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Programa de Doctorado en Ciencias Biomédicas

"Participación de la fructosa 1,6-bifosfato en la inducción del efecto Crabtree en la levadura Saccharomyces cerevisiae"

TESIS

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1. Lista de abreviaciones y simbología.

$\Delta\mu_{H+}$: Potencial químico de los protones.

$\Delta\Psi$: Potencial transmembranal mitocondrial.

$\Delta G_{O/R}$: Potencial de óxido-reducción.

ΔG_p: Potencial fosfato.

Δp: Fuerza protónmotriz.

ADH: Alcohol deshidrogenasa.

ANT: Translocador de nucleótidos de adenina.

BSA: Albúmina sérica de bovino.

CCCP: Carbonil cianuro 3-clorofenilhidrazona.

D.E: Desviación estándar.

EGTA: Acido etilen glicol-bis(2-aminoetileter)-N,N,N'.N'-tetracético.

F16bP: fructosa 1,6-bifosfato.

F6P: fructosa 6-fosfato

$Fe(CN)_6^{3+}$: Ferricianuro.

G6P: glucosa 6-fosfato.

HK: Hexocinasa.

JO_2: Flujo respiratorio.

L: Coeficiente fenomenológico.

LDH: Lactato deshidrogenasa.

PDC: Piruvato descarboxilasa.

PDH: Complejo de la piruvato deshidrogenasa.

PDHK: Cinasa de la piruvato deshidrogenasa.

PDP: Fosfatasa de la piruvato deshidrogenasa.

Pi: Fosfato inorgánico.

q: Constante de acoplamiento.

Rh-123: Rodamina 123.

T6P: Trehalosa 6-fosfato.

TMPD: N,N,N',N'-tetrametil-p-fenilendiamina.

Tris: Tris(hidroximetil) aminometano.

TPS1: Trehalosa 6-fosfato sintasa.

VDAC: Canal aniónico dependiente de voltaje.

X: Fuerza termodinámica.

2. Resumen.

Cuando la levadura *Saccharomyces cerevisiae* crece de forma aerobia, la respiración es inmediatamente reprimida al añadir glucosa al medio de cultivo. A este fenómeno se le ha denominado "efecto Crabtree". Se ha reportado que la inhibición del flujo respiratorio es concomitante con la acumulación citoplásmica de las hexosas fosfato derivadas de la glucólisis. En este trabajo se empleó a *S. cerevisiae* para investigar los eventos regulatorios a corto plazo asociados al efecto Crabtree y el posible papel de las hexosas fosfato en la inhibición de la respiración. Empleando mitocondrias aisladas de levadura se encontró que la glucosa 6-fosfato y la fructosa 6-fosfato estimulan el flujo respiratorio. Este efecto fue contrarrestado por concentraciones fisiológicas de fructosa 1,6-bifosfato, la cual además inhibe la respiración en ausencia de las otras dos hexosas fosfato. Esto ocurre *in situ*, ya que el efecto mediado por la fructosa bifosfato también se observó en esferoplastos permeabilizados de levadura. La represión del flujo respiratorio mediada por la fructosa 1,6-bifosfato es debida a una inhibición de la actividad de los complejos respiratorios III y IV. Los resultados sugieren que la fructosa 1,6-bifosfato podría ser uno de los inductores del efecto Crabtree en la levadura. En mamíferos, también es posible que esta hexosa bifosfato regule el metabolismo de las células tumorales, en donde también se ha observado el efecto Crabtree.

3. Introducción.

3.1 Las vías metabólicas y su interacción.

Las células requieren de energía para el mantenimiento de procesos esenciales tales como la síntesis de proteínas, lípidos y ácidos nucleicos. De esta forma, aseguran su supervivencia aún en condiciones adversas. La producción de energía es función del estado energético de la célula, el cual es definido por el balance entre su producción y su consumo, es decir, entre el catabolismo y el anabolismo. La interrelación entre ambos tipos de metabolismo depende a su vez de la disponibilidad de nutrientes (en los microorganismos) o de estímulos hormonales (en el caso de organismos multicelulares).

A través del catabolismo, la célula genera energía empleando secuencias de reacciones químicas que degradan a los nutrientes y que conservan la energía contenida en ellos en forma de intermediarios químicos específicos: el trifosfato de adenosina (ATP) y el NAD(P)H. Estos son posteriormente empleados para llevar a cabo procesos de biosíntesis (anabolismo), de mantenimiento de las estructuras celulares y de otras funciones específicas de las células (por ejemplo, la motilidad celular).

Por lo tanto, la intercomunicación de las vías metabólicas debe modificarse de acuerdo a las necesidades energéticas de la célula (1). A nivel molecular existen distintos mecanismos por los cuales puede regularse esta interacción: modulación alostérica de la actividad de enzimas clave, modificaciones post-traduccionales, control del flujos metabólicos y la canalización de sustratos (2). También existe una regulación termodinámica. De hecho, es posible cuantificar y analizar el metabolismo energético celular (ver sección 3.2) y explicar cómo están acoplados los flujos metabólicos a través de distintas vías al emplear las herramientas de la termodinámica fuera del equilibrio (ver sección 3.2).

La intercomunicación entre vías metabólicas puede verse alterada en condiciones patológicas como en la diabetes o el cáncer. Por esto, es importante analizar este aspecto del metabolismo, ya que una descripción detallada de estos fenómenos ayudaría a desarrollar estrategias terapéuticas más efectivas para el tratamiento de enfermedades asociadas con trastornos metabólicos.

3.2 El metabolismo energético de la célula

La capacidad de la célula para regenerar el ATP y los equivalentes reductores define el estado energético de la célula. Con base en la termodinámica fuera del equilibrio, estos estados pueden ser cuantificados por medio de dos parámetros: el potencial fostato (ΔG_p) y el potencial de óxido-reducción ($\Delta G_{O/R}$) (2, 3). Estos potenciales también son denominados como "fuerzas termodinámicas", y son matemáticamente definidos por las ecuaciones 1 y 2:

$$\Delta G_p = \Delta G_p^{0'} + RT \ln [ATP]/[ADP]\,[Pi] \quad \text{(Ec.1)}$$

$$\Delta G_{O/R} = \Delta G_{O/R}^{0'} + RT \ln [NAD(P)H]/[NAD(P)^+] \quad \text{(Ec. 2)}$$

$\Delta G_p^{0'}$ y $\Delta G_{O/R}^{0'}$ son los cambios de energía libre de las respectivas reacciones en condiciones estándar a pH 7.0. Debido a que en la célula ambas reacciones están alejadas del equilibrio, el valor numérico de las fuerzas termodinámicas difiere de sus valores de referencia en condiciones estándar (4). Haciendo uso de la termodinámica fuera del equilibrio también se puede definir una relación matemática entre el flujo metabólico (J) y las fuerzas termodinámicas (X):

$$J = \sum LX \quad \text{(Ec. 3)}$$

En donde L es una constante de proporcionalidad denominada "coeficiente fenomenológico". En el caso de una célula, X representa ΔG_p o $\Delta G_{O/R}$. Puesto que el flujo metabólico está definido por ambos parámetros y no por sólo uno de ellos, se dice que ambas fuerzas están acopladas. Esto es cuantificado por medio de una "constante de acoplamiento" (q) (4). De esta forma, la modificación de cualquiera de estos dos parámetros (ΔG_p o $\Delta G_{O/R}$) afectará el metabolismo y la interacción entre las vías metabólicas. Por ejemplo, para favorecer la fermentación se requiere de una disminución del potencial fosfato y un incremento en la concentración de NAD^+ citoplásmico (3). En la célula, estas dos fuerzas son a su vez definidas por el balance entre los procesos que producen energía y aquellos que la consumen (ver sección 3.1).

En las células eucariontes existe un organelo especializado en la producción de energía: la mitocondria. Es en este sitio donde se lleva a cabo principalmente la síntesis de ATP y la re-oxidación del NADH citoplásmico. Por lo tanto, su función es crucial para el mantenimiento de los potenciales fosfato y de óxido reducción.

La fosforilación oxidativa es el mecanismo por el cual se lleva a cabo la producción de ATP en la mitocondria (2). En la membrana interna de este organelo existen tres complejos multi-proteicos (dos en el caso de las mitocondrias de levadura) que llevan a cabo la oxidación de equivalentes reductores acoplada a la translocación de protones hacia el espacio intermembranal, creando así una diferencia en el potencial electroquímico de esta especie química ($\Delta\mu^{+}$). Esto es aprovechado por la ATP sintetasa mitocondrial para catalizar la conversión de ADP y Pi en ATP (5). En términos termodinámicos, el $\Delta G_{O/R}$ es transducido a ΔG_p a través de un intermediario: la fuerza protónmotriz (Δp) (5).

Además de la fosforilación oxidativa, existe otra forma de mantener el potencial fosfato en la célula: a través de las reacciones de fosforilación a nivel de sustrato (6). En la glucólisis existen dos enzimas que catalizan esta reacción: la fosfoglicerato cinasa y la piruvato cinasa. De esta forma, cuando la función mitocondrial es reprimida, el metabolismo energético se lleva a cabo principalmente en el citoplasma de la célula. Esto ha sido observado en distintos modelos experimentales: en las células tumorales (7), en células con una alta tasa de proliferación (8), en algunas bacterias (9) y en ciertas especies de levaduras (10) (ver sección 3.4).

3.3 El metabolismo fermentativo en la levadura

Cuando se encuentra en su ambiente natural, la levadura *Saccharomyces cerevisiae* está sujeta a fluctuaciones en la disponibilidad de nutrientes. Por lo tanto, este microorganismo posee la habilidad de adaptar su metabolismo energético de acuerdo a la naturaleza y a la concentración de los sustratos presentes en el medio. Experimentalmente se ha observado que en presencia de glucosa, *S. cerevisiae* emplea la glucólisis como su principal vía metabólica. En cambio, cuando crece en un medio que contiene fuentes de carbono no fermentables (tales como acetato, etanol, glicerol o lactato), lleva a cabo producción de su energía a través de la fosforilación oxidativa (metabolismo oxidativo).

La glucosa es la fuente de carbono preferida por la levadura, cuando se añade a cultivos de células que crecen de forma oxidativa, éstas dejan de respirar inmediatamente y experimentan una transición hacia el metabolismo fermentativo (11). A este proceso se le ha denominado represión catabólica (12). Esta adaptación se logra por medio de mecanismos de regulación que actúan a corto y a largo plazo (11).

Con respecto a estos mecanismos, se han observado cambios en el patrón de expresión de los transportadores de glucosa de la membrana plasmática (13). Al mismo tiempo, se incrementan los niveles de prácticamente todas las enzimas de la glucólisis y de la piruvato descarboxilasa (14). Esta última enzima cataliza la conversión de piruvato a acetaldehído, el cual es reducido por la alcohol-deshidrogenasa (ADH) para producir etanol. Esta vía es importante para el mantenimiento del $\Delta G_{O/R}$ en condiciones de fermentación, ya que contribuye a la regeneración del NAD^+ requerido por la glucólisis (15).

Durante la fermentación, los genes que codifican a las proteínas del metabolismo oxidativo dejan de expresarse (16). Por ejemplo, en presencia de glucosa se reprime la expresión del citocromo c (17) y de algunos componentes del complejo respiratorio II (succinato deshidrogenasa) (18). También se han observado disminuciones en la actividad de enzimas del ciclo de Krebs como la malato deshidrogenasa y la isocitrato deshidrogenasa (19).

Los procesos de regulación transcripcional se han sido descrito con detalle y se han identificado las cascadas de eventos regulatorios que conducen a la represión catabólica, así como a algunos de los elementos que participan en ellas (12, 20). Por otra parte, los mecanismos que actúan a corto plazo no se han esclarecido. En relación a esto, se ha encontrado que existen algunas vías de transducción de señales que son activadas en respuesta a glucosa (21). Sin embargo, aún no se sabe exactamente cómo están coordinados estos sistemas y tampoco se han identificado sus blancos relevantes.

Un tipo de mecanismo regulatorio a corto plazo puede ser la modificación de los potenciales termodinámicos en la célula (ver sección 3.2). Desafortunadamente, en el caso de la levadura aún no se ha llevado a cabo la cuantificación de ΔG_p o de $\Delta G_{O/R}$. También se ha propuesto la existencia de intermediarios metabólicos que podrían funcionar como “mensajeros” en la respuesta a glucosa a corto plazo (ver sección 3.5.6).

3.4 El efecto Crabtree

El efecto Crabtree se define como la represión inmediata de la fosforilación oxidativa en respuesta a la presencia de glucosa y fue inicialmente descrito en células tumorales (7). Esto podría ser consecuencia de la reprogramación metabólica que sufre este tipo de células, ya que poseen un metabolismo fermentativo a pesar de la presencia de oxígeno (un fenómeno denominado "glucólisis aerobia" o "efecto Warburg") (22). Se ha propuesto que este fenotipo confiere a los tumores la capacidad de evadir los mecanismos de muerte celular programada (23, 24) y de adaptarse a condiciones potencialmente dañinas para las células tumorales, como la hipoxia (25). La caracterización de los mecanismos que originan el efecto Crabtree es importante, ya que podrían indicar posibles blancos terapéuticos para el tratamiento del cáncer (26,27).

Se ha encontrado que el efecto Crabtree no está restringido a las células tumorales, pues se ha observado en algunas bacterias (9) y en ciertas especies de levadura como *Saccharomyces cerevisiae* (10). En todos estos casos, la presencia de glucosa induce una transición hacia el metabolismo fermentativo. Debido a la analogía con el efecto observado en tumores, se propuso que estos microorganismos podrían servir como modelo para encontrar los mecanismos del efecto Crabtree y también para la posible evaluación de fármacos antitumorales que aprovechen el metabolismo de los tumores como blanco (9,27).

Las causas del efecto Crabtree son desconocidas. En las células tumorales se ha propuesto que es causado por la sobreexpresión de las enzimas de la glucólisis y un daño irreversible en las mitocondrias (22). Sin embargo, no todas las líneas celulares tumorales presentan estas características (28). En las levaduras, la sobreexpresión de las enzimas de la glucólisis tampoco es un pre-requisito para la aparición del efecto Crabtree, ya que éste ocurre de forma inmediata en una célula que posee un metabolismo puramente oxidativo. Existen diversas teorías que intentan explicar los eventos que dan origen al efecto Crabtree (Revisado en 29). Debido a que éstas han sido propuestas para las células tumorales, a continuación se hace una breve revisión de ellas y se hace una comparación con lo que se ha observado en *Saccharomyces cerevisiae*.

3.5 Los mecanismos que conducen al efecto Crabtree.

3.5.1 La limitación de ADP y de Pi: Posible papel del potencial fosfato.

La hipótesis que ha sido más aceptada es que el ADP citoplásmico libre disminuye después de la adición de glucosa, lo que provoca una competencia entre las enzimas de la glucólisis y la mitocondria por este nucleótido (30, 31, 32). Efectivamente, cuando se mide la respiración de mitocondrias aisladas en ausencia de ADP, el flujo respiratorio es muy bajo debido que no se está llevando a cabo la fosforilación oxidativa. A este estado estacionario se le denomina "estado de no fosforilación" o "estado 4" (33).

Para demostrar la hipótesis de la limitación del ADP se reconstituyó la glucólisis *in vitro* en presencia de mitocondrias aisladas y se demostró que al aumentar la concentración de las enzimas glucolíticas la respiración de las mitocondrias decrecía (31). Resultados similares fueron obtenidos empleando sólo la enzima piruvato cinasa para consumir ADP (30). Lo anterior también llevó a proponer que el incremento en la actividad de esta enzima podía ser clave para la inducción del efecto Crabtree (30). Esto también podría aplicarse al efecto Crabtree en la levadura, ya que se ha observado que esta enzima se sobreactiva durante el metabolismo fermentativo en *S. cerevisiae* (34).

A pesar de esto, también existe evidencia experimental que descarta esta hipótesis. En células tumorales se ha cuantificado la concentración de ADP después de la adición de glucosa y se encontró que ésta no cambia (35). Se han observado resultados similares para *S. cerevisiae* (36). Incluso, en este caso se ha reportado que los niveles de ADP incrementan al iniciar la fermentación (37).

Con respecto a las determinaciones de ADP es importante considerar que existe una fracción de los niveles totales de nucleótidos de adenina que se encuentra unida a proteínas (38). Por lo tanto, sólo la fracción libre podría participar en esta competencia entre las enzimas de la glucólisis y la mitocondria. Sin embargo, la cuantificación de esta fracción no ha sido considerada en los estudios del efecto Crabtree. Esto podría confirmar si en realidad existe esta limitación de ADP durante la represión del metabolismo respiratorio.

Otro punto importante a considerar es que el translocador de nucleótidos de adenina de la mitocondria (ANT), que es la proteína que capta el ADP citoplásmico para transportarlo hacia la matriz mitocondrial, tiene una afinidad mucho más alta por el ADP (K_m = 1-4 μM) que las

enzimas de la glucólisis ($K_m \approx 300$ μM) (38). Lo anterior implica que aunque los niveles de ADP disminuyan, la mitocondría capta al nucleótido con mayor avidez que la fosfoglicerato cinasa y la piruvato cinasa. Esto cuestiona la validez de la hipótesis de la limitación de ADP.

Se ha propuesto que el verdadero inductor del efecto Crabtree es el Pi (39). En relación a esto, se encontró que cuando las células tumorales eran incubadas en presencia de bajos niveles de Pi se observaba un efecto Crabtree, mismo que podía ser eliminado al incrementar la concentración de este anión en el medio de incubación (39). Una posible explicación es que al disminuir el Pi se esté afectando la glucólisis debido a la inhibición de la reacción catalizada por la gliceraldehído 3-fosfato deshidrogenasa. Puesto que en esta reacción se produce NADH, habría una disminución concomitante en la provisión de sustrato respiratorio para las mitocondrias. Otra consecuencia de la disminución de fosfato es la limitación de uno de los sustratos para llevar a cabo la fosforilación oxidativa. En ambos casos, disminuiría la respiración. Apoyando esta hipótesis, se ha encontrado que después de la adición de glucosa existe una disminución de Pi citoplásmico tanto en células tumorales (35) como en levaduras (40).

El Pi, el ADP y el ATP son especies químicas que definen el potencial fosfato en la célula (ΔG_p) (Ec 1). Como se mencionó anteriormente, este parámetro determina el estado energético celular y el flujo a tavés de los procesos metabólicos que se llevan a cabo en ella (ver sección 3.2). Debido a esto, se propuso que el efecto Crabtree no es consecuencia de los cambios de Pi o de ADP *per se*, sino que es inducido por alteraciones en el ΔG_p (35, 41). Para que el flujo glucolítico incremente tiene que disminuir el valor del ΔG_p, lo cual se logra al disminuir los procesos que producen ATP (3). Con respecto a esto, se ha observado que en células tumorales el ΔG_p efectivamente disminuye durante el efecto Crabtree (42).

3.5.2 La reoxidación de los equivalentes reductores citoplásmicos y el potencial de óxido-reducción.

Se ha propuesto que la disminución de la respiración observada en el efecto Crabtree es inducida por la sobreactivación de las enzimas citoplásmicas que reoxidan al NADH. Esto afectaría la provisión de sustratos respiratorios a la mitocondria. En las células de mamífero, el principal mecanismo de re-oxidación de NADH citoplásmico es por medio de la enzima lactato deshidrogenasa (LDH), que cataliza la reducción de piruvato a lactato. Se ha observado que en

las células tumorales existe una sobreexpresión de esta enzima (43). Cuando se interrumpe la expresión de la LDH se elimina el efecto Crabtree (44).

En *S. cerevisiae*, existen dos isoformas de la lactato deshidrogenasa y están localizadas en la mitocondria (45). Una de éstas participa en la cadena respiratoria donando los electrones provenientes de la oxidación del lactato al citocromo c (46). Aunque se ha identificado un gen que codifica para una isoforma citoplásmica de la LDH, su función en el metabolismo fermentativo es aún desconocida (47).

En la levadura, el principal mecanismo de reoxidación de NADH citoplásmico es la síntesis de etanol. Esto se lleva a cabo por la actividad secuencial de dos enzimas: la piruvato descarboxilasa (PDC) y la alcohol deshidrogenasa (ADH) (6). La Pdc cataliza la conversión de piruvato a acetaldehído, el cual es reducido a etanol por la ADH. Durante la fermentación se ha observado un incremento en los niveles de expresión de ambas enzimas (11,12).

Inmediatamente después de la adición de glucosa, hay un incremento en la actividad de la piruvato descarboxilasa (10). Con base en lo anterior, se propuso que el efecto Crabtree está mediado por esta enzima (48). Sin embargo, después se encontró que la PDC también es importante durante el metabolismo oxidativo de la levadura al funcionar como una vía alterna de provisión de sustratos para el ciclo de Krebs (49).

Los cambios en el potencial de óxido-reducción citoplásmico ($\Delta G_{O/R}$) podrían ser importantes en la inducción del efecto Crabtree. En relación a esto, se ha demostrado que después de la adición de glucosa existe una disminución de los niveles de NADH en células de levadura que llevan a cabo la fosforilación oxidativa (37). En células tumorales, la interrupción de la expresión de la LDH elimina el efecto Crabtree y favorece un aumento en la concentración de NADH (44). Los resultados observados en ambos modelos sugieren que el incremento en los niveles de NAD^+ puede ser uno de los fenómenos a corto plazo que favorecen la represión de la respiración. Sin embargo, la función de los equivalentes reductores y del $\Delta G_{O/R}$ no han sido exploradas con detalle en los estudios que se han llevado a cabo en relación al efecto Crabtree.

3.5.3 La restricción de la entrada del piruvato hacia el ciclo de Krebs.

El piruvato, al ser el producto final de la vía glucolítica es susceptible de ser transportado hacia la matriz mitocondrial y posteriormente ser oxidado en el ciclo de Krebs, pero también puede ser sustrato de enzimas citoplásmicas como la lactato deshidrogenasa o la piruvato descarboxilasa. Estas últimas están sobreexpresadas durante el metabolismo fermentativo en las células tumorales y en las levaduras, respectivamente (ver sección 3.5.2). Su sobreactivación, además de restingir el NADH que capta la mitocondria, también limitaría la cantidad de piruvato que entra al ciclo de Krebs. En relación a esto, en la levadura se han cuantificado los flujos metabólicos en ausencia y en presencia de glucosa, y se encontró que durante la fermentación el flujo metabólico a través del ciclo de Krebs decrece considerablemente (50). Aunque en ese reporte no se hizo una correlación con la actividad de la PDC, existen otros estudios donde se ha observado que esta enzima es activada de forma concomitante con la represión de la respiración (10). De forma similar, cuando en las células tumorales se interrumpió la expresión de la LDH, se observó una disminución en la producción de lactato al mismo tiempo que la respiración incrementó (44).

También existen otros fenómenos regulatorios fisiológicos que actúan a corto plazo y que controlan la entrada del piruvato hacia el ciclo de Krebs. En mamíferos y en levaduras la actividad del complejo de la piruvato deshidrogenasa (PDH) está modulada por fosforilación (51, 52). La cinasa de la piruvato deshidrogenasa (PDHK) es la proteína encargada de fosforilar a la Pdh ocasionando que su actividad sea inhibida. Esto puede ser revertido por la actividad de la piruvato deshidrogenasa fosfatasa (PDP) (51). Se encontró que en las células tumorales la PDHK está sobre-expresada y se sugirió que la glucólisis aerobia en los tumores se debe a la inhibición del complejo PDH (53). Sin embargo, en este estudio no se realizaron los análisis de la expresión de la PDP y de la actividad del complejo de la PDH para corroborar esta propuesta. En la levadura, la expresión de la PDHK incrementa en condiciones de fermentación (54). También se encontró que una mutante que carece una de las dos isoformas de la PDP crece de forma más lenta en fuentes de carbono no fermentables (55). Esto también podría explicar porqué el flujo a través del ciclo de Krebs disminuye durante la fermentación (50). Aún no se han llevado a cabo estudios para ver si existe una correlación entre la fosforilación de la PDH y la disminución de su actividad con el efecto Crabtree.

3.5.4 La permeabilidad de la membrana externa mitocondrial.

Otro factor que podría explicar la inhibición de la fosforilación oxidativa en respuesta a la presencia de glucosa es la disminución de la permeabilidad de la membrana externa mitocondrial. En este sitio se encuentra un canal aniónico dependiente de voltaje (VDAC, por sus siglas en inglés) que permite el paso inespecífico de pequeñas moléculas (hasta 1.5 kDa) hacia el espacio intermembranal (56). Se ha demostrado que el estado de apertura de este canal puede variar dependiendo de distintos factores (56).

