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ADAPTACIONES METABÓLICAS A LA RESPUESTA A GLUCOCORTICOIDES DURANTE LA EXPRESIÓN DEL OSCILADOR SINCRONIZADO POR ALIMENTO

TESIS

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PRESENTA

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RESUMEN

Una característica importante de los seres vivos es la presencia de variaciones periódicas en parámetros diversos, desde los conductuales hasta los fisiológicos, que se denominan ritmos biológicos. Cuando el periodo de los ritmos es cercano a las 24 h, a los ritmos se les conoce como circadianos. Los ritmos circadianos son elementos clave en la evolución, ya que facilitan la adaptación y la anticipación a cambios periódicos en el ambiente. Estos ritmos se sustentan por la existencia de relojes u osciladores internos, los cuales se sincronizan a través de eventos ambientales como luz, temperatura y en nuestro modelo, por acceso restringido al alimentación. A este reloj se le ha denominado oscilador sincronizado por alimento (OSA). Para que el OSA se exprese, es necesario someter a los animales a restricción de alimento, 2 h cada día (12:00-14:00 h) durante 3 semanas. Se propone que el OSA está constituido por un sistema en que participan tanto estructuras centrales como periféricas involucradas en la regulación de la ingestión del alimento. En este contexto, el hígado es un órgano importante ya que es un centro integrador de nutrientes y de respuestas endócrinas. En animales bajo horario restringido de alimentación se presenta un estado fisiológico que contrasta con los estados de alimentación *ad libitum* y de ayuno agudo, en el cual los animales se adaptan a nuevas condiciones de manejo de nutrientes, en un nuevo equilibrio cronostático. Durante la expresión del OSA, se han reportado un gran número de adaptaciones fisiológicas que anticipan al organismo a la llegada del alimento para hacer más eficiente el aprovechamiento de nutrientes y de energía. Pensando en que esa anticipación podía constituir una fuente de estrés, ya que existe una elevación de glucocorticoides circulantes previo a la presentación de la comida, formulamos la hipótesis de que pudiera presentarse una respuesta inmunológica propia del hígado, conocida como respuesta de fase aguda asociada a la restricción alimenticia. Por otro lado, debido los glucocorticoides son también reguladores del metabolismo, se estudió también el ciclo de la urea como una vía de salida. Con el desarrollo de este proyecto, descartamos que la anticipación implemente una respuesta de fase aguda, pero si un estado pro-inflamatorio local (hígado), promovido por NFκB. Además, de cambios en la ritmicidad de IL-1α, IL-6, TNF-α, y fibrinógeno. Durante la anticipación encontramos aumento de glucocorticoides circulantes, que sugieren cambios en la regulación metabólica por éstos mismos. También encontramos un aumento en la presencia del receptor a glucocorticoides en citoplasma; cambios en el ritmo de urea y actividad de las enzimas mitocondriales, carbamoil fosfato sintasa 1 (CPS1) y ornitina transcarbamilasa (OTC). La modificación del ciclo de la urea a

través de glucocorticoides representa una importante vía de comunicación entre el OSA y el metabolismo hepático.

ABSTRACT

Under a protocol of restricted food, the circadian rhythmicity is not only controlled by the suprachiasmatic nucleus but also by a different biological clock known as the Food Entrained Oscillator (FEO). In order to express FEO, the food is restricted during photophase few of hours (12:00-14:00 h) every day along 3 weeks. Animals expressing FEO, show increased activity and other behavioral changes shortly before the time that food becomes available, what is known as the anticipatory activity (AA). Despite these similarities to the SCN oscillator, the FEO is anatomically distinct, because SCN lesions do not abolish the rhythmic food-anticipatory behavior. The difficulty in conclusively identifying the location of the FEO has raised the possibility that the FEO may not reside in a single structure but may rather be distributed among many sites. In this sense, the liver is important because plays a central role in metabolism and endocrine responses. This new physiological state to adapt to food availability is called it chronostasis. During the AA, a rise in plasmatic corticosterone is observed which is considered stress marker and also an internal synchronizer . To corroborate the AA was a stressful condition we decided to study an immunological response typical of the liver (acute phase response). To corroborate glucocorticoids can modulate metabolism, we decided study urea cycle, like an output way. The aims of our project were to explore if an acute phase response (APR) or a pro-inflammatory state occurred during FEO expression and to characterize the presence and cellular distribution of glucocorticoid receptor (GR) (because glucocorticoids effects on metabolism are mediated by intracellular GR as well as urea formation and the activity of several enzymes of urea cycle in the liver. The stress condition associated with FEO expression is not enough to induce an APR, but it could be related to a local stress response within the liver (NFκB increased). And also, food restriction modified the rhythmic 24-h fluctuations of IL-1α, IL-6, TNF-α, and fibrinogen. On another hand, the results showed that restricted feeding led to a significant increase in cytosolic and a decrease in nuclear GR levels compared to ad libitum animals. There were no significant changes in average values of urea levels and both Carbamoyl-phosphate synthase I (CPS1), Ornithine transcarbamylase (OTC) activities during the FEO expression. However, important modifications were observed in the rhythmic patterns of urea and CPS1 and OTC activities. All of these changes in urea cycle represent an important communication way between glucocorticoids and hepatic metabolism.

LISTA DE ABREVIATURAS

11 β HSD 1	**11 β hidroxiesteroide deshidrogenasa tipo 1**
AA	**Actividad anticipatoria**
ADN	**Ácido desoxirribonucleico**
ARC	**Núcleo arcuato**
ARG	**Arginasa**
ARN	**Ácido ribonucleico**
ASL	**Arginosuccinato liasa**
ASS	**Arginosuccinato sintasa**
ATF	**Activating transcription factor**
ATP	**Adenosine triphosphate**
BMAL1	**Brain and muscle Art-like protein 1**
BSA	**Bovine serum albumin**
CKIε	**Caseína cinasa epsilon**
CLOCK	**Circadian locomotor output kaput**
CPSI	**Carbamoylphosphate Synthetase**
CREB	**Cyclic AMP response element-binding protein**
CREM	**Cyclic adenosine monophosphate response element modulator**
CRY	**Cryptochrome**
DBP	**Albumin D-binding protein**
ELISA	**Enzyme-linked-I of immuno-sorbent assay**
GC	**Glucocorticoides**
GRE	**Glucocorticoid response element**
H6PDH	**Hexosa 6 fosfato deshidrogenasa**

HDM	**Hipotálamo dorsomedial**
HL	**Hipotálamo lateral**
HLF	**Hepatocyte leukemia factor**
HNF-4	**Hepatocyte Nuclear Factor 4**
HPV	**Hipotálamo paraventricular**
HSF-1	**Heat shock factor 1**
HVM	**Hipotálamo ventromedial**
IL-1 α	**Interleucina 1 α**
IL-6	**Interleucina 6**
LPS	**Lipopolisacárido**
NFkB	**Nuclear factor kappa-light-chain-enhancer of activated B cells**
NSQ	**Núcleo supraquiasmático**
OSA	**Oscilador sincronizado por alimento**
OTC	**Ornitina transcarbamilasa**
PEPCK	**Fosfoenolpiruvato carboxicinasa**
PER	**Period**
PFA	**Proteínas de fase aguda**
REV ERV α	**Reverse erythroblastosis virus α**
RFA	**Respuesta de fase aguda**
RGC	**Receptor a glucocorticoides**
RORE	**Retinoid acid receptor-related orphan receptor response element**
TEF	**Thyrotroph embryonic factor**
TNF-α	**Factor de necrosis tumoral α**
ZT	**Zeitgeber**

1. INTRODUCCIÓN

1.1 Ritmos biológicos

La palabra circadiana deriva del griego circa (cerca) y diano (día). Dado que nosotros vivimos en un ambiente cíclico creado por la rotación de la tierra, no es de sorprenderse que la adaptación haya involucrado ciclos circadianos en actividades fisiológicas y conductuales. Muchos parámetros biológicos presentan variaciones cercanas a las 24 h y están presentes tanto en eucariontes como en procariontes. La mayoría de estos ritmos son endógenos, es decir están presentes en ausencia de señales externas y cuando tienen una periodicidad cercana a las 24 h, se les denomina ritmos circadianos (Sehgal, 2004). Entre algunas de las características de los ritmos, encontramos el periodo (lapso en que ocurre dicha repetición), amplitud (la magnitud de la variación del fenómeno en estudio), mesor (el promedio de todos los valores observados) y fase (hora en que ocurre un hecho característico dentro del ciclo de estudio) (Gruart *et al*., 2002).

Estos ritmos son generados por un reloj circadiano. En los mamíferos, el sistema está organizado en jerarquías donde el reloj maestro es el núcleo supraquiasmático (NSQ) localizado en el hipotálamo lateral, asociado a otros relojes secundarios o periféricos ubicados en otras regiones cerebrales, así como órganos periféricos (ver figura 1) (Sehgal, 2004).

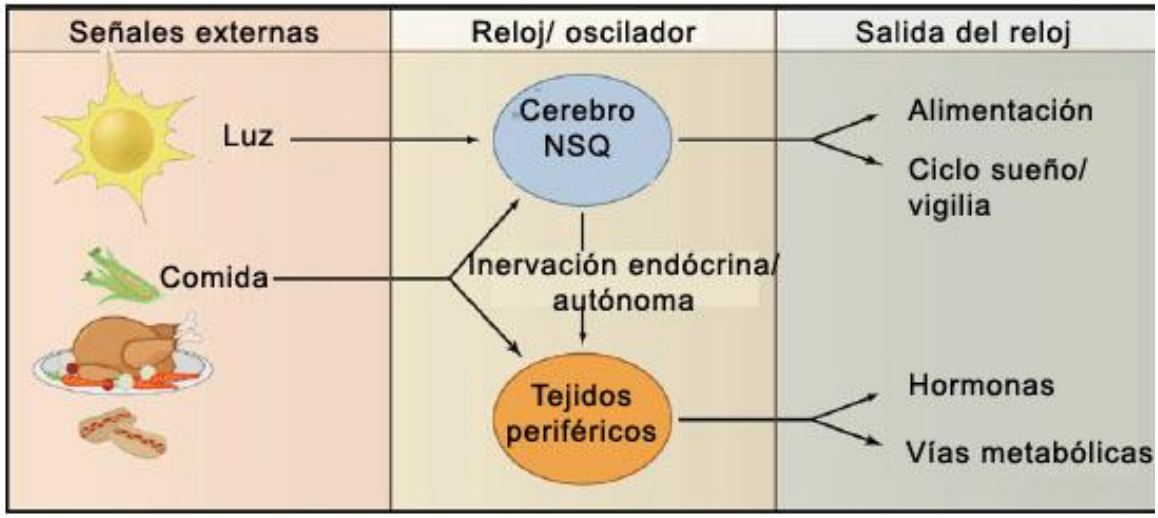

Figura 1. Sistema circadiano. Entre señales externas o sincronizadores podemos encontrar a la luz y a la comida. El reloj que se sincroniza por luz reside en el núcleo supraquiasmático (NSQ) y el que se sincroniza por comida se cree forma una asa de retroalimentación entre estructuras del sistema nervioso central y tejidos/órganos periféricos involucrados con manejo de nutrientes. Modificada de Green *et al*., 2008

Los relojes circadianos de los mamíferos presentan propiedades que se conservan entre las especies: una es la periodicidad de 24 h, aunque la longitud del periodo es variable. Otra característica, es que son endógenos, ya que persisten en ausencia de señales ambientales. La sincronización por señales ambientales es otra de las propiedades, siendo el sincronizador dominante la luz. Jürgen Aschoff acuñó el término zeitgeber (vocablo alemán que significa "dador de tiempo") para nombrar a aquella señal ambiental que sincroniza un ritmo circadiano. La mayoría de los ritmos son sincronizados a ciclos ambientales, por lo que los cronobiólogos se refieren al tiempo del sincronizador como como tiempo del zeitgeber (o en inglés como zeitgeber time ZT), de tal forma que ZT0 corresponde al encendido de la luz y ZT12 al apagado de la misma.. Aunque en los homeotermos es menos claro su valor adaptativo, los ritmos circadianos en heterotermos y poiquilotermos demuestran otra característica, que es la compensación de temperatura, en donde se mantiene un periodo constante sobre un rango de temperatura amplio (Sehgal, 2004).

Los relojes circadianos son parte de un sistema que incluye componentes de entrada y de salida. En la entrada encontramos a las señales ambientales/externas, que sincronizan al reloj. Las señales generadas por el reloj deben ser transmitidas por una vía de salida hacia otras partes del organismo para generar cambios conductuales o fisiológicos con un patrón rítmico acoplados en fase y periodo con el sincronizador (Sehgal, 2004) (Figura 1).

1.2 Reloj molecular

A nivel molecular el reloj circadiano tiene fundamentalmente un asa de elementos activadores y otra de elementos represores. Los componentes activadores son las proteínas CLOCK y BMAL1 que, como dímeros, inducen la transcripción de un par de genes represores, que dan lugar a las proteínas PER y CRY, y que a su vez son regulados por una caseína cinasa (CKIε) (Figura 2). Los elementos activadores pertenecen a la familia PAS (Period circadian protein, Aryl hydrocarbon receptor nuclear translocator protein, Single-minded protein) de las proteínas bHLH (basic helix-loop-helix), las cuales reconocen secuencias diana dentro de los promotores de los genes *Per*. Estos promotores pertenecen a una familia general de elementos E-box, pero existen otros como RORE y el motivo E-box, los cuales también tienen un papel importante en la generación de patrones circadianos a nivel de transcripción. Además, BMAL1 es regulado negativamente por la proteína REV-ERV α. Como se mencionó, la salida del reloj es dictada por la compleja integración entre activadores y represores (Kohsaka y Bass, 2006; Crumbley y Burris, 2011).

1.3 Interacción reloj molecular-metabolismo

En los últimos años han aumentado el número de trabajos que ponen en evidencia el control que tiene el reloj molecular sobre el metabolismo, pues se ha demostrado que un gran porcentaje del transcriptoma de diferentes tejidos muestran perfiles de expresión génica circadianos; como por ejemplo, el hígado, músculo esquelético, tejido adiposo café y blanco. Algunos de los genes identificados están involucrados con procesos metabólicos, como en la síntesis de lípidos y colesterol, glucólisis y gluconeogénesis, fosforilación oxidativa, así como procesos de detoxificación. Muchas de las enzimas que presentan regulación circadiana son las que funcionan como pasos limitantes de las vías metabólicas. Otro aspecto interesante, es que el patrón rítmico de expresión varía dependiendo del tejido, lo que sugiere que la regulación es tan fina que permite a cada célula/tejido/órgano llevar a cabo la función que le corresponde al tiempo más adecuado (Green *et al*., 2008; Panda *et al*., 2002; Reddy *et al.,* 2006).

