AMP from adenylosuccinate in the purine nucleotide cycle. Other pathway for oxaloacetate synthesis is the transamination of aspartate catalyzed by aspartate aminotransferase (AST), which is of special importance in liver and skeletal muscle. The catabolism of odd-chain fatty acids can derive in the production of succinyl-CoA in a reaction catalyzed by the biotin-dependent enzyme propionyl-CoA carboxylase.

3. ANAPLEROSIS IN CANCER

3.1. The Existence of Functional Mitochondria in Cancer?

According to Warburg's original hypothesis, mitochondria ought to be irreversibly damaged [3]. Although this seems to be the case for some malignancies such as phaeochromocytomas and leiomyomas (see Section 1), it has been well documented that several cell lines derived from a wide variety of tumors rely mainly on oxidative phosphorylation (Reviewed in [19]). For instance, breast cancer cells obtain their energy mainly from mitochondria [38], and this seems to be the case for other epithelial cancers as well, for which the activity of cytochrome oxidase (complex IV) is up-regulated causing a hyperactivation of mitochondrial metabolism [39]. A similar feature has been observed in liver and brain cancer, where a fully functional oxidative phosphorylation has been characterized [40,41]. Mitochondrial oxidative metabolism probably prevails in brain tumors *in vivo*, as it has been demonstrated that glioblastomas display a preference for glucose oxidation when implanted in mouse brain [42]. Further evidence comes from the study of lipolysis in cancer, as the hydrolysis of triglycerides is essential for cancer-associated cachexia induced by lung carcinoma and melanoma implantation in mice [43]. This clearly points out that cancer cells can use fatty acids (FA) as fuels *in vivo*. A similar mechanism occurs in ovarian cancer, as adipocytes provide malignant cells with lipids as substrates in order to support metastasis and invasion [44]. Of note, FA metabolism is carried out through the β-oxidation pathway in mitochondria, which is a process highly dependent on a functional oxidative phosphorylation.

Furthermore, some tumors seem to display metabolic flexibility, i.e. they can switch between aerobic glycolysis and oxidative metabolism depending on their microenvironment [45,46]. These cells trigger adaptive mechanisms (e.g. mitochondrial biogenesis and the formation of an extensive mitochondrial network) in order to optimize their oxidative phosphorylation according to the substrate supply and their energy demand [47]. In the vast majority of the *in vitro* studies, functional mitochondria and metabolic adaptation mechanisms could have been masked because of the cell culture conditions, in which a defined metabolic program is imposed on the cells as a function of the media composition and the optimal growth conditions. However, it has been shown that tumor microenvironments are highly heterogeneous due to glucose and oxygen gradients [47]. In the *in vivo* scenario, the metabolic transitions could be crucial for tumor physiology. For instance, experimental evidence shows a "lactate shuttling" between glycolytic and oxidative cells in lung carcinoma and colorectal adenocarcinoma [48]. This might facilitate a symbiosis between different populations within a tumor: the glycolytic cells would function as the "substrate providers" for those who are able to synthesize ATP through oxidative phosphorylation. A similar phenomenon, termed "the reverse Warburg effect", has been characterized between stromal fibroblasts and breast cancer cells where the former provide lactate, ketones, glutamine and free fatty acids (being the four of them substrates for the mitochondrial respiratory chain) to the oxidative malignant cells [49].

Originally, Warburg's observations posed a conundrum for researchers in the cancer field: how come a highly proliferative cell would support its proliferation using mainly glycolysis, a relatively inefficient pathway in terms of energy yield? One possible explanation is that, even if glycolytic ATP production is lower, it might be accelerated in order to meet the energy demand imposed by the fast proliferation rate of tumors [50]. Another non-exclusive possibility is that glycolytic flux enhancement brings as consequence the accumulation of metabolites subsequently used as building blocks for biosynthetic processes [26]. Some experimental evidence supports this theory. For instance, there is an alternative glycolytic pathway that uncouples ATP production at the pyruvate kinase step in order to promote metabolite accumulation for biosynthetic purposes [51]. In this regard,

a possible source of intermediates for serine and glycine biosynthesis might be at the phosphoglycerate dehydrogenase step, which function as a shunt from glycolysis and whose gene it is commonly amplified in tumors [52]. Moreover, the overall glycolytic intermediate accumulation may be linked to the tyrosine kinase deregulated signaling pathways commonly observed in tumors [53].