En relación a esto, se ha encontrado que existe una diferencia en la permeabilidad hacia el ADP entre las mitocondrias aisladas y las células permeabilizadas, ocasionando que la K_m por este nucleótido incremente un orden de magnitud en este último modelo (57). Al comparar mitocondrias aisladas de levadura con esferoplastos permeabilizados, se observó un efecto similar para el NADH (58). En ambos casos se propuso que esto es provocado por un cierre del canal VDAC (57, 58). Por lo tanto, si este canal se encuentra cerrado podría impedir el paso de sustratos respiratorios o de ADP hacia el espacio intermembranal, lo que conduciría a la aparición del efecto Crabtree.

Experimentos de reconstitución de membranas en donde se incluyó al VDAC mostraron que el NADH induce una disminución de la conductancia a través de este canal (59). Posteriormente se encontró que este nucleótido de pirimidina indujo una disminución en el consumo de oxígeno de mitocondrias aisladas de tubérculos de papa que empleaban succinato como sustrato respiratorio (60). Debido a que sólo el β-NADH fue capaz de inducir este efecto, se propuso que los equivalentes reductores generados en la vía glucolítica favorecen el cierre del VDAC y de esta forma favorecen la aparición del efecto Crabtree al restringir el acceso del ADP hacia la matriz mitocondrial (60). Sin embargo, este modelo no es aplicable en la levadura *S. cerevisiae*, ya que estudios en esferoplastos permeabilizados mostraron que el NADH producido en la glucólisis es canalizado a través del VDAC hacia el espacio intermembranal en donde se encuentran las NADH deshidrogenasas (61).

3.5.5 El papel inhibitorio del calcio sobre la respiración.

Otro posible inductor del efecto Crabtree es el Ca^{2+}. Se ha observado que la adición de glucosa a células de hepatoma favorece la acumulación de este catión en la matriz mitocondrial (62). Empleando mitocondrias aisladas de esta línea celular, se demostró que el Ca^{2+} induce una disminución de la respiración debido a la inhibición de la ATP sintetasa mitocondrial (63). Sin embargo, se ha cuestionado si este mecanismo es aplicable a otros modelos experimentales ya que en otra línea celular de hepatoma distinta no pudo ser detectada esta acumulación de Ca^{2+} intramitocondrial (35).

En el caso de la levadura, se ha observado que la adición de glucosa a levaduras que llevan a cabo la respiración induce un incremento en los niveles de calcio citoplásmico (aproximadamente 100 μM) (64). Se encontró que en mitocondrias aisladas de *S. cerevisiae* existe un efecto inhibitorio de este catión sobre la respiración (65). A diferencia de las mitocondrias de hepatoma, la inhibición que ejerce el Ca^{2+} sobre la respiración parece estar mediada por el canal VDAC (66). Sin embargo, la concentración de calcio requerida para lograr este efecto es bastante elevada con respecto a la que se ha determinado en el citoplasma celular (aproximadamente 500 μM) (65).

3.5.6 Los mensajeros metabólicos.

Se ha encontrado que algunos intermediarios metabólicos pueden tener una función regulatoria adicional (67, 68). A éstos se les denominó "mensajeros metabólicos" (11). Por ejemplo, en la levadura se encontró que se requiere de la acumulación de ciertos metabolitos de la glucólisis para inducir la expresión de algunas de las enzimas pertenecientes a esta vía (67). En las células de mamífero se encontró que la presencia de algunos oxoácidos endógenos contribuye a la correcta expresión de los genes inducidos en condiciones de hipoxia (68).

Es posible que los mensajeros metabólicos también participen en la regulación a corto plazo. Por ejemplo, en la levadura se ha visto que la ausencia de las enzimas que fosforilan a la glucosa afecta los procesos de señalización que son activados al inicio de la fermentación (62, 69, 70). Esto ha llevado a proponer que la glucosa 6-fosfato es uno de los mensajeros más importantes involucrados en la represión catabólica de la levadura *S. cerevisiae* (71).

Otro posible mensajero metabólico en la levadura podría ser la trehalosa 6-fosfato (T6P), que es un intermediario en la vía de síntesis de trehalosa. Se ha encontrado que *in vitro* la T6P inhibe la actividad de las dos isoformas de la hexocinasa (HK) de levadura (72). A diferencia de las enzimas de mamífero, las HK de la levadura no son inhibidas por glucosa 6-fosfato (73). Con base en esto, se propuso que la T6P funciona como regulador del flujo glucolítico (72). Para comprobar esto, se demostró que en ausencia de la trehalosa 6-fosfato sintasa existe una sobreacumulación de las hexosas fosfato de la glucólisis después de la adición de glucosa, sugiriendo que la glucólisis se encuentra sobreactiva (74).

La adición de glucosa a células tumorales y de levadura induce un incremento en los niveles de las hexosas fosfato de la glucólisis (glucosa 6-fosfato, fructosa 6-fosfato y fructosa 1,6-bifosfato) (75,76). Esta acumulación es concomitante con el efecto Crabtree. Sin embargo, no se ha investigado si estas hexosas pueden funcionar como mensajeros metabólicos e inducir una represión de la respiración. En el presente estudio se buscó esta relación. Debido a que la mitocondria es el sitio donde se lleva a cabo la mayor parte del consumo de oxígeno en la célula, se examinó la posibilidad de que las hexosas fosfato derivadas de la glucólisis sean efectivamente mensajeros metabólicos y actúen de forma directa sobre este organelo. Este es el primer estudio donde se intenta demostrar de forma directa la existencia de los mensajeros metabólicos.

4. Objetivo:

- Determinar la posible función de las hexosas fosfato de la glucólisis como mensajeros metabólicos durante la inducción del efecto Crabtree en la levadura *Saccharomyces cerevisiae*.

5. Objetivos particulares:

- Examinar el posible efecto de las hexosas fosfato de la glucólisis sobre la respiración de mitocondrias aisladas e *in situ*.
- Identificar el mecanismo de acción regulatoria de las hexosas fosfato.
- Demostrar el papel de estos intermediarios como mensajeros metabólicos.

6. Hipótesis

- Si al inicio del metabolismo fermentativo de la levadura existe una represión de la respiración de forma concomitante con la acumulación de las hexosas fosfato derivadas de la glucólisis, entonces éstas pueden funcionar como mensajeros metabólicos al inducir la inhibición del flujo respiratorio durante el efecto Crabtree.

7. Materiales y métodos.

7.1 Cepas y medios de cultivo.

Para realizar los aislamientos de mitocondrias y la obtención de esferoplastos se utilizó una cepa industrial (Yeast Foam) de la levadura *Saccharomyces cerevisiae*. Para la evaluación del efecto Crabtree y los estudios en células íntegras se empleó la cepa de laboratorio W303-1A (Mat a; *ura*3-52; *trp*1Δ2; *leu*2-3_112; *his* 3-11; *ade*2-1; *can1*-100) y una cepa mutante derivada de ésta, que carece de la enzima trehalosa 6-fosfato sintasa *(tps1*Δ).

Las células de la cepa Yeast Foam se sembraron en medio de cultivo YPL (extracto de levadura 1 %. Peptona 1 %, KH_2PO_4 0.1 %, NH_4SO_4 0.12 % y lactato 2 %). Para las células derivadas de W303 se empleó el medio YPGal (extracto de levadura 2%. Peptona 2%, KH_2PO_4 0.1 %, NH_4SO_4 0.12 % y galactosa 2%) adicionado con adenina (100 mg/mL). En todos los casos se colectaron las células en fase media logarítmica de crecimiento.

7.2 Obtención de esferoplastos.

Se prepararon de acuerdo a un método descrito anteriormente (58). Después de colectar las células de levadura, se hicieron tres lavados con agua bidestilada en una centrífuga refrigerada a 3900 g durante 5 minutos. Se resuspendieron en amortiguador de preincubación (β-mercaptoetanol 0.5M / Tris 0.1M, pH 9.3) y se mantuvieron en agitación constante por 10 minutos a 28ºC. Posteriormente, se lavó 3 veces a 3900 g durante 5 minutos en un amortiguador Tris/KCl (KCl 0.5M / Tris 10 mM, pH 7.0). Después de los lavados se procedió a realizar la digestión de la pared celular empleando citohelicasa. Para esto, se resuspendió la biomasa en el amortiguador de digestión A (Sorbitol desionizado 1.35M, EGTA 1 mM, Na_2HPO_4 10mM, citrato de sodio 10mM, pH 5.8) y se añadió la enzima (0.17g/g de peso seco) manteniendo en

agitación constante a 32 ºC. Se monitoreó la digestión por medio de la disminución de la absorbancia a 600 nm y se detuvo cuando se alcanzó aproximadamente el 80% de la digestión. A continuación, se lavó tres veces en un amortiguador para esferoplastos (Sorbitol desionizado 1M, NaCl 1.7 mM, EGTA 0.5 mM, Tris 10mM, BSA 1%, pH 6.8) a 700 g durante 5 minutos. Al final, la biomasa se resuspendió delicadamente en amortiguador para esferoplastos. Se determinó la concentración de proteína por medio del método Biuret (77)

7.3 Aislamiento de mitocondrias

Se realizó de acuerdo al método reportado por Guérin y cols (78). Después de lavar las células, se llevaron a cabo una preincubación y tres lavados similares al método de obtención de esferoplastos descrito en la sección 7.2. Posteriormente se realizó la digestión de la pared celular empleando la enzima zimoliasa 20T. Para esto, se resuspendió la biomasa en el amortiguador de digestión B (Sorbitol desionizado 1.35 M, EGTA 1mM, amortiguador di-monofosfato 0.2M, pH 7.0) y se añadió la enzima a una concentración de 10 mg de enzima por gramo de peso seco, manteniendo en agitación constante y a 32 ºC. Se monitoreó la digestión por medio de la disminución de la absorbancia a 600 nm y se detuvo cuando se alcanzó aproximadamente el 80% de la digestión. Inmediatamente, se realizaron tres lavados a 16000 g durante 10 minutos empleando un amortiguador para protoplastos (Sorbitol desionizado 0.75M, manitol desionizado 0.4 M, Tris/maleato 10 mM, BSA 1% pH 6.8). A continuación, se resuspendió la biomasa en amortiguador de homogenización (Manitol desionizado 0.5M, EGTA 2mM, Tris/Maleato 10mM, BSA 0.2%, pH 6.8) y se rompieron los esferoplastos en frío, empleando un homogenizador Waring Blendor (3 ciclos de 20 segundos con pausas de 40 segundos). El homogenado fue centrifugado a 700 g durante 10 minutos, y el sobrenadante resultante se recuperó para ser nuevamente centrifugado por 10 minutos a 16000 g. El sedimento obtenido de esta última centrifugación fue resuspendido en un amortiguador de recuperación (Manitol desionizado 0.6M, EGTA 2mM, pH 6.8) y se sometió a otras dos centrifugaciones similares a las anteriores (700 y 16000 g, respectivamente). Al final, las mitocondrias se resuspendieron en amortiguador de recuperación y fueron congeladas en nitrógeno líquido para ser almacenadas a -80 ºC. Para cada preparación se determinó la concentración de proteína por medio del método Biuret (77).

7.4 Determinación del consumo de oxígeno.

Se realizó a través de un método polarográfico empleando un electrodo de Clark selectivo para oxígeno. Este electrodo se acopló a una cámara de temperatura constante a 28 ºC y se conectó a un aparato graficador. En cada experimento se obtuvo el cambio de la concentración de oxígeno con respecto al tiempo.

Cuando se emplearon esferoplastos se hizo una preincubación en amortiguador C (Sorbitol desionizado 1M, NaCl 1.7 mM, EGTA, 0.5 mM, Pi 1mM, yodoacetato 4mM, Tris-Maleato 20mM, pH 6.8) en presencia de nistatina (20 μg/mL), a 28 ºC y con aireación constante. Esto se realizó con el propósito de permeabilizar la membrana de los esferoplastos y para permitir que escapen los sustratos endógenos a fin de que no interfieran en las determinaciones posteriores (58, 79). Para las mediciones del consumo de oxígeno se utilizó una concentración de 1 mg de proteína/mL y NADH 10 mM como sustrato respiratorio.

Para los experimentos con mitocondrias, éstas fueron previamente descongeladas con agitación suave. En este caso se usó el amortiguador D (Manitol desionizado 0.6M, EGTA 2mM, Pi 1 mM, Tris-Maleato 10mM, pH 6.8), y se emplearon a una concentración de 0.3 mg proteína/mL. Se usaron NADH 1 mM y etanol 100 mM como sustratos respiratorios. Cuando se evaluó la respiración en condiciones de fosforilación (estado 3) se incluyó ADP 2mM en el amortiguador. Para cada preparación de mitocondrias se verificó que existiera un buen control respiratorio, esto es, un coeficiente respiratorio igual o mayor a 2.

Para medir la respiración de células intactas se tomó una alícuota de 1 mL de un cultivo de células de *S. cerevisiae* en fase logarítmica (ver sección 7.1) y se colocaron directamente en la cámara del oxímetro. Antes de cada lectura se burbujeó aire con el fin de saturar el medio con oxígeno. Se realizó la determinación de la respiración endógena y para inducir el efecto Crabtree se adicionó glucosa 30 mM. Para determinar la rapidez del consumo de oxígeno en el estado desacoplado se incluyó CCCP 8 μM en presencia de etanol 100 mM.

7.5 Cuantificación del potencial transmembranal mitocondrial (ΔΨ).

El ΔΨ se determinó empleando como indicador el compuesto fluorescente rodamina 123 (Rh-123) de acuerdo al método descrito por Emaus y cols (80). Este método se basa en la correlación entre el valor del potencial transmembranal y el apagamiento de la fluorescencia de la

Rh-123. En el laboratorio se realizó previamente la calibración de este método y se obtuvo una relación lineal entre el valor del potencial transmembranal y el cambio en la fluorescencia relativa de la Rh-123.

Se suspendieron las mitocondrias (0.3mg prot/mL) en una celda de fluorómetro (Kontron SFM) empleando el amortiguador D en presencia de Rh-123 2 μM y con agitación constante a una temperatura de 28 ºC. Para la detección de los cambios de fluorescencia se emplearon longitudes de onda de excitación y de emisión de 485 y 525 nm, respectivamente. Empleando una curva de calibración se extrapoló el valor de las lecturas para así obtener el potencial transmembranal mitocondrial ($\Delta\Psi$).

7.6 Determinación indirecta de la actividad del complejo III

Se monitoreó a través de un método espectrofotométrico. Las mitocondrias (0.3 mg prot/mL) se suspendieron en el amortiguador D en presencia de $Fe(CN)_6^{3+}$ 2 mM, KCN 1 mM y etanol 100 mM como sustrato respiratorio. Se siguieron los cambios de absorbancia a 437 nm en un espectrofotómetro (Safas, Mónaco) y se registró el valor del cambio de absorbancia con respecto al tiempo para cada condición. Es importante mencionar que en estas condiciones los electrones provienen del NADH generado por la oxidación del etanol en la reacción catalizada por la alcohol deshidrogenasa intramitocondrial. Debido a que las mitocondrias de levadura carecen de complejo I (81), en estas condiciones la oxidación del etanol refleja la actividad del complejo III.

7.7 Determinación de la actividad de la citocromo oxidasa (complejo IV)

Se siguió por medio de la determinación del consumo de oxígeno (ver sección 7.4). Las mitocondrias aisladas (0.3 mg prot/mL) se incubaron en amortiguador D en presencia de antimicina A (2.5 μg/mg prot), arscorbato 2.5 mM y TMPD (N,N, N',N'-tetrametilfenilendiamina) 100 μM. En estas condiciones, el consumo de oxígeno es mediado únicamente por la actividad del complejo IV. La velocidad de consumo de oxígeno se calculó como se describe en la sección 7.4.

8. Resultados

En las levaduras que crecen de forma aerobia, la adición de glucosa induce una transición hacia el metabolismo fermentativo (ver sección 3.3). En estas condiciones la glucólisis incrementa mientras que la respiración y la fosforilación-oxidativa son reprimidas. Durante esta transición, se han detectado cambios en los niveles de algunos metabolitos en el citoplasma (40, 75, 82). En particular existe un incremento considerable en los niveles de las hexosas fosfato derivadas de la vía glucolítica (75). Con base en esto, se ha sugerido que la G6P podría participar en eventos que regulan la transición al metabolismo fermentativo (mensajero metabólico) (ver sección 3.5.6). Sin embargo, aún no se ha identificado un blanco específico para ésta, ni su posible mecanismo de acción.

Debido a esto, se decidió probar si había un efecto directo de las hexosas fosfato de la glucólisis sobre la respiración de mitocondrias aisladas. En primer lugar se evaluó esta adición en condiciones donde la fosforilación oxidativa no está activa (estado 4), con el fin de observar si hay un efecto directo de estos intermediarios sobre la cadena respiratoria mitocondrial.

En la Fig 1 se muestra el efecto de la glucosa 6-fosfato (G6P), la fructosa 6-fosfato (F6P) y la fructosa 1,6-bifosfato (F16bP) sobre la respiración de mitocondrias aisladas. Puede observarse que hubo un efecto diferente para cada una de éstas. En presencia de G6P y de F6P la respiración del estado 4 fue estimulada en función de la concentración de hexosa. Este incremento fue considerable al emplear G6P, en donde se alcanzó hasta 2.5 veces el valor del flujo respiratorio. La estimulación mediada por la F6P fue mucho menor (incrementó aproximadamente 25% con respecto al nivel basal) y se observó una saturación de este efecto a una concentración de 7.5 mM. La F16bP tuvo un efecto contrario, esto es, indujo una inhibición en el flujo respiratorio de las mitocondrias aisladas. Al emplear la concentración más elevada de esta hexosa se observó una inhibición de 30% de la respiración basal (Fig 1). Sin embargo, también se obtuvo un efecto de saturación a partir de una concentración de F16bP 7.5 mM.

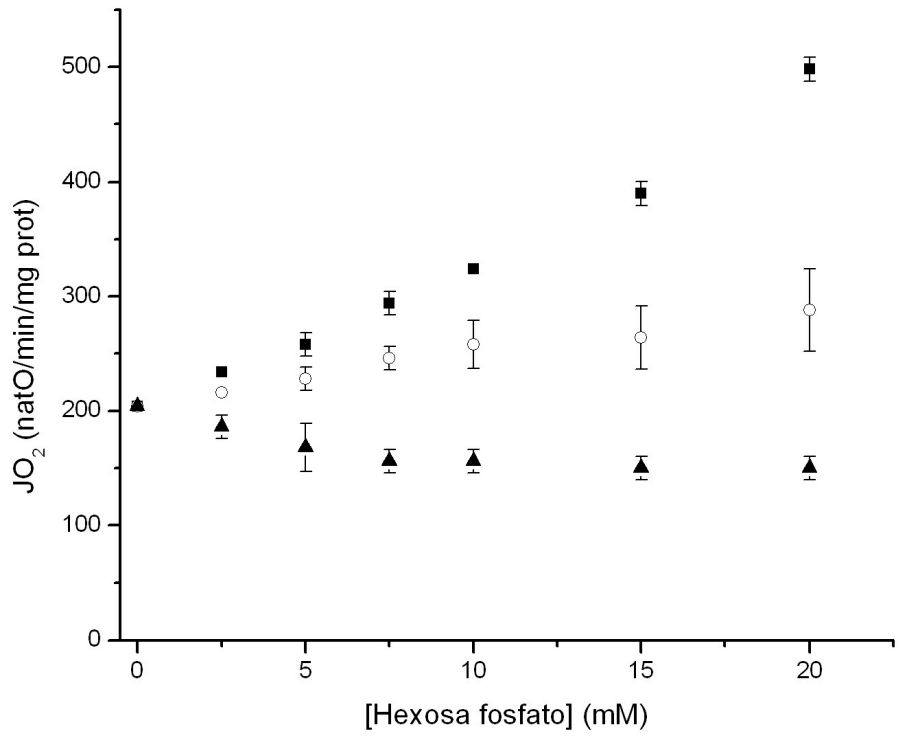

Figura 1. Efecto de las hexosas fosfato derivadas de la glucólisis sobre la respiración (JO_2) de mitocondrias aisladas de levadura en condiciones de no-fosforilación (estado 4). Mitocondrias aisladas de la cepa industrial Yeast Foam (0.3 mg prot/mL) se suspendieron en amortiguador D (Manitol 0.6 M, EGTA 2 mM, Pi 1 mM, Tris-Maleato 10mM, pH 6.8) en presencia de NADH 1 mM como sustrato respiratorio y se monitoreó el consumo de oxígeno (ver la sección 7.4). Se adicionaron distintas concentraciones de glucosa 6-fosfato (■), fructosa 6-fosfato (○) y fructosa 1,6-bifosfato (▲). Se muestra el promedio de tres experimentos independientes ± D.E. (n=3).

Los efectos inducidos por las hexosas fosfato de la glucólisis sobre la respiración de mitocondrias aisladas no fueron dependientes del sustrato respiratorio empleado, ya que se obtuvieron resultados similares al realizar estos experimentos usando etanol como sustrato (No mostrado). Es importante mencionar que en los experimentos de la Fig 1 se emplearon niveles no fisiológicos de cada uno de estos intermediarios. La concentración de G6P que ha sido determinada *in vivo* es de 1-6 mM, mientras que la F6P es menor a 1 mM (75). Con base en la Fig 1, puede apreciarse que en presencia de concentraciones fisiológicamente relevantes de hexosas monofosfato la estimulación de la respiración de las mitocondrias aisladas no es tan

elevada, e incluso es nula en el caso de la F6P. Por otra parte, la F16bP alcanza niveles mucho mayores, llegando a concentraciones entre 5 y 10 mM en el citoplasma (75, 83, 84). Por lo tanto, es posible que la inhibición del flujo respiratorio inducida por la F16bP sea fisiológicamente más importante que la estimulación mediada por las otras dos hexosas fosfato.

Con el fin de observar el efecto de estos intermediarios en condiciones que se asemejen a las de la célula intacta en donde se lleva a cabo la fosforilación oxidativa, se midió la respiración del estado 3 (condiciones de fosforilación) de mitocondrias aisladas en presencia de cada uno de estos metabolitos (Fig 2).

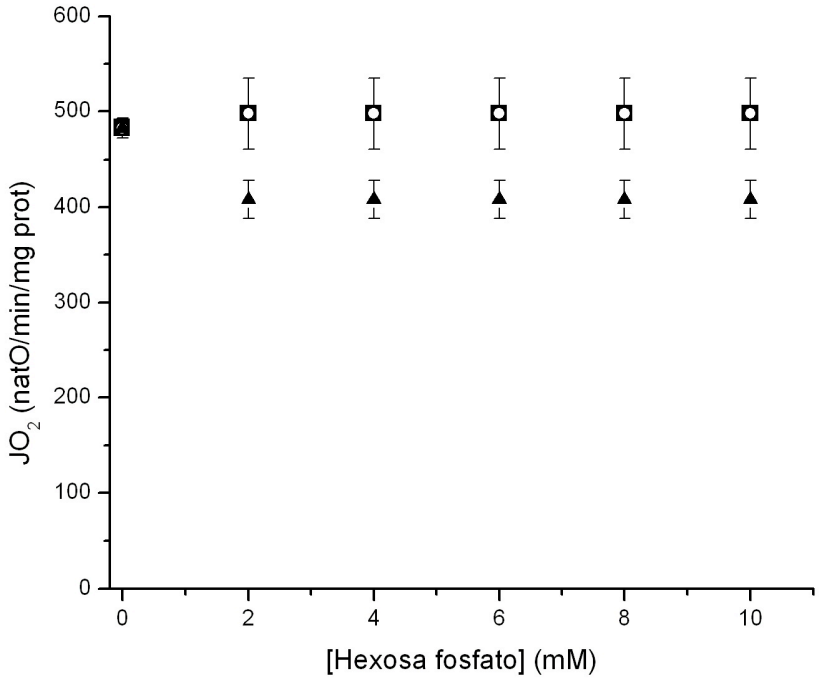

Figura 2. Efecto de las hexosas fosfato derivadas de la glucólisis sobre la respiraciónn (JO_2) de mitocondrias aisladas de levadura en condiciones de fosforilación (estado 3). Las condiciones son similares a las de la Fig 1 excepto que se incluyó ADP 1 mM. Se usaron distintas concentraciones de glucosa 6-fosfato (■), fructosa 6-fosfato (○) y fructosa 1,6-bifosfato (▲). En la gráfica se muestra el promedio de tres experimentos independientes ± D.E. (n=3).