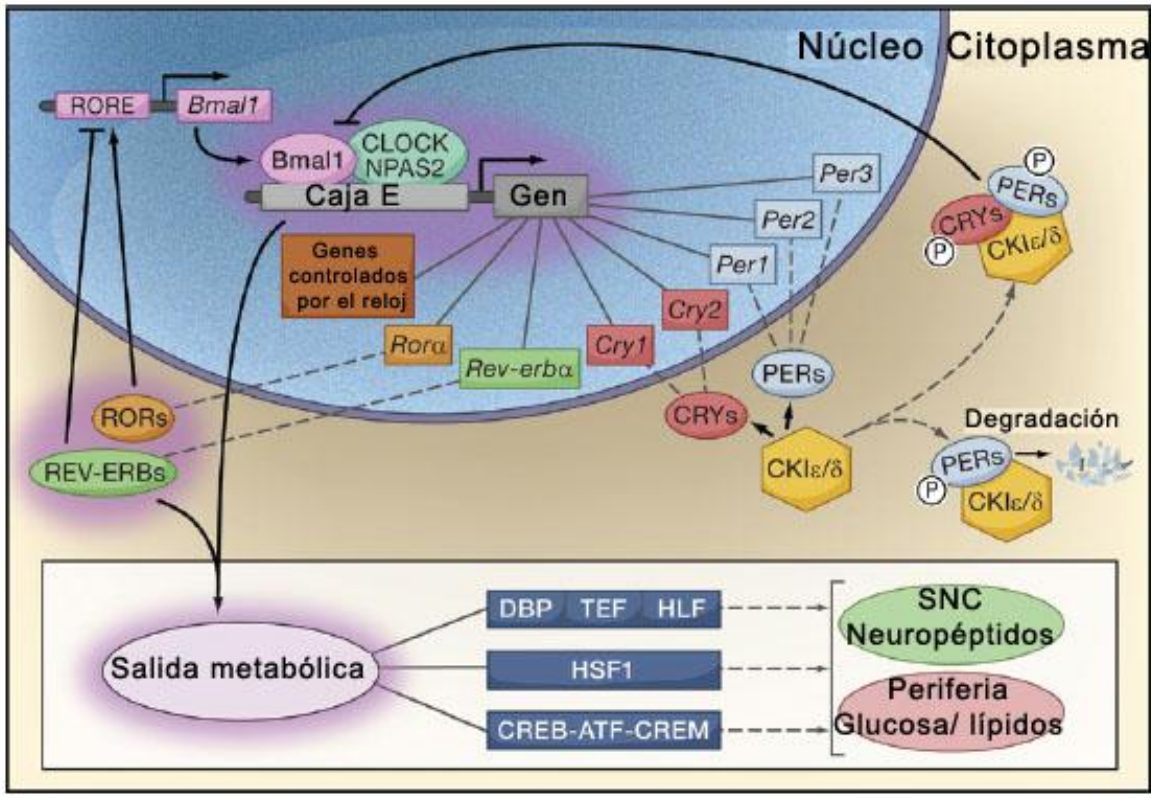

Figura 2. Reloj molecular. La salida del reloj, ya sea en tejidos neurales o periféricos se lleva a cabo a través de proteínas reloj o a través de cambios en la expresión rítmica de factores de transcripción que son modulados por proteínas reloj. DBP, Albumin D-binding protein; TEF, thyrotroph embryonic factor; HLF, hepatocyte leukemia factor; HSF-1, heat shock factor 1; CREB, cyclic AMP response element-binding protein; ATF, activating transcription factor; CREM, cyclic adenosine monophosphate response element modulator. Adaptada de Green *et al*., 2008

Aunque es claro que existe una fuerte interacción entre el reloj molecular y el metabolismo, es importante estudiar más acerca de cómo se lleva a cabo esta interacción y cómo se hace más específica dependiendo del tejido.

Para corroborar la relación entre el reloj molecular y el metabolismo, se han generado animales con mutaciones en diferentes genes reloj. Los ratones mutantes de CLOCK (C57BL/6J) presenta alteraciones severas en el balance energético, con características similares a las reconocidas en el síndrome metabólico, incluyendo obesidad, hiperlipidemia, esteatosis hepática, glucosa circulante alta y bajos niveles de insulina circulante (Turek *et al*. 2005). Sin embargo, cuando el ritmo de alimentación está modificado, los animales comen más durante el día, probablemente debido por alteraciones en la ritmicidad de neuropéptidos en el hipotálamo (Green *et al*., 2008).

En el mutante de *Bmal1* -/- ha sido más complicado de estudiar el aspecto metabólico, pues los animales desarrollan artritis degenerativa y su salud decae rápidamente (Kohsaka y Bass, 2007).

Mientras se sigue estudiando cómo el reloj molecular puede alterar el metabolismo, hay muy poca información de cómo el metabolismo puede alterar el reloj molecular. Lo que se sabe, es que cambios en el potencial redox pueden afectar la actividad de proteínas reloj (Rutter *et al*., 2001). Además, se conoce que algunos factores de transcripción relacionados con metabolismo, como REV-ERBα y RORα pueden regular la transcripción de Bmal1 (Sato *et al*., 2004).

Como hemos visto, hay una fuerte interacción reloj molecular y metabolismo que podría ayudar a la optimización de energía y lo que brindaría cierta ventaja adaptativa a los organismos.

1.4 Oscilador Sincronizado por Alimento

Además del núcleo supraquiasmático, se acepta la existencia de un reloj alternativo que es sensible a la restricción de alimento , por lo que se le ha denominado oscilador sincronizado por alimento (OSA). En condiciones de libre acceso al alimento, el OSA no se expresa, y la ritmicidad circadiana se controla por el NSQ. Para que el OSA se exprese, es necesario someter al animal a horarios restringidos de alimentación. Cuando se restringe el alimento ocurren cambios fisiológicos y conductuales previos a la presentación de la comida conocidos como actividad anticipatoria al alimento (AA) (Mistlberger, 1994). Dentro de estos cambios, se han caracterizado aumentos en actividad

motriz, de temperatura corporal y una activación del metabolismo energético (Escobar *et al*., 1998; Davidson y Stephan, 1999).

Entre algunas de las características útiles para identificar células y órganos que pudieran formar parte del OSA están: el poder 1) anticipar al alimento; 2) estimular la actividad en ausencia de alimento; 3) promover la ingesta de alimento; 4) la eliminación de la señal de salida o su receptor debe eliminar o atenuar la actividad anticipatoria; 5) estar bajo control circadiano; 6) presentar ritmicidad en luz constante; 7) o en condiciones constantes de nutrientes; 8) estar sincronizado al tiempo de presentación del alimento (Silver y Balsam, 2010).

Se han realizado numerosos trabajos para identificar el substrato anatómico del OSA. Muchos se han realizado en estructuras cerebrales indispensables para la AA. Experimentos de lesión en hipocampo, amígdala y núcleo accumbens no eliminaron la AA (Mistlberger y Rusak, 1988; Mistlberger y Mumby, 1992). Lesiones en el núcleo parabranquial provocaron una disminución en la AA, lo que implica que esta región pudiera servir como una vía de salida de OSA o una vía de información del sincronizador hacia el sistema nervioso central (Davidson *et al*., 2000).

Otras estructuras hipotalámicas que regulan el metabolismo son el hipotálamo ventromedial (HVM), núcleo paraventricular (HPV), núcleo arcuato (ARC) e hipotálamo lateral (HL). Lesiones en estas áreas afectan el metabolismo y la ingesta de comida ocasionando pérdidas o ganancias de peso (Mistlberger, 2011).

Por otro lado, las eferentes neurales del NSQ proyectan al hipotálamo dorsomedial (HDM), núcleo que al ser lesionado produjo la atenuación de los ritmos circadianos de ingesta de alimento. Sin embargo, en experimentos posteriores la presencia de actividad anticipatoria en ratas con daño colateral extensivo, descartaron al HDM como substrato crítico para la expresión del OSA. Otras lesiones, incluyendo la parte dorsal del HDM a través del HVM y el ARC, atenuaron los ritmos de anticipación (menos en el hígado) al alimento por una semana. Estas observaciones sugieren que las estructuras mediales hipotalámicas, incluyendo el ARC y el HDM pudieran constituir parte del OSA (Mistlberger, 2011).

A nivel hormonal, se reconocen tres glándulas importantes en la producción sistémica de señales circulantes, la glándula adrenal, el pancreas y el estómago, sabiendo que aunque ninguna es necesaria para la actividad anticipatoria, cada una provee información temporal para la respuesta anticipatoria a la comida Dentro de estas señales

circulantes se incluyen a la corticosterona, insulina, grelina, leptina, adiponectina, ácidos grasos libres, glucagon, hormonas tiroideas, etc. (Silver y Balsam, 2010).

La idea más aceptada a la fecha es que el OSA está constituido por un sistema en que participan tanto estructuras centrales como periféricas involucradas en la regulación de la ingestión del alimento, debido a que órganos como intestino, riñón o hígado expresan genes reloj y pueden ser sincronizados por horario restringido de alimentación (Figura 1) (Mistlberger, 2011).

1.5 EL OSA y el hígado

El hígado lleva a cabo funciones una gran variedad de funciones, entre las que encontramos el metabolismo de lípidos, carbohidratos, proteínas, funciones de detoxificación, inmunológicas y de depósito. Siendo este un importante procesador de nutrientes, se han caracterizado un gran número de adaptaciones bioquímicas y fisiológicas en el modelo de restricción de alimento. Por ejemplo, se ha demostrado que existen señales metabólicas asociadas con el balance de energía corporal, que sugieren un incremento en la movilización de lípidos del tejido adiposo y un aumento de su metabolismo en el hígado. Dentro de estas señales encontramos que previo al acceso al alimento hay una disminución en los niveles de glucagon, triacilglicéridos y un incremento en los ácidos grasos y cuerpos cetónicos circulantes (Escobar *et al*., 1998). Además, previo a la presentación de la comida se encontró un estado redox oxidado tanto en el citoplasma como en la mitocondria, un incremento en los niveles de los nucleótidos de adenina y un aumento en los niveles de ATP. Después de comer, encontramos un estado redox hepático reducido tanto en citoplasma como en mitocondria y una reducción en los niveles de ATP (Díaz-Muñoz *et al*., 2000; Báez-Ruiz *et al*., 2005). Éstos cambios representan la anticipación metabólica y fisiológica al alimento.

Sin embargo, no todas las adaptaciones ocurren antes y después de la presentación de la comida, sino a lo largo de las 24 h. Un ejemplo de esto, es la constante reducción de las reacciones pro-oxidantes (dienos conjugados y peroxilípidos) en la mayoría de las fracciones subcelulares hepáticas, así como un incremento persistente en el potencial de membrana mitocondrial ($\Delta\Psi$) (Báez-Ruiz *et al*., 2005; Luna-Moreno *et al*., 2007).

Las condiciones fisiológicas en los animales con acceso restringido al alimento contrastan con los estados de alimentación *ad libitum* y de ayuno agudo. En la condición

de restricción, se reconoce un conjunto de cambios metabólicos coordinados para sostener un nuevo estado fisiológico en función del ciclo de 24 h, que se conoce como cronostasia (Mrosovsky, 1990). Durante la expresión del OSA, el hígado presenta estos cambios para adaptarse de manera óptima al manejo del alimento durante el horario restringido de alimentación.

En este punto es conveniente explicar algunas definiciones que han sido usadas para explicar el equilibrio dinámico que caracteriza las adaptaciones bioquímicas y metabólicas durante la expresión del OSA. Por un lado, está la homeostasis, la cual mantiene dentro de ciertos límites la composición del medio interno; la reostasis, permite el ajuste de las funciones orgánicas en función de las necesidades orgánicas o a los cambios ambientales (Mrosovsky, 1990) y finalmente, la cronostasis (Aguilar-Roblero y Diaz-Muñoz, 2010), que coordina los procesos fisiológicos mediante la existencia de una organización temporal interna y la anticipación a fenómenos cíclicos ambientales.

1.6 Respuesta de fase aguda

No se puede descartar que esta adaptación fisiológica del hígado implique un estado de estrés por la restricción de alimento a la cual se someten los animales. Entre algunos factores que pudieran representar estrés para los animales con alimentación restringida están: el ayuno de 22 h al que son sometidos diariamente o por lo contrario la hiperfagia que experimentan después de sólo 2 h de disponibilidad al alimento. El estrés por ayuno estaría respaldado por la disminución de tejido adiposo y el aumento de corticosterona previo al acceso al alimento, la cual es una hormona marcadora de estrés (Díaz-Muñoz *et al*., 2000; Martínez-Merlos *et al*., 2004). Debido a que los animales sometidos a restricción de alimento comen en 2 h una cantidad similar de comida a la que comen animales con libre acceso a alimento en un ciclo de 24 h experimentan distensión estomacal, la que podría representar otro factor de estrés (Martínez-Merlos y col., 2004).

Datos preliminares de actividad transcripcional obtenida por microarreglos sugirieron que el hígado pudiera estar implementando una respuesta inmunológica primaria conocida como respuesta de fase aguda (RFA) (Báez-Ruíz *et al*., 2005). Entre algunos ejemplos están aumento en la expresión de genes que codifican para interleucina-6, interleucina-12, apoliproteínas B, C3 y C1 durante la AA.

La RFA es una respuesta sistémica del organismo que se implementa en el hígado, pero también con la participación de sistemas como el neuroendocrino, el hematopoiético, el inmune y el muscular. La respuesta a estrés comienza cuando en células especializadas (macrófagos, células T, fibroblastos, células B, células dendríticas, neutrófilos, células de Kupffer, entre otras) se activa el factor NFkB (Nuclear factor kappa-light-chain-enhancer of activated B cells), un activador transcripcional también llamado sensor de estrés. El NFkB regula la transcripción de genes que codifican para citocinas, las cuales son factores clave en la activación de la RFA. Las principales citocinas implicadas son: las interleucinas 1 y 6 (IL-1α, IL-6), interferones y el factor de necrosis tumoral (TNF-α). Al instalarse la RFA, se promueve la síntesis y secreción de un conjunto de proteínas hepáticas que se conocen como proteínas de fase aguda (PFA) (Figura 3) (Ramadori y Armbrust, 2001). También se ha determinado que durante la RFA hay alteraciones importantes en el metabolismo de lípidos que se asocian a una elevación de colesterol plasmático principalmente por un aumento de lipoproteínas de muy baja densidad (Liao *et al*., 1996) (Figura 3).