Nevertheless, in order to cope with the increased demand for biosynthesis intermediates, those ones derived solely from glycolysis are most probably insufficient to sustain a high anabolic flux. In normal cells the overall anabolic flux is heavily dependent on mitochondria and the Krebs cycle functioning (see Section 2). In support of this hypothesis, it is known that some molecules previously identified as essential for cancer cell proliferation, such as citrate [25] and heme prosthetic groups [54], are exclusively derived from Krebs cycle intermediates. Although anaplerosis in cancer remains ill-defined, recent findings suggest that mitochondria-localized metabolism is of special relevance for cancer. Some of these processes are linked to anaplerotic pathways and their relevance is beginning to be elucidated.

3.2 Glutaminolysis

Based on FDG-PET scan observations it was previously considered that tumors avidly consumed only glucose. This observation was supported by the detected overexpression of glucose transporters in several cancer cell lines [55]. Nevertheless, glucose is not the only highly consumed substrate in tumors, as *in vivo* studies demonstrated a considerable glutamine uptake by malignant overgrowths [56]. Although glutamine *per se* can serve as a biosynthetic intermediate (for a review see [57]), glutaminolysis is nowadays a well-characterized process inherent of several tumors. In accordance to this, both L- and K-type glutaminase isoforms were found to be simultaneously expressed at high levels in leukemia cells, although the predominance of the latter was evident [58]. The relevance of the K isoform was further verified in lymphomas and on colorectal adenomas and carcinomas [59]. Based on these findings, it has been suggested that K-glutaminase has a defined role for tumor cell proliferation, although more studies need to be conducted in this regard.

The overexpression of glutaminase is observed in other malignancies as well, such as liver, brain and breast tumors [60-62]. Of note, these tumors possess an active mitochondrial metabolism *in vitro* as characterized by other studies (see Section 3.1).

Glutamine uptake and metabolism have been detected in proliferating non-tumor lymphocytes and in HeLa cells. In these studies it was discovered that glutaminase expression and activity is regulated according to the cell cycle stage, being most active during the replication (S) phase [63,64]. Interestingly, during this same cell cycle phase, mitochondrial biogenesis and oxidative phosphorylation are up-regulated [65]. Furthermore, it has been demonstrated that glutaminase expression depends on MYC, which is a commonly activated oncogene in a wide variety of tumors [66]. It is noteworthy the fact that MYC also promotes mitochondrial biogenesis [67]. This experimental evidence highlights the dependence of glutamine metabolism on functional mitochondria. Moreover, it points out the possible existence of a specific program in which MYC, mitochondrial biogenesis and anaplerosis are coordinated as central elements of energy metabolism in some malignancies (see below).

As pointed out earlier, glutaminase serves to replenish α-ketoglutarate to the Krebs cycle. It has been proposed as the only anaplerotic route in tumors [68]. Although there are no studies focused to elucidate the anaplerotic function of glutaminolysis in cancer, recent reports indirectly demonstrate its implication in metabolic processes in some malignant cells. For instance, glutamine consumption feeds the Krebs cycle yielding fumarate, malate and citrate in an overall process coordinated by MYC in Burkitt lymphoma cells [69]. Citrate can be exported to cytosol where it serves as substrate of ATP citrate lyase (ACL) producing cytosolic acetyl-CoA, which is required as an intermediate for *de novo* fatty acid synthesis. ACL is found to be essential for tumor cell survival as acetyl groups provider for histone acetylation in brain cancer [25], and our own findings demonstrate that ACL is activated in primary glioblastomas but not in normal human astrocytes (Diaz-Ruiz, R. Unpublished results). The involvement of anaplerosis as a compensating mechanism of mitochondrial citrate production in brain cancer is an issue that will be subject of further research.