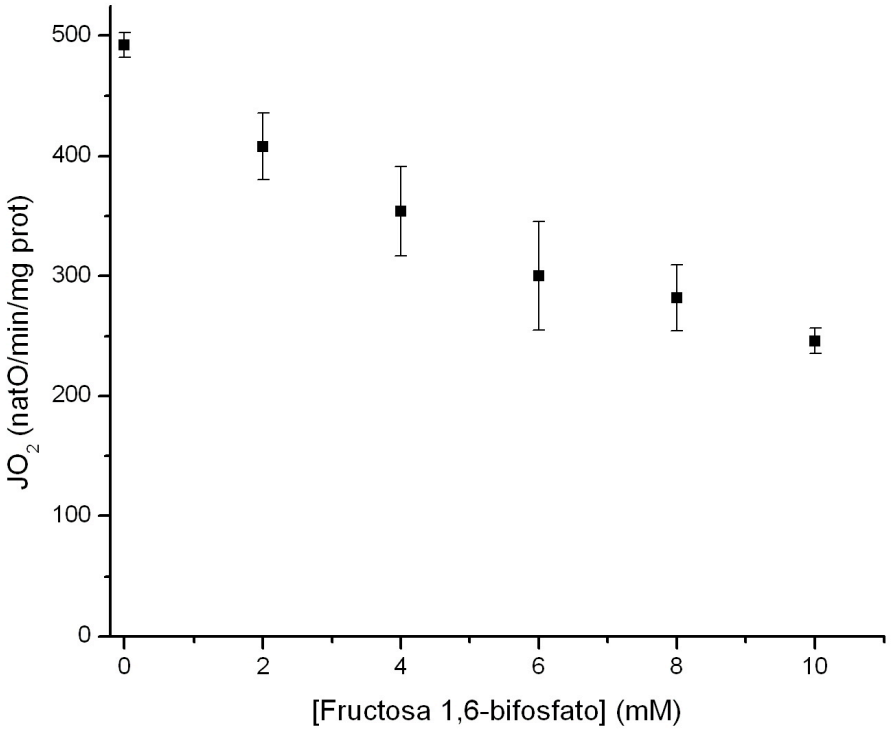

Fig 3. Efecto de la fructosa 1,6-bifosfato sobre el incremento del flujo respiratorio (JO_2) mediado por la glucosa 6-fosfato. Las condiciones experimentales fueron similares a la Fig 1 en presencia de NADH 1 mM como sustrato respiratorio y glucosa 6-fosfato 20 mM. Se muestra el resultado de tres experimentos independientes ± D.E. (n=3).

En estas condiciones la adición de las hexosas monofosfato no tuvo efecto alguno sobre la respiración. Por otra parte, se siguió observando la inhibición de la respiración inducida por la F16bP. El grado de inhibición fue similar al que se observó en condiciones de no fosforilación: aproximadamente una disminución del 20% del valor del consumo de oxígeno inicial. (ver Fig 1). También es importante señalar que la saturación de este efecto se observó a concentraciones muy bajas (2 mM), el cual se encuentra dentro del intervalo de concentraciones que ha sido medido en la célula intacta. También se investigó si la F16bP podría influir en el rendimiento de la fosforilación oxidativa, sin embargo su presencia no alteró la relación ADP/O (No mostrado).

El hecho que no se haya observado una estimulación de la respiración mediada por las hexosas monofosfato en condiciones de fosforilación, refuerza la hipótesis de que probablemente éstas no ejerzan este efecto *in vivo*. Por otra parte, empleando las concentraciones fisiológicas de F16bP se observó la inhibición de la respiración de mitocondrias aisladas sin importar el estado respiratorio (estado 3 o 4). Esto sugiere que este metabolito podría ser un efector importante para la inducción del efecto Crabtree en las levaduras.

Al iniciar la fermentación, la concentración de las tres hexosas incrementa de forma simultánea y la mitocondria estaría en presencia de los tres intermediarios en la célula intacta. Dado que la acumulación de G6P, F6P y F16bP coincide con una inhibición del flujo respiratorio, se esperaría que el efecto predominante sea el de la fructosa 1,6-bifosfato. Para demostrar esto, se evaluó el efecto de la adición simultánea de G6P (que indujo el efecto estimulante más significativo) y de F16bP (Fig 3).

	$\Delta\Psi_m$ (mV)		
Concentración (mM)	**Glucosa 6P**	**Fructosa 6P**	**Fructosa 1,6P$_2$**
0	132 ± 0	128 ± 1	124 ± 4
2	128 ± 0	126 ± 2	126 ± 2
4	128 ± 3	130 ± 4	125 ± 4
6	126 ± 2	130 ± 2	122 ± 5
8	128 ± 3	130 ± 4	120 ± 4
10	130 ± 2	131 ± 1	119 ± 3
20	128 ± 0	131 ± 1	117 ± 3

Tabla 1. Potencial transmembranal mitocondrial ($\Delta\Psi_m$) en presencia de las hexosas fosfato de la glucólisis. Mitocondrias aisladas (0.3 mg prot/mL) fueron incubadas en presencia de rodamina 123 1µM etanol 100 mM y diferentes concentraciones de glucosa 6-fosfato, fructosa 6-fosfato y fructosa 1,6-bifosfato en amortiguador D (Manitol 0.6 M, EGTA 2 mM, Pi 1 mM, Tris-Maleato 10mM, pH 6.8) con agitación constante a 28ºC. Los valores del potencial en milivoltios (mV) se obtuvieron como se describió en la sección 7.5.

En los experimentos de la Fig 3 se empleó la concentración de G6P (20 mM) con la que se observó una estimulación máxima en la Fig 1. Puede verse que la aceleración del consumo de oxígeno mediada por la G6P fue completamente contrarrestada por la adición de F16bP. Esto demuestra que los efectos de las hexosas monofosfato sobre la respiración son reversibles, ya que se restauró el valor control de la respiración con F16bP 10 mM (Fig 3). También se hizo el mismo experimento usando una concentración de G6P similar a la que ha sido determinada en las células intactas de levadura (5 mM) (74). En este caso, el incremento de la respiración mediado por la hexosa monofosfato fue completamente inhibido al incluir F16bP 2 mM en el medio de incubación (No mostrado). Lo anterior indica que en condiciones fisiológicas, el efecto neto de la acumulación de las tres hexosas fosfato de la glucólisis es en realidad el de la F16bP.

A continuación se buscó el mecanismo por el cual las hexosas fosfato ejercen su efecto sobre la respiración. Ya que las hexosas monofosfato incrementaron el flujo respiratorio, es posible que esto sea debido a un desacoplamiento. Para verificar lo anterior, se cuantificó el potencial transmembranal mitocondrial ($\Delta\Psi_m$) en presencia de estos intermediarios metabólicos. Si efectivamente existe un efecto desacoplante mediado por las hexosas monofosfato, se esperaba que su presencia disminuyera del valor del $\Delta\Psi_m$. En la Tabla 1, se muestra el resultado de estas determinaciones. Puede verse que la adición de G6P o de F6P no modificó de forma significativa el valor del potencial transmembranal. Por otra parte, la F16bP indujo una leve disminución del $\Delta\Psi_m$ cuando se empleó a concentraciones superiores a 10 mM (aproximadamente 7 mV) (Tabla 1). Esto descarta que las hexosas monofosfato tengan un efecto sobre la permeabilidad de la membrana interna mitocondrial. Por otra parte, el hecho que la F16bP inhiba la respiración y disminuya el potencial transmembranal sugiere que esta hexosa actúa sobre uno de los complejos respiratorios de la mitocondria.

A continuación se buscó el sitio de la cadena respiratoria donde la F16bP podría actuar para inhibir la respiración. En relación a esto, es importante señalar que en una secuencia de reacciones metabólicas (incluida la cadena respiratoria) existen ciertos pasos que tienen mayor importancia que otros para controlar el flujo a través de toda la vía (85, 86, 87). Al modular la actividad de dichos pasos existe mayor probabilidad de alterar toda una vía metabólica. En las mitocondrias de levadura se ha determinado que la citocromo oxidasa (complejo IV) controla en

gran parte el flujo respiratorio en los estados 3 y 4 (88). Por esta razón se buscó si la fructosa 1,6-bifosfato podía afectar la actividad del complejo IV (Fig 4).

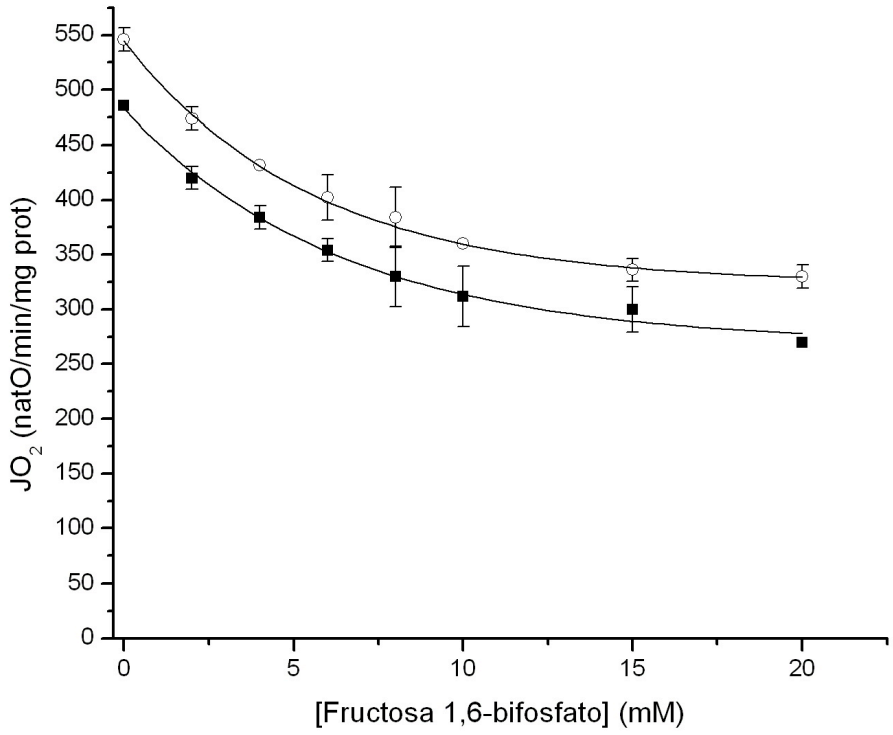

Fig 4. Actividad del complejo IV (citocromo oxidasa) en presencia de fructosa 1,6-bifosfato en condiciones de fosforilación (estado 3) y de no fosforilación (estado 4). Mitocondrias aisladas (0.3 mg prot/mL) se suspendieron en amortiguador D (Manitol 0.6 M, EGTA 2 mM, Pi 1 mM, Tris-Maleato 10mM, pH 6.8) y se incubaron en presencia de antimicina A (2.5 µg /mg prot), ascorbato 2.5 mM y N,N,N',N'-tetratmetil-p-fenilendiamina (TMPD) 100 µM. Se midió el consumo de oxígeno en presencia (○) y ausencia (■) de ADP 1 mM. Se añadieron las concentraciones de fructosa 1,6-bifosfato que están indicadas. Se muestra el resultado promedio de tres experimentos independientes ± D.E. (n = 3).

La adición de esta hexosa difosfato indujo una disminución de la actividad del complejo IV en forma dependiente de su concentración. La magnitud del efecto inhibitorio máximo fue similar en ambos estados respiratorios (inhibieron aproximadamente un 55% de la actividad

inicial). Esta disminución de la respiración es considerable aún en presencia de la más bajas concentración de F16bP empleadas en este experimento (aproximadamente, una disminución del 20% del valor control). De forma similar a las Figs 1 y 2, el efecto de la hexosa bifosfato fue saturable y se alcanzó el efecto máximo con una concentración de 10 mM. También se probó la adición de las hexosas monofosfato, sin embargo éstas no ejercieron ningún efecto (No mostrado).

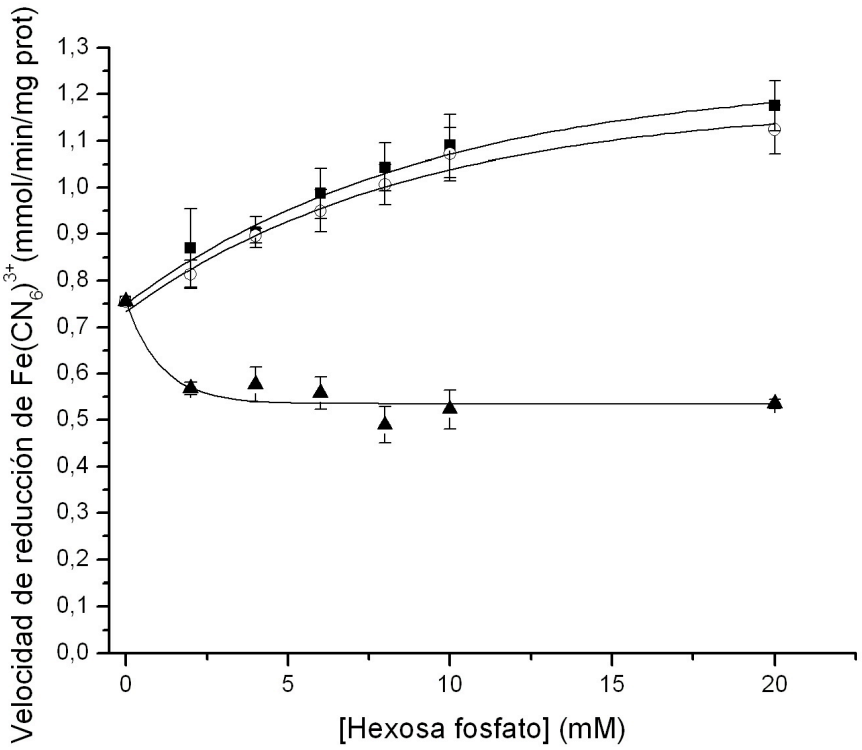

Fig 5. Efecto de las hexosas fosfato de la glucólisis sobre la actividad del complejo III. Mitocondrias aisladas (0.3mg prot /mL) se suspendieron en amortiguador D (Manitol 0.6 M, EGTA 2 mM, Pi 1 mM, Tris-Maleato 10mM, pH 6.8) en presencia de KCN 1 mM, ferricianuro 2 mM y etanol 100 mM. Se monitoreó la reducción del ferricianuro por medio de un espectrofotómetro a 436 nm. Se añadieron distintas concentraciones de glucosa 6-fosfato (■), fructosa 6-fosfato (○) y fructosa 1,6-bifosfato (▲). Se muestra el resultado promedio de tres experimentos independientes ± D.E. (n =3).

Esto demuestra que la F16bP inhibe a la citocromo oxidasa y de esta forma inhibe el consumo de oxígeno de mitocondrias aisladas. Sin embargo, aún no es claro el mecanismo por el cual la G6P y la F6P ejercen un efecto contrario. Por lo tanto, se decidió evaluar el efecto de éstas sobre otro de los complejos respiratorios. Debido a que las mitocondrias de levadura no poseen complejo I (80), y a que el complejo II se encuentra en la matriz mitocondrial, se decidió evaluar el efecto de las hexosas fosfato de la glucólisis sobre la actividad del complejo III (Fig 5).

La actividad de este complejo respiratorio se incrementó en respuesta a la adición de las hexosas monofosfato derivadas de la glucólisis. El efecto fue similar para las dos, ya que en ambos casos la actividad basal se aumentó 1.5 veces (Fig 5). Por otra parte, la F16bP indujo una disminución de la actividad de este complejo respiratorio, reprimiendo un 35% de la actividad inicial. Es importante mencionar que en estos experimentos, los efectos mediados por cada una de las hexosas fosfato fueron sensibles a la presencia de antimicina A, indicando que estos intermediarios únicamente afectaron la actividad del complejo III (Datos no mostrados).

Por lo tanto, la estimulación de la respiración de mitocondrias aisladas mediada por las hexosas monofosfato que fue observada en la Fig 1 es debida a una activación del complejo III respiratorio. Este fue un resultado inesperado, debido a que el complejo IV es considerado como el paso más importante para el control de la respiración (88) y se pensó que los efectos de las hexosas monofosfato tendrían que ocurrir a este nivel. Sin embargo, hay ocasiones en donde el complejo III puede contribuir de forma importante a la modulación del flujo respiratorio (89) (ver Discusión). Por otra parte, estos datos muestran que la inhibición de la respiración inducida por la F16bP es debida a una inhibición de los complejos III y IV.

En la Fig 3 se encontró que los efectos de la G6P y de la F16bP sobre la respiración son antagónicos. Debido a que las hexosas monofosfato no afectan la actividad de la citocromo oxidasa, se determinó si esto podía deberse a los efectos que ejercen sobre el complejo III. En la Fig 6 se muestra la actividad del complejo III en presencia de G6P y de F16bP. Para realizar estos experimentos se empleó la concentración de G6P con la que se obtuvo una activación máxima de este complejo respiratorio (20 mM) (ver Fig 5).

En ausencia de F16bP se observó una actividad elevada con respecto a las condiciones control en ausencia de G6P (1.35 y 0.75 mmol de Fe(CN) /min/mg prot, respectivamente) (ver también Fig 5). Conforme se hizo la adición de F16bP, se observó una disminución de la actividad de este complejo. Cuando la concentración de ambas hexosas fosfato fue idéntica se encontró que la actividad del complejo III fue similar al control (0.85 mmoles de $Fe(CN)_6^{3+}$/min/mg prot). Esto indica que la F16bP contrarrestó la activación del complejo III mediada por la G6P. Por lo tanto, es posible que el antagonismo entre ambas hexosas que se observó previamente (ver Fig 3) se deba a una modulación de la actividad del complejo III.

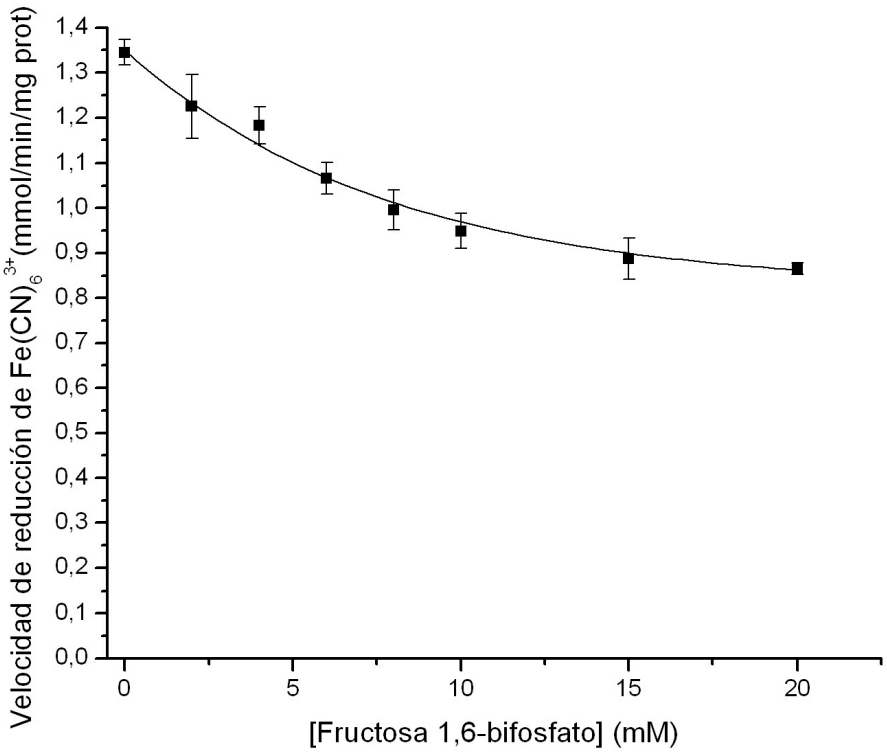

Figura 6. Efecto de la fructosa 1,6-bifosfato sobre la aceleración de la actividad del complejo III inducida por la presencia de glucosa 6-fosfato. Mitocondrias aisladas se suspendieron en amortiguador D (Manitol 0.6 M, EGTA 2 mM, Pi 1 mM, Tris-Maleato 10mM, pH 6.8) en presencia de KCN 1 mM, ferricianuro 2 mM, etanol 100 mM y glucosa 6-fosfato 20 mM. La reducción de ferricianuro se siguió espectrofotométricamente a 436 nm. Se adicionaron diferentes concentraciones de fructosa 1,6-bifosfato. Se muestra el resultado promedio de tres experimentos independientes ± D.E. (n=3).

Hasta el momento, todos los experimentos han sido llevados a cabo con mitocondrias aisladas. Sin embargo, las propiedades observadas en el organelo aislado podrían no reflejar los mecanismos de control a los que está sujeto en su contexto fisiológico dentro de las células intactas (89). Otros reportes han empleado células permeabilizadas como modelo experimental con el fin de evaluar la regulación de la mitocondria *in situ* (57, 58, 79). Este modelo posee la ventaja de conservar la organización de los componentes de la célula intacta, además de que es posible manipular fácilmente la concentración de metabolitos o de otras sustancias de interés. En el caso de la levadura *S. cerevisiae*, se han establecido las condiciones para la obtención de esferoplastos permeabilizados y se han estudiado los mecanismos por los cuales se modula la fosforilación oxidativa *in situ* (57, 78, 90, 91).

Con el fin de determinar si el efecto de la F16bP es fisiológicamente significativo, se decidió evaluar la respiración de esferoplastos permeabilizados en presencia de esta hexosa (Fig 7). Debido a que los efectos mediados por las hexosas monofosfato no son fisiológicamente relevantes, no fueron consideradas al llevar a cabo estudios posteriores.

Antes de cada experimento se llevó a cabo una incubación de diez minutos con nistatina con el fin de permeabilizar los esferoplastos y de vaciar la célula de metabolitos endógenos que pudiesen interferir. Para evitar el catabolismo de la F16bP por los esferoplastos, se incluyó yodoacetato (un inhibidor de la gliceraldehído 3-fosfato deshidrogenasa) en el medio de incubación (ver sección 7.4).

El efecto de la F16bP en esferoplastos permeabilizados fue similar al que se observó en las mitocondrias aisladas, esto es, indujo una disminución del flujo respiratorio. Este fenómeno ocurrió en ausencia (7A) o presencia (7B) de la fosforilación oxidativa. La inhibición de la respiración fue considerable en el estado 4 (alcanzó un 40% del valor control) (Fig 7A). En estas condiciones, no se observó efecto alguno al emplear la concentración más baja de F16bP (2 mM), pero a partir de 4 mM la respiración decreció en forma dependiente de la concentración. En condiciones de fosforilación el efecto inhibitorio fue menor (aproximadamente una disminución del 20%) (7 B). Lo anterior es comparable con lo que se observó en la respiración de mitocondrias aisladas en el estado 3 (Fig 2).

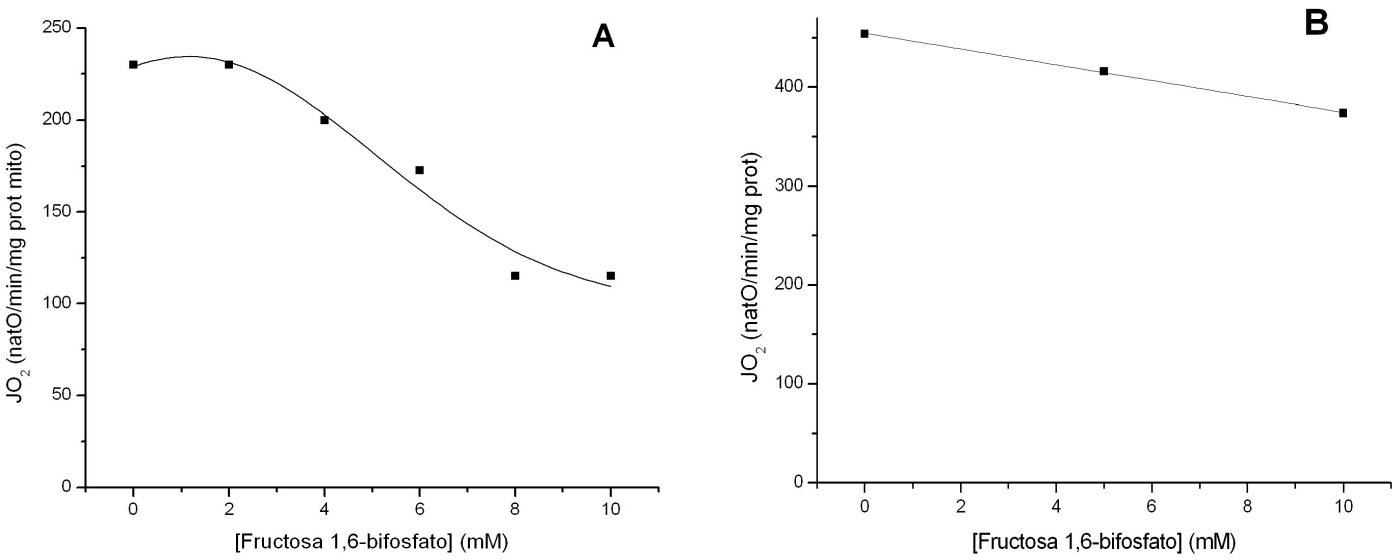

Figura 7. Efecto de la fructosa 1,6-bifosfato sobre la respiración de esferoplastos permeabilizados de levadura en los estados 4 (A) y 3 (B). Esferoplastos obtenidos de *S. cerevisiae* (1 mg prot/mL) fueron previamente incubados con nistatina (20 µg/mL) y suspendidos en el amortiguador C (Sorbitol desionizado 1M, NaCl 1.7 mM, EGTA, 0.5 mM, Pi 1mM, yodoacetato 4mM, Tris-Maleato 20mM, pH 6.8) en presencia de NADH 10 mM a 28 ºC. En B, se incluyó ADP 2 mM en el medio de incubación. Se determinó la rapidez del consumo de oxígeno de acuerdo a lo descrito en la sección 7.4. Se muestra un resultado representativo de al menos tres experimentos independientes (n=3).