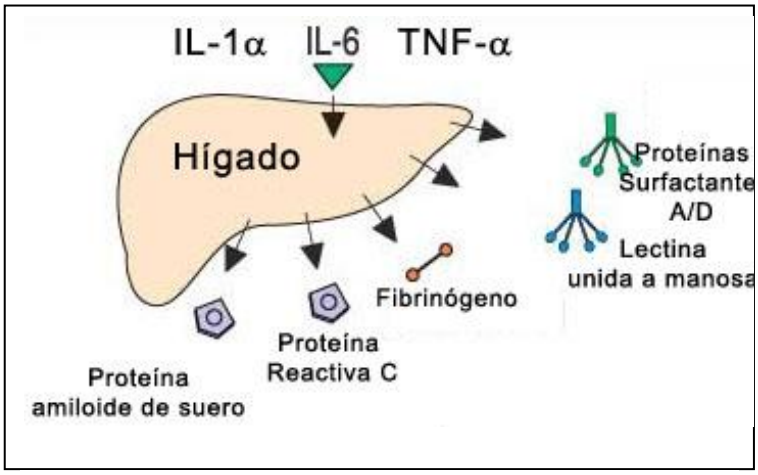

Figura 3. Efecto de citocinas pro-inflamatorias durante la respuesta de fase aguda. HTTP://EPIDEMIOLOGIAMOLECULAR.COM

1.7 Glucocorticoides como sincronizadores

La sincronización del NSQ hacia el hígado emplea vías que involucran señales neurales y humorales. Dentro de las señales hormonales, los glucocorticoides juegan un papel muy importante (Buijs *et al*., 2003) (Figura 4). En el modelo de sincronización por alimento hay una elevación plasmática de estas hormonas (Díaz- Muñoz *et al*., 2000) resultado de la activación del eje hipotálamo-pituitaria-adrenal, el cual es parte fundamental de la respuesta al estrés. Los glucocorticoides, además de estar asociados a estrés, son importantes en el mantenimiento de la homeostasis de la glucosa a través de la regulación de la expresión hepática de genes involucrados en vías gluconeogénicas, glicolíticas y en el ciclo de la urea, incluyendo la tirosina aminotransferasa, la fosfoenolpiruvato carboxicinasa (PEPCK) y la piruvato cinasa.

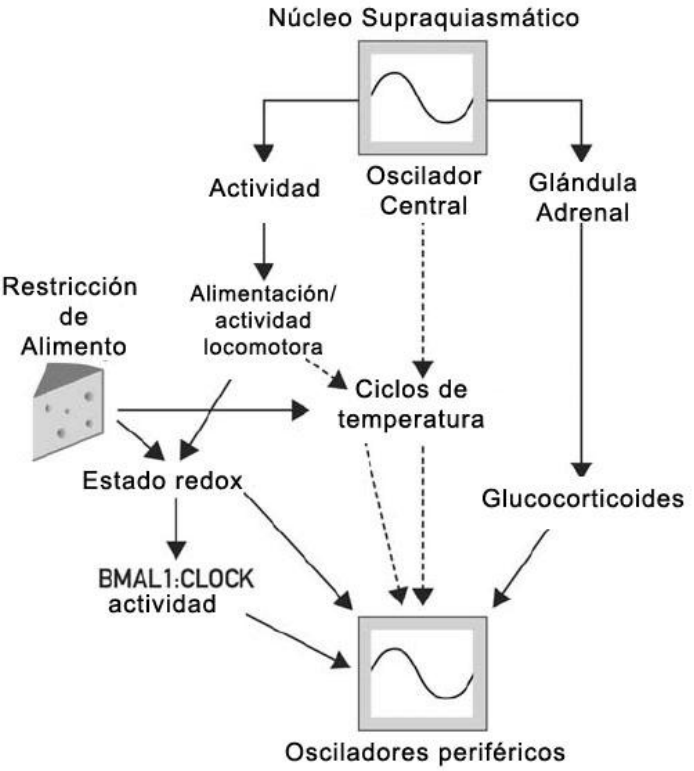

Figura 4. Vías de sincronización de osciladores periféricos. Modificada de Stratmann y Schibler, 2006.

Otra función muy importante de los glucocorticoides es que pueden regular también el reloj molecular, por lo que tienen un papel como sincronizadores (Figura 4). Una exposición única de agonistas a glucocorticoides (como la dexametasona) pone en fase a un conjunto de células asincrónicas; además, la señalización de glucocorticoides *in vivo* induce la expresión del gen reloj *mPer1* en el hígado (Balsalobre *et al*., 2000). Reddy *et al*., (2007) demostraron que en ausencia de señales rítmicas dependientes del sistema nervioso central, los glucocorticoides son suficientes para sincronizar cerca del 60 % del transcriptoma circadiano. Sin embargo, la inyección de dexametasona altera la fase de expresión del gen CLOCK en el hígado y otros tejidos periféricos, pero no en el NSQ. Esta acción selectiva de los glucocorticoides es debida a la expresión diferencial del receptor en estos tejidos. Los receptores a glucocorticoides pueden actuar directamente sobre elementos del reloj molecular (Per1, Rev erv-α) a través de elementos de respuesta a glucocorticoides (GRE) (Schmutz *et al*., 2011) (Figura 5).

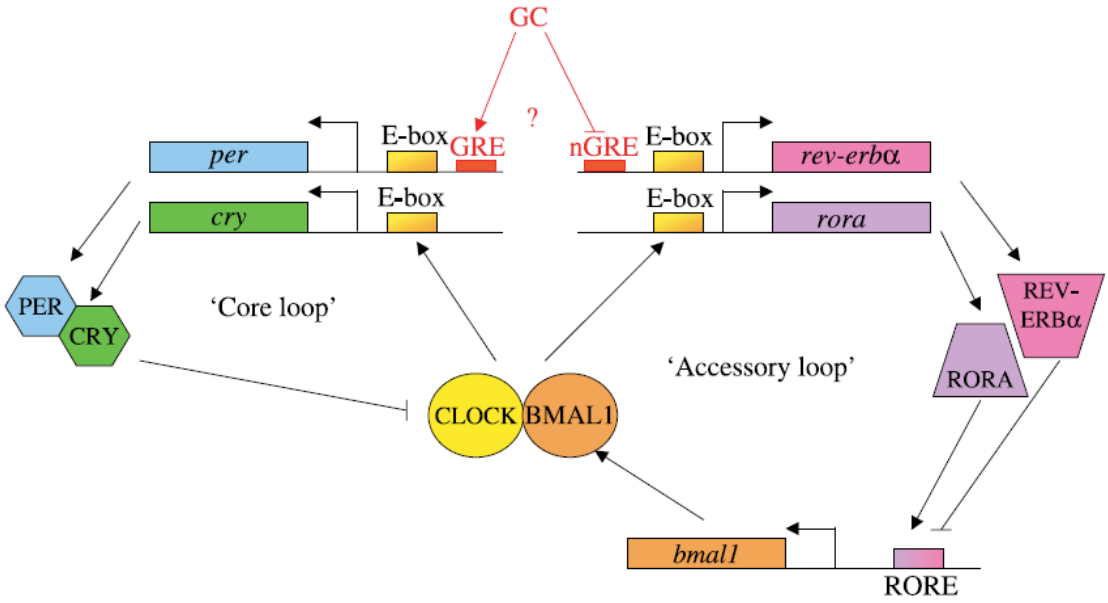

Figura 5. Glucocorticoides sobre el reloj molecular. Los glucocorticoides (GC) pueden modular la transcripción de ciertos genes al unirse a elementos responsivos a glucocorticoides (GRE) en sus promotores. Un GRE positivo induce la expresión de per1 y en contraste, rev-erba contiene un GRE negativo (nGRE) lo cual inhibe su expresión. Dickmeis, 2009.

Los glucocorticoides promueven una gran variedad de respuestas en el hígado debido a que su receptor puede interactuar con cerca del 30 % de los genes hepáticos. A nivel celular, los glucocorticoides median su efecto fisiológico a través de la unión a un receptor intracelular específico, el receptor a glucocorticoide (RGC). Si el glucocorticoide está presente en el citosol, el RGC es liberado de una proteína de choque térmico, y se traslocan al núcleo donde funcionan como regulador transcripcional específico para una secuencia de DNA. Además de interactuar con el DNA, el RGC tiene reconoce otros reguladores transcripcionales por lo que extiende su control sobre otros genes. Animales knock out para el receptor a glucocorticoide no son viables, lo que demuestra su importancia para la sobrevivencia (Vegiopoulos *et al*., 2007).

Hay 3 elementos clave que pueden afectar la regulación por glucocorticoides: disponibilidad del ligando, el receptor y el reclutamiento de cofactores y otras proteínas. De estos 3, la disponibilidad del ligando ha tomado gran importancia en los últimos años, debido a que hay 2 receptores que tienen afinidad para glucocorticoides, uno es el RGC y el otro el receptor a mineral corticoides, éste último con 10 veces más afinidad que el propio RGC. La concentración del ligando fluctúa entre 0.5 nM y 100 nM, y es clave en la

respuesta que ejerza uno u otro receptor (a altas concentraciones, el RGC es dominante) (Trapp y Holsboer, 1996). Esta regulación es tejido-específica. La manera en cómo se regula gran parte de la disponibilidad local del ligando, está dada por la actividad de la enzima 11 β hidroxiesteroide deshidrogenasa tipo 1 (11 β HSD 1), ya que sintetiza corticosterona. La actividad de esta enzima, se controla por la presencia del cofactor NADPH, el cual es generado por la enzima microsomal hexosa 6 fosfato deshidrogenasa (H6PDH) (Lu *et al*., 2006).

1.8 Ciclo de la urea

Durante la conversión de aminoácidos en glucosa, hay liberación de amonio el cual es convertido a urea para evitar su toxicidad. La inducción de enzimas gluconeogénicas se coordina en ocasiones con la inducción de enzimas del ciclo de la urea. Durante la AA se observó que hay una disminución en la expresión de algunos genes que codifican para proteínas clave en el ciclo de la urea, como son la carbamoil fosfato sintasa 1 (CPSI), la arginosuccinato liasa (ASL) y la arginasa (ARG), lo que sugiere que hay una disminución en la formación de urea (Báez-Ruiz *et al*., 2005).

Reddy y colaboradores (2007), analizaron un grupo de genes de expresión circadiana sensibles a glucocorticoides, y observaron que había una alta frecuencia de genes blanco para el factor nuclear hepático 4 alfa (HNF-4α). HNF-4α es un factor de transcripción que regula numerosas vías metabólicas como la producción de proteínas del suero, la actividad de citocromos P450 y la función más conocida, la formación de urea. Las enzimas del ciclo de la urea son las responsables de neutralizar el amonio tóxico originado de la degradación de aminoácidos. Junto con otros genes que codifican para enzimas gluconeogénicas y que catabolizan aminoácidos, los genes del ciclo de la urea son expresadas en la región periportal del hígado y son activados por glucagon (vía AMPc) y hormonas glucocorticoides (Reddy *et al*., 2007).

Los glucocorticoides son reguladores importantes del ciclo de la urea. Este ciclo abarca 5 enzimas, la carbamoil fosfato sintasa (CPSI), la ornitina transcarbamilasa (OTC), la arginosuccinato liasa (ASL), la arginosuccinato sintasa (ASS) y la arginasa (ARG) (Almada *et al*., 2006). La enzima mitocondrial CPSI es la enzima limitante en el ciclo y es la encargada de catalizar la unión del amonio y el HCO^{-3}, en presencia de ATP, para la formación de carbamoil fosfato. La expresión específica en el hepatocito de la CPSI es regulada por un potenciador distal, localizado 6.3 kb río arriba del sitio de inicio de la transcripción, en combinación con la región promotora. Este enhancer distal está compuesto de 2 unidades funcionales, una unidad de respuesta a AMPc (150-200 pb) y una unidad de respuesta a

glucocorticoides (80 pb) (Hoogenkamp *et al*., 2007). La OTC es la segunda enzima de la síntesis de la urea y se localiza también en la matriz de la mitocondria hepática de animales ureotélicos. Cataliza la síntesis de citrulina a partir de carbamoil fosfato y ornitina. En contraste a las otras 4 enzimas del ciclo, la actividad de la OTC es ligeramente inducida en cultivos primarios de hepatocitos de rata por los glucocorticoides o por glucagón (Takiguchi y Mori, 1995).

2. PLANTEAMIENTO DEL PROBLEMA

Durante la expresión del OSA, se han reportado un gran número de adaptaciones metabólicas y fisiológicas que anticipan al organismo a la llegada del alimento para hacer más eficiente el aprovechamiento de los nutrientes y de la energía. No descartamos que esa anticipación constituya una fuente de estrés, puesto que hay una elevación de glucocorticoides importante previo a la presentación de la comida, una hiperfagia, peso corporal casi constante, una disminución de los depósitos lipídicos retroperitoneales y epididimales, así como una posible respuesta inmunológica de fase aguda. Tampoco descartamos que durante la restricción alimenticia los glucocorticoides también actúen como sincronizadores metabólicos, y modulen la actividad cíclica del ciclo de la urea.

3. HIPÓTESIS GENERAL

Si durante la expresión del OSA, los animales presentan un aumento en glucocorticoides (corticosterona), los cuales son indicadores de estrés e importantes reguladores metabólicos, entonces habrá adaptaciones en el hígado a nivel inmunológico y metabólico.

4. OBJETIVO GENERAL

Caracterizar el papel de los glucocorticoides en la respuesta inmunológica (de fase aguda) y metabólica (ciclo de la urea) del hígado durante la expresión del oscilador sincronizado por alimento.

5. RESULTADOS

Artículo A. ¿La expresión del oscilador sincronizado por alimento se acompaña de una respuesta de fase aguda?

I. Hipótesis.

Ya que en animales que expresan el OSA en restricción de alimento muestran adaptaciones metabólicas y fisiológicas en el hígado que son sugestivas de una situación de estrés, entonces el hígado podría implementar una respuesta de fase aguda.

II. Objetivos particulares

a. Determinar si hay una respuesta de fase aguda durante la expresión del oscilador sincronizado por alimento.

b. Caracterizar las variaciones diurnas de citocinas y proteínas de fase aguda, así como si hay una respuesta de estrés celular en el hígado.

c. Cuantificar en los hepatocitos el marcador pro-inflamatorio NFκB.

III. Materiales y métodos

Animales y condiciones experimentales. Ratas macho Wistar con un peso de 180–220 g al inicio del experimento*s* fueron mantenidas en un ciclo luz-oscuridad 12:12-h (encendido de luz 08:00 h) y una temperatura constante (22± 1° C). La intensidad de luz fue de 350 lux. Todos los experimentos fueron aprobados por las normas de la Guía para el Cuidado y Manejo de Animales para Experimentación (Universidad Nacional Autónoma de México).