In this regard, another report proposed a "reductive" metabolic pathway in which the α-ketoglutarate derived from glutamine metabolism is used in order to produce cytosolic acetyl-CoA for lipid synthesis in a process dependent on the activity of the cytosolic form of isocitrate dehydrogenase (IDH1) [23]. There are some inconsistencies with this model, as they propose IDH1 as a central element of this reductive pathway. However, glutamine needs to be processed in mitochondria in order to produce α-ketoglutarate, whether if it is being supplied through the oxidation of the glutamate produced by glutaminase activity, or through glutamate transamination by alanine aminotransferase (ALT). And even if α-ketoglutarate is being carboxylated to isocitrate, the latter is not used as lipogenic precursor, being citrate the one used for such purposes. Moreover, citrate efflux from mitochondria is mandatory for cytosolic acetyl-CoA production and this feature has been clearly identified in hepatomas [70]. An alternative explanation is that glutaminolysis is functioning in an anaplerotic fashion in or-

der to compensate mitochondrial citrate efflux and IDH1 may be supplying the NADPH cofactors required by two of the six steps in *de novo* fatty acid biosynthesis (**Figure 2**).

According to the reductive carboxylation hypothesis, it is proposed that a reversed Krebs cycle is the source of citrate and acetyl-CoA for biosynthesis [71]. The drawback in this proposal is of thermodynamic nature, as the reversal of the Krebs cycle implies a build-up of intramitochondrial NADH that would inhibit the pyruvate dehydrogenase complex thus inducing the dampening of pyruvate supply to mitochondria thereby inhibiting the Krebs cycle. It has been demonstrated by other reports that exogenous glutamine yields citrate [69], therefore it is more plausible that cytosolic citrate and acetyl-CoA are derived from the normal functioning of the Krebs cycle supported by an enhanced anaplerosis from glutamine (**Figure 2**).

In spite of the caveats mentioned above, it is clear that glutamine metabolism is being carried out in order to contribute with intermediates for biosynthesis in cancer. A more detailed study focusing on its anaplerotic role might clarify the inconsistencies of the reductive carboxylation hypothesis and it could offer some insight into the overall tumor energy metabolism. Anaplerotic glutaminolysis could be a therapeutic target as well. Several *in vitro* studies show that glutaminase inhibition affect tumor cell viability [72,73]. Moreover, by interfering with the signaling routes linked with glutaminase it is possible to selectively eliminate malignant cell such as lymphomas and breast cancer cells [74]. Although anaplerosis assessments in these conditions are lacking, most probably they are being affected as well. In order to confirm this, studies on the impact of anaplerosis inhibition on tumor cell viability need to be addressed.

3.3 Pyruvate Carboxylase

As mentioned in Section 2, pyruvate carboxylase (PC) is the main anaplerotic enzyme in normal cells. Early reports proposed that it has no participation in tumor cell metabolism as its activity is low in gliomas [75] and also in hepatomas [76]. However, an up-regulation of biotin-binding enzymes have been found in pancreatic tumors, particularly in ductal adenocarcinomas [77], increased levels of PC have been identified in several tumor cell lines [78], and perfused livers of tumor-bearing