También se verificó si la G6P podía inducir un incremento del flujo respiratorio en los esferoplastos permeabilizados. En este caso, se requirió de una concentración demasiado elevada para observar un efecto significativo (aproximadamente 120 mM) (No mostrado).

Se ha reportado que los esferoplastos permeabilizados requieren de concentraciones de ADP o de NADH superiores a las que se emplean en las mitocondrias aisladas (aproximadamente un orden de magnitud) (58, 59). Esto puede deberse a que la permeabilidad de la membrana externa de la mitocondria *in situ* es menor que en las mitocondrias aisladas (58, 59). En la Fig 7 puede verse que no fue necesario incrementar la concentración de F16bP para inhibir la respiración de los esferoplastos. En contraste con esto, se requirió elevar la concentración de G6P un orden de magnitud para estimular la respiración. Esto implica que la F16bP podría ser

canalizada de forma específica a través de la membrana externa mitocondrial y así tener acceso al espacio intermembranal para poder inhibir a los complejos respiratorios III y IV.

	W303-1A		**Δ*tps1***	
	JO_2 (natO/min/u.d.o)	**%**	**JO_2 (natO/min/u.d.o)**	**%**
Espontánea	160 ± 7	100	120 ± 10	100
+ Glucosa 30 mM	103 ± 10	64	47 ± 2	39
+ Etanol / CCCP (100mM / 8 μM)	190 ± 2	119	137 ± 17	114

Tabla 2. El efecto Crabtree en la cepa Δ*tps1*. Se tomó una alícuota de 1 mL de los respectivos cultivos cuando éstos se encontraban en la fase logarítmica de crecimiento (Densidad óptica = 1) y se determinó el consumo de oxígeno como se describió en la sección 5.4. Se hicieron las adiciones indicadas. Se muestra el resultado de tres experimentos independientes ± D.E. (n = 3).

Finalmente, se decidió medir el efecto Crabtree en células intactas. Para observar el posible papel de la F16bP, se empleó una cepa de levadura que carece de la enzima trehalosa 6-fosfato sintasa (Δ*tps1*). Se ha reportado que estas células sobre-acumulan F16bP en respuesta a la adición de glucosa (92, 93). Se midió la respiración endógena de las células Δ*tps1* y de la respectiva cepa silvestre. Se encontró que las mutantes respiran menos que la cepa control (Tabla 2). En ambos casos, la adición de etanol 100 mM estimuló la respiración, indicando que bajo las condiciones de crecimiento, las mitocondrias se encuentran limitadas por la disponibilidad de sustrato respiratorio. De acuerdo a reportes anteriores (10), se observó que la adición de glucosa indujo una disminución del consumo de oxígeno en ambos tipos de células (efecto Crabtree). Esta represión fue mayor en las células Δ*tps1* (40% de inhibición en la silvestre y 60% en la mutante). También se determinó la capacidad máxima respiratoria en presencia de etanol y un agente desacoplante de la fosforilación oxidativa (CCCP). Se encontró que las mutantes poseen menor capacidad respiratoria. Lo anterior puede ser debido a que las células Δ*tps1* posean menor cantidad de mitocondrias. Esto mismo también explicaría porqué la cepa mutante posee una respiración endógena menor (Tabla 2).

9. Discusión.

La represión del metabolismo aerobio es un fenómeno bien caracterizado en algunos modelos experimentales, entre ellos la levadura *S. cerevisiae*. Uno de los mecanismos que conducen a esta represión es el efecto Crabtree, que es definido como la inhibición inmediata de la respiración y la fosforilación oxidativa por la presencia de glucosa. Aunque se han propuesto varios modelos para explicarlo (ver sección 1.4), ninguno de ellos ha sido claramente demostrado.

Uno de los fenómenos más importantes que ocurre después de la adición de glucosa a células Crabtree-positivas es la aceleración de la glucólisis. Esto ocurre tanto en levaduras como en células de mamíferos. En ambos casos se ha encontrado que incrementa la concentración de algunos de los intermediarios de la vía. En particular, los niveles de las hexosas fosfato aumentan de forma significativa (75, 76). En relación a esto, se ha propuesto que en la levadura la glucosa 6-fosfato es un mensajero metabólico que contribuye a la regulación de eventos a largo plazo en la represión catabólica (ver sección 1.4.6) (71). Sin embargo, esta es sólo una hipótesis y no existe evidencia directa de que algún intermediario metabólico tenga una función regulatoria.

Puesto que la acumulación citoplásmica de las hexosas fosfato derivadas de la glucólisis es concomitante con la inhibición de la respiración en *S. cerevisiae*, en este trabajo se verificó si estos metabolitos podían funcionar como mensajeros metabólicos en la inducción del efecto Crabtree. La mitocondria es el organelo donde se lleva a cabo la respiración, como parte del proceso de la fosforilación oxidativa. Por lo tanto, se esperaría un efecto de estos intermediarios sobre mitocondrias aisladas.

La adición de fructosa 1,6-bifosfato induce un efecto Crabtree en mitocondrias, tanto aisladas como *in situ* (Figs 1, 2 y 7). Es importante tomar en cuenta que para lograr esto se requieren concentraciones comparables a las que han sido determinadas en las células intactas (2-10mM) (75, 94, 95). También se demostró que en células que sobreacumulan F16bP (Δ*tps1*) el efecto Crabtree es de mayor magnitud (Tabla 2). En esta cepa la concentración de F16bP es alrededor de 40 mM (93). Estos resultados confirman que este intermediario, a diferencia de las otras dos hexosas fosfato derivadas de la glucólisis, está efectivamente cumpliendo una función de mensajero metabólico. Este es el primer reporte que demuestra de forma concreta la existencia de una regulación mediada por este tipo de intermediarios.

Se ha señalado que la permeabilidad a través de la membrana externa es un proceso importante para el control de la función mitocondrial (96). Por lo tanto, si la F16bP ejerce su inhibición *in vivo*, debe de tener acceso al espacio intermembranal y pasar a través del canal VDAC, la proteína que regula la permeabilidad en este sitio. En relación a esto, se ha observado que en células permeabilizadas de músculo y de hígado es necesario incrementar la concentración de ADP un orden de magnitud para inducir la respiración del estado 3 (57). Este efecto se eliminó después de un tratamiento con proteasas, lo cual sugirió que hay una proteína que está limitando el paso del ADP hacia el espacio intermembranal (probablemente el VDAC) (96). Un efecto similar se encontró para el NADH en las mitocondrias de levadura (58). Todo esto sugirió que en las células permeabilizadas el canal VDAC se encuentra cerrado, mientras en la mitocondria aislada está abierto (58).

Al comparar las Figs 1,2 y 7, puede apreciarse que la concentración empleada para inhibir la respiración de los esferoplastos permeabilizados fue idéntica a la requerida para ejercer este mismo efecto en las mitocondrias aisladas. En cambio, esto no ocurrió para la G6P (No mostrado). En este caso fue necesario incrementar su concentración en un orden de magnitud para estimular el flujo respiratorio. Esto indica que, en contraste con la G6P, la F16bP puede ser canalizada de forma específica a través de la membrana externa mitocondrial.

Las hexosas mono-fosfato de la glucólisis tuvieron un efecto estimulatorio. Sin embargo, esto sólo ocurrió a concentraciones muy elevadas que no reflejan sus niveles fisiológicos en la célula intacta. La F16bP revierte completamente la estimulación de la respiración mediada por las hexosas monofosfato (Figs 3 y 6). Los datos anteriores indican que el efecto que predomina es el de la F16bP, a pesar de que las tres hexosas fosfato sean acumuladas al mismo tiempo en el citoplasma.

Con base en lo anterior, se descartó que los efectos inducidos por la glucosa 6-fosfato y la fructosa 6-fosfato sobre la mitocondria tengan alguna importancia fisiológica. Aunque esto no excluye su posible participación en otros fenómenos regulatorios durante la represión catabólica en las levaduras, como se ha sugerido anteriormente (71).

Existe evidencia experimental que apoya los resultados que se obtuvieron en este estudio. Por ejemplo, las células de levadura que sobreexpresan a la fosfofructocinasa y que poseen niveles más altos de F16bP, respiran menos que las células de la respectiva cepa silvestre (97).

Por otra parte, se demostró que en levaduras que carecen de la enzima hexocinasa II (Δ*hxk2*), poseen niveles más bajos de este intermediario y respiran más que la cepa silvestre (83).

Hay levaduras que se han clasificado como Crabtree-negativas, en las cuales la adición de glucosa no afecta su consumo de oxígeno (10). Estas pueden ser una herramienta útil para estudiar los mecanismos detrás del efecto Crabtree. Por ejemplo, se probó el efecto de la adición de fructosa 1,6-bifosfato a mitocondrias aisladas de *Candida utilis* (clasificada como Crabtree-negativa) y no se observó un efecto inhibitorio sobre la respiración (94). Esto indica que la susceptibilidad de las mitocondrias a la inhibición mediada por la F16bP es una de las características propias de las células Crabtree-positivas.

El mecanismo de acción de la fructosa 1,6-bifosfato es la inhibición de la actividad de los complejos respiratorios III y IV (Figs 4 y 5). Se considera que este último controla en gran parte el proceso de transferencia de electrones en la cadena respiratoria mitocondrial de la levadura (88). Esto puede explicar porqué la F16bP actúa a bajas concentraciones y ejerce una inhibición importante del flujo respiratorio. Es muy probable que el blanco de esta hexosa bifosfato sean el citocromo c o el dominio CuA de la subunidad II del complejo IV, mientras que en el complejo III los posibles sitio de acción pueden ser el citocromo c_1 o la proteína de Rieske. Todos estos componentes se localizan en el espacio intermembranal (98). Aún tienen que realizarse otros estudios para confirmar esto y para describir con más detalle el mecanismo de inhibición de la F16bP.

Las hexosas monofosfato ejercen su efecto a través del complejo III (ubiquinol:citocromo c oxidasa) (Fig 5). Esto puede parecer contradictorio, ya que se ha encontrado que el coeficiente de control de este complejo sobre la cadena respiratoria es bastante bajo (87). Esto implica que la reacción catalizada por el complejo III es muy rápida y cualquier efecto sobre éste no tendría ningún impacto sobre la respiración. Sin embargo, se ha demostrado que cuando la fuerza iónica del medio se ajusta a las condiciones fisiológicas, el coeficiente de control del complejo III incrementa de forma considerable (89). Esto es debido a que la cadena respiratoria se comporta como una unidad y cualquier efecto sobre el complejo III afecta también al complejo IV (89). Es posible que esto ocurra en nuestras condiciones experimentales y que la G6P y la F6P en realidad estimulen al complejo IV de forma indirecta. Para apoyar esta hipótesis, se ha encontrado que en

las mitocondrias de levadura estos dos complejos interactúan físicamente y forman un complejo supramolecular (99).

En las células tumorales fue donde se caracterizó el efecto Crabtree en primer lugar (7). Se propuso que debía existir un daño irreversible en las mitocondrias para que esto pudiera ocurrir (22). En el citoplasma de estas células también se acumula una cantidad importante de F16bP como consecuencia de una vía glucolítica sobreactiva (76). De acuerdo a lo que se observó en las mitocondrias de levadura se podría pensar que ocurre lo mismo en las células tumorales. En apoyo a lo anterior, se encontró que es posible inducir un efecto Crabtree en mitocondrias aisladas de hígado de rata al añadir concentraciones de F16bP similares a las que se han encontrado en líneas celulares de hepatoma de rata (94). Esto indica que el daño a las mitocondrias no es un pre-requisito necesario para la inducción el efecto Crabtree en las células tumorales. Estos resultados también demuestran que la función de la F16bP como mensajero metabólico no se restringe a las levaduras.

El efecto Crabtree es inducido por una variedad de factores (35). Es posible que los cambios del potencial fosfato tengan una participación importante en la inducción de este fenómeno (ver secciones 1.1 y 1.4.1) (41), lo cual podría explicar porqué se inhibe la síntesis de ATP al iniciar la fermentación, ya que la F16bP no tuvo ningún efecto sobre la eficiencia de la fosforilación oxidativa (No mostrado). Con base en los resultados obtenidos en este estudio se puede afirmar que la acumulación de F16bP es uno de los factores que conducen al efecto Crabtree.

Esto tiene implicaciones importantes en el caso de las células tumorales. Se ha encontrado que la glucólisis aerobia en estas células es determinante para que los tumores evadan los mecanismos de muerte celular (24). De manera que si se busca reducir de alguna manera la concentración de F16bP en la célula se podría revertir el efecto Crabtree y volver a la célula cancerosa más sensible a fármacos anti-tumorales. Este punto será investigado posteriormente.

10. Conclusiones

- En la levadura, las hexosas fosfato derivadas de la glucólisis tienen funciones adicionales a su participación en la vía metabólicas. Cada una de ellas ejerce un efecto sobre las mitocondrias aisladas al modular su respiración.
- La fructosa 1,6-bifosfato funciona como mensajero metabólico en la inducción del efecto Crabtree en la levadura *Saccharomyces cerevisiae*.
- La F16bP es canalizada de forma específica a través de la membrana externa mitocondrial para llegar al espacio intermembranal e inhibir a los complejos respiratorios III y IV.
- En la levadura, la acumulación de fructosa 1,6-bifosfato que ocurre como consecuencia de la aceleración de la glucólisis es uno de los eventos que conducen al efecto Crabtree.

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Mitochondrial Oxidative Phosphorylation Is Regulated by Fructose 1,6-Bisphosphate

*A POSSIBLE ROLE IN CRABTREE EFFECT INDUCTION?**

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In numerous cell types, tumoral cells, proliferating cells, bacteria, and yeast, respiration is inhibited when high concentrations of glucose are added to the culture medium. This phenomenon has been named the "Crabtree effect." We used yeast to investigate (i) the short term event(s) associated with the Crabtree effect and (ii) a putative role of hexose phosphates in the inhibition of respiration. Indeed, yeast divide into "Crabtree-positive," where the Crabtree effect occurs, and "Crabtree-negative," where it does not. In mitochondria isolated from these two categories of yeast, we found that low, physiological concentrations of glucose 6-phosphate and fructose 6-phosphate slightly (20%) stimulated the respiratory flux and that this effect was strongly antagonized by fructose 1,6-bisphosphate (F16bP). On the other hand, F16bP by itself was able to inhibit mitochondrial respiration only in mitochondria isolated from a Crabtree-positive strain. Using permeabilized spheroplasts from Crabtree-positive yeast, we have shown that the sole effect observed at physiological concentrations of hexose phosphates is an inhibition of oxidative phosphorylation by F16bP. This F16bP-mediated inhibition was also observed in isolated rat liver mitochondria, extending this process to mammalian cells. From these results and taking into account that F16bP is able to accumulate in the cell cytoplasm, we propose that F16bP regulates oxidative phosphorylation and thus participates in the establishment of the Crabtree effect.

In aerobic organisms, glycolysis and oxidative phosphorylation are coordinated to fulfill the cell energy demand. In some conditions, such as glucose addition to the cells, one can observe an increase in glycolytic flux, whereas respiration is inhibited. This has been observed in tumoral cells (1), nontumoral proliferating cells (2), some bacteria (3), and some yeast species (4). In all of these cases, glucose induces a transition to a mostly fermentative metabolism. This phenomenon has been named the "Crabtree effect," after its discoverer (1).

The physiological events that could clearly explain the occurrence of the Crabtree effect are currently unknown, although many hypotheses have been laid (4–7). It has been proposed, for instance, that it could originate from a competition between mitochondria and glycolytic enzymes for free ADP and inorganic phosphate (5, 8). Indeed, the respiration of isolated mitochondria is decreased in the presence of ADP-consuming systems, such as reconstituted glycolysis or the phosphocreatine/creatine kinase system (5). Nevertheless, after glucose addition, ADP levels remain constant or even increase in yeast (6, 9) and hepatoma cells (7). Furthermore, in both models, there is a transient decrease in cytoplasmic P_i levels (7, 10), pointing to a possible role of P_i or phosphate potential (ΔG_p) in this process.

It has been proposed that one of the short term events leading to the Crabtree effect is an overflow through pyruvate decarboxylase, since it has been observed that in Crabtree-positive yeast strains, its activity increases after a glucose pulse (4). Nonetheless, pyruvate decarboxylase seems to be an important bypass of pyruvate dehydrogenase during oxidative metabolism (11).

It was also proposed that changes in mitochondrial outer membrane permeability could be critical for the regulation of the Crabtree effect (12, 13). From results obtained with reconstituted systems (12) and with mitochondria isolated from potato tubers (13), it was suggested that cytosolic NADH produced by glycolysis could close the voltage-dependent anionic channel and consequently limit the passage of molecules such as ADP toward the intermembrane space. In permeabilized yeast cells, it has been shown that NADH is not involved in the voltage-dependent anionic channel closure and that *in situ* produced NADH is channeled through voltage-dependent anionic channel to the intermembrane space, where the external NADH dehydrogenases are located (14).

Another possible effector involved in the Crabtree effect is Ca^{2+} (15). In Ehrlich ascites tumors and in Zajdela hepatoma cells, it has been observed that there is a glucose-induced increase in cytoplasmic calcium levels along with an enhanced mitochondrial uptake of this cation. Inside the mitochondria, Ca^{2+} would inhibit ATP synthase by enhancing the interaction with IF1, its inhibitory subunit (16). However, is not clear whether this Ca^{2+} accumulation is a common event in all Crabtree-positive cells, since in AS-D30 hepatoma cells, cal-

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cium levels are not modified after glucose addition (7). Thus, to date, no clear experimental results have allowed determination of the early event leading to the Crabtree effect.

With regard to this Crabtree effect, yeast species are either negative (*e.g. Candida utilis*) or positive (*e.g. Saccharomyces cerevisiae*). Yeast thus constitutes a good experimental model to study this effect. Under its alternative designation of glucose repression, the Crabtree effect has indeed been thoroughly studied in *S. cerevisiae*, in which short term and long term events have been defined. Regarding the latter class of events, when *S. cerevisiae* grows using high glucose concentrations as carbon source, it represses oxidative metabolism by down-regulating the synthesis of mitochondrial respiratory chain components and by inhibiting enzymatic activities of the Krebs and glyoxylate cycles. At the same time, the expression of glycolytic enzymes is enhanced (reviewed in Ref. 17). Whereas long term effects have been thoroughly studied and are very well understood, the origin of the short term events is ill defined.

It has been proposed that during glucose repression, metabolic intermediates could have a regulatory role in both short and long term events, functioning as "metabolic messengers" (18). For instance, in mutants that accumulate different glycolysis metabolites, the transcription of glycolytic genes increases, pointing to a relationship between internal metabolite levels and enzyme expression (19) (*e.g.* glucose phosphorylation to glucose 6-phosphate (G6P)[4] seems to be important for signaling processes induced by glucose (20–22); also, trehalose 6-phosphate (T6P) might regulate the glycolytic flux by modulating hexokinase activity (23)). Therefore, a yeast mutant that lacks trehalose 6-phosphate synthase (Tps1p) overaccumulates glycolysis hexose phosphates in response to glucose (24). However, it has not been clearly demonstrated whether metabolic intermediates could function as messengers in long or short term regulatory events.

In *S. cerevisiae*, cytoplasmic levels of glycolysis hexose phosphates increase after glucose addition to the culture medium (25, 26). Given the possible role of glycolysis intermediates as metabolic messengers, we investigated whether some of these could contribute to the short term Crabtree effect (*i.e.* function as signaling molecules during glucose-induced repression of oxidative metabolism). In order to study such a process, we took advantage of the existence of Crabtree-positive (*S. cerevisiae*) and Crabtree-negative (*C. utilis*) yeast strains. In both kinds of isolated mitochondria, we found that low, physiological concentrations of G6P and fructose 6-phosphate (F6P) stimulated the respiratory flux (JO_2), and this effect was strongly antagonized by fructose 1,6-bisphosphate (F16bP). On the other hand, F16bP by itself inhibited mitochondrial respiration only in mitochondria isolated from *S. cerevisiae*. We also observed that in the yeast mutant Δ*tps1*, which accumulates F16bP in response to glucose addition, the Crabtree effect is enhanced as compared with the parental strain. Moreover, F16bP-mediated inhibition of respiratory flux was also observed in isolated rat liver mitochondria. Based on these results and taking into account that F16bP accumulates in the cell cytoplasm under certain conditions, we propose that F16bP has an effector role in the repression of oxidative metabolism observed in the course of the Crabtree effect.

[4] The abbreviations used are: G6P, glucose 6-phosphate; T6P, trehalose 6-phosphate; F6P, fructose 6-phosphate; JO_2, respiratory flux; F16bP, fructose 1,6-bisphosphate.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—*S. cerevisiae* (Yeast Foam, an industrial strain) and *C. utilis* (laboratory strain) were used for mitochondria preparation. Cultures were obtained by growing cells in YPL medium (1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate, supplemented with 2% lactate as carbon source, pH 5.5). Yeast cells were harvested in midlog growth phase for spheroplasts and mitochondria preparation. The laboratory strains W303 1-A (wild type: Mat a, *ade* 2-1, *trp*1-1, *leu* 2-3/112, *his* 3-11-15, *ura* 3-1, *can* 1-100, *GAL*, *SUC2*) and YSH 648 (Mat a, *ade* 2-1, *trp*1-1, *leu* 2-3/112, *his* 3-11-15, *ura* 3-1, *can* 1-100, *GAL*, *SUC*, *tps1*::*TRP1*) (27) were grown in YPGal medium (1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate, pH 5.5, supplemented with 2% galactose as carbon source) and collected at 1 unit of optical density.

Spheroplasts and Mitochondria Preparation—Spheroplasts were obtained according to Avéret *et al.* (28) and were resuspended in buffer A (1 M sorbitol, 1.7 mM NaCl, 0.5 mM EGTA, 10 mM KCl, 1 mM potassium phosphate, 10 mM Tris-HCl, 4 mM iodoacetate, and 1% bovine serum albumin, pH 6.8). Yeast mitochondria were isolated from spheroplasts as described elsewhere (29), and they were suspended in buffer B (0.6 M mannitol, 5 mM MES, 10 mM KCl, 1 mM potassium phosphate, pH 6.8). Rat liver mitochondria were obtained according to Saavedra-Molina *et al.* (30) from male Wistar rats weighing 180–200 g and suspended in buffer C (250 mM sucrose, 10 mM Tris-HCl, 1 mM EGTA, pH 7.2). Protein determination was done using the biuret method with BSA as a standard.

Respiration Assay—The rate of oxygen consumption was measured in a thermostatically controlled chamber at 28 °C equipped with a Clark electrode connected to a recorder. Spheroplasts (1 mg of protein/ml) or mitochondria (0.3 mg of protein/ml) were suspended in buffer A or B, respectively. NADH (1 mM for mitochondria or 10 mM for spheroplasts) or 100 mM ethanol was used as respiratory substrate. For rat liver mitochondria, 5 mM glutamate/malate was used. In order to achieve proper permeabilization, spheroplasts were incubated for 10 min with nystatin (20 μg/ml) at 28 °C before each experiment. For cytochrome *c* oxidase-mediated respiration, mitochondria (0.3 mg of protein/ml) were incubated in the presence of antimycin (2.5 μg/mg of protein), 2.5 mM ascorbate, and 100 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine. To induce state 3 respiration, ADP was added at the concentration indicated in the figure legends. To determine cellular respiration, galactose-grown cells (wild type W303 1-A and Δ*tps*1) or lactate-grown cells (Yeast Foam and *C. utilis*) collected in midlog phase were placed in an oxygraph.

Determination of Mitochondrial Complex III (Ubiquinol:Cytochrome c Oxidoreductase) Activity—Mitochondria (0.3 mg of protein/ml) were incubated in buffer B in the presence of 1 mM KCN, 2 mM ferricyanide, and 100 mM ethanol as respiratory substrate. Since isolated *S. cerevisiae* mitochondria do not have

a complex I (31) but rather internal and external NADH dehydrogenases that donate their electrons to the quinone pool, the ferricyanide reduction rate in these conditions is representative of the ubiquinol:cytochrome *c* oxidoreductase activity. Absorbance changes were followed at 436 nm in a Safas spectrophotometer (Monaco). The rate of ferricyanide reduction was calculated from the slope of absorbance change as a function of time. A molar extinction coefficient (ϵ) of 0.21 $mM^{-1}\ cm^{-1}$ was used.