Las ratas fueron mantenidas con libre acceso a alimento (Purina Chow) y aclimatadas a las condiciones ambientales del laboratorio durante 3 días, antes de iniciar el experimento**.** Fueron asignadas aleatoriamente a: 1) un grupo *ad libitum* (AL) el cual tuvo libre acceso al alimento (Purina Chow), 2) un grupo sincronizado por alimento (RF) con acceso al alimento por 2 h al día (12:00 a 14:00 h), 3) un grupo con un ayuno único de 22 h (Ay) y 4) un grupo realimentado por 2 h después de un ayuno único de 22:00 h seguido por un periodo de acceso al alimento de 2 horas (Re). El grupo 3 y 4 son controles de alimentación, tomando en cuenta que los animales en restricción alimenticia están alternando entre un ayuno de 22 h y un periodo de alimentación de 2 h, hay que compararlos con un ayuno de 22 h justo antes de que coman y una vez alimentados hay que hacer la comparación con un grupo realimentado de 1 día. Esto es para descartar que los cambios observados sean ocasionados por la condición de ayuno o la de realimentación. La duración del protocolo fue de 3 semanas para los animales con horario restringido y alimentación *ad libitum* (Díaz-Muñoz *et al*., 2000). El control positivo de respuesta de fase aguda consistió en animales inyectados intraperitonealmente con el lipopolisacárido (LPS) de *Escherichia coli* 055:B5 intraperitonealmente: 7mg/kg para

determinación de citocinas y 500 µg/100g para determinación de fibrinógeno, colesterol, proteína reactiva C y NFκB (Kitoh *et al*., 2005; Liao *et al*., 1996).

Los grupos AL y RF se sacrificaron en diferentes puntos temporales (8:00, 11:00, 14:00, 17:00, 20:00, 23:00, 2:00 y 5:00 h). Las ratas del grupo Ay se sacrificaron a las 11:00 h y las del grupo Re a las 14:00 h (Díaz-Muñoz *et al*., 2000).

Métodos. Las ratas fueron sacrificadas por decapitación. La sangre fue colectada en 2 partes: Una fue centrifugada a 5,000 rpm durante 5 min para la obtención del suero; la otra en tubos con buffer de citratos y se centrifugó a 3,500 rpm durante 10 min para la obtención del plasma. El hígado también dividió en 2 partes, en una se tomaron 5 g de hígado para el método de fraccionamiento celular (Aguilar-Delfín *et al*., 1996) y en otra se cortaron cuidadosamente trozos pequeños de 1-2 mm^3 y se utilizaron para inmunohistoquímica.

Los 5 g de hígado se homogeneizaron en buffer de sacarosa 225 mM, Tris/HCl 10 mM, BSA 0.2 %, EGTA 0.3 mM e inhibidores de proteasas (1:10 w/v). Posteriormente, el homogenado fue centrifugado A 3,500 rpm por 15 min. El sobrenadante fue centrifugado a 7,500 rpm por 20 min, y el sobrenadante fue re-centrifugado a 36,000 rpm por 60 min y siendo el sobrenadante final la fracción citosólica.

Las fracciones nucleares fueron preparadas de la siguiente manera: 1 g de tejido fue homogeneizado en 5 ml de sacarosa 0.5 M, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, y solución 25 mM KCl. El homogenado fue separado en sacarosa 0.9 M, Tris-HCl 50 mM (pH 7.5), EDTA 1 mM, y solución KCl 25 mM. Después de la centrifugación a 4500 rpm por 20 min, los núcleos fueron resuspendidos en glicerol 40%, Tris-HCl 50 mM (pH 8), $MgCl_2$ 25 mM, y EDTA 0.1 mM y almacenados a -70 °C (Escher *et al*., 2001).

Las muestras para inmunohistoquímica se fijaron en formalina al 10% (formaldehido al 37% 100 ml, agua destilada 900 ml, fosfato de sodio monobásico 4 g y fosfato de sodio dibásico 6.5 g). Una vez fijados, se deshidrataron en un tren de alcoholes (60% 2 h, 70% 2 h, 80% 2 h, 96% 1 h, alcohol absoluto 2 h, ETOH 50%- Xileno 50% 2 h, xileno 2 h. Los tejidos se incluyeron en parafina y se cortaron en micrótomo de rotación. Los cortes se recogieron en portaobjetos y se metieron al horno a 60 °C por 1 h para desparafinar. Para continuar desparafinando, las muestras se metieron en los siguientes solventes: xilol 100%, xilol 100%, etanol absoluto, etanol 95%, etanol 95%, etanol 50%, agua destilada. Las muestras se bloquearon con leche baja en grasa 2.5% durante 1 h. El agente bloqueador de lavó con TBST (TBS (Tris/HCl 20 mM, pH 7,4; NaCl 150 mM, Tritón X100 10ml). Las muestras se incubaron toda la noche con el anticuerpo primario cabra

anti-NFκB p65 (1:250 dilución en TBST, Santa Cruz). Al día siguiente, las muestras se lavaron con TBST y se incubaron con el anticuerpo secundario anti-cabra acoplado a rojo tejas (1:200 dilución en TBST). Se conservaron laminillas sin anticuerpo primario como control negativo y para verificar la fluorescencia endógena. Para conservar las preparaciones, se adicionó PBS-glicerol. Las muestras se sellaron y se analizaron en el microscopio. Finalmente, se cuantificó la fluorescencia por medio del programa Image Pro-Plus© (Media Cybernetics, U.S.A.).

Determinación de citocinas y de corticosterona. Las citocinas pro- inflamatorias TNF-α, IL-1α, IL-6 así como también la corticosterona (marcador de la expresión del OSA) fueron cuantificadas usando ensayos de ELISA (Enzyme-linked- of immuno- sorbent assay). Kits BioSource Rat UltraSensitive fueron usados para la determinación de citocinas y el kit Assay Design para corticosterona. Anticuerpos monoclonales específicos para IL- 6, IL-1α, TNF-α, y corticosterona fueron colocados en los pozos de acuerdo a las instrucciones del proveedor. La intensidad del producto coloreado fue directamente proporcional a la cantidad de IL-6, IL-1α, TNFα, e inversamente proporcional a la cantidad de corticosterona. La absorbancia fue leída a 450 nm para todos los casos (Khan *et al.*, 2004).

Determinación de fibrinógeno. El fibrinógeno es una glicoproteína que se eleva considerablemente durante procesos pro-inflamatorios y durante la RFA. Se determinó por el método de Clauss (1957) (turbidimétrico). Se emplearon muestras de plasma, a las cuales se les agregó trombina. La trombina ataca los enlaces arginina-glicina presentes en los fibrinopéptidos de la molécula de fibrinógeno, con lo que se convierten en moléculas de fibrina soluble. La agregación del factor XVIII activado por la trombina, activa a la enzima transglutaminidasa, la cual forma enlaces covalentes entre monómeros de fibrina, produciendo un coágulo visible e insoluble. El tiempo que pasa entre la adición de la trombina y la formación del coágulo, es inversamente proporcional a los niveles de fibrinógeno (Rumley *et al*., 2003).

Determinación de proteína reactiva C. La proteína reactiva C pertenece a la familia de las pentraxinas. Sintetizada en el hígado es una de las proteínas de fase aguda más sensibles y su nivel aumenta hasta 2,000 veces durante procesos pro-inflamatorios. Para la determinación de la proteína reactiva C, se empleó un ensayo de aglutinación (PCR LÁTEX, prueba directa; BIO RAD), en donde concentraciones superiores a los 6 mg/L provoca la aglutinación de las partículas de látex recubiertas con anti-proteína reactiva C (Senju *et al*., 1986).

Cuantificación de PER1. La proteína Per1 del hígado en la fracción nuclear (marcador de sincronización) fue detectada por western blotting. La concentración de proteína fue determinada por el método de Lowry. Las muestras fueron tratadas con agentes reductores y separadas por peso molecular por SDS-PAGE 12% con un buffer de Tris 25 mM, glicina 192 mM y SDS 0.1% y transferidas a una membrana de nitrocelulosa con un buffer de Tris 100 mM, glicina 192 mM y metanol 20% durante 35 min. Después del bloqueo de sitios de unión no específicos con leche al 5% en PBS-Tween-20 0.1% por 1 h a temperatura ambiente, las membranas fueron incubadas toda la noche con anti Per1 cabra policlonal (1:500; Santa Cruz Biotech. Inc.), y β-actina ratón monoclonal (1:500; Santa Cruz Biotech. Inc.). Las membranas fueron lavadas e incubadas por 2 h con el anticuerpo secundario conejo anti- cabra y burro anti ratón IgG acoplado a fosfatasa alcalina (1:5000; Santa Cruz). Las proteínas fueron detectadas añadiendo buffer para fosfatasa alcalina (Tris 100 mM pH 9.5; NaCl 100 mM; $MgCl_2$ 5 mM) (BioRad). La reacción de color fue parada con H_2O.Las bandas fueron normalizadas de acuerdo a la señal correspondiente a la banda de β-actina El análisis de densitometría fue hecho con el programa KODAK ID, Scientific Imaging System (Nadjar *et al.,* 2003).

Análisis estadístico. Los datos se reportaron como medias ± error estándar. Las diferencias entre animales AL, RF y tratados con LPS fueron detectadas por medio de una prueba t con un nivel de significancia $P<0.05$. Las diferencias significativas entre los diferentes puntos temporales fueron detectadas por medio de ANOVA de una vía, seguidas por una prueba Tukey post hoc con un nivel de significancia $P<0.05$. Las diferencias entre animales control y bajo horario restringido de alimentación fueron detectadas por medio de ANOVA de 2 vías, seguidas por una prueba Tukey post hoc con un nivel de significancia $P<0.05$. Las diferencias entre controles de condición y animales bajo horario restringido de alimentación fueron detectadas por medio de una prueba t con un nivel de significancia $P<0.05$. El análisis estadístico fue realizado con el programa GraphPad PRISMA.

IV. Resultados

ARTÍCULO A: Restricted Feeding entrains rhythms of inflammation related factors without promoting an acute phase response

B. Adaptaciones en la señalización y en la respuesta metabólica a glucocorticoides durante la expresión del Oscilador Sincronizado por Alimento

I. Hipótesis

Si durante la expresión del OSA hay un aumento de glucocorticoides previo al acceso al alimento, y éstos son importantes sincronizadores de los ritmos de diversos parámetros hepáticos, el número y distribución intracelular de los receptores hepáticos a glucocorticoides, así como elementos del ciclo de la urea se sincronizarán al horario de disponibilidad del alimento.

II. Objetivos particulares

Utilizando el protocolo de alimentación restringida, evaluar en hígado a lo largo del ciclo de 24 h los siguientes parámetros:

a. Los niveles y distribución intracelular del receptor a glucocorticoides.
b. La presencia y actividad enzimática de la 11β-HSD1 y de la H6PDH.
c. Los niveles urea circulante y la actividad de las enzimas mitocondriales del ciclo de la urea: la carbamoil fosfato sintasa y la ornitina transcarbamilasa.

III. Materiales y métodos

Animales y condiciones experimentales. El protocolo fue el mismo que el de la sección pasada (ver página 20).

Toma de muestras biológicas. Las ratas fueron sacrificadas por decapitación. La sangre fue colectada en tubos vacutainer y centrifugada a 5,000 rpm durante 5 min para la obtención del suero (para determinación de urea). El hígado se dividió en 2 partes, en una se tomaron 5 g de hígado para fraccionamiento (mitocondrial, nuclear y microsomal) y en otra se apartó 1 g para la obtención de núcleos (Aguilar-Delfín *et al*., 1996). La primera fracción para la cuantificación de las actividades enzimáticas, de la CPSI y de la OTC, la nuclear para la cuantificación del receptor a glucocorticoide y la microsomal para la cuantificación de la 11β-HSD1 y de la H6PDH.

Los 5 g de hígado se homogeneizaron en buffer de sacarosa 225 mM, Tris/HCl 10 mM, BSA 0.2 %, EGTA 0.3 mM e inhibidores de proteasas (1:10 w/v). Posteriormente, el homogenado fue centrifugado a 3,500 rpm por 15 min. El sobrenadante fue llevado a

7,500 rpm por 20 min y el sobrenadante fue re-centrifugado a 36,000 rpm por 60 min y siendo el sobrenadante final la fracción citosólica.

Las fracciones nucleares fueron preparadas de la siguiente manera: 1 g de tejido fue homogeneizado en 5 ml de sacarosa 0.5 M, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, y solución 25 mM KCl. El homogenado fue separado en sacarosa 0.9 M, Tris-HCl 50 mM (pH 7.5), EDTA 1 mM, y solución KCl 25 mM. Después de la centrifugación a 1,800 *g* por 20 min, los núcleos fueron resuspendidos en glicerol 40%, Tris-HCl 50 mM (pH 8), $MgCl_2$ 25 mM, y EDTA 0.1 mM y almacenados a -70 °C (Escher *et al*., 2001).

Cuantificación de urea en suero. Se determinó por método enzimático (ELITech, Francia), en donde la urea es desdoblada por la acción específica de la ureasa, en CO_2 y NH_3. En una segunda etapa el NH_3 reacciona con fenol e hipoclorito en medio alcalino para producir un compuesto de color azul, que se determina colorimétricamente en el espectrofotómetro a 340 nm.

Cuantificación de la actividad de la carbamoil fosfato sintasa en hígado. La mezcla de reacción consistió en NH_4HCO_3 10 µmol, ATP 1 µmol, acetato de magnesio 2 µmol, N-acetil glutamato 1 µmol, ditiotreitol 0.2 µmol, trietanolamina 10 µmol (pH 8) y 200 µg de proteína mitocondrial en un volumen final de 0.6 ml. La reacción se corrió 10 min a 37 °C y el carbamoil fosfato resultante fue convertido a hidroxi-urea por la adición de 0.03 ml de hidroxilamina 2 M seguido de una incubación por 10 min a 95 °C. Para cuantificar la hidroxi-urea se añadieron 2.4 ml de reactivo cromogénico seguido de la incubación por 15 min a 95 °C. Después de enfriar las muestras a temperatura ambiente, la absorbancia fue medida a 458 nm en el espectrofotómetro. La concentración de hidroxi-urea (carbamoil fosfato) fue determinada con el uso de una curva estándar (Pierson, 1980).

Cuantificación de la actividad de la ornitina transcarbomilasa en hígado. La actividad de la OTC fue medida siguiendo el método de Bagrel *et al*., (1975). La mezcla de reacción consistió en 400 µl de una solución ornitina/ureasa 20 mM y 100 µg de proteína mitocondrial. La mezcla se incubó a 37°C por 5 min y se añadieron 400 µl de solución Carbamoil Fosfato 50 mM para incubarse otros 20 min a 37 °C. La reacción se detuvo con 1 ml de ácido tricloroacético (100g/l). Posteriormente, los tubos se centrifugaron a 5,000 rpm por 10 minutos y se tomó 1 ml de sobrenadante, al cual se le agregó 1 ml de diacetil monoxima (100 mM) y 4 ml del reactivo antipirina-fosfoférrica (antipirina 65 mmol, $FeCl_3$ 12.5 mmol, 625 ml deH_3PO_4 y 375 ml de agua destilada) como compuestos que reaccionan con la citrulina formada por la acción de la OTC. La mezcla se incubó a 95 °C en baño María por 20 min, seguida de 10 min de incubación a temperatura ambiente. Por último la

mezcla fue leída en un espectofotómetro, a 460 nm. Para obtener tubos con ensayo control se sustituyó la muestra por la misma cantidad de Buffer Hepes 20 mM.