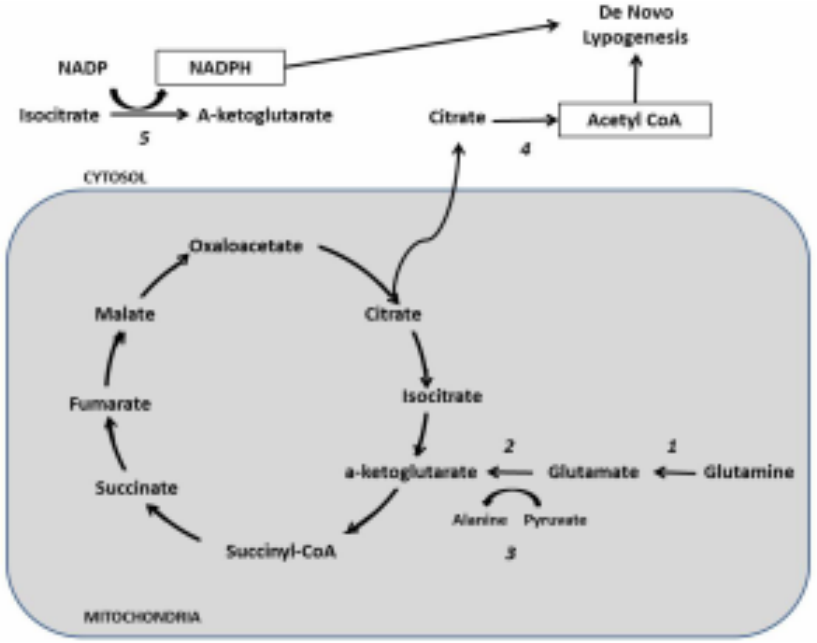

Figure 2. Proposed model for glutamine reductive metabolism for fatty acids biosynthesis based on anaplerotic reactions. Glutaminase (1) is the entry point of this pathway by catalyzing the deamination of glutamine to glutamate, which subsequently enters the Krebs cycle as α-ketoglutarate after either being oxidized by Glutamate dehydrogenase (2); or after being transaminated by Alanine aminotransferase (3); Citrate is generated after substrate influx. The latter can be exported to cytosol where it serve as the substrate of ATP Citrate lyase (4); producing the acetyl-CoA required for *de novo* fatty acid synthesis. Isocitrate dehydrogenase 1 (5) generates NADPH, the other substrate needed for fatty acid biosynthesis.

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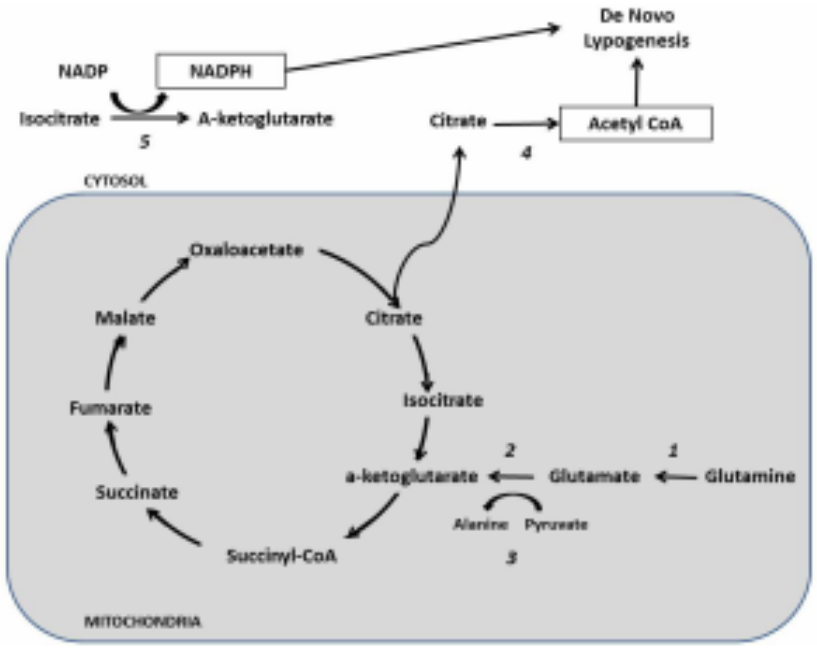

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but to the tumorigenic process as well (e.g. IDH1 and IDH2). All these discoveries force us to adopt more rigorous approaches in order to clearly identify potential "druggable" targets that might be used in future therapies.

The current biosynthesis model suggests anabolic processes as an attractive therapeutic target in cancer, but we just recently began to understand the anabolic deregulations in malignancies. The steady supply of metabolic intermediates is mandatory in order to synthesize *de novo* macromolecules. In this scenario, anaplerosis is crucial by keeping the availability of anabolic precursors. Based on recent discoveries, it is becoming clear that both glutaminolysis and pyruvate carboxylation can fulfill this role in tumors. Furthermore, anaplerosis might be implicated in important aspects of tumor physiology such as metastasis. Based on this and given the therapeutic implications, anaplerotic pathways in cancer deserve further study.

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