Measurement of Mitochondrial Transmembranal Electrical Potential Difference ($\Delta\psi$) by Fluorescent Probe Distribution—$\Delta\Psi$ was estimated from fluorescent quenching of the lipophilic cationic dye rhodamine 123 (32). Isolated mitochondria (0.3 mg/ml) were incubated in the mitochondrial buffer supplemented with rhodamine 123 (Sigma) (0.5 μg/ml) in the presence of 100 mM EtOH as respiratory substrate. The rhodamine fluorescence signal at each steady state was recorded with a Kontron SFM 25 fluorimeter at 28 °C. The excitation wavelength was 485 nm, and fluorescence emission was continuously collected at 525 nm.

Metabolite Separation and Quantification by HPIC (High Performance Ionic Chromatography)—High Performance Ionic Chromatography (HPIC) was carried out on a DX 500 chromatography work station (Dionex, Sunnyvale, CA) equipped with GP50 gradient pump and ED_{50} electrochemical and UV detectors. System management and data acquisition were controlled through Peaknet 4.3 software (Dionex). Separation and quantification of sugar phosphates was carried out on a CarboPac PA10 column (250 × 4 mm) equipped with a Dionex PA1 guard column according to Ref. 33.

RESULTS

In yeast cells, the addition of large amounts of glucose results in a metabolic shift toward fermentation and in the accumulation of glycolysis hexose phosphates (25, 26). Since accumulation of hexose phosphates occurs concomitantly with an inhibition of respiration, we tested the possibility of a direct role of these intermediates in the regulation of mitochondrial oxidative phosphorylation. Each one of the three glycolysis hexose phosphates (G6P, F6P, and F16bP) was tested. Fig. 1 shows the effect of G6P, F6P, and F16bP on nonphosphorylating respiration. The respiration was stimulated in a concentration-dependent manner in the presence of G6P and F6P. Although the stimulation mediated by G6P was considerable, it should be stressed that cytosolic concentrations from 1 to 6 mM have been reported; thus, higher concentrations of G6P are not physiological, and only the stimulation induced by up to 5 mM G6P is physiologically meaningful. In the presence of F6P, this increase was less important, and for physiological concentrations (less than 1 mM), there was no effect. On the other hand, F16bP, at physiological concentrations (2–10 mM) (25, 26, 34) induced an inhibition of the respiratory rate that reached 25%. This effect is not dependent on the respiratory substrate, since the same effect was also observed when using ethanol as substrate instead of NADH (data not shown). Under phosphorylation conditions, G6P and F6P had no significant effect on the respiratory rate (Fig. 2), whereas the inhibition in the presence of F16bP was still observed (20%). This indicates that the F16bP-mediated inhibition is present regardless of the respiratory state.

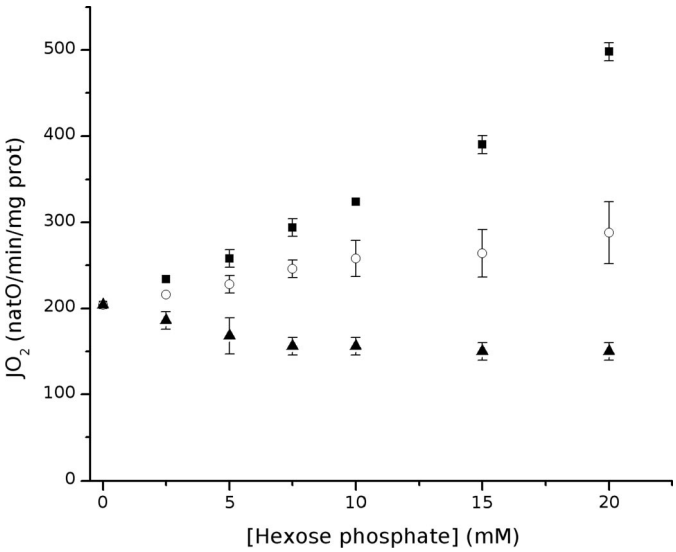

FIGURE 1. **Effect of glycolysis-derived hexose phosphates on the JO_2 of isolated yeast mitochondria under nonphosphorylating conditions.** Yeast Foam mitochondria (0.3 mg of protein/ml) were suspended in buffer B (see "Experimental Procedures") using 1 mM NADH as respiratory substrate, and oxygen consumption was monitored as described under "Experimental Procedures." Various concentrations of glucose 6-phosphate (■), fructose 6-phosphate (○), and fructose 1,6-bisphosphate (▲) were added as indicated. Results are expressed as mean values ± S.D. (n = 3). *natO*, nanoatoms of oxygen.

In order to determine whether the stimulation mediated by G6P and F6P was due to uncoupling, the mitochondrial transmembrane electrical potential ($\Delta\Psi_m$) was assessed in the presence of each glycolysis hexose phosphate. Up to 20 mM, the addition of either G6P or F6P did not change the transmembrane potential value (Table 1). Furthermore, the $\Delta\Psi_m$ did not vary up to 8 mM F16bP and slightly (10 mV) decreased upon the addition of 20 mM F16bP (Table 1). Based on these results, the hypothesis of a possible uncoupling effect induced by G6P and F6P was ruled out. The F16bP-mediated decrease of the respiratory rate in either state, plus the null to slight decrease in $\Delta\Psi_m$ strongly suggested that at least one of the mitochondrial respiratory complexes is inhibited.

During the fermentative shift, every hexose phosphate increases in the cytosol. Thus, the mitochondria are in the presence of all of these hexoses at the same time. Therefore, we tested whether the G6P-induced stimulation of the respiratory rate could be inhibited by F16bP. G6P (3 mM)-induced stimulation was reverted by 1.5 mM F16bP (Fig. 3). Moreover, the F16bP (7 mM)-induced inhibition was present (*i.e.* 25%; 154 nanoatoms of oxygen/min/mg of protein *versus* 204 nanoatoms of oxygen/min/mg of protein). This indicates that under physiological conditions, the effect of glycolysis hexose phosphates on oxidative phosphorylation is that of F16bP (*i.e.* an inhibition of the respiratory rate).

As mentioned above and in view of the results observed for flux and $\Delta\Psi_m$ in the presence of each hexose phosphate (see above), we identified the respiratory chain complex(es) whose activity is affected by these hexose phosphates. Complex IV (cytochrome *c* oxidase) catalyzes an irreversible step of the respiratory chain and has been identified as an important step for controlling the respiratory fluxes both in phosphorylating and

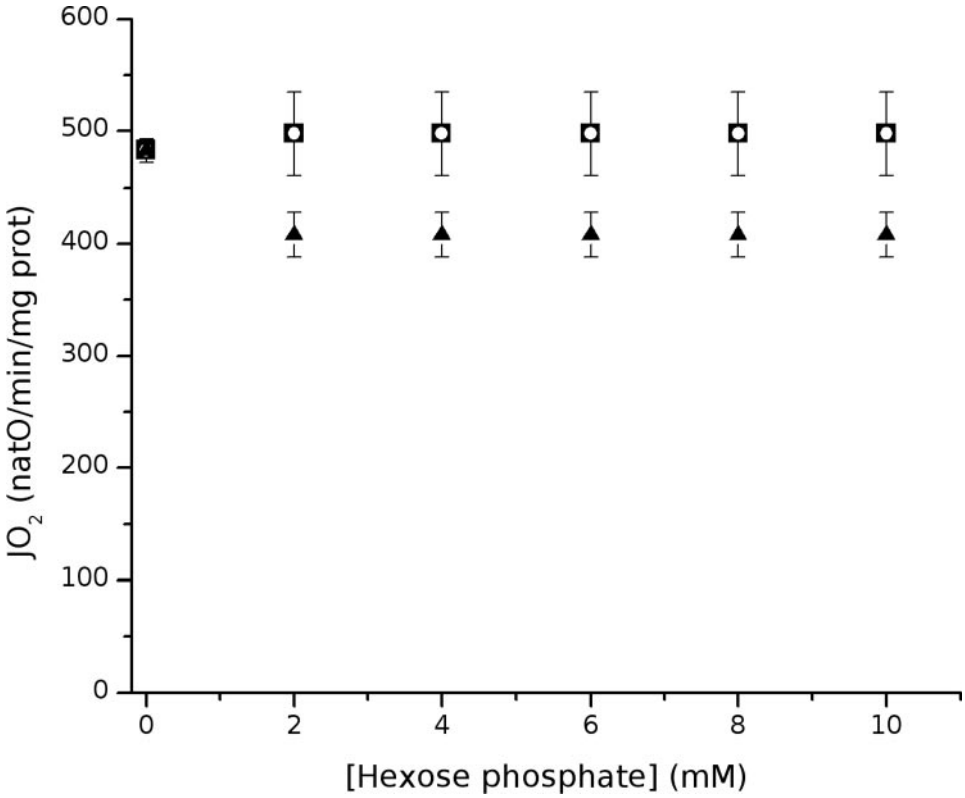

FIGURE 2. **Effect of glycolysis-derived hexose phosphates on the respiratory flux of isolated yeast mitochondria under phosphorylating conditions.** Reaction mixture and respiratory substrate were the same as in Fig. 1 except that 1 mM ADP was added. Respiratory rates were measured after the addition of various concentrations of glucose 6-phosphate (■), fructose 6-phosphate (○), and fructose 1,6-bisphosphate (▲), as indicated. Results are expressed as mean values ± S.D. ($n = 3$). *natO*, nanoatoms of oxygen.

TABLE 1

Mitochondrial transmembranal electrical potential difference in the presence of hexose phosphates

$\Delta\Psi$ was estimated from fluorescent quenching of the lipophilic cationic dye rhodamine 123 as described under "Experimental Procedures." $\Delta\Psi$ was expressed in mV. Results are means ± S.D. of at least three independent experiments.

	$\Delta\Psi$						
	0 mM	2 mM	4 mM	6 mM	8 mM	10 mM	20 mM
	mV						
G6P	178 ± 4	178 ± 3	175 ± 5	177 ± 4	174 ± 5	175 ± 4	173 ± 3
F6P	178 ± 4	177 ± 5	178 ± 5	180 ± 4	180 ± 3	180 ± 5	178 ± 3
F16bP	178 ± 4	177 ± 5	175 ± 3	175 ± 4	176 ± 7	171 ± 6	168 ± 3

nonphosphorylating conditions (35, 36). Complex IV activity is strongly inhibited by F16bP (Fig. 4). This inhibition, which is similar regardless of the respiratory state, is important at low concentrations and reaches ~30% of inhibition for 5 mM F16bP. Furthermore, G6P and F6P had no effects on cytochrome *c* oxidase activity (data not shown).

We then assessed complex III (ubiquinol:cytochrome *c* oxidoreductase) activity in the presence of these glycolysis intermediates. Both hexose monophosphates stimulated the basal activity of complex III (Fig. 5). This activation was similar for both hexose monophosphates, reaching a maximal stimulation at ~150% of the basal value. However, this maximal stimulation was obtained for nonphysiological concentrations of these intermediates, and only a slight stimulation was observed in the presence of physiological (3 mM G6P and 1 mM F6P) concentrations of hexose monophosphates. An F16bP-induced inhibition was also observed on this respiratory complex. Furthermore, hexose phosphate-mediated effects shown in Fig. 5 were sensitive to antimycin A (data not shown), indicating that the ferricyanide reduction rate assessed here was indeed mediated by complex III.

Since hexose monophosphates and F16bP have, respectively, stimulatory and inhibitory effects over complex III activity (Fig. 5), we decided to determine whether these antagonistic effects could be observed on the functionally isolated complex III. Indeed, G6P-mediated stimulation of complex III could be reverted by F16bP, and basal activity was almost restored (Fig. 6).

To this point, we have used mitochondria isolated from *S. cerevisiae* as an experimental model. However, to extrapolate our results to the *in vivo* situation, it was necessary to study mitochondria in a more physiological context. One approach that has been used to undertake bioenergetic studies *in situ* is the use of permeabilized cells (28, 37). Nystatin-permeabilized spheroplasts have been successfully employed to study yeast energetic metabolism (28, 38, 39). To determine the effect of F16bP on the mitochondrial respiratory rate *in situ*, we evaluated nystatin-permeabilized spheroplast respiration in the presence of this hexose. To avoid F16bP metabolism, we omitted the cofactors required for adequate function of the glycolytic enzymes and added iodoacetate in respiration buffer. Fig. 7 shows that in nystatin-permeabilized spheroplasts, F16bP inhibited the nonphosphorylating respiratory rates by 50%. The range of concentrations used to obtain the maximum inhibition was similar to that employed on isolated mitochondria: 6–10 mM for state 4 and 2–4 mM for state 3 (data not shown). Of note, these concentrations are within the physiological range (25, 26) (see Table 3). The additions of G6P and F6P were also tested. However, an exceedingly high concentration was required to observe a stimulation of respiratory flux (~120 mM) (data not shown), indicating that there might be a constraint for metabolite diffusion toward mitochondria or the intermembrane space. A similar situation was observed in permeabilized cells for NADH in yeast (28) and in hepatocytes (37). In each of these cases, it was found that closure of the voltage-dependent anion channel was responsible for restraining molecule flux to the intermembrane space (14, 40). Since the voltage-dependent anionic channel is closed in permeabilized spheroplasts, it is possible that the hexose monophosphate diffusion is constrained. According to our results, this would also imply that

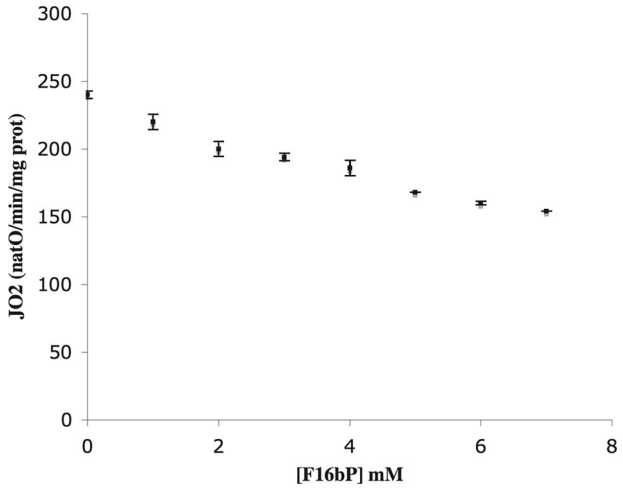

FIGURE 3. **Effect of fructose 1,6-bisphosphate on the glucose 6-phosphate induced increase in the respiratory flux (JO_2).** Yeast Foam mitochondria (0.3 mg of protein/ml) were incubated in buffer B with 1 mM NADH in the presence of 3 mM glucose 6-phosphate. Different concentrations of fructose 1,6-bisphosphate were added, as indicated. Results are expressed as mean values ± S.D. ($n = 3$). *natO*, nanoatoms of oxygen.

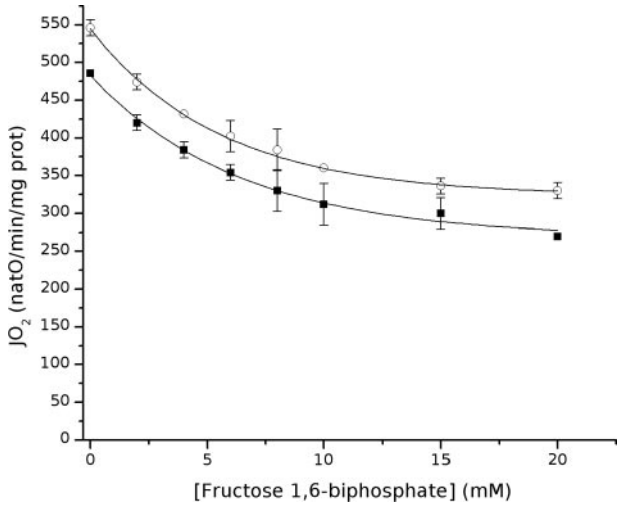

FIGURE 4. **Cytochrome *c* oxidase activity in the presence of fructose 1,6-bisphosphate in both phosphorylating and nonphosphorylating conditions.** Mitochondria (0.3 mg of protein/ml) were incubated in respiration buffer B in the presence of 2.5 μg of antimycin A/mg of protein, 2.5 mM ascorbate, and 100 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. Oxygen consumption was monitored in the absence (■) or in the presence (○) of 1 mM ADP. Fructose 1,6-bisphosphate was added at the indicated concentrations. Results are expressed as mean values ± S.D. ($n = 3$). *natO*, nanoatoms of oxygen.

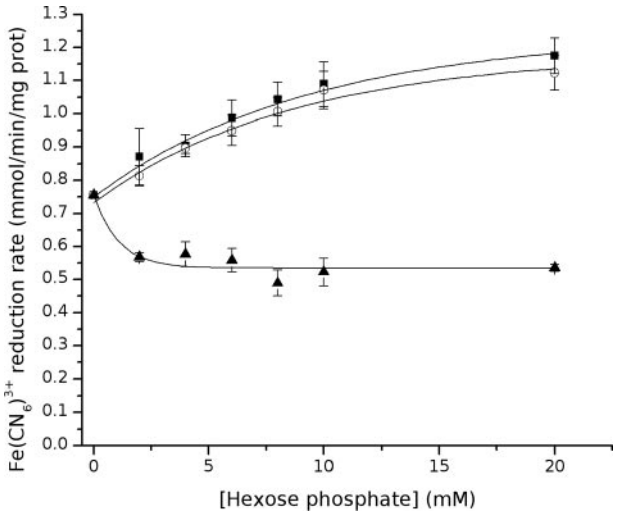

FIGURE 5. **Effect of glycolysis-derived hexose phosphates on the activity of mitochondrial complex III.** Mitochondria (0.3 mg of protein/ml) were incubated in buffer B in the presence of 1 mM KCN, 2 mM ferricyanide, and 100 mM ethanol. The reduction of ferricyanide was monitored spectrophotometrically at 436 nm. Different concentrations of glucose 6-phosphate (■), fructose 6-phosphate (○) and fructose 1,6-bisphosphate (▲) were used as indicated. Results are expressed as mean values ± S.D. ($n = 3$).

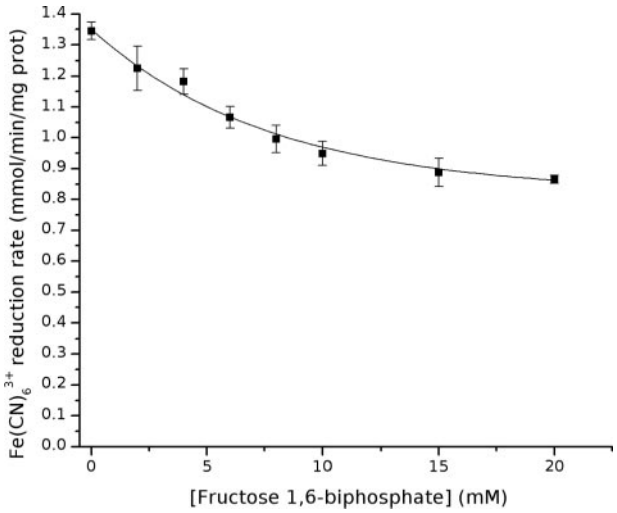

FIGURE 6. **Effect of fructose 1,6-bisphosphate on the glucose 6-phosphate-induced stimulation of complex III activity.** Mitochondria (0.3 mg of protein/ml) were incubated in buffer B in the presence of 1 mM KCN, 2 mM ferricyanide, 100 mM ethanol, and 20 mM glucose 6-phosphate. Ferricyanide reduction was measured at 436 nm. Various concentrations of fructose 1,6-bisphosphate were added as indicated. Results are expressed as mean values ± S.D. ($n = 3$).

F16bP would be specifically channeled through the outer membrane in permeabilized spheroplasts.

Once F16bP had been shown to regulate mitochondrial oxidative phosphorylation *in situ*, it was necessary to determine respiration of intact cells in conditions where F16bP accumulates. We thus induced the Crabtree effect in galactose-grown cells and in the mutant Δ*tps1*, which lacks the enzyme trehalose 6-phosphate synthase. Several reports have demonstrated that this mutant overaccumulates glycolysis hexose phosphates, particularly F16bP, in response to glucose addition (24, 41, 42). Spontaneous and uncoupled respiration values were decreased in the Δ*tps1* strain (Table 2). Furthermore, when comparing the percentage of spontaneous respiration inhibition for each strain, glucose addition (30 mM) inhibited by 36 and 62% the respiration of wild type and Δ*tps1* strains, respectively (Table 2). We assessed the intracellular concentration of each of the considered hexose phosphates after glucose addition. Table 3 shows that in the wild type strain, the intracellular concentration of both G6P and F6P did not significantly change after glucose addition. On the other hand, the concentration of F16bP largely increased. In the mutant strain (Δ*tps1*), there is a significant increase in the concentration of G6P and F6P and a drastic (10-fold) increase in F16bP concentration. Taking into account our data indicating that F16bP inhibits the respiratory flux of isolated mitochondria, permeabilized spheroplasts, and whole cells, we propose that the accumulation of F16bP is

responsible for the difference observed between the two strains (wild type and Δ*tps1*) in the observed extent of glucose-induced inhibition of respiration.

In some Crabtree-negative yeast species, where glucose repression (Crabtree effect) does not occur, glucose addition does not inhibit respiration (4, 43). *C. utilis* is one such species. The respiratory activity of cells growing on lactate was not affected by the addition of 30 mM glucose (not shown), whereas the concentrations of G6P and F6P were doubled, and that of F16bP was 10-fold increased (see Table 3). In mitochondria isolated from *C. utilis*, G6P and F6P stimulated the respiratory rate (Fig. 8). This effect is reverted by F16bP. Moreover, it is noteworthy that there was no F16bP-mediated inhibition of the respiratory chain in isolated mitochondria (Fig. 8). This result reinforces our hypothesis that the respiration inhibition by F16bP plays a crucial role in the glucose-induced Crabtree effect.

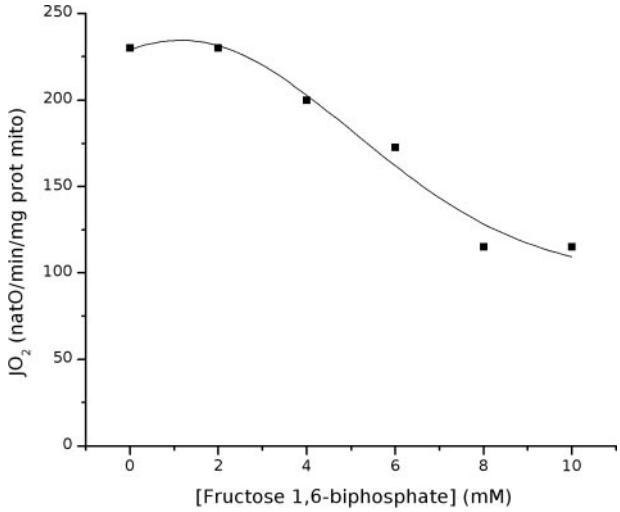

FIGURE 7. **Effect of fructose 1,6-bisphosphate on the rate of oxygen consumption in nystatin-permeabilized yeast spheroplasts in nonphosphorylating conditions.** Spheroplasts (1 mg of protein/ml) were preincubated with nystatin (20 μg/ml) in buffer A. The substrate was 10 mM NADH. Different concentrations of fructose 1,6-bisphosphate were used as indicated. Respiratory rates on permeabilized spheroplasts were expressed as nanoatoms of oxygen (*natO*)/min/mg of mitochondrial protein by considering the following relationship: 1 mg of mitochondrial protein = 0.32 mg of spheroplast protein. Results are representative of one typical experiment; similar data were obtained for two other spheroplast preparations.

In mammals, it has been demonstrated that hepatoma cells exhibit a Crabtree effect concomitantly with a 30-fold increase in cytoplasmic F16bP amount (7). This metabolite can exhibit a cytosolic concentration between 7 and 25 mM (7, 44). We thus tested whether the F16bP-mediated inhibition could also take place in isolated mammalian mitochondria. As hypothesized, this accumulation was important for the glucose-induced repression of respiratory fluxes, since we were able to inhibit the respiration of mitochondria from a nontumorous rat liver using concentrations comparable with those found in hepatoma cells. Phosphorylating respiration was 75% inhibited by 5 mM F16bP (Fig. 9), indicating that this compound is probably implicated in the Crabtree effect in mammalian cells. Furthermore, whereas G6P and F6P by themselves had no effects on the mitochondrial respiratory rate, the F16bP-induced inhibition was slightly decreased in their presence (Fig. 9).

DISCUSSION

In aerobic organisms, the phosphate potential is maintained both through the glycolytic and respiratory pathways. Glycolysis provides reducing equivalents to the mitochondrial respiratory chain. In the presence of high concentrations of glucose, facultative aerobic organisms shift their metabolism toward fermentation, hence implicating a modification of the glycolysis/respiration balance. The glucose-induced shift toward fermentation associated with respiration inhibition was named the "Crabtree effect." As mentioned in the Introduction, different hypotheses have been laid out, and no clear mechanisms apply to all Crabtree-positive cells. It has been stressed that this phenomenon is induced by different factors, such as changes in the phosphate potential or in pH (7). Indeed, since glycolytic and respiratory fluxes both depend on the phosphate potential,

TABLE 2

The Crabtree effect is enhanced in the yeast Δ*tps1* mutant strain

The rate of oxygen consumption by yeast cells grown in YPL medium was measured as described under "Experimental Procedures." Additions were as indicated. Mean values of three independent experiments are shown. natO, nanoatoms of oxygen; DW, dry weight.