Cuantificación de la actividad de la 11 β HSD 1 en hígado. La actividad enzimática fue determinada usando el método de Thurston *et al*., (2007). Se usaron 30 mg de proteína microsomal, la cual fue transferida a tubos de ensayo con 700 µl de PBS. Como blanco se usaron 100 µl de BSA 1 mg/ml PBS. Cada ensayo se hizo por triplicado. Los tubos fueron incubados por 30 min a 37° C en baño maría. Para iniciar la reacción, se añadieron 100 µl de PBS con NADPH 4 mM (Sigma, USA), [1,2-^{3}H] cortisona 0.1 µCi (Perkin Elmer, Rodgau-Jugesheim, Germany), y cortisona fría (Sigma, USA) a una concentración final de final de 100 nM. Los tubos se volvieron a incubar a 37° C en baño maría por 60 min y la reacción fue detenida con 2 ml cloroformo frío (J.T. Baker, México). La separación de las fases, orgánica y acuosa, se llevó a cabo por centrifugación (7,000 rpm por 30 min). Después de la aspiración del sobrenadante, los extractos orgánicos fueron evaporados toda la noche a temperatura ambiente. Los residuos esteroides fueron resuspendidos en 20 µl de etil acetato que contenía corticosterona y cortisona fría 1 mM (Sigma) y fueron separados a través de cromatografía en capa fina (TLC), usando placas de Silica 60 TLC (Merck) en un ambiente saturado de 92:8 (v/v) cloroformo:95% (v/v) etanol (Merck). Las manchas correspondientes a corticosterona fueron raspadas y analizadas mediante un contador de centelleo (Bioscan 200 TLC radiochromatogram scanner; LabLogic, Sheffield, UK) para la cuantificación de [^{3}H]-corticosterona.

Cuantificación de la actividad de la Hexosa-6-Phosphato Deshidrogenasa. La actividad de la H6PDH fue medida después de una pre-incubación a 4°C con buffer RIPA para lograr la lisis de membranas microsomales. La mezcla de reacción consistió en 3 mg de proteína microsomal, glucosamine-6-fosfato 0.3 mM, glicina-NaOH 100 mM (pH 10), y BSA 1% en un volumen total de 900 µl. La reacción se inició con la adición de 100 µl de $NADP^{+}$ 1 mM. La reacción se incubó a 37°C en baño maría. El incremento en absorbancia a 340 nm fue monitoreado durante 5 min usando un espectofotómetro (Ultrospec 3,300; Pharmacia Biotech). La cantidad de NADPH producido fue calculado usando el coeficiente de extinción de 6.22. La actividad específica fue expresada como µmol de NADPH por min, por mg de proteína (Nammi *et al*., 2007).

Cuantificación del receptor a glucocorticoide y de la 11 β HSD 1 en hígado. Por medio de inmunoblot el receptor a glucocorticoide (RGC) de hígado fue detectado en la fracción nuclear y la 11βHSD1 en la fracción microsomal. La concentración de proteína fue determinada por método de Lowry. Las muestras fueron tratadas con agentes reductores

y separadas por peso molecular por SDS-PAGE 10% (RGC) y 12% (11βHSD-1) con un buffer de Tris 25 mM, glicina 192 mM y SDS 0.1% y transferidas a una membrana de nitrocelulosa con un buffer de Tris 100 mM, glicina 192 mM y metanol 20% durante 35 min. Después del bloqueo de sitios de unión no específicos con leche al 5% en PBS-Tween-20 0.1% por 1 h a temperatura ambiente, las membranas fueron incubadas toda la noche con anti receptor a glucocorticoide conejo policlonal (1:200; Santa Cruz) o con anti 11 β HSD 1 (1:200; Santa Cruz), y β-actina ratón monoclonal (1:500; Santa Cruz). Las membranas fueron lavadas e incubadas por 2 h con el anticuerpo secundario conejo anti-conejo y burro anti-ratón IgG acoplado a fosfatasa alcalina (1:5000; Santa Cruz). Las proteínas fueron detectadas añadiendo buffer para fosfatasa alcalina (Tris 100 mM pH 9.5; NaCl 100 mM; $MgCl_2$ 5 mM) (BioRad). La reacción de color fue parada con dH_2O. Las bandas fueron normalizadas de acuerdo a la señal correspondiente a la banda de β-actina (Nadjar *et al.,* 2003). El análisis de densitometría fue hecho con el programa Quantity One program version 4.6.9. (BioRad Laboratories Software, U.S.A.).

Análisis estadístico. Los datos se reportaron como medias ± error estándar. Las diferencias significativas entre los diferentes puntos temporales fueron detectadas por medio de ANOVA de una vía, seguidas por una prueba Tukey post hoc con un nivel de significancia $p<0.05$. Las diferencias entre animales control y bajo horario restringido de alimentación fueron detectadas por medio de ANOVA de dos vías, seguidas por una prueba Tukey post hoc con un nivel de significancia $p<0.05$. Las diferencias entre controles de condición y animales bajo horario restringido de alimentación fueron detectadas por medio de una prueba t con un nivel de significancia $p<0.05$. El análisis estadístico fue realizado con el programa GraphPad PRISMA (GraphPad Software, U.S.A.).

IV. Resultados

ARTÍCULO B: Daytime restricted feeding modifies 24-h rhythmicity and subcellular distribution of liver glucocorticoid receptor and the urea cycle in rat liver

6. DISCUSIÓN GENERAL

Como se mencionó anteriormente, el OSA está constituido tanto por estructuras del sistema nervioso central como por tejidos periféricos involucrados con manejo de nutrientes, las cuales se coordinan para mantener la sincronización interna en una fase dependiente de la presencia del alimento. Por lo tanto es sumamente importante conocer cómo éstos se relacionan y cuáles son las adaptaciones que cada uno tiene durante la restricción de alimento. Es por ello que la caracterización de las adaptaciones que tiene el hígado durante la restricción de alimento nos ayuda a un mejor entendimiento de la respuesta fisiológica del OSA.

Durante la expresión del OSA, ya se han caracterizado varias adaptaciones fisiológicas en el hígado como estado redox, carga energética, respiración mitocondrial y reacciones pro-oxidantes (Báez-Ruiz *et al*., 2005; Díaz-Muñoz *et al*., 2000; Luna-Moreno *et al*., 2006). También se han demostrado alteraciones en factores endócrinos tales como insulina, glucagon y hormonas tiroideas las cuales ejercen una regulación directa sobre el funcionamiento hepático (Aceves *et al*., 2003; Díaz-Muñoz *et al.,* 2000). El hecho de encontrar alteraciones en el ritmo de citocinas, proteínas de fase aguda, así como cambios en el ciclo de la urea ayuda a entender mejor cómo se adapta en este tipo de protocolo la fisiología del organismo en general y el hígado en particular.

Glucocorticoides y respuesta de fase aguda

Con el desarrollo de este proyecto, pese a la hipótesis propuesta con base en los resultados de microarreglos, en este trabajo descartamos que la restricción alimenticia y la expresión del OSA asociada, promuevan una fase de respuesta aguda. Sin embargo, la elevación de NFκB, asociada a un estado pro-inflamatorio en el hígado, sugiere también cambios en vías de señalización que involucran a este factor, como son proliferación celular, apoptosis y homeostasis de calcio (Yates y Górecki, 2006). Además, con este trabajo se pone en evidencia los cambios en ritmicidad de IL-1α, IL-6, TNF-α, así como del fibrinógeno los cuales nos están diciendo que hay adaptaciones inmunológicas gracias a la restricción de alimento. Por otro lado, la elevación de glucocorticoides previo al acceso al alimento parece no estar relacionada con una respuesta de fase aguda, sin embargo esta elevación puede formar parte de la comunicación sistema nervioso central-órganos periféricos lo que sugiere cambios a nivel metabólico en órganos o tejidos periféricos, en el caso de hígado a nivel del ciclo de la urea y gluconeogénesis (Desvergne *et al*., 2006).

Glucocorticoides y ciclo de la urea

Tomando en cuenta que ya se había demostrado un aumento de glucocorticoides circulantes en el modelo de restricción de alimento (Luna-Moreno *et al*., 2009) y que éstos son importantes reguladores metabólicos era obvio pensar que habría cambios en las diferentes vías reguladas por los mismos. Lo que no era obvio era la clase de cambios pues la regulación por glucocorticoides es muy compleja, por un lado, se sabe que además de la producción de GC por las glándulas adrenales, hay una liberación tejido específica a partir de la enzima βHSD1. Además de esto, que dependiendo de los niveles locales del ligando iba a ser el tipo de receptor que estuviera involucrado, llámese mineralocorticoide a concentración baja o glucocorticoide a alta (Seckl y Walker, 2001). En este trabajo se observó que los niveles de glucocorticoides circulantes tienen una correlación alta con la presencia del receptor a GC en citosol y con los niveles de urea en animales bajo restricción de alimento, lo que podría representar una vía de sincronización a metabolismo hepático. Otro aspecto interesante es, que el aumento en la presencia del receptor a glucocorticoide en el citoplasma durante la expresión del OSA, en complemento a la actividad transcripcional puede involucrar la modulación de canales iónicos dependientes de voltaje y ligando, proteínas G y la vía ERK1/2, aunque habría que realizar más experimentos para corroborar esta hipótesis (Prager y Johnson, 2009).

Por otro lado, es necesario estudiar con más profundidad el papel de los glucocorticoides como sincronizadores, puesto que nosotros sólo nos enfocamos en los cambios en una vía de salida (el ciclo de la urea), y no en la regulación transcripcional que tienen los glucocorticoides en coordinación con sus receptores sobre los genes que codifican para la OTC y CPS1. También sería interesante, conocer las posibles modificaciones postraduccionales en éstas mismas proteínas en el modelo de la restricción de alimento para poder argumentar que es lo que ocurre a nivel de actividad de la mismas. Esto último debido a que como se observó en este proyecto, los cambios a nivel del mensajero en el caso de OTC y CPS1 sugeridos por el estudio de microarreglos (Báez-Ruiz *et al*., 2005) no van de la mano con la actividad de la proteína.

Con este proyecto queda claro que otra de las adaptaciones del OSA es el cambio en el ritmo de la dinámica del receptor a glucocorticoide, así como de la actividad de la OTC, CPS1 y urea; que éstas adaptaciones cronostáticas en el hígado forman parte de la expresión del OSA.

El Oscilador Sincronizado por Alimento

Encontrar el substrato anatómico del OSA, ha sido uno de los desafíos de gran interés en el área de cronobiología. El candidato más fuerte siempre ha sido el sistema nervioso central, en áreas bien localizadas del hipotálamo (ARC, PVN, DMN, LH por mencionar

algunos) por tener vías de entrada del NSQ, pero ninguna lesión ha eliminado completamente la actividad locomotriz anticipatoria, característica importante de la expresión del OSA. Tomando en cuenta que el acceso restringido y programado al alimento parece ser el único estímulo que controla la fase de ritmos en periferia y que hay una comunicación SNC-periferia a través de sistema nervioso simpático/ parasimpático, por lo que la siguiente hipótesis era que el OSA estaba también constituido por tejidos periféricos. Sin embargo, con el tiempo y complicados experimentos, el nervio vago también fue descartado. El punto es que además del sistema nervioso central y tejidos periféricos está la participación hormonal u otros factores que pueden servir de enlace y ayudar a la sincronización.

Este proyecto permite seguir explorando la posible vía de comunicación SNC-periferia, en este caso, cómo a través de glucocorticoides se podría estar regulando el metabolismo hepático (Reddy *et al.*, 2007). Obviamente hay mucho que estudiar al respecto, pues aunque se sabe que los glucocorticoides pueden poner en fase la expresión de varios genes reloj, la interrupción de la señalización de este receptor sólo altera el ritmo de actividad. Todo parece indicar que hay algún mecanismo compensatorio y que la señalización por glucocorticoides es más compleja, que hay otros elementos involucrados.

Es muy cierto también, que no sólo los glucocorticoides pueden modular al sistema circadiano y así contribuir a la expresión del OSA existen otros factores adicionales glucosa y ácidos grasos. También es sabido que hay genes reloj que pueden sensar el estado energético, como es el caso de *Bmal1* y *Rev-erb* α a través de PPARα y PGC1α (propios de metabolismo).

Lo cierto es que, con el desarrollo de nuevos proyectos como este, se puede entender mejor la naturaleza y el funcionamiento del OSA, que si bien está integrado por importantes núcleos hipotalámicos y órganos o tejidos periféricos involucrados en el manejo de nutrientes la comunicación entre ambos es muy compleja y delicada, por lo que es necesario seguir estudiando las vías humorales. Es crucial conocer cómo la maquinaria molecular del reloj está sensando y transmitiendo el mensaje al metabolismo. Por otro lado, también es importante conocer la adaptación de otros tejidos además del hígado, como por ejemplo el tejido adiposo, pues una fuente importante tanto de metabolitos energéticos como de señales humorales.

8. CONCLUSIONES

El desarrollo de este proyecto permitió caracterizar adaptaciones importantes a nivel inmunológico y metabólico que ocurren durante la expresión del Oscilador Sincronizado por Alimento. Entre éstas, encontramos cambios en la señalización por glucocorticoides, que a su vez sugieren una regulación más fina a nivel de metabolismo hepático.

A nivel hepático, una de las adaptaciones previas al acceso al alimento es un estado pro-inflamatorio local promovido por NFκB. Esto tiene implicaciones importantes pues sugiere cambios a nivel transcripcional de proteínas pro- y anti-inflamatorias, lo que impactaría directamente sobre vías de señalización relacionadas con dicho factor transcripcional como son proliferación celular, apoptosis y homeostasis de calcio (Yates y Górecki, 2006). El estrés local después del acceso al alimento podría estar relacionado con la disminución en los niveles de ATP (Díaz-Muñoz *et al.,* 2000), así como un aumento en las reacciones pro-oxidantes en la fracción microsomal (Luna-Moreno *et al*., 2006).

El hecho de que los glucocorticoides sean importantes sincronizadores, hace más interesante cómo están regulando metabolismo hepático. Por lo tanto, el hecho de encontrar una correlación entre el nivel circulante de glucocorticoides, el receptor a glucocorticoides en citoplasma y urea, da cabida a seguir estudiando el mecanismo de cómo los glucocorticoides sincronizan ritmos metabólicos. Los cambios en la señalización por glucocorticoides es otra de las adaptaciones importantes de la expresión del OSA.

Otra de las adaptaciones cronostáticas encontradas con este proyecto son los cambios en la ritmicidad de marcadores inmunológicos (citocinas, proteína de fase aguda), así como de ciclo de la urea (OTC, CPS1 y urea), que demuestran que hay una perfecta comunicación entre diferentes órganos y tejidos; que el sistema inmune y metabolismo hepático son capaces de percibir el cambio y adaptarse a la restricción de alimento.

9. PERSPECTIVAS

Durante la expresión del Oscilador Sincronizado por Alimento:

- Estudiar la regulación de NFkB sobre genes que codifican para proteínas pro y anti-inflamatorias para entender sobre el balance de éstos frente al alimento.
- Conocer qué papel tienen los receptores de glucocorticoides y mineralocorticoides en la regulación transcripcional por glucocorticoides en el modelo del OSA. Lo que ya se conoce son aspectos aislados, por un lado, la competencia de ambos receptores por el mismo ligando y por otro, como los glucocorticoides pueden sincronizar la expresión de genes hepáticos (Reddy *et al*., 2007).
- Conocer cómo es que los glucocorticoides regulan el metabolismo hepático.
- Investigar qué ocurre con la presencia de substratos, cofactores y otras proteínas que pudieran modificar la actividad de las enzimas del ciclo de la urea.
- Si se entiende bien la comunicación entre reloj molecular-metabolismo, sería más fácil diseñar terapias para mejorar no sólo los problemas de sueño, sino también para enfermedades metabólicas como síndrome metabólico, obesidad y diabetes mellitus.

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RESTRICTED FEEDING ENTRAINS RHYTHMS OF INFLAMMATION-RELATED FACTORS WITHOUT PROMOTING AN ACUTE-PHASE RESPONSE

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A restricted schedule of food access promotes numerous metabolic and physiological adaptations to optimize the biochemical handling of nutrients. The restricted feeding activates responses in hypothalamic and midbrain areas, as well as in peripheral organs involved in energy metabolism. A restricted feeding schedule (RFS) is associated with marked behavioral arousal coincident with the food anticipatory activity (FAA) and extreme hyperphagia during food access. Food restriction is also accompanied by changes in an array of stress-related parameters, such as increase in corticosterone, slower rate in body weight gain, and reduction in retroperitoneal and epididymal adipose tissue. During RFS, the liver shows a diversity of biochemical and physiologically adaptations that are advantageous for food ingestion and processing, as well as for adequate nutrient distribution to other tissues. Taking into account the probable relationship between stressful conditions and the metabolic adaptations in the liver, we addressed whether an acute-phase response (APR), or a pro-inflammatory state, occurred after three weeks of 2 h food restriction. First, we compared the circulating levels of inflammation markers (interleukin-1α, interleukin-6, tumor necrosis factor-α), and APR proteins (C-reactive protein and fibrinogen) in rats under food restriction to those in rats treated with lipopolysacharide, a strong inducer of the APR. Second, the influence of RFS on the daily rhythms of systemic cytokines and APR proteins was characterized. Third, we tested if the feeding condition (22 h fasting and 2 h refeeding) influences these parameters. Finally, we assessed if a local stressed state was established in the liver associated with the restricted feeding by measuring the activation of the transcriptional factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). The results showed that the following occurred during RFS: no APR was implemented; food restriction modified the rhythmic 24 h fluctuations of IL-1α, IL-6, TNF-α, and fibrinogen; simple fasting-refeeding modulated the level of IL-1α, IL-6, and fibrinogen, but this effect was not observed before and after food access in rats with restricted food; and food

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restriction produced a significant peak in NF-κB signal in the liver (including its translocation into the nuclei of hepatocytes) that was dependent on feeding condition, as it was coincident with the time after food access. In conclusion, the stress condition associated with RFS is not sufficient to induce an APR, but it could be related to a local stress-response within the liver (Author correspondence: mdiaz@inb.unam.mx).

Keywords Food-entrainable oscillator, Food anticipatory activity, Stress, Blood cytokines, Liver

INTRODUCTION

A timing system in most organisms controls a set of physiological adaptations to rhythmic environmental changes. In mammals, the suprachiasmatic nuclei (SCN), located in the anterior and ventral hypothalamus, acts as a major pacemaker to command the metabolic, endocrine, and behavioral functions that vary in a circadian (∼24 h) fashion (Ralph et al., 1990). The SCN is synchronized by light-dark alternation, and its rhythmic output is communicated to peripheral oscillators that adopt the phase dictated by the master clock (Dardente & Cermakian, 2007; Guo et al., 2006). However, the phase of peripheral clocks can be completely uncoupled from the SCN pacemaker by restricted feeding (Mistlberger, 1994). Under this condition, a different oscillator is revealed, one that is entrained not by light, but by food (i.e., food entrained oscillator or FEO). Indeed, it has been demonstrated that this oscillator functions even when the SCN is ablated (Marchant & Mistlberger, 1997).

When food access is restricted for few hours per day during several consecutive days, a behavioral arousal and increased locomotor activity 2–3 h before food presentation, known as anticipatory activity (FAA), appears (Stephan, 2002). Restricted feeding induces a shift in the circadian phase of many physiological variables in peripheral organs according to the schedule of food access, including the peak expression of clock genes, which are the molecular core of the timing system (Mendoza, 2007). The anatomic localization of the oscillator that makes possible the physiological response to restricted feeding schedule (RFS) is still controversial, but it may involve a distributed system activated as a rheostatic adjustment in the timing system when energy-sensor areas in the brain and nutrient-handling peripheral organs adopt a new set-point control due to the timed restriction of food availability (Báez-Ruiz et al., 2005; Díaz-Muñoz et al., 2000).

Because of the primary role that the liver plays in the metabolic handling of nutrients and their delivery to the rest of tissues in the organism, it is accepted that the liver is one of the principal components in the physiological response to restricted feeding (Díaz-Muñoz et al., 2000; Stokkan et al., 2001). Our group has previously reported upon the entrainment of

metabolic parameters in the liver during the RFS, such as redox state, energetic charge, mitochondrial respiration, and pro-oxidant reactions (Báez-Ruiz et al., 2005; Díaz-Muñoz et al., 2000; Luna-Moreno et al., 2006). We also demonstrated significant changes in fundamental endocrine factors, such as insulin, glucagon, and thyroid hormones, which regulate liver physiology (Aceves et al., 2003; Díaz-Muñoz et al., 2000). Taken together, these results indicate that substantial modifications before (during the FAA) and after food presentation of the biochemical and physiological performance of the liver are associated with the restricted access to food.

Interestingly, RFS conveys some potentially stressful conditions that could influence the physiological response of the entire organism and, in particular, the activity of the liver when the food is restricted for three weeks:

1. there is an extreme hyperphagia and stomach distention associated with the 2 h period of food intake (Martínez-Merlos et al., 2004);
2. body weight remains almost constant during the treatment;
3. retroperitoneal and epididymal lipid deposits decrease (Martínez-Merlos et al., 2004); and
4. a peak of glucocorticoids accompanies the arousal and expectancy associated with restricted food access (Díaz-Muñoz et al., 2000).

The combination of all these factors suggest that restricted feeding might be associated with a chronic state of stress. Stress is considered an adverse effect of the ambient environment or internal milieu that forces adaptive changes in an animal's physiology and behavior (Black, 2006).

Recurring stress is related to the synthesis and release of pro-inflammatory cytokines by several organs and tissues, but mainly by immune cells, adipose tissue, endothelia, and liver. The liver is a specialized sensor of metabolically demanding states and is responsible for mounting a primary defensive strategy known as the acute-phase response (APR) (Bengmark, 2004), which is part of the innate immune system. According to the degree of instability produced by the stressful conditions, the APR can be systemic or confined to only one or a few organs (Moshage, 1997). Hence, the present study aimed to elucidate if during the RFS an APR-like state is raised, and, if so, to characterize the diurnal variations of cytokines and APR-related proteins during RFS, and whether there is a local stress-response in the liver associated with food restriction.

METHODS

Animals and Housing Conditions

Male Wistar rats (180–220 g when experiments started) were kept on a cycle of 12 h light/12 h dark (light-on at 08:00 h; average light intensity at

the surface of the cages 350 lux), at constant temperature (~21°C), and with free access to food (5001 rodent diet from LabDiet, Brentwood, Missouri, USA) and water, unless otherwise stated. All experimental treatments were conducted following the norms approved by the "Guide for care and use of animal experimentation" from our university (Universidad Nacional Autónoma de México) and conformed to international ethical standards previously recommended (Portaluppi et al., 2008).

Experimental Design

Control and experimental groups were similar to those reported by Ángeles-Castellanos et al. (2005). To determine daily and food-entrained rhythmicity, rats were randomly assigned for three weeks to one of two feeding conditions:

1. control animals fed ad libitum with free access to food and water during the 24 h period; and
2. experimental animals with restricted feeding, in which food availability was limited manually to 2 h daily from 12:00 to 14:00 h.

At the end of the feeding protocol, different subgroups of animals (n = 6) were sacrificed at 3 h internals, starting at 08:00 h, over a complete 24 h cycle to obtain blood and liver tissue samples.

To determine that the observed effects were not due to either an acute 21 h food-deprivation interval or to the refeeding after fasting, two additional control groups were included (each n = 6):

1. Animals in 21 h simple fasting were maintained with free food access for three weeks; on the last day of the experiment, food was removed at 14:00 h, and they were sacrificed after 21 h of food deprivation (at 11:00 h).
2. A second group of rats was similarly deprived of food for 21 h and then refed for 2 h (from 12:00 to 14:00 h) before tissue sampling.

As positive control for systemic APR, a group of animals were injected intraperiotoneally at 11:00 h with lipopolysaccharide (LPS) from *E. coli* 055:B5, using 7 mg LPS/kg for cytokines and 5 mg LPS/kg for C-reactive protein and fibrinogen determinations (Kitoh et al., 2005). Rats treated with LPS were sacrificed after 1 h for cytokines and after 24 h for C-reactive protein and fibrinogen quantifications.

Blood and Liver Sampling

Rats were sacrificed and then decapitated for trunk blood collection. Blood was collected in two fractions. The first fraction was centrifuged at

5000 rpm for 5 min to obtain serum; the other fraction was collected in tubes with 1% citrate and centrifuged at 3500 rpm for 10 min for plasma. The liver sample was also divided into two portions: 5 g was processed for subcellular fractionation, and 0.5 g was cut into fragments of $\sim$1 mm^3 for immunohistochemistry.

The subcellular fractionation was processed according to the report of Aguilar-Delfín et al. (1996). Briefly, the liver was homogenized in 10 mM Tris-HCl (pH 7.4; 1:10 w/v); the homogenate was centrifuged at 1500 g for 15 min, and the pellet was kept for further isolation of the nuclear fraction. The supernatant was centrifuged at 10,000 g for 20 min to sediment the mitochondrial fraction. The new supernatant was ultracentrifuged at 100,000 g for 60 min yielding the microsomal (pellet) and cytosolic fractions (supernatant). The nuclear fraction was prepared from the first pellet using the citric acid method reported by Reiners and Busch (1980).

Determination of Food-Entrainment Markers

Levels of the hepatic clock-protein Per-1 and serum corticosterone were quantified to assess synchronization with the time of food access during the protocol of food restriction (from 12:00 to 14:00 h).

Per-1 was assayed by Western blotting, according to Chilov et al. (2001). Total protein was measured using Folin phenol reagent (Lowry et al., 1951). Equal amounts of proteins were mixed with 2× Laemmli (electrophoresis) sample buffer (Bio-Rad) and incubated at 80°C for 10 min. The nuclear protein was separated on a 12% polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and then incubated overnight with primary N-terminus targeted antibody anti-PER1 (Santa Cruz, California, USA) at 1:500 dilution. Membranes were washed and incubated for 2 h with alkaline phosphatase (AP)-conjugated rabbit anti-goat secondary antibody at 1:5000 dilution (Santa Cruz, California, USA), and the bands were visualized using the AP conjugate substrate kit (Bio-Rad) according to the manufacturer's instructions.

Corticosterone was quantified by an ELISA method using a commercial kit (Assay Design, Michigan, USA), following the manufacturer's instructions. The intensity of the signal was inversely correlated with the amount of corticosterone in the sample.

Cytokines Determination

Circulating IL-1α, IL-6, and TNF-α were quantified by ELISA assays (BioSource Europe, Belgium) based on specific monoclonal antibodies according to the method of Khan et al. (2004).

Quantification of Acute-Phase Response Proteins

C-reactive protein (CRP) and fibrinogen were determined as pro-inflammatory state markers characteristic of a systemic APR. CRP was measured by an agglutination assay following the technique of Senju et al. (1986). The presence of fibrinogen was determined by an assay of clottable fibrinogen (prothrombin-time derived) according to the method of Rumley et al. (2003).

Immunochemical Detection of NF-κB in Liver

NF-κB in hepatocytes was quantified in liver slices. Active NF-κB was detected using a rabbit polyclonal antibody directed against the epitope mapping within the amino terminal domain of human NF-κB p65; the antibody also recognizes active rat NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, California, USA). Following a modification reported by Luna et al. (2008), the tissue was incubated for 20 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween, pH 6) at 95–100°C. Samples were washed with buffered saline, blocked with milk, and incubated overnight with anti NF-κB primary antibody, 1:200 dilution. The slides were then incubated for 1 h with rabbit anti-goat polyclonal antibody conjugated to Texas Red (Santa Cruz Biotechnology, Santa Cruz, California, USA), 1:250 dilution. Controls were prepared by omitting the primary antibody. The quantification of the fluorescent signal (with the Image Pro-Plus program) was done in two ways: one included the total fluorescence from at least 10 sections/liver slice, being analyzed 3–5 slices/rat, to calculate the fluctuations of NF-κB in a 24 h cycle; in the other, having detected a significant peak at 14:00 h in the rats under RFS, the number of nuclei with positive signal was counted in more than 100 hepatocytes (from 144 to 199) from different fields in liver slices. The data were compared with the percentage of nuclear NF-κB in animals fed ad libitum at the same time and with rats treated with the pro-inflammatory agent LPS (5 mg/kg i.p.) after 24 h.

Data Analysis

Data were classified for group and time and are presented as mean $\pm$ standard error of the mean (SEM). All graphs were drawn using the Sigmaplot curve-fitting program (Jandel Scientific). Data were compared using a two-way ANOVA for independent measures, with a factor for group (two levels) and a factor for time (eight levels). In order to determine significant time effects for each curve, a one-way ANOVA was performed for individual groups. The one- and two-way ANOVAs were followed by a Tukey post hoc test with significant values set at $p<0.05$.