	W303-1A		Δ*tps1*	
	JO_2	Percentage	JO_2	Percentage
	natO/min/mg (DW)	*%*	*natO/min/mg (DW)*	*%*
Spontaneous	160 ± 7	100	120 ± 10	100
With 30 mM glucose	103 ± 10	64	47 ± 2	39
With 100 mM ethanol, 8 μM carbonyl cyanide *m*-chlorophenylhydrazone	190 ± 2	119	137 ± 17	114

TABLE 3

Hexose phosphate concentration in the different strains

Cells were harvested 5 min after 30 mM glucose addition, and metabolites were extracted as described in Ref. 33. Hexose phosphates were assessed as described under "Experimental Procedures." Mean values of three independent experiments are shown.

Strain	G6P		F6P		F16bP	
	Without glucose	With glucose	Without glucose	With glucose	Without glucose	With glucose
			mM			
W303	0.53 ± 0.04	0.50 ± 0.01	0.42 ± 0.1	0.43 ± 0.08	2.32 ± 0.03	6.0 ± 0.6
Δ*tps1*	0.82 ± 0.12	1.68 ± 0.04	0.45 ± 0.1	1.01 ± 0.04	3.54 ± 0.2	39.0 ± 2.2
C. utilis	0.14 ± 0.04	0.26 ± 0.09	0.20 ± 0.1	0.55 ± 0.1	0.72 ± 0.03	6.8 ± 1.9
Yeast Foam	0.41 ± 0.08	0.46 ± 0.002	0.56 ± 0.07	0.43 ± 0.03	0.83 ± 0.1	6.3 ± 1.9

it is possible that changes in ATP or P_i could modify the glycolysis/oxidative phosphorylation coordination in aerobic conditions. In this regard, during the Crabtree effect in hepatoma cells (7) and in yeast (10), a glucose-induced decrease of cytoplasmic phosphate has been measured. Moreover, a cytoplasmic accumulation of fructose 1,6-bisphosphate has been determined as well for hepatoma, Ehrlich ascites, and yeast Crabtree-positive cells (7, 25, 45). Further, in yeast cells, it has been reported that the addition of 100 mM glucose induced an increase in cytosolic F16bP concentration from 2 mM up to 9 mM in a wild type strain (46), which is in good agreement with concentrations presented in this work (see Table 3). Furthermore in a Δ*tps1* strain, this concentration increases largely upon glucose addition (see Table 3). In mammalian cells and more precisely hepatocytes, this metabolite exhibits a cytosolic concentration lower than 1 mM. However, in hepatoma cells, this concentration is highly increased and reaches values up to 25 mM (42). This is clearly within the range of concentrations used in this study.

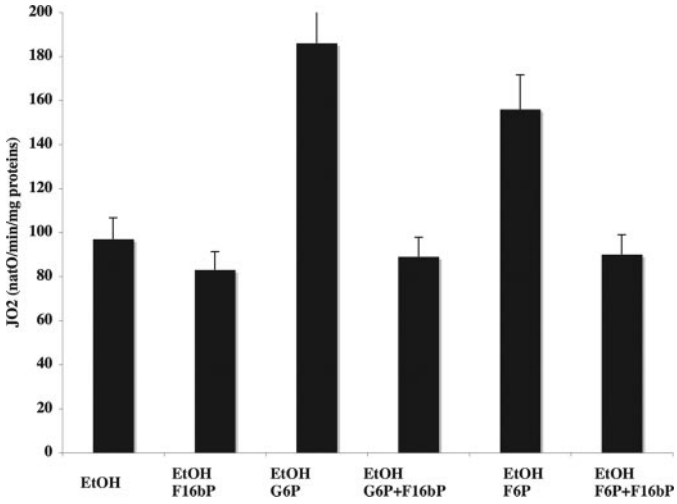

FIGURE 8. **Effect of glycolysis-derived hexose phosphates on the respiratory flux of *C. utilis* mitochondria.** *C. utilis* mitochondria (0.3 mg of protein/ml) were suspended in buffer B (see "Experimental Procedures") using 100 mM ethanol as respiratory substrate. Fructose 1,6-bisphosphate, glucose 6-phosphate, and fructose 6-phosphate (10 mM each) were added, as indicated. Results are expressed as mean values ± S.D. ($n = 3$). *natO*, nanoatoms of oxygen.

Thus, in this paper, we clearly show that the mitochondrial respiratory rate is inhibited by physiological concentrations of F16bP both in nonphosphorylating and phosphorylating conditions. This is true for both isolated mitochondria and permeabilized spheroplasts from the Crabtree-positive yeast *S. cerevisiae*. Whatever the respiratory state, when hexose mono- and bisphosphates are used together, the resulting effect is an inhibition of the respiratory rate by F16bP. We have shown that electron transfer through complex III was slightly inhibited by F16bP and that this metabolite inhibited cytochrome *c* oxidase activity. These results point to an *in situ*, F16bP-mediated regulation of the mitochondrial respiratory chain.

This effect seems specific to Crabtree-positive yeast species. Indeed, in mitochondria isolated from the Crabtree-negative species *C. utilis*, hexose monophosphates are able to stimulate the respiratory rate, and this stimulation is inhibited by F16bP. However, in this strain, the respiratory rate itself is never inhibited by F16bP. Altogether, these results indicate that in yeast, F16bP probably participates in the Crabtree effect.

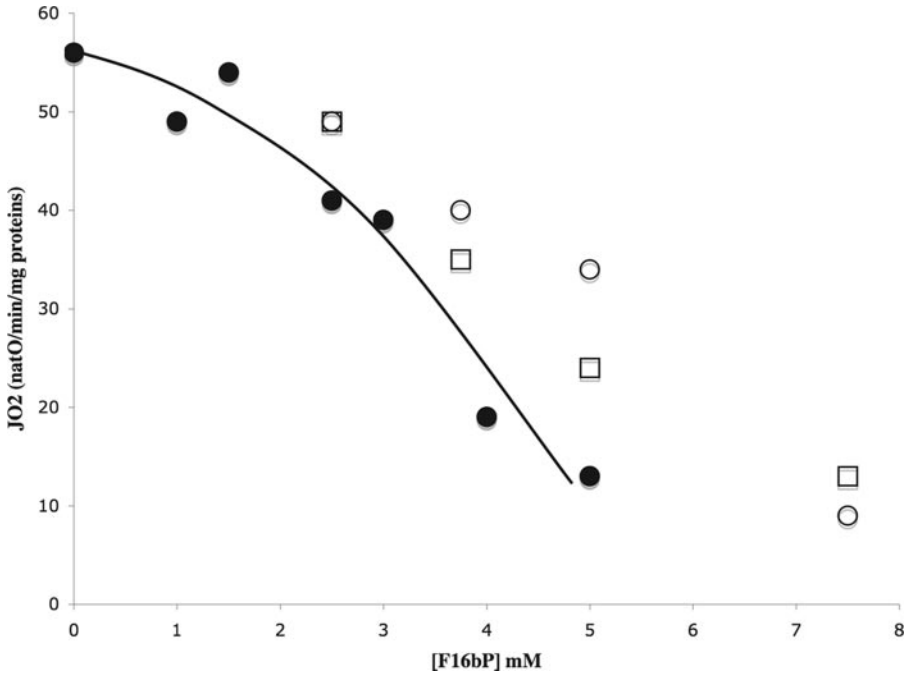

FIGURE 9. **Effect of fructose 1,6-bisphosphate on the respiratory flux in rat liver mitochondria under phosphorylating conditions.** Rat liver mitochondria (0.5 mg of protein/ml) were suspended in buffer C in the presence 10 mM P_i, 1 mM ADP, and 5 mM glutamate/malate. Respiratory rates were measured after the addition of different concentrations of fructose 1.6-phosphate in the absence (●) or in the presence of either 10 mM glucose 6-phosphate (○) or 10 mM fructose 6-phosphate (□). Results are representative of one typical experiment, similar data were obtained for at least two other isolated mitochondria preparations. *natO*, nanoatoms of oxygen.

We clearly show that F16bP induces an inhibition of the respiratory rate, acting directly on two complexes of the mitochondrial respiratory chain. We propose that *in vivo*, this hexose acts as an inhibitor of the respiratory flux. Indeed, here, we bring to light several elements that sustain this hypothesis. Several reports in the literature show that the respiration of cells that accumulate F16bP to different degrees changes proportionally. It has been shown that the rate of oxygen consumption of the Δ*hxk2* mutant, lacking isoform 2 of hexokinase, which has lower levels of F16bP, is not inhibited by high glucose concentrations (25, 47). A strain overexpressing phosphofructokinase has lower JO_2 values (48). Furthermore, the Δ*tps1* strain, well known for accumulating hexose phosphates (24, 26, 41, 42), has an enhanced Crabtree effect (see Tables 2 and 3).

In mammalian cells, we have demonstrated that physiological concentrations of F16bP inhibited the respiration of isolated rat liver mitochondria as well. One of the early theories on the Crabtree effect in cancer cells was the impairment of oxidative phosphorylation (reviewed in Ref. 49). This was challenged by the finding that hepatoma cells maintained their phosphate potential mainly through respiration (50). Our results point to the fact that an impairment of mitochondrial function is not a prerequisite for Crabtree effect induction since the same arrest of respiration caused by F16bP can be observed in mitochondria obtained from a nontumor source. G6P is another hexose phosphate that seems to have an impact on mitochondrial function (51). The presence of this hexose-monophosphate is important for the release of mitochondria-bound hexokinase and for enhancing the Bax-mediated release of cytochrome *c* (51). Thus, the participation of hexose phosphates as metabolic messengers and their influence on tumor cell oxidative metabolism needs to be further evaluated.

In conclusion, we propose that F16bP acts as a true metabolic messenger, inducing a decrease in respiratory flux during the transition to fermentative metabolism (*i.e.* participating in the induction of the Crabtree effect). F16bP accumulation must be one of several events (*e.g.* changes in pH or phosphate potential) that lead to this inhibition of respiration.

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Review

Tumor cell energy metabolism and its common features with yeast metabolism

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ABSTRACT

During the last decades a considerable amount of research has been focused on cancer. A number of genetic and signaling defects have been identified. This has allowed the design and screening of a number of anti-tumor drugs for therapeutic use. One of the main challenges of anti-cancer therapy is to specifically target these drugs to malignant cells. Recently, tumor cell metabolism has been considered as a possible target for cancer therapy. It is widely accepted that tumors display an enhanced glycolytic activity and oxidative phosphorylation down-regulation (Warburg effect). Therefore, it seems reasonable that disruption of glycolysis might be a promising candidate for specific anti-cancer therapy.

Nonetheless, the concept of aerobic glycolysis as the paradigm of tumor cell metabolism has been challenged, as some tumor cells use oxidative phosphorylation. Mitochondria are of special interest in cancer cell energy metabolism, as their physiology is linked to the Warburg effect. Besides, their central role in apoptosis makes these organelles a promising "dual hit target" for selectively eliminate tumor cells.

Thus, it is desirable to have an easy-to-use and reliable model in order to do the screening for energy metabolism-inhibiting drugs to be used in cancer therapy. From a metabolic point of view, the fermenting yeast *Saccharomyces cerevisiae* and tumor cells share several features. In this paper we will review these common metabolic properties and we will discuss the possibility of using *S. cerevisiae* as an early screening test in the research for novel anti-tumor compounds used for the inhibition of tumor cell metabolism.

Contents

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1. Tumor cell metabolism: possible target for cancer treatment

1.1. The Warburg effect

Pioneer work done by Otto Warburg suggested that most tumor cells rely on the glycolytic pathway for their energy conversion processes [1]. This was referred to as the "aerobic glycolysis" as a variety of tumor cells display a decreased respiration rate and an enhancement of lactate production rate in the presence of glucose, in spite of oxygen availability [2]. It is probable that this phenotype endows tumor cells with some advantages in order to survive and proliferate in harsh microenvironments, e.g. in hypoxic conditions and within poorly vascularized tumors [3]. This metabolic adaptation could also help malignant cells to overcome programmed cell death mechanisms [4]. For some tumors a correlation between invasiveness and their glycolytic capacity was observed [5,6].

The exact molecular mechanisms underlying the "Warburg effect" are unknown. Nonetheless, it is possible that the enhanced expression of glycolysis enzymes and glucose transporters along with a down-regulation of mitochondrial metabolism could be at the basis of the glycolytic phenotype of tumor cells (see Fig. 1) [1]. Regarding this, the hypoxia-induced metabolic reprogramming mediated by the HIF-1 transcription factor has been pointed out as the key regulator for the "aerobic glycolysis" in tumors, as the expression of glycolysis enzymes, glucose transporters and several other tumor-related genes are controlled by HIF-1 [7]. It is also proposed that Warburg effect appears as consequence of an irreversible damage to mitochondria and to the proteins involved in oxidative metabolism [1]. Nowadays, the "aerobic glycolysis" is considered the paradigm of tumor cell metabolism. Nevertheless, this hypothesis was challenged by the finding that some tumor cell lines possess an efficient oxidative metabolism [8–10]. Moreover, tumor cell metabolism is a function of its microenvironment. When near vascularised zones they may behave as aerobic, but if they are located in hypoxic zones (e.g. the inner core of a solid overgrowth) they switch to lactic fermentation [3,11]. It is important to fully understand the Warburg effect in order to gain insight into the mechanisms of tumor cell energy conversion processes. This will impact the establishment of specific therapies for successful cancer treatment (See below).

1.2. The Crabtree effect

An important feature of some tumor cells is their ability to repress respiration and oxidative phosphorylation in response to glucose

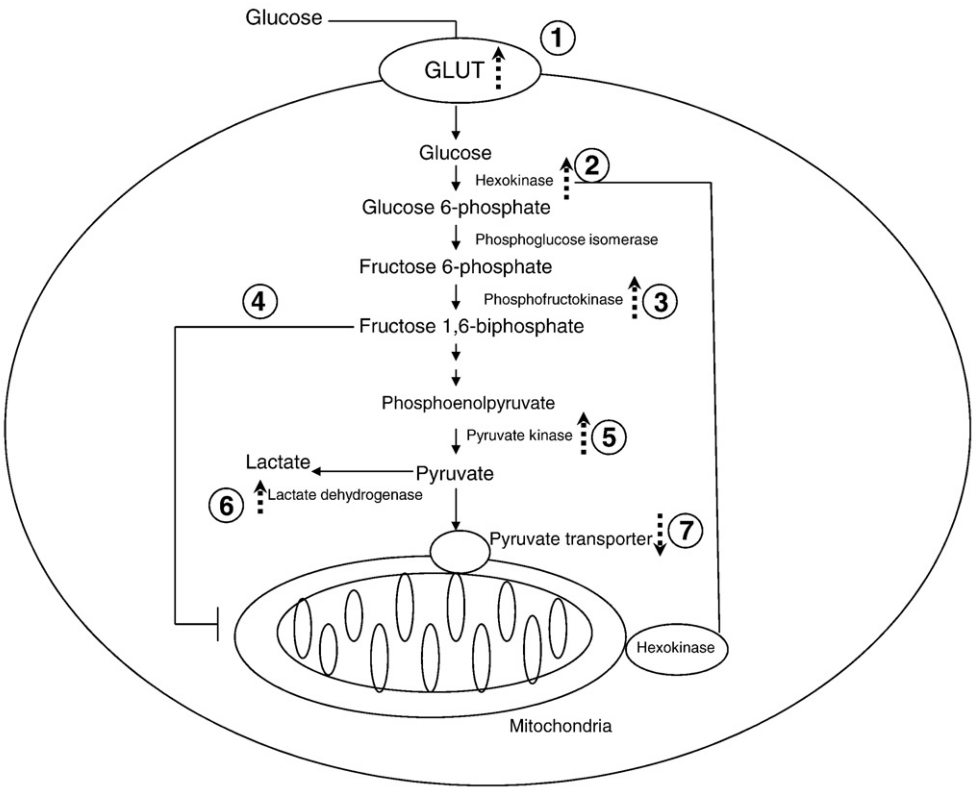

Fig. 1. Metabolic alterations that might lead to the Warburg effect in tumor cells. Upward arrows indicate an upregulated step and the contrary is shown by downward arrows. 1: Overexpression of the high-affinity glucose transporters. 2: Higher levels of isoforms I and II of hexokinase that can physically interact with mitochondria. 3: Predominance of L-phosphofructokinase isoform that is more sensistive to fructose 2,6-biphosphate-mediated activation. 4: Inhibition of respiratory complexes III and IV by an overaccumulated fructose 1,6-biphosphate. 5: Overactivation of pyruvate kinase. 6: Overexpression of a lactate dehydrogenase insensitive to pyruvate-mediated inhibition (M isoform). 7: Impairment of pyruvate translocation to the mitochondrial matrix. See text for further details.

presence by short-term mechanisms. This has been termed the "Crabtree effect" after its discoverer [2]. Mechanistically it is different from the Warburg effect, as the latter is given by several long-term adaptations that reprogram cell metabolism to aerobic glycolysis. Both effects are part of the general mechanism that redirects metabolic flux towards fermentation in tumors. The elucidation of the Crabtree effect could explain why some tumors display a preference for fermentation in spite of the oxygen availability on a short-term basis. Several mechanisms have been proposed for the Crabtree effect induction, such as limitation of respiratory substrate [12], inhibition of ATP synthase [13] and changes in cytoplasmic phosphate potential and pH [14]. Although it seems that this induction is due to a combination of several factors rather than only one of them [14] (See below).

Recently, we showed that there is a link between glycolysis and the Crabtree effect. One of the intermediates of this pathway (fructose 1,6-biphosphate) inhibits mitochondrial respiratory chain and induces a decrease in respiration [15]. Moreover, this phenomenon was observed in non-tumor mitochondria suggesting that mitochondrial impairment is not a prerequisite for the Crabtree effect [15]. This may also provide a relationship between the Warburg and the Crabtree effects, as during the "aerobic glycolysis" fructose 1,6-biphosphate is highly accumulated in tumor cell cytoplasm [16]. So, it is possible that the Crabtree effect is induced as one of the early metabolic events in tumorigenesis, leading to the establishment of the Warburg effect. This could also provide the grounds for an integrative model for explaining the glycolytic phenotype of tumors.

1.3. Metabolic therapy: the inhibition of tumor cell energy metabolism

Some features of tumor aerobic glycolysis are actually used as a marker for cancer diagnosis and treatment procedures. The fluoro-deoxyglucose-based positron emission tomography (FDG-PET scan) is widely employed for these purposes [17]. This technique is based on the increased capacity of tumor cells for glucose uptake. As a consequence of the Warburg effect, malignant cells display an increased glucose uptake, thus the non-metabolizable probe will be highly accumulated in such tissues, facilitating the identification of tumors and potentially malignant overgrowths [17]. As mentioned above, it has recently been demonstrated that not all tumor cell lines use glycolysis as their main energy-yielding pathway and oxidative phosphorylation is completely functional in these cases [9,10,18]. However, these cell lines are more sensitive to oxidative phosphorylation-inhibiting drugs than their normal counterparts [8]. This has raised the idea of using the inhibition of tumor cell energy metabolism for cancer treatment. This approach is known as "Metabolic therapy" [10]. To support this proposal, it has been shown that inhibition of glycolysis and/or oxidative phosphorylation results in delayed growth or in the impairment of tumor cell viability in xenograft models and cell cultures in vitro [8]. Metabolic therapy would have two advantages regarding other therapeutic approaches: First, it is more specific, as tumor cells are more sensitive to metabolism inhibitors than their normal counterparts [8,10]. And second, in contrast to oncogene targeting approaches, this therapy is independent of the specific signaling or epigenetic dysfunctions that might occur as the origin of cancer. Further, a combination of energy metabolism inhibitors with other anti-tumor drugs could increase the effectiveness of the treatment (dual hit approach) [19].

2. The fermentative metabolism of *Saccharomyces cerevisiae*

S. cerevisiae is subjected to fluctuations of nutrient availability in its natural environment. Thus, these cells must be able to successfully adapt their metabolism in order to meet the energy demand and survive. In a glucose-rich environment, fermentation is the main metabolic pathway for carbon and energy metabolism. In contrast, when a non-fermentable carbon source (i.e., glycerol, ethanol, lactate or acetate) is present, oxidative phosphorylation is the main energy-yielding pathway [20].

Glucose is the preferred carbon source for yeast, regardless the presence of oxygen or other non-fermentable carbon sources. In fact, fermenting yeasts grow at higher rates compared to those that grow oxidatively [21]. If glucose is added during yeast aerobic growth, they immediately arrest respiration (Crabtree effect) and accelerate glycolysis at the same time that ethanol is produced [22]. This transition is modulated by short- and long-term events. The latter involves the down-regulation of gluconeogenic and oxidative metabolism-related proteins and the enhanced transcription of glycolytic enzymes and glucose transporters [20,23,24].

From a metabolic point of view, there are similarities between the glucose-induced repression of oxidative metabolism (catabolite repression) in yeast and the "Warburg phenotype" of tumor cells. In both cell types, there are mechanisms that enhance glycolytic flux concomitantly with the repression of oxidative phosphorylation, and fermentation is preferred even in the presence of oxygen (Table 1).

Saccharomyces cerevisiae display a Crabtree effect, thus providing a good model for studying this phenomenon. Another advantage is the existence of yeasts that are classified as Crabtree-negative (such as *Candida utilis* and *Kluyveromyces lactis*) [22]. Hence, the identification of the differences between both kinds of yeast may provide some insight into the mechanism leading to Crabtree effect. The metabolic features shared between *S. cerevisiae* and tumors have been previously identified [25]. In view of these similarities and given the fact that this yeast is an easy-to-use experimental model, the possibility of the utilization of yeast as a tool for testing anti-tumor drugs for metabolic therapy is discussed below.

3. Metabolic similarities between tumor cells and fermenting yeast

3.1. Glucose transport

In both yeast and mammalian cells, an important control of the glycolytic pathway resides at the glucose uptake step [26,27]. This sugar is one of the most consumed substrates by tumors in vivo [28]. This may be caused by the overexpression of glucose transporters in cytoplasmic membrane (Fig. 1). Accordingly, an overexpression of GLUT1 transporter has been identified for several tumor cell lines [29]. GLUT3, another high affinity glucose transporter, is overexpressed in HeLa and Ehrlich ascites tumor cells [30,31]. Moreover, the K_m value for these transporters is between 2–5 mM [32], which is approximately the value for blood glucose concentration in vivo [33,34].

As in solid tumors there are poorly vascularised zones, it is possible that tumor cells make this adaptation to successfully compete with normal cells for substrate supply [35]. Taking this into account, glucose transport has been proposed as a target of anti-tumor therapy [29,36]. Disruption of GLUT1 expression arrests cell growth and invasiveness of tumor cells in vitro [37]. It has also been demonstrated that drug-mediated glucose transport inhibition increased the effectiveness of several anti-tumor drugs and effectively induced growth arrest and apoptosis in lung, breast cancer and leukemia cell lines [29,38].

Yeast, in their natural environment are subjected to fluctuations of glucose level, from the millimolar to the molar range. As a consequence, they adapt their transporter expression profile in a reversible fashion according to glucose availability [39]. When glucose levels are too low, only the high affinity isoforms of glucose transporter are expressed, while those with low affinity are the main isoforms when its level increases [40]. In the absence of glucose carrier, yeast does not grow on this carbon source [41,42]. Hence, yeast would be helpful for the screening of glucose transport-inhibiting drugs for metabolic therapy.

3.2. Glycolytic flux enhancement

Glycolysis and the Krebs cycle function in a coordinated fashion as the central route by which carbon substrates are catabolized in

Table 1
Comparison of metabolic features between yeast and tumor cells.