Statistical analysis was performed with the program Statistica version 4.5 (StatSoft, Inc., Chicago, Illinois, USA).

RESULTS

Restricted Feeding Schedule and Corticosterone Levels

Figure 1 shows the fluctuations of hepatic Per1 and serum corticosterone over the 24 h period. As expected, rats fed ad libitum showed a clear peak in nuclear Per1 levels during the dark period, when the animals are awake and active (panels A & B, left side). In contrast, the group with restricted food access exhibited a similar elevation of this clock protein, but in the middle of the light period, corresponding to the FAA (11:00 h) and after food intake (14:00 h) (panels A & B, right side).

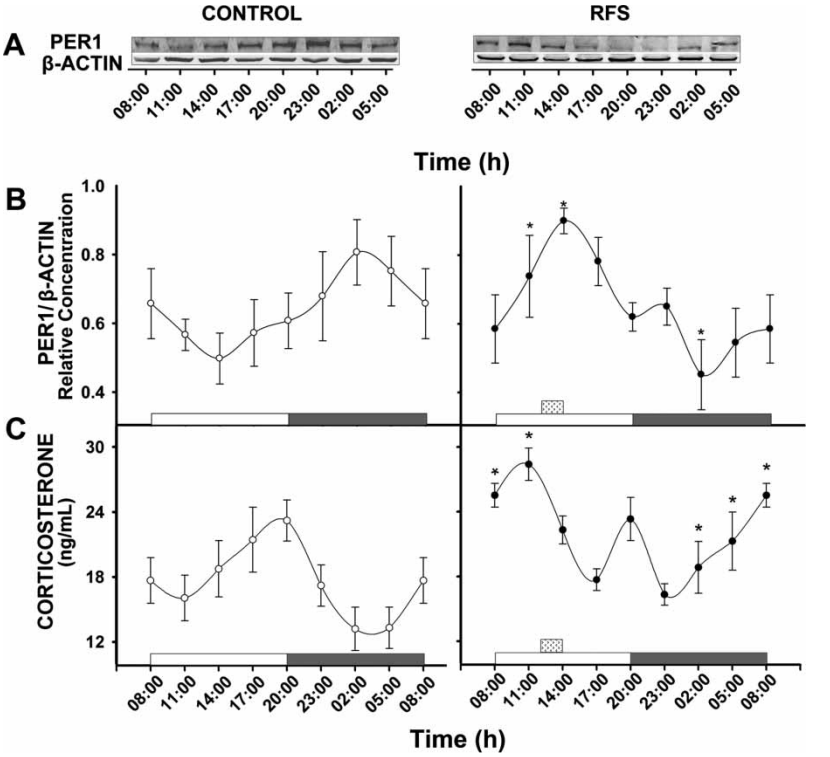

FIGURE 1 Levels of liver Per1 and circulating corticosterone during the expression of the food-entrainable oscillator. Panel A: Western Blot of 24 h cycle for Per1 and β-actin in rats fed ad libitum (control) and under restricted feeding schedules (RFS). Panel B: 24 h cycle of the ratio Per1/β-actin. Panel C: blood levels of corticosterone. Open symbols, control group fed ad libitum; filled symbols group under RFS. Lights-on at 08:00 h and off at 20:00 h. Box with arrowheads indicates mealtime (12:00–14:00 h). Data are expressed as mean $\pm$ SEM of at least eight independent observations. *Timepoints with a significant difference between control and RFS groups (Tukey post hoc test, $p < 0.05$).

TABLE 1 Comparison between groups fed ad libitum (control) and under restricted feeding schedules (RFS) of average values and the ratio of data corresponding to the light and dark periods

Parameters	Control mean	% Change light/dark	RFS mean	% Change light/dark
Corticosterone (ng/mL)	17.58	↑ 10	21.71	↑ 17
Per1 (relative concentration)	0.64	↓ 20	0.66	↑ 32
IL-6 (pg/mL)	94.52	↑ 56	101.66	↑ 5
IL-1α (pg/mL)	134.34	↑ 46	111.97	↓ 6
TNF-α (pg/mL)	16.56	↑ 3	14.82	↑ 48
Fibrinogen (mg/dL)	139.03	↓ 33	118.04	↑ 55
NF-kB (arbitrary units)	1.23	↑ 58	1.64	↑ 135

Averages were taken from data in Figures 1, 3, and 4. Up arrow indicates increase and down arrow decrease in the percentage of change between values of the light and dark periods.

These data indicate a clear phase shift of Per1 protein promoted by the RFS (see Table 1). Similarly, the control group fed ad libitum showed a significant peak of circulating corticosterone ($\sim 23 \pm 2$ ng/ml), corresponding to the time of the transition from the light to the dark period (at 20:00 h) (panel C, left side) and corroborating the increase of this glucocorticoid prior to awakening in nocturnal animals. In contrast, the RFS promoted a marked enhancement in this hormone ($\sim 29 \pm 2$ ng/ml) before food access (during FAA, 11:00 h). In this group, it was still possible to detect an increase in blood corticosterone at 20:00 h, similar to that observed in the rats fed ad libitum (panel C, right side). It is noteworthy that the blood levels of corticosterone in rats with restricted food access were higher during the 24 h cycle ($\sim$23%) in comparison with the control group fed ad libitum (see Table 1). These findings are in agreement with previous results (Díaz-Muñoz et al., 2000; Stokkan et al., 2001) and confirm the efficacy of our experimental protocol to induce the physiological responses promoted by the restricted food access.

Acute-Phase Response Parameters

As expected, treatment with LPS promoted a clear systemic APR evidenced by the significant increase in the level of circulating pro-inflammatory cytokines and acute-phase proteins (APPs) (see Figure 2). Indeed, the LPS-treated group showed a several-fold elevation in the blood levels of IL-1α (panel A), IL-6 (panel B), and TNFα (panel C) (2, 75, and 15 times, respectively). Further support that LPS induces the development of a pro-inflammatory state was the detection of circulating CRP in these rats, but not in any of the control or other experimental groups (panel D). Another APP, fibrinogen, was also increased by LPS, but to a much lesser extent (panel E). When the substantial changes associated with the

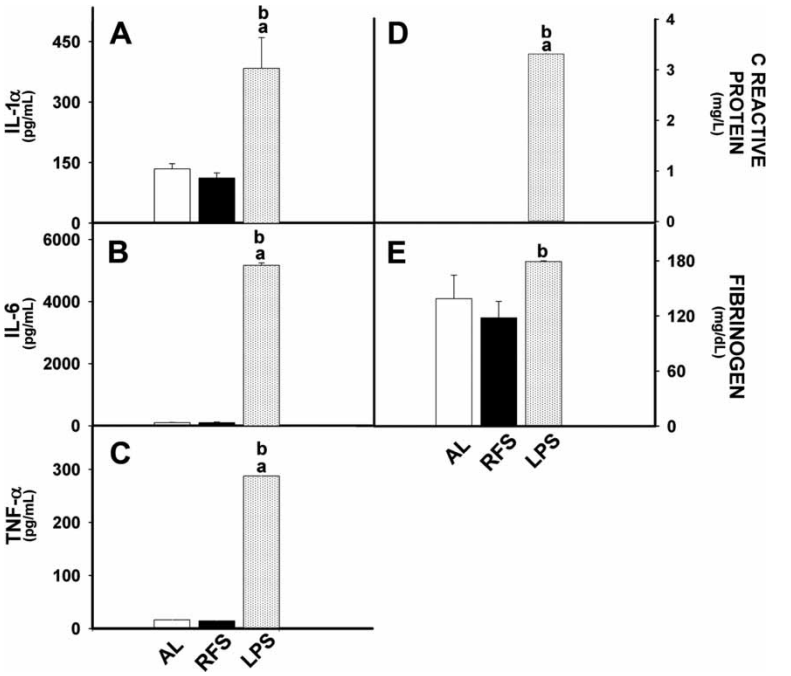

FIGURE 2 Comparison between lipopolysaccharide treatment and the expression of the food-entrainable oscillator on circulating cytokines, C-reactive protein, and fibrinogen. Panel A: interleukin 1α; panel B: interleukin 6; panel C: tumor necrosis factor α; panel D: C-reactive protein; panel E: fibrinogen. All data from the 24 h cycle of the groups fed ad libitum and under RFS were averaged and combined in a single bar. White bars, group fed ad libitum (AL); black bars, group under RFS; dotted bars, group treated with LPS (7 mg/kg for cytokines and 5 mg/kg for C-reactive protein and fibrinogen). Data are expressed as mean $\pm$ SEM of at least eight independent observations. [a]Significant difference for control vs. LPS; [b]RFS vs. LPS (Student's t-test, $p < 0.05$).

LPS-induced APR were compared with the moderate levels of cytokines and APPs in ad libitum fed and food-restricted rats, it was apparent that FEO expression does not involve a systemic APR-like response. The variations of IL-1α, IL-6, TNFα, and fibrinogen showed by the control group fed ad libitum and the group with restricted food access were further analyzed by chronobiological criteria.

24 h Rhythmicity of Circulating Cytokines and Fibrinogen during the Restricted Feeding Schedule

Although cytokines and APPs are usually associated with inflammatory processes, they also exhibit non-pathological roles, acting as physiological regulators of endocrine responses. Indeed, it has been reported that IL-1 and TNFα participate in the modulation of insulin levels as well as in the consolidation of non-rapid eye movement sleep (Krueger et al., 2001; Matsuki et al., 2003). Our results confirmed that IL-1α, IL-6, TNFα, and fibrinogen fluctuated in diurnal patterns in control rats fed ad

libitum, as previously reported by other groups (Hayashi et al., 2007; Sakao et al., 2003) (see Figure 3, left panels). The three cytokines showed complex rhythms, with peaks in the light and dark periods. Higher levels of IL-1α and IL-6 were observed in the light period (see Table 1), whereas TNFα displayed a clear peak at 11:00 h. The diurnal rhythms of these cytokines were markedly modified by the RFS (see Figure 3, right panels): the ultradian pattern was reinforced, as one additional peak appeared in the three variables (two peaks in the light period for IL-1α and IL-6, and two peaks in the dark period for TNFα); the proportion of IL-1α and IL-6 in the light and dark periods became similar; and the levels of TNFα were higher during the light period (see Table 1).

The diurnal variation in blood fibrinogen level is shown at the bottom of Figure 3. As reported (Sakao et al., 2003), control rats fed ad libitum exhibited a 24 h rhythm with a minimum at 08:00 h and maximum at 23:00 h, with the rest of the timepoints showing a similar intermediate value (left panel). In contrast, rats with restricted access to food showed a different rhythmicity in blood fibrinogen level: the peak disappeared, and the trough was observed during the dark period, at 23:00 h, at the same time when the control group showed the peak in fibrinogen (right panel). Consequently, fibrinogen levels became higher during the light period in the rats under RFS, in contrast to the control group fed ad libitum (see Table 1).

Taken together, these results demonstrate that the 24 h rhythms of IL-1α, IL-6, TNFα, and fibrinogen are entrained by RFS.

24 h Rhythmicity and Response to LPS-Treatment of Liver NF-κB

NF-κB is a key transcription factor that regulates the genes responsible for innate and adaptive immune responses, but it also acts as a major cellular coordinator in tissues that react to stressful metabolic conditions (Brasier, 2006). We measured the levels of NF-κB in the livers of rats fed ad libitum and under RFS to evaluate a possible state of local biochemical stress. Figure 4 (panel A) shows the 24 h variation of the liver NF-κB immunohistochemical signal. In rats fed ad libitum, the NF-κB signal is high mainly during the dark phase, when the rodents are awake and active (from 02:00 to 08:00 h). During the light (rest) phase, the NF-κB signal declines gradually, showing minima at 20:00 and 23:00 h (top of panel A). These results confirm previous reports showing temporal fluctuations of NF-κB levels in several tissues, including the liver (Sillitoe et al., 2007). Compared with the hepatic NF-κB levels after LPS treatment, control rats fed ad libitum had lower signal within the nuclei of the hepatocytes, indicating a transcriptionally active form of NF-κB ($\sim$50% of the LPS-treated rats, panel B and image 1 and 3 in panel C). In contrast,

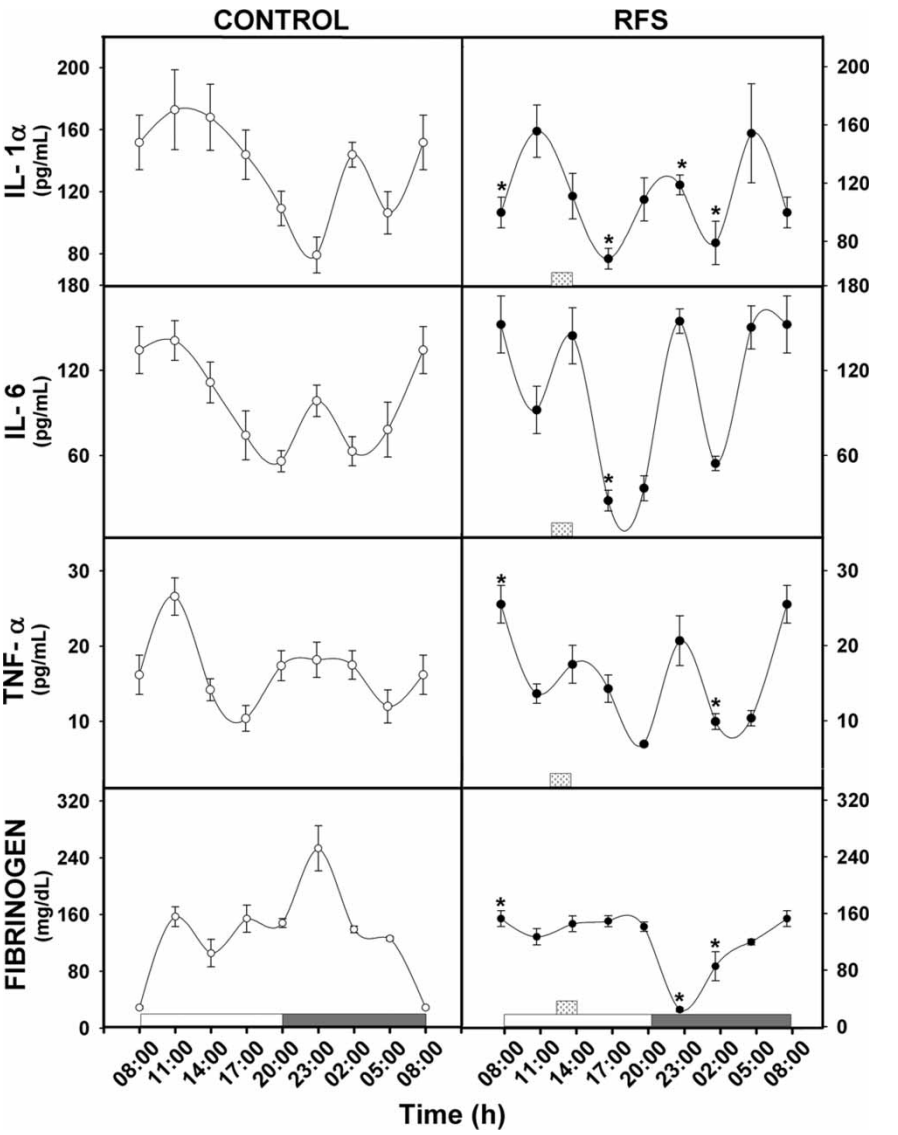

FIGURE 3 24 h rhythmicity of circulating cytokines and fibrinogen during the expression of the food-entrainable oscillator. First panel, interleukin 1α; second panel, interleukin 6; third panel, tumor necrosis factor α; fourth panel, fibrinogen. Open symbols, group fed ad libitum; filled symbols, group under RFS. Lights-on at 08:00 h; lights-off at 20:00 h. Box with arrowheads indicates mealtime (12:00–14:00 h). Data are expressed as mean $\pm$ SEM of at least eight independent observations. *Timepoints with a significant difference between control and RFS group (Tukey post hoc test, $p < 0.05$).

significant changes in the 24 h rhythmicity of liver NF-κB were noticed in the rats with restricted feeding (bottom of panel A). A clear peak was detected at 14:00 h, after the intense 2 h period of food access. The NF-κB signal depicted during this peak was significantly greater ($\sim$70%) than the peak value showed by the group fed ad libitum. The NF-κB

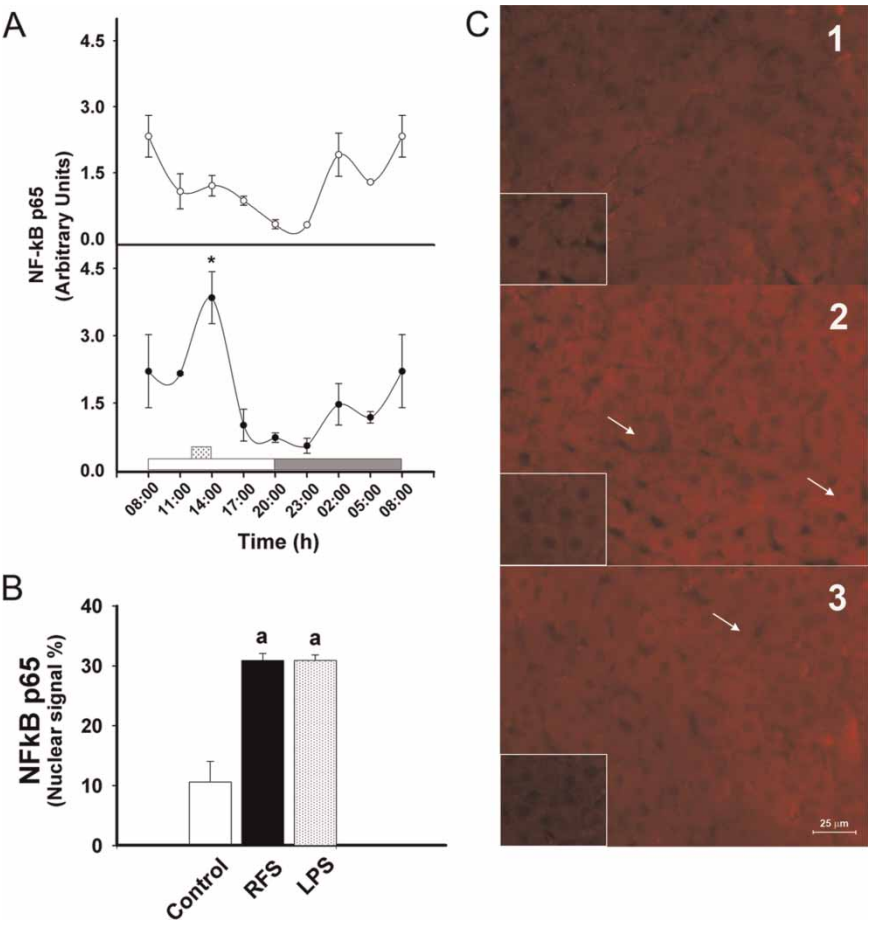

FIGURE 4 24 h rhythmicity of NF-κB p65 in liver and quantification of its nuclear presence during the expression of the food-entrainable oscillator. Panel A: 24 h cycle of immunohistochemical signal of liver NF-κB p65 factor; group fed ad libitum at top with open symbols; group under RFS at bottom with filled symbols. Lights-on at 08:00 h; lights-off at 20:00 h. Box with arrowheads indicates mealtime (12:00–14:00 h). Data are expressed as mean ± SEM of at least eight independent observations. *Timepoints with a significant difference between control and RFS group (Tukey post hoc test, $p < 0.05$). Panel B: quantification of NF-κB p65 signal within the nuclei of hepatocytes. Nuclei with positive signal were counted in 10 fields chosen at random. The results are expressed as percentage (control, 14/141; RFS, 61/199, and LPS 58/188). White bar, group fed ad libitum (control); black bar, group under RFS; dotted bar, group treated with LPS (5 mg/kg and sacrificed after 24 h). The experiment was done at 14:00 h (i.e., the time corresponding to the peak in NF-κB p65 signal detected in rats under RFS, see panel A). Panel C: representative images of six independent experimental observations. Experiments are those quantified in panel B. Insets are images obtained in absence of the antibody against NF-κB. Image 1, control fed ad libitum; image 2, RFS; image 3, LPS. White arrows indicate nuclei with positive NF-κB p65 signal.

signal declined markedly at 17:00 h, and remained low until 23:00 h. At 02:00 h, the level of NF-κB gradually increased in a pattern similar to that seen in the control group fed ad libitum. Another difference shown by the rats with restricted food was that at 11:00 h (during the FAA), the

signal continued to be high, prior to the peak at 14:00 h. On average, the NF-κB signal in the rats under RFS was 33% higher than that of the control group of rats fed ad libitum (see Table 1). It can be seen in panel C that the NF-κB immunohistochemical signal at the 14:00 h peak in the liver of the rats with restriction to food access (image 2) was similar in magnitude to the signal elicited by the LPS treatment (image 3). Consistently, the percentage of positive nuclei in both conditions was similar (RFS and LPS in panel B). Hence, the data suggest a possible state of metabolic stress in the liver during the processing of the large amount of food ingested in the daily 2 h period of food access.

Feeding-Condition Effects on Circulating Cytokines and Fibrinogen and Liver NF-κB

Besides the influence of the timing system, the experimental protocol of RFS involves successive episodes of 22 h of fasting with 2 h of intense and abundant feeding. To explore the effect of the feeding condition, we compared the blood levels of IL-1α, IL-6, TNFα, and fibrinogen (see Figure 5, panels A to D), as well as the liver NF-κB signal (see Figure 5, panel E) of rats under RFS before and after food access (at 11:00 and 14:00 h), with those of rats exposed exclusively to a single cycle of 22 h fasting and 2 h of refeeding. Data from control groups confirmed earlier reports establishing that a ∼24 h fasting period is sufficient to increase the levels of blood cytokines and fibrinogen. Refeeding after fasting reduced the circulating levels of these parameters (whitish bars) (McMillan et al., 1996; Nishio et al., 2003. The fasting effect was more evident with IL-6 (panel B), followed by IL-1α (panel A), and fibrinogen (panel D), and only modest with TNFα (panel C). In contrast, rats under RFS did not show any significant change in the levels of circulating IL-1α, IL-6, TNFα, or fibrinogen, comparing the times before (11:00 h) and after food access (14:00 h) (dark bars). Overall, the levels of circulating cytokines in the rats with restricted food access were more similar to the refeeding condition at the two times tested. Fibrinogen was the exception: in this case, restricted feeding promoted a notorious increase (∼4 times over control groups), without effect by the fasting and refeeding condition (dark bars in panel D). The hepatic NF-κB signal showed a different response to the feeding condition: simple fasting and refeeding did not affect it (whitish bars in panel E), but the restricted feeding promoted a significant enhancement of the NF-κB signal after feeding (dark bars in panel E). Interestingly, when the groups in fasting (simple fasting and restricted feeding at 11:00 h) were compared, the NF-κB level was still higher in the rats under RFS. Hence, restricted feeding is associated with an increased NF-κB signal in the liver at the two times studied, but it is still dependent on the feeding condition.

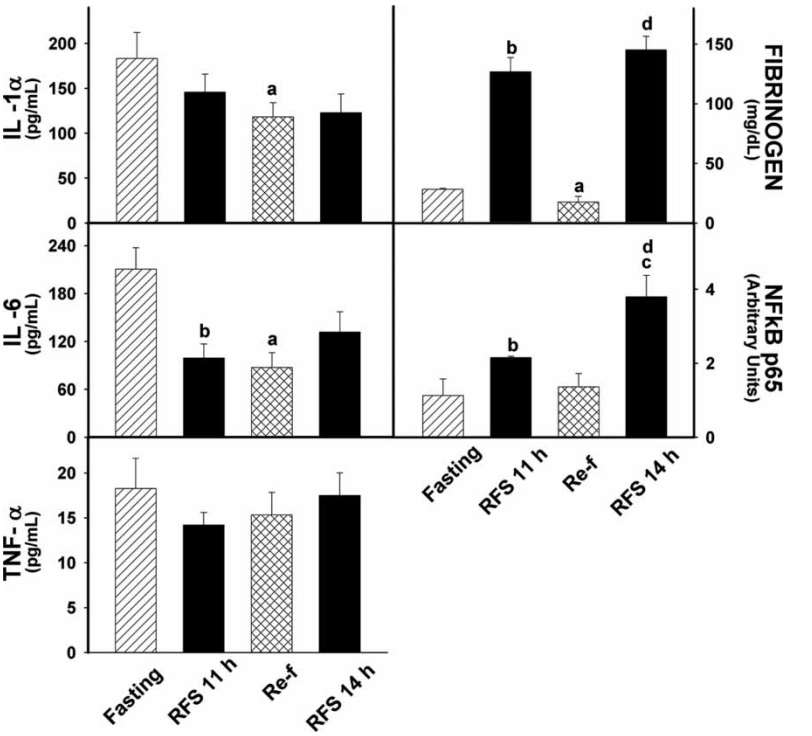

FIGURE 5 Effect of fasting and re-feeding on circulating cytokines and fibrinogen, as well as liver NF-κB p65 during restricted feeding schedules. Comparison between simple 21 h fasting (fasting, white bars with diagonal pattern), simple 21 h fasting and 2 h refeeding (Re-f, white bars with crossing pattern), RFS before food access (RFS 11, black bars), and RFS after mealtime (RFS 14, black bars). Data are expressed as mean ± SEM of at least eight independent observations. [a]Significant difference for fasting vs. refed; [b]significant difference for fasting vs. RFS 11 h; [c]significant difference for RFS 11 h vs. RFS 14 h; [d]significant difference for refed vs. FRS 14 h (Tukey post hoc test, $p < 0.05$).

DISCUSSION

Our results clearly indicate that restricted food access schedules did not promote a systemic APR. However, RFS was associated with changes in the 24 h rhythmic variations of pro-inflammatory cytokines and fibrinogen, an accepted acute-phase protein (APP). At the same time, elevation of the NF-κB factor suggested a probable local stress-response in the liver as part of the metabolic and physiological adaptations associated with a protocol of daily 2 h restricted feeding.

Is There a Stressful Condition Associated with Restricted Feeding Schedule?

The interest in studying the relationship between restricted feeding and the stress-response was based on previous studies highly suggestive

that an organism under food restriction could be in a metabolically and physiologically challenging condition—daily 2 h access to food. The successive eating episodes during the RFS (Martínez-Merlos et al., 2004), in which a large amount of food is eaten very quickly, leads to stomach distention, as the food needed in 24 h must be eaten in just a couple of hours. In general, eating disorders that are accompanied by severe gastric distention, loss of adipose tissue, and metabolic stress in nutrient handling have been related to an activation of the neuroendocrine system for stress-response, as well as to a possible deregulation of the balance between anti- and pro-inflammatory cytokines (Corcos et al., 2003; Lo Sauro et al., 2008). Previously, Stephan (1997) reported that stomach distention is not sufficient to act as a zeitgeber for the FEO, and that the caloric content of the food ingested is more important. Hence, the hyperphagia and associated stomach distention might participate in a stress-response that could influence several physiological responses, but independently of the physiological adaptations associated with the RFS.

Altered Corticosterone Levels

Glucocorticoids are endocrine messengers that play an important role in the stress-response centered in the hypothalamic-pituitary-adrenal axis. These steroidal hormones are also part of the fasting response, modulating important metabolic pathways such as gluconeogenesis and urea synthesis. They are synthesized and secreted as a primary response to metabolically demanding stimuli, acting as protective agents against stress and also as anti-inflammatory factors (Tsigos & Chrousos, 2002). The average level of corticosterone in the blood of rats expressing the FEO during the 24 h period of this study was increased >20% in comparison to the control group fed ad libitum (see Figure 1, panel C). Elevated levels of circulating corticosterone are associated with chronic stress and with the occupation of a larger fraction of active glucocorticoid receptors (Karten et al., 1999). It has been postulated that altered glucocorticoid signaling could be one of the events that precipitates the establishment of pro-inflammatory ailments, such as metabolic syndrome-like conditions, including insulin resistance, obesity, and APR (Black, 2006; Brindley, 1995).

In spite of this background, our experimental protocol of limited access to food involved neither a generalized stress-response nor a systemic APR similar to the one induced by LPS treatment, suggesting that organisms under RFS can avoid the stressful stimuli and assume a distinctive physiological status that could be considered a rheostatic adaptation. A rheostatic response is one in which the system adopts a new functional state that is different from a simple on/off response. This new functional state is reached by a gradual set of changes in the set-point

of key physiological variables (Rossi et al., 2000). Other examples have been postulated to illustrate the rheostatic characteristics associated with food restriction: the characteristic patterns of hepatic mitochondrial activities and pro-oxidant reactions in rats under restricted feeding in comparison with those observed in control groups fed ad libitum or with a simple 24 h-fasting period (Báez-Ruiz et al., 2000; Luna-Moreno et al., 2006).

Is There a Stress-Response in the Liver during the Food Anticipatory Activity?

The liver is one of the most important organs for nutrient processing, and under RFS it undergoes important metabolic adaptations (Báez-Ruiz et al., 2000; Díaz-Muñoz et al., 2000). Previously, we reported local adjustments in the liver relating to thyroid status in rats under