	Tumor cells	Fermenting yeast
Glucose transporters	• Increased expression of high affinity transporters (GLUT)	• Predominance of low-affinity transporters
Hexokinase	• Overexpression isoforms HKI and HKII. • Binding to mitochondrial outer membrane	• Overexpression of only isoform II. • No binding with mitochondria
Phosphofructokinase	• Predominance of P- and L- isoforms. Resistance to citrate-mediated inhibition.	• Expression pattern do not change. • Mainly regulated by allosteric effectors
Pyruvate kinase	• Mainly isoform M2 is present. • Reversible association with other glycolysis enzymes.	• Mainly isoform 1 which is strongly activated by F16bP
Pyruvate transport	• Decrease of pyruvate transport kinetic parameters (K_m and V_{max}) regarding normal cells.	
Pyruvate dehydrogenase kinase	• Overexpressed	• Overexpressed and active
Krebs cycle enzymes	• Isocitrate dehydrogenase defect. • High citrate efflux.	• Inactivation of malate dehydrogenase and isocitrate dehydrogenase
Mitochondrial respiratory chain	• Low expression of complexes II and IV.	• Glucose-induced repression of cytochrome *c* and complex II expression.
Mitochondrial ATP synthesis	• Overexpression of ATP synthase inhibitory unit. • Lower ADP transport rate.	• Decrease in ATP synthase content during fermentation • Lower respiratory control ratio

eukaryotic cells. Moreover, cell energy status depends on the correct functioning of both pathways [43]. Glucose, once taken from the external medium is processed by glycolysis and split in two pyruvate molecules. This pathway yields two molecules of ATP and two of NADH. During oxidative metabolism, pyruvate is taken up by mitochondria and further oxidized in the Krebs cycle, whereas in fermentation it remains in the cytosol and is subsequently reduced in order to reoxidize cytoplasmic NADH. Complete combustion of glucose through glycolysis and oxidative phosphorylation has a better yield than fermentation. Under fermentation conditions, there must be an acceleration of both glucose uptake and catabolism, in order to cope with cell energy demand.

From Warburg's discoveries it has been demonstrated that a number of tumor cell lines use fermentative metabolism [2,44]. Similarly to yeast, when glucose supply is high, these cells mainly use glycolysis for energy conversion processes [23]. Both cell types achieve this adaptation by up-regulating glycolytic enzymes expression and by modulating their activity in order to increase the fermentative flux (Fig. 1).

Metabolic flux determinations demonstrate that glycolysis is several-fold increased in a number of tumor cell lines and in tumors in vivo [16,34]. One of the possible explanations for this flux enhancement in both yeast and tumors is the overexpression of virtually all enzymes of the glycolytic pathway [45–47].

Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) are considered the main controlling steps for glycolysis flux modulation as they catalyze the three irreversible reactions in the glycolytic sequence [48]. Indeed, an overexpression and/or over-activation of these three enzymes has been described for a number of tumor cells and in fermenting yeast (Fig. 1) [10,45,49,50].

Another feature that may impact on fermentative flux is the change of isoenzyme expression pattern of glycolysis enzymes [5,51]. Normally, specific enzyme isoforms are confined to a particular tissue, but in tumor cells this pattern is altered and this seems to happen for every enzyme in glycolysis [45,51]. In yeast, the expression pattern of these enzymes also changes in fermentation [20,47]. For both models, we will focus our discussion on the three key enzymes mentioned above (HK, PFK and PK).

3.2.1. Hexokinase

In mammals, hexokinase exists in four isoforms (I,II, III and IV), each one with particular kinetic properties and tissue distribution [52]. Isoform IV (glucokinase) levels decrease in hepatoma cells compared with normal liver, while the other three isoforms are increased [53]. In rapid-growth hepatomas, a loss of isoforms III and IV was detected [54], and hexokinase I is the predominant form in brain tumors [55]. Both, I and II HK are high affinity isoforms that have been observed bound to mitochondrial external membrane (Fig. 1) [55,56]. This association renders hexokinase less sensitive to glucose 6-phosphate-mediated inhibition and it may allow a maximal fermentative flux [57]. This could provide an explanation for the Warburg effect, as hexokinase would have preferential access to mitochondria-generated ATP allowing a constant supply for glycolysis [57]. Nevertheless, recently it was reported that at physiological temperature and pH, the mitochondria-bound HK is effectively inhibited by glucose 6-phosphate [16]. As glucose 6-phosphate promotes the disruption of the mitochondria-hexokinase interaction, it remains to be demonstrated if this inhibition is observed on the mitochondria-bound isoform or over a detached form [55,58]. As mitochondrial-bound HK also hinders the translocation of the pro-apoptotic protein Bax to mitochondrial outer membrane, it may be involved on the cell death resistance mechanisms of tumor cells [58].

In *S. cerevisiae* hexokinase activity increases upon glucose addition [49]. There are three glucose-phosphorylating enzymes in yeast (Hexokinases I, II, and glucokinase) and none of these are inhibited by glucose 6-phosphate [59]. During fermentation, HKII is the main isoform present, whereas during oxidative metabolism HKI and glucokinase are the predominant forms [60]. Apparently, this adaptation is made in order to make an efficient transition towards fermentation, as it was demonstrated that HKII is needed during glucose-induced catabolite repression participating in a signal transduction pathway [61]. In contrast to their mammalian counterparts, yeast HK does not bind to mitochondrial outer membrane as it lacks a N-terminal segment that is required for its interaction with mitochondrial porin [62].

3.2.2. Phosphofructokinase

Some studies point out Phosphofructokinase (PFK) as the "rate-controlling" step in glycolysis in both yeasts and tumors [63,64]. Its activity is modulated by several metabolites such as AMP, citrate, ATP, Pi, Fructose 1,6-biphosphate (F16bP) and fructose 2,6-biphosphate (F26bP) [65]. The levels of all these metabolites change in response to external stimuli and therefore PFK activity is modulated according to the energy demand [65]. In hepatoma cells there is an over-accumulation of F16bP (which stimulates PFK activity) and a decrease of one of its inhibitors (ATP) [14], supporting previous evidence showing an over-activation of this enzyme in tumors [66]. In mammals, PFK is a homo- or hetero-tetramer composed of subunits M, L and P, which are differentially expressed in a tissue-dependent fashion [67]. In several leukemia and lymphoma cell lines the main isoform of PFK is that of liver and platelets (L and P isoforms, respectively) [68]. This adaptation seems to be carried out in order to enhance glycolysis, as the L isoform is less sensitive to its inhibitors (ATP and citrate) while it responds more effectively to the allosteric activator fructose 2,6-biphosphate [66]. PFK in yeast, seems not to be subjected to transcriptional regulation as it is constitutively expressed [69]. Increased activity of this enzyme has been detected during fermentation, but this may be due to changes of its allosteric modulators, especially F26bP [65].

3.2.3. Pyruvate kinase

Pyruvate kinase (PK) catalyzes the third irreversible step in the glycolytic pathway and it is the second ATP-generating site in the glycolytic sequence. Some studies show a correlation between tumor cell degree of malignancy and the over-activation of PK [70]. This enzyme is allosterically activated by F16bP and ADP, while it is inhibited by ATP. As F16bP is highly accumulated and ATP levels decrease, PK is expected to be fully active during aerobic glycolysis in tumor cells. However, there are reports that suggest that the contrary occurs and in tumor cells, there may be specific mechanisms preventing activation: one such mechanism is the expression of the E7 oncoprotein in human papillomavirus [71–73].

This enzyme exists as a L-, R-, M1- or M2 isoforms, which are tissue-specific and differ in their kinetic properties [71]. Tumor cells mainly express isoform M2, that is present in tissues with a high biosynthetic activity [71]. Moreover, a regulation of PK activity is proposed based on its quaternary structure, as M2-PK is found as a dimer or as a tetramer [72]. The latter is a very active form of the enzyme and it physically interacts with all other enzymes in the glycolytic pathway, whereas the dimer is inactive and it is found in a free non-associated form in the cytosol [73]. It is proposed that dimeric form promotes biosynthetic processes while the tetramer favors an increased glycolytic flux. Presumably, the former is the main isoform present in tumor cells [71]. However, as mentioned above, PK is crucial for metabolic flux modulation, and this proposal seems to be contradictory with the glycolytic flux assessments that indicate a several-fold increase in glucose consumption and lactate production [16]. Furthermore, the physiological trigger for the re-association of PK into a tetramer is fructose 1,6-biphosphate [71]. This metabolite can reach very high levels in tumor cell cytoplasm [16], pointing out to a possible predominance of the tetramer during tumor aerobic glycolysis. It is probable that PK oligomerization state may be a consequence of the tumor cell energy demand. This issue needs to be clarified in order to solve the disagreement mentioned above.

In yeast, there are two isoforms of pyruvate kinase (Pyk1 and Pyk2) [74]. The former seems to be expressed constitutively, although its levels are much higher during fermentation [46]. Pyk2 is less sensitive towards fructose 1,6-biphosphate and its expression seems to be repressed by glucose [74]. So, the presence of only isoform 1 during fermentation could be interpreted as an adaptation that leads to the complete activation of the enzyme and hence allows a maximal glycolytic flux.

3.3. Pyruvate metabolism

In eukaryotic cells, pyruvate metabolism is a crossroad between oxidative and fermentative pathways. This intermediate can be oxidized, reduced, undergo carboxylation or decarboxylation, the latter process being exclusive of microorganisms. Pyruvate is transported into mitochondrial matrix in order to be oxidized, or it can remain in cytosol to undergo either reduction, carboxylation or decarboxylation, depending on the organism and the enzyme that acts upon it. In addition, this intermediate can undergo transamination to produce alanine, thus it functions as a link with amino acid metabolism.

The inhibition of pyruvate oxidation is proposed as a metabolic deviation that could explain both the Warburg effect of tumor cells [75] and the glucose-induced repression of yeast oxidative metabolism [76]. This can be achieved at three levels: a) limitation of pyruvate transport into mitochondrial matrix, b) the decrease of pyruvate dehydrogenase complex (PDH) activity, and c) the enhancement of cytosolic pyruvate metabolism.

3.3.1. Inhibition of pyruvate transport

In both, mammalian and yeast mitochondrial inner membrane a specific transport of pyruvate was identified [77,78]. However, the isolation and characterization of this carrier has remained elusive and this has hindered further studies regarding its regulation and thus, its role in metabolism in vivo. To our knowledge there is only one report where the isolation of this carrier was achieved [79]. However, no further characterization was made. Recently, promising results were obtained as the gene coding for the *S. cerevisiae* pyruvate carrier was identified [80].

It was proposed that pyruvate transport is deficient in tumor cell mitochondria (Fig. 1) [81,82]. Indeed, a limitation of pyruvate transport into mitochondria would limit the activity of Krebs cycle and would increase the cytoplasmic levels of pyruvate that will serve as substrate for the lactate dehydrogenase (see below). This seems in accordance with the observation that pyruvate transport was one order of magnitude lower in mitochondria isolated from Ehrlich ascites cells compared to rat liver mitochondria [81]. Later, an increased K_m and a decreased V_{max} for pyruvate translocation was detected in mitochondria isolated from Morris hepatoma cell lines [82]. It is to be confirmed whether this phenomenon is present in other tumor cell lines, as for hepatoma AS-30D cells an enhanced pyruvate uptake and oxidation was reported [83].

As mentioned above, our knowledge of pyruvate transport regulation is limited and it is vital to solve this issue in order to gain some insight into the possible participation of pyruvate transport in physiological conditions and in cancer.

3.3.2. Inhibition of pyruvate oxidation

Oxidation of this intermediate is catalyzed by the pyruvate dehydrogenase complex (PDH) in mitochondria. This reaction yields NADH and Acetyl-CoA. The latter is fed into the Krebs cycle and the reducing equivalents obtained are oxidized by mitochondrial respiratory chain, where ATP is synthesized through oxidative phosphorylation. However, in situations where mitochondrial pyruvate oxidation is down-regulated, the fermentative pathway allows energy generation through glycolysis (see below). The decrease of oxygen consumption in both, tumor cells and glucose-repressed yeasts could be due to the inhibition of pyruvate oxidation. This would redirect metabolic flux to cytosolic pyruvate metabolism, and consequently, to lactate or ethanol production, respectively.

PDH complex activity in vivo is tightly regulated in order to modulate carbon metabolism according to the energy availability/demand in the cell. PDH is regulated by several mechanisms: thermodynamic phosphate and redox potentials, Acetyl-CoA and reversible phosphorylation [84,85]. It is believed that the latter is the main regulatory mechanism of PDH activity in vivo [84]. Therefore, special attention has been focused on the specific enzymes that catalyze the phosphorylation/dephosphorylation of the PDH complex and the mechanisms by which they are regulated.

In mammals, there are four tissue-specific isoforms of the pyruvate dehydrogenase kinase (Pdhk) (coded by the *PDHK1*, *PDHK2*, *PDHK3* and *PDHK4* genes) and two of the pyruvate dehydrogenase phosphatase (Pdp1 and Pdp2) [86]. Pdhks phosphorylate three serine residues located in the complex E1 subunit [85]. This component contains the pyruvate dehydrogenase activity, the main rate-controlling step of the overall reaction catalyzed by the complex in vitro [87]. Pdhks-mediated phosphorylation renders PDH inactive, while dephosphorylation completely reverts this inhibition [88].

Pdhk activity is modulated as a function of the intramitochondrial energy and redox states: it is inactive in the presence of high levels of ADP, NAD^+, CoA and Pi, and it is stimulated by NADH and Acetyl-CoA [85]. Changes in PDHK expression are also detected in response to different hormonal stimulation and nutritional conditions [89,90]. Whether Pdp protein levels are also affected by these same stimuli is unknown. It is important to stress that the fully active and the inhibited forms of PDH complex do not occur in vivo [84]. Thus, a balance between the activities of Pdhk and Pdp modulates PDH activity and hence the metabolic flux into the Krebs cycle.

In renal carcinoma cells and in a human lymphoma cell line, it has been demonstrated that expression of the isoform 1 of Pdhk is mediated by the Hypoxia-Induced Factor 1 (HIF-1) [91,92]. Disruption of Pdk1 by siRNA decreased lactate production in head and neck squamous cancer cells [93]. As mentioned above, HIF-1 is also involved in the over-expression of the glycolytic enzymes in several tumor cell lines [7]. It is tempting to speculate that the HIF1-mediated expression of Pdkh1 is somewhat responsible for a decreased oxidation of pyruvate and the subsequent lactate production in tumor cells. Nonetheless, metabolic flux determinations and pyruvate dehydrogenase complex activity assessments are still lacking in order to make a more precise interpretation of these data. Moreover, an increase of Pdk activity would indeed explain a reduced pyruvate oxidation, but this must be concomitant with a decrease in the activity and expression of the pyruvate dehydrogenase phosphatase. Information about this issue is still missing. In addition, Pdhk overexpression would not apply to all types of tumor cells since oxidation of exogenous pyruvate by PDH complex is enhanced in mitochondria isolated from AS-30D hepatoma cells regarding their normal counterpart [83].

In yeast, the existence of homologues of the mammalian Pdhk was initially discarded as no phosphorylation was detected in isolated pyruvate dehydrogenase complex [94]. However, it was later demonstrated that isolated Pdh could be phosphorylated and dephosphorylated in vitro by a purified bovine Pdkh and Pdp, respectively. Similar to the mammalian homologue, phosphorylation led to the inactivation of the complex and its dephosphorylation restored its activity [95]. Furthermore, the phosphorylated peptide sequence of yeast Pdh is similar to that of mammalian PDH complex in response to Pdk activation in vivo [95].

This led to the identification of two kinases and two phosphatases in yeast [96,97]. The dephosphorylated (active) form of the Pdh complex seems to predominate in yeasts grown non-fermentable carbon source (i.e. lactate) [96]. Additionally, in a mutant lacking one of the two phosphatases, growth on ethanol was delayed [97], in contrast to growth on glucose where this deletion had no effect. Altogether, these data suggest that during fermentation the pyruvate dehydrogenase complex is mainly present in its phosphorylated (inhibited) state, while the contrary would occur in oxidative metabolism.

An alternative mode of pyruvate oxidation inhibition was reported for tumor cells. The formation of acetoin (a by-product of the non-oxidative decarboxylation of pyruvate) was detected in Ehrlich tumor ascites and AS-30D mitochondria [98]. This compound was shown to inhibit pyruvate oxidation in isolated mitochondria in vitro [99]. Some questions arise about this particular mechanism, as it is not clear if acetoin is indeed generated by tumors in vivo. Besides, when exogenous pyruvate is added to Ehrlich ascites tumor cells, a stimulation of respiration is appreciated along with its oxidative decarboxylation [100–102].

In yeast, mitochondrial oxidation of pyruvate could be decreased in fermentative conditions. Indeed, some metabolic flux assessments demonstrate that after glucose addition to glucose-limited cultures there is an increased flux in glycolysis while the contrary is observed for Krebs cycle [103]. The glucose-induced transcriptional repression of *LPD1*, encoding E3 subunit (lipoamide dehydrogenase) of pyruvate dehydrogenase seems to explain this [104]. Moreover, it has been shown that pyruvate decarboxylase can function as a by-pass of pyruvate dehydrogenase during oxidative metabolism [105,106]. Consequently, pyruvate transport does not seem to be a controlling step of its metabolism.

3.3.3. Cytoplasmic pyruvate metabolism

In conditions where oxygen is scarce and hence oxidative metabolism is limited, pyruvate serves as a substrate for cytoplasmic enzymes in order to reoxidize NADH. Another possible explanation for the Warburg effect is a metabolic overflow at the level of pyruvate metabolism [107]. An increase of the expression and/or activity of the cytosolic pyruvate-dependent enzymes would decrease the amount of the substrate to be transported to mitochondria. Indeed, this has been detected in both tumors and yeast [22,47,108,109].

In mammals, pyruvate can be reduced by lactate dehydrogenase for the maintenance of a constant supply of NAD^+ required in order to drive glycolysis (specifically, the glyceraldehyde-3-P-dehydrogenase step). Lactate dehydrogenase is a homo- or hetero-tetramer composed of H or M subunits. The latter isoform is not inhibited by pyruvate and it is the predominant form in muscle [110]. In several human tumors, an overexpression of the M subunit was detected (Fig. 1) [111]. This could explain the lactate overproduction detected in a number of tumors. In colorectal adenocarcinomas and in breast malignant cells the overexpression of lactate dehydrogenase isoform is correlated with tumor invasiveness and is proposed as a marker of tumor progression [112,113]. Disruption of lactate dehydrogenase expression stimulates respiration and decreases tumor cell viability in hypoxic conditions, pointing out the importance of cytosolic pyruvate metabolism during aerobic glycolysis [107].

Yeast possesses a D-lactate dehydrogenase and a L-lactate dehydrogenase, both are localized in mitochondria inner membrane and irreversibly catalyze pyruvate formation [114,115]. A cytoplasmic isoform of lactate dehydrogenase was detected but its role in fermentation is currently unknown [116]. During glucose-induced repression of oxidative metabolism in *S. cerevisiae*, cytoplasmic pyruvate is rather metabolized by pyruvate decarboxylase, which is an enzyme only present in microorganisms. Through this pathway, pyruvate is decarboxylated to acetaldehyde, which is subsequently reduced to ethanol by the cytoplasmic isoform of alcohol dehydrogenase in order to regenerate NAD^+ [117]. Yeast possesses two pyruvate decarboxylase isoforms (Pdc1 and Pdc5) [118]. A mutant lacking both isoforms is unable to grow on fermentable carbon [106]. An increase of pyruvate decarboxylase activity has been observed during the onset of fermentative metabolism and it may provide an explanation for Crabtree effect in yeast (see below) [22,103].

3.3.4. Pyruvate metabolism, amino-acids and anaplerotic reactions

Cells undergoing rapid growth need to produce proteins and nucleotides at a high rate. This is true for tumor and for yeast cells alike. It has been observed that rapidly growing rhabdomyosarcoma cells exhibit higher concentrations of glutamate, aspartate, ribose and pyrimidines than normal myocytes [119]. The differences in metabolic rates in proliferating cells as compared to quiescent cells have been examined and it has been observed that aerobic glycolysis, lipid biosynthesis and glutamine-dependent anaplerosis are very active in an active cell cycle [120]. These activities result in a high requirement for glucose and glutamine [121]. The metabolic rates in proliferating cells are controlled by cytoplasmic tyrosine kinases, while a Myc signaling pathway both activates glutamine metabolism and promotes nucleotide synthesis [121,122]. In yeast, the mechanisms controlling the growth rate are only beginning to be understood: when carbon sources dwindle in yeast cultures, the cells switch to gluconeogenic metabolism. Here enzymes such as carboxykinase are activated through acetylation [123]. Also under respiratory conditions, alanine aminotransferase-1 can both synthesize and use alanine but when cells switch to fermentation, this enzyme assumes a catabolic role only [124].

Pyruvate is an important intermediate in aminoacid metabolism as it can be converted to alanine by transamination. This reaction is catalyzed by alanine aminotransferase, which also converts glutamate to α-ketoglutarate. In some tumor cell lines, alanine is identified as one of their main excretory products in vitro [125]. This may be explained by an abnormal glutamine metabolism. This amino acid is one of the main carbon sources assimilated by malignant cells [126]. In consequence, they overexpress glutaminase, an enzyme that deaminates exogenous glutamine to yield glutamate. An accelerated glutaminolysis would allow for a constant glutamate supply that drives the alanine transaminase reaction towards alanine synthesis.

Glutaminolysis also accounts for pyruvate synthesis, as its metabolism can also yield malate that may serve as substrate for the mitochondrial $NADP^+$-dependent malic enzyme [127,128]. This enzyme is overexpressed in several tumor cell lines and this feature is correlated with the malignancy degree in hepatomas [127]. Glutamine-derived pyruvate is also susceptible to reduction by the lactate dehydrogenase. This is corroborated by the fact that lactate and alanine are observed as excretion products of glutamine-grown cells [128].

Nonetheless, this issue is yet not clear because some evidence suggest that glutamine is ultimately converted to aspartate by the sequencial activity of glutaminase and aspartate aminotransferase, and that it is converted to pyruvate only when exogenous malate is present [125,129].

A possible explanation for these discrepancies may lie on the carbon source used in the respective experiments, as it is expected that glutamine-derived pyruvate would only be significant when cells grow on glutamine as substrate. In spite of this, some of the reports use glucose as the only carbon source in growth media [125]. In fact, it is demonstrated that the replacement of glucose for glutamine impact on cell growth and energy metabolism [8]. A further elucidation of this issue is vital, as the glutamine-derived pyruvate and the mitochondrial malic enzyme might be valuable targets in metabolic therapy.

In yeast, pyruvate transamination to alanine is carried out by the alanine transaminase coded by the *ALT1* gene. There is another isoform of enzyme (Alt2), but its expression seems to be repressed in all conditions tested [130]. Alt1 carries out alanine metabolism in both, aerobic and fermentative conditions, but its activity during fermentation seems to be restricted only to alanine catabolism, and thus it participates in pyruvate supply [130].

Glutaminolytic pathway is not quite clear in yeast. Presumably, the *SNO1* gene codes for a glutaminase that is co-expressed with Snz1, a glutamine amidotransferase [131]. The expression of both genes is induced during nutrient deprivation and the diauxic shift and they may participate in pyridoxine synthesis [132].

Yeast also possess a mitochondrial $NADP^+$-dependent malic enzyme, which is present in both, oxidative and fermentative conditions, but is further activated during the latter [133]. However, this enzyme seems to be more implicated in anaplerosis along with pyruvate carboxylase, as deletion of malic enzyme did not affect the Krebs cycle flux [133].

Studies regarding the role of glutaminolysis during the oxidative and fermentative conditions need to be done in order to establish a comparison with tumor cell metabolism. In tumor cells, glutaminolytic pathway account for energy production in oxidative conditions but in yeast this has not been explored.

3.4. Krebs cycle and mitochondrial respiratory chain down-regulation

3.4.1. Krebs cycle limitation

Pyruvate is the end-product of glycolysis and in normal respiratory conditions it enters mitochondria through a specific carrier) [77,78] (see above). Once in the mitochondrial matrix, it is further catabolized through the Krebs cycle (also known as the tricarboxylic acid cycle). After its complete oxidation, one pyruvate molecule yields 4 NADH, 1 $FADH_2$, 1 GTP (or 1 ATP depending on the tissue and the organism) and 3 CO_2 molecules. The reducing equivalents obtained in the Krebs cycle are re-oxidized by mitochondrial respiratory chain and eventually yield around 9 ATP molecules synthesized through oxidative phosphorylation per one turn of the cycle. Indeed, it is often thought that 1 turn of Krebs cycle gives 14 or 11.5 ATP molecules [134,135]. However such values are largely overestimated since oxidative phosphorylation do not reach an optimal yield in vivo. From more realistic studies, we can estimate the yield, in vivo, to be 80% of the maximal one [136,137]. Thus, Krebs cycle dehydrogenases activity is crucial for metabolic flux modulation and cell energy production [138,139]. Another possible metabolic deviation that might originate the Warburg effect could be the down-regulation of the Krebs cycle. This would decrease oxidative phosphorylation rate because of a reduced respiratory substrate supply.

Using isolated mitochondria from fast-growth Morris hepatoma cells it was demonstrated that there is a considerable citrate efflux from these organelles [140]. Additionally, in these mitochondria a negligible respiration in phosphorylating conditions (state 3) was observed when citrate or pyruvate was used as respiratory substrates [141]. These results were interpreted as a deviation of carbon flux towards cholesterol synthesis as a consequence of a truncated Krebs cycle [141]. However, this was not confirmed through the assessment of the Krebs cycle enzymes maximal capacity in comparison to their normal counterpart. Furthermore, another study with a different hepatoma cell line (AS-30D) showed no existence of a truncated Krebs cycle [83]. This difference may be explained as the hepatoma cell line used by Parlo and Coleman [140], was a poorly differentiated fast-growth hepatoma cell that behaves as glycolytic because they present a decreased expression of mitochondrial oxidative enzymes [10]. In addition, this cell line has a reduced mitochondrial ADP transport that might well explain the lack of state 3 respiration [142]. AS-30D cell line, in contrast, mainly uses oxidative phosphorylation for energy conversion processes [8]. Breast cancer cells display this same dependency on mitochondrial metabolism [9]. Thus, disruption of the Krebs cycle cannot be taken as a general mechanism of the Warburg effect.

It is possible that in some conditions oxidative tumor cell lines down-regulate Krebs cycle enzymes activity and oxidative phosphorylation through short-term mechanisms. In this regard thermodynamic driving forces (phosphate and redox potentials) might have an important role for metabolic flux modulation [143]. This may well explain why some tumor cells display a Warburg effect in vivo in spite of producing ATP through oxidative phosphorylation in vitro [144]. Unfortunately there are no metabolic studies of tumor cells based on thermodynamic forces.

Yeast represents a good model to study such transition as it undergoes a similar process when adapting its metabolism to nutritional conditions. When it grows using a non-fermentable carbon source, mitochondrial metabolism is of central importance, but in the presence of glucose it represses oxidative phosphorylation in order to enhance fermentation [20,23]. Accordingly, the enzymes from the Krebs cycle (such as malate dehydrogenase, isocitrate dehydrogenase and aconitase) decrease their activity immediately after glucose addition [145,146]. This could be due to two well described processes: the inhibition of respiratory chain by fructose 1,6-biphosphate [15] and the inhibition of intramatricial NADH oxidation by an increase in cytosolic NADH [147].

3.4.2. Down regulation of mitochondrial respiratory function

Warburg originally hypothesized that in addition to glycolysis acceleration there is an irreversible damage to mitochondria in tumor cells as the origin of "aerobic glycolysis" [1]. This seems to be correct for some tumors. For instance, deficiencies in complex I are linked with tumor cell progression and metastasis in lung carcinoma and fibrosarcoma cell lines [148]. In paragangliomas and phaeochromocytomas there is a deficiency of succinate dehydrogenase (complex II) [149]. Complex II and IV activities decrease in some hepatoma cell lines regarding normal hepatocytes [150]. However, deficiencies in the content of mitochondrial respiratory chain components may not apply to all tumors as in some cases an overexpression of respiratory complexes is observed [151].

Another possibility is the down-regulation of oxidative phosphorylation components. In this case ATP could not be synthesized by mitochondrial ATP synthase and this would induce a decreased respiration and a high mitochondrial transmembrane potential ($\Delta\Psi$) (State 4). This may be due to an inhibition of mitochondrial ATP synthase or adenine nucleotide translocase (ANT). In some tumors both situations have been reported to occur. In hepatomas, lower levels of F1 subunit of ATP synthase have been reported [152] and in fast-growth Morris hepatomas mitochondrial ADP transport rate was decreased regarding normal hepatocytes [142]. The latter could

explain why some tumor cell lines have high mitochondrial $\Delta\Psi$ and no state 3 respiration [107,140]. However, in slow-growth hepatomas the kinetic parameters of ANT were undistinguishable from their normal counterpart [142]. This seems into accordance with the observations that in some hepatoma cell lines ATP is generated by oxidative phosphorylation [8,10]. In fact, when isolated, hepatoma cell mitochondria have high respiratory control values and ADP/O ratios compared to those obtained from normal liver cells [153].

Most of the studies in mitochondria have been done at a transcriptional level and rigorous bioenergetical and biochemical studies are lacking in order to fully understand the role of mitochondria in the Warburg effect. In this case a metabolic control analysis is also required as only the rate-controlling steps downregulation could explain a decrease in respiratory flux (see below).

It was proposed that tumor metabolism depends on nutritional status and consequently tumor cells are able to switch between oxidative metabolism and fermentation [154]. Recently it was shown that mono-layer cultures of hepatoma cells rely on oxidative metabolism, but when they aggregate to form three-dimensional spheroids they display a Crabtree effect [144]. Importantly, these 3D structures resemble the morphology of tumors in vivo [11]. So it is possible that even those tumor cells with a competent respiratory chain switch to fermentation depending on its microenvironment, especially when they aggregate form a solid overgrowth. In addition, this transition will be favored by hypoxia as these formations are poorly vascularised. It is possible that short-term regulatory mechanisms have an important role for this transition. Unfortunately, this type of regulation has not been taken into account in tumor cell metabolism studies.

Yeast cells when grown in the presence of oxidative carbon sources (e.g. lactate, acetate or glycerol) contain competent and well differentiated mitochondria [155]. In contrast, when glucose is present, mitochondrial morphology changes and oxidative phosphorylation is inhibited [156,157]. This process is termed the carbon-catabolite induced repression [20]. and from the metabolic point of view is very similar to the Warburg effect.

This metabolic adaptation is regulated by short- and long-term mechanisms. The latter class of events is relatively well defined and they involve the transcriptional repression of mitochondrial respiratory chain components expression [20]. Specifically, a decreased activity of complexes II and IV has been detected in yeast mitochondria upon transfer to a glucose-rich medium [49,145]. Corresponding down-regulation of Complex II and cytochrome *c* expression has been demonstrated to occur in these conditions [158,159]. As cytochrome oxidase is the main controlling step in mitochondrial respiratory chain in yeast [160], this might explain the long-term adaptation to fermentative conditions. The short-term regulatory mechanisms remain ill-defined, however some results point out that cytoplasmic metabolite levels may be responsible for the short-term metabolic flux modulation (see below).

3.5. The Crabtree effect

As mentioned above, one of the main features of fast-proliferating cells is a glucose-induced repression of oxidative metabolism: the Crabtree effect [2]. This was identified in the 1920's for tumor cells [2], but the same phenomenon has been observed for *S. cerevisiae* and some other yeasts [22]. The precise mechanism by which the Crabtree effect is triggered is unknown, although several mechanisms have been proposed to explain its induction [161].

The most accepted proposal is that glycolysis enzymes and mitochondria compete for free cytoplasmic ADP [5,162]. Upon glucose addition, glycolysis is activated and thus the ADP-consuming steps in this pathway could avidly trap free ADP and thereby force mitochondria to enter in a non-phosphorylating state and consequently decrease respiration [162]. The same mechanism has also been proposed for the Crabtree effect in yeast [64]. Although this competition might occur, these studies do not take into account the free and protein-bound pools of adenine nucleotide since only the free form could account for this competition [43]. Furthermore, experiments in yeast demonstrate that ADP levels either increase or remain constant after glucose addition [155,163]. A further evaluation of this issue is necessary to clarify the existence of this competition.

Phosphate (Pi) has been proposed as the actual trigger of the Crabtree effect [164]. In tumor cells after glucose addition, respiration was partially restored upon external Pi addition [164]. In both tumors and yeast a dramatic decrease of cytoplasmic Pi has been detected during the onset of the Crabtree effect [14,165,166]. However, it is possible that the ratio ATP/ADP.Pi, i.e. the phosphate potential (ΔG_p), may be one of the driving forces involved in the Crabtree effect instead of Pi or ADP themselves [14,166].

Ca^{2+} could be another trigger of the Crabtree effect [13]. In hepatoma cells, it has been demonstrated that glucose induces an increase in mitochondrial Ca^{2+}-uptake [13], and in mitochondrial matrix, this cation inhibits ATP synthase causing a decrease of respiration [167]. However, this finding was challenged by the finding that Ca^{2+} levels remain constant in response to glucose in hepatoma cells [14].

There are similar events that occur in yeast. It has been shown that glucose addition induces a rise in cytoplasmic Ca^{2+} levels [168]. Moreover, Ca^{2+} induces a respiratory decrease in isolated yeast mitochondria [169]. Although the mechanism of repression is different from that of hepatoma cells, as this Ca^{2+}-mediated inhibition was also obtained in conditions where ATP synthase is not operating (state 4) [169]. Furthermore, Ca^{2+} target seems to be the mitochondrial outer membrane VDAC channel [170].

Another possibility is a decrease of mitochondrial outer membrane permeability towards respiratory substrates or to ADP [12]. This could be an important regulatory mechanism of oxidative phosphorylation in both mammalian cells and yeast [171,172]. However, this possibility has not been taken into account for tumor cell metabolism studies. Although it has been suggested that respiratory substrate availability may decrease in these conditions [173].

Recently we have found a link between glycolysis and Crabtree effect induction, as fructose 1,6-biphosphate (F16bP) inhibits mitochondrial respiratory chain [15]. In both, fermenting yeasts and tumors, F16bP is highly accumulated in the cell cytoplasm during fermentative metabolism [15,16,166] it is thus possible that this hexose phosphate has an important role for Crabtree effect induction in both cell types in vivo. We also observed that if isolated mitochondria from normal rat liver are incubated in the presence of high concentrations of F16bP (similar to those measured in hepatoma cells), they display a Crabtree effect [15]. This might explain why some hepatoma cell lines repress respiration when glycolysis is stimulated despite having an intact oxidative phosphorylation [144].

F16bP thus has an additional regulatory function besides its participation in the glycolytic pathway. A regulatory role has been also observed for other metabolic intermediates such as glucose 6-phosphate in yeast [174] and for oxaloacetate and pyruvate in glioma cells [175]. The term “metabolic messengers” has been coined to classify these kinds of metabolites [23].

Yeast also provides a useful model for studying the Crabtree effect as there are some yeasts that are classified as Crabtree-negatives (e.g. *C. utilis*) [22]. A characterization of the differences between those two types of cells might shed some light into the mechanism leading to Crabtree effect. For instance F16bP induce a respiratory decrease on mitochondria isolated from *S. cerevisiae* whereas this inhibition is not observed for *C. utilis* mitochondria [15].

4. The design of metabolic therapies using metabolism inhibitors

4.1. Metabolism-inhibiting drugs

As mentioned above, the study of the Warburg effect has offered a new perspective for cancer treatment: the inhibition of tumor cell

metabolism [10]. As all tumors are considered as glycolytic, a lot of attention has been put into this metabolic pathway as a target for cancer treatment [45]. A number of glycolysis-inhibiting drugs are being successfully tested for tumor cell growth inhibition in vitro [45]. Furthermore, some of these drugs (e.g. 2-deoxyglucose) are currently being tested in clinical trials [176]. However, not all tumor cells have an impaired oxidative metabolism [9]. For these cases, glycolysis inhibition alone might not be as efficient for inducing tumor cell growth arrest, and oxidative phosphorylation-inhibiting drugs such as rotenone, rhodamines and oligomycin seem to be more appropriate for therapeutic purposes [10], although it may be necessary to design drugs with a wider safety margin than those available to date.

Furthermore, some results suggest that oxidative phosphorylation inhibitors may not be suitable for therapeutic purposes, as in vitro experiments show that tumor cell growth is not significantly affected by them [177]. However, care must be taken interpreting these kind of results, as some tumor cells switch between fermentative and oxidative metabolism depending on the carbon source in growth media (see above) [154]. If grown on glucose-containing medium, tumor cells may down-regulate the oxidative phosphorylation machinery and decrease their mitochondrial content [154], thus the oxidative phosphorylation inhibitors are ineffective in these conditions.

For oxidative tumor cells a transition between fermentation and respiration (Crabtree effect) has been detected [144]. This shift depends on cellular microenvironment since when cultured as monolayer the tumor cells displayed an oxidative metabolism, but when in three-dimensional spheroids a Crabtree effect was induced [144]. In this case, the drug-mediated impairment of both glycolysis and oxidative phosphorylation is even more effective for suppressing tumor cell proliferation [10]. This originated in the presence of different cell populations with distinctive metabolic features within the tumor [11]. Those cells in the outer region are more oxygenated as they lay near vascularized zones whereas those residing in the inner core undergo hypoxia [11,178]. This has an impact in cellular metabolism because of oxygen and nutrient availability. Thus, tumor cell metabolism cannot be suppressed by using glycolysis or oxidative phosphorylation inhibitors alone. Furthermore, a "dual hit" approach comprising one of the tumor key signal transduction pathways and energy metabolism inhibition has been proposed [19]. Yet another possibility is to exploit the central role of mitochondria in programmed cell death processes and apoptosis stimulation [179]. All these approaches deserve to be further explored in the future.

One of the main issues of the metabolic therapy approach is to establish the appropriate doses of the metabolism-inhibiting drugs, as these could eventually affect normal cells. For instance, oligomycin treatment successfully arrested the growth of leukemia at very low concentrations that do not affect normal cells [180]. Another study revealed that 3-bromopyruvate suppresses cell growth in tumor xenografted rabbits [181]. But the high concentrations of this drug that were used (50–100 μM) in this study would limit its usefulness for therapeutic application in patients. In this regard, it will be interesting to explore if the simultaneous application of both substances may reduce the effective dose for one of them.

As the hypoxia-induced factor 1 (HIF-1) was identified as a key element for the Warburg effect induction, it was proposed that tumor cell metabolism could be specifically targeted by HIF-1 targeted inhibition [7]. However, targeting a transcriptional factor is a difficult approach that can only be achieved through indirect pathways that may compromise the specificity of the treatment. Moreover, based on results obtained with the spheroid model it has been suggested that HIF targeting efficiency also depends on the nutrient status and the microenvironment of the tumor cell [182].

4.2. Lactate metabolism inhibition

Lactate is commonly considered as a waste product of glucose metabolism during fermentation in mammalian cells. However, lactate metabolism in vivo is highly relevant as it participates in carbon and reducing equivalent shuttling between organs. Lactate can be translocated to cytoplasm to be oxidized by a cytoplasmic lactate dehydrogenase. A classical example is the Cori cycle, where lactate produced by skeletal muscle is taken by the liver where it serves as gluconeogenic substrate. Glucose synthesized in liver subsequently goes back to skeletal muscle thus closing the cycle [183]. Besides liver there are other organs, such as brain and heart, where lactate shuttling is important [184]. This reveals that lactate plays a relevant role for interorgan communication and energy metabolism in non-pathological conditions.

Recently, it has been demonstrated that this may occur within tumors. As described above, in malignant overgrowths there is heterogeneity regarding cell metabolism depending on its location [11]. Oxidative and fermentative tumor cells may communicate through lactate shuttling [178,185]. It has been demonstrated that lactate excreted by hypoxic tumor cells can be trapped by the oxidative ones even if glucose is present [185]. When in cytoplasm, lactate can be oxidized to pyruvate by an overexpressed lactate dehydrogenase and thus it feeds Krebs cycle for energy generation through oxidative phosphorylation in these cells [185].

Monocarboxylate transporter proteins (MCT) play a prominent role for this shuttling. They are located in plasmatic membrane and they mediate the symport of monocarboxylates (pyruvate, lactate and ketone bodies) with a proton through facilitative diffusion [186]. Four MCT functional isoforms have been identified and MCT1 and 2 seem to be overexpressed in several tumor cell lines [185,187]. These carriers may be therapeutic targets for anti-cancer treatment [187]. This has been experimentally explored by interrupting MCT expression and through drug-mediated inhibition. Using both strategies it has been demonstrated that lactate shuttling inhibition reduce tumor cell viability and rendered them radiosensitive [185,188,189]. Moreover, when lactate transport of fermentative tumor cells is inhibited they immediately die, whereas the oxidative ones switch to fermentation [185].

An additional advantage of MCT inhibition may be the decrease of tumor cell invasiveness, as lactate export can serve to the generation of an acidic microenvironment that favors tumor cell proliferation while inhibiting that of normal cells [3]. Presumably, this acidification process may contribute to metastasis by rendering endothelium basal membranes more susceptible to proteolytic degradation [3]. In this regard, disruption of LDH expression decreased tumor cell growth and metastasis [107].

Taking this into account, α-cyano-4-hydroxy cinnamic acid (ACCA), a MCT inhibitor, is also proposed as a drug for metabolic therapy [185,189]. In vitro and in vivo tests have shown the effectiveness of ACCA by reducing tumor cell viability [185,189]. Although ACCA is a known inhibitor of the mitochondrial pyruvate carrier [77], it was demonstrated that in the conditions tested it remained in extracellular space and thus did not interfere with mitochondrial metabolism [189]. Thus lactate shuttle inhibition offers an attractive possibility for cancer treatment, especially in combination with other energy metabolism-inhibiting drugs. However, it remains to be shown how ACCA doses affect the lactate shuttling that occurs between other organs (e.g. in brain and heart) and if this represents a possible risk for patients.

4.3. Metabolic control analysis

Tumor cell metabolism study is based on the observation of gene expression [45]. For instance, the overexpression of glycolysis enzymes has been interpreted as an evidence for fermentative metabolism acceleration. Nevertheless, expression profiles alone cannot be taken as an evidence of the metabolic flux modulation. In some studies, the isolation of enzymes or the use of reconstituted systems has allowed to gain some insight regarding the mechanisms of glycolysis acceleration during aerobic glycolysis. Still, it is difficult to extrapolate these results for the description of glycolysis modulation in vivo.

Moreover, there are several phenotypic differences between tumor cell lines, even between those derived from the same tissue [10,53].

This raises the necessity of a quantitative approach that could be applied to describe the metabolism in complex systems such as intact cells. Metabolic control analysis (MCA) is a rigorous mathematical ground that provides quantitative information about individual metabolic steps through the assessment of a control coefficient [190,191]. This is a numerical parameter that is experimentally obtained and it represents the influence of an individual reaction on the overall metabolic flux. Those steps with higher control coefficient (close to one) are the slowest, and thereby the rate-controlling steps in the pathway. This challenges the existence of the "rate-limiting step" as it has been experimentally demonstrated in several models that metabolic flux control is shared between two or more steps in a number of metabolic pathways. For a detailed description of MCA the reader is referred elsewhere [192].

By applying MCA on normal liver and muscle cells it has been demonstrated that the control of glycolysis is shared between hexokinase and phosphofructokinase, i.e., they have the highest control coefficient [193,194]. MCA has also been applied to hepatoma cells and it was observed that glucose transport and HK bear the highest control coefficients in the glycolytic sequence [16], whereas in Ehrlich ascites tumor cells hexokinase and phosphofructokinase have the highest coefficient controls [195]. In virtually all tumor cell metabolism studies information concerning flux control coefficients is missing. The implementation of MCA as a cancer research tool would contribute to precisely understand its metabolism and for the identification of the relevant molecular targets for metabolic therapy.

4.4. Interrelationship between energy metabolism and apoptosis

Mitochondrial respiratory chain defects have been described for some tumors [148,151,196]. As a consequence, these organelles have been generally considered as non-functional entities in tumor cells and they are not viewed as an attractive target. However, recent findings challenge this consideration as not all tumors display these defects and they are successfully killed by oxidative phosphorylation inhibitors (see above). In contrast, the targeting of glycolytic tumor cell mitochondria is not straightforward and the role of these organelles in cell death induction mechanisms may be exploited in those cases [179].

After the cell receives an apoptotic stimulus, mitochondrial outer membrane is permeabilized by one of two distinct phenomena: a) through the action of pro-apoptotic proteins such as Bax and Bak, or b) by the opening of the mitochondrial permeability transition pore [197]. Both processes induce the release of cytochrome *c* and other pro-apoptotic proteins from the mitochondrial intermembrane space to the cytoplasm. So far, the relationship between mitochondrial energy state and apoptosis triggering has been frequently overlooked. However, there are some findings in this regard. For instance, it has been shown that glycolytic tumors display an elevated mitochondrial transmembrane electrical potential value ($\Delta\Psi$), and this feature is correlated with tumor cell invasiveness [198]. When $\Delta\Psi$ value was decreased, these cells underwent cell death [107,198].

This elevated $\Delta\Psi$ may reflect a downregulation of oxidative phosphorylation (non-phosphorylating state). According to this, during aerobic glycolysis in tumors, there is a decrease of ATP levels and an increase of the $\Delta\Psi$ value [107]. This may support the hypothesis that a functional oxidative phosphorylation is required for mitochondria-mediated cell death [4], and that apoptosis triggering requires ATP [197,199]. Nevertheless, more rigorous bioenergetic studies are required in order to elucidate this issue.

A recent report provides a direct link between the Warburg effect and apoptosis inhibition [200]. It was demonstrated that accelerated glucose metabolism induces a reduction of cytochrome *c*, which in contrast to its oxidized form, is unable to trigger cell death in spite of being released from the mitochondrial intermembrane space [200]. The reduction of cytochrome *c* may be consequence of a reduced state of the cytoplasm due to high levels of NADPH generated by an overactive pentose phosphate pathway [200]. Further evidence that support this model comes from our laboratory [15]. We found that fructose 1,6-biphosphate, which is highly accumulated in tumor cell cytoplasm, inhibits the activity of cytochrome oxidase and thus it may favor the reduction of cytochrome *c* [15]. This reveals a previously overlooked link between mitochondria and glycolysis intercommunication and it deserves further evaluation as it offers an interesting possibility for mitochondrial targeting based therapy.

Another evidence for the dependence of apoptosis on energy metabolism was found using yeast as experimental model [201]. It was demonstrated that heterologue Bax expression in yeast induces cell death mediated by releasing cytochrome *c* from mitochondrial intermembrane space [202]. The effectiveness of Bax depends on yeast metabolic state, as in fermentation the cells were less sensitive to Bax-induced cell death compared to those who possesses an oxidative metabolism [201]. A possible role of the thermodynamic phosphate potential (ΔG_p) was suggested in this case [201].

One of the main goal for cancer treatment is to specifically induce apoptosis in tumor cells. Thus, targeting tumor cell metabolism can be used for this purpose as well. Therefore, it is crucial to establish the exact relationship between energy metabolism and apoptosis induction for a further improvement of the metabolic therapy.

5. Concluding remarks: yeast as a model for metabolism-inhibitor drugs

Tumor cell display a wide variety of genetical or epigenetical dysfunctions that renders them unable to control their cell cycle. This is accompanied by the loss of their differentiation, and in some instances they acquire the ability to invade neighboring tissues (metastasis). Due this heterogeneity of mechanisms, tumor cells display different metabolic features in vitro. For instance, some cell lines do display an elevated glycolysis in spite of an excess of oxygen and they continually take glucose and produce lactate, as originally seen by Warburg (e.g. renal carcinoma cell lines) [203]. But some others mainly use oxidative phosphorylation for energy conversion processes (e.g. some hepatomas, HeLa and breast cancer cells) [9,16]. Moreover, in the latter category a shift between fermentative and oxidative metabolism is observed depending on the nutritional and environmental conditions [144,154,204]. The growth rate of a particular tumor cell line has an impact on metabolism as well. In slow-growth Morris hepatomas oxidative phosphorylation seems to be intact whereas those with higher growth rates display mitochondrial down-regulation and an overactive glycolysis, suggesting a Warburg effect [53,54,142].

In addition, tumor cells in vitro have distinct properties from those found in vivo [11,144,204], and probably the oxidative ones behave as glycolytic in vivo. This may be a consequence of hypoxic regions within tumors and the activation of the HIF-1 pathway [7]. Some short-term adaptation mechanisms may participate as well, such as changes in cellular thermodynamic driving forces and/or metabolic messengers [15].

Most of the studies addressed to establish a metabolic therapy are done using monolayer cell cultures in vitro as experimental model. Nevertheless, the outcome may vary from model to model depending on the specific defects of the cell line. Therefore, a more suitable model is required for screening purposes. A simple model may be the fermenting yeast *Saccharomyces cerevisiae*. In presence of glucose, yeast invariably uses fermentation as its main metabolic pathway and share some metabolic features observed in tumors in vivo (see Table 1). These similarities may be used in order to establish yeast as a screening model to design metabolic therapies.

In addition, due to its amenability and its easy genetical manipulation, yeast may be used to mimic tumors by mutating some yeast homologue tumor-related genes [205]. Some of these yeast genes and their correspondent homologues in tumors have been previously identified [206]. The utilization of such mutants could be also very useful for testing metabolism-inhibitor drugs.

A possible inconvenient of using yeast as a metabolic model is that differences regarding the effectiveness of metabolism-inhibiting drugs may arise because of enzyme variations between yeast and human. However, the drugs tested in metabolic therapy so far are well-known metabolic inhibitors (e.g. 2-deoxyglucose, iodoacetate, arsenite, oligomycin, rotenone) that act upon enzymes from both species [8,10]. When testing new drugs for their possible use in metabolic therapy, a comparison of its effects on both, yeast and tumor cell lines, is mandatory.

A set of experiments will be addressed in order to validate the use of yeast as a model for the screening of metabolism-inhibiting drugs. Together with the studies of yeast-related tumor genes, fermenting yeast offers an easy-to-use and reliable model to rapidly assess the effectiveness of metabolic therapy and other anti-tumor drugs.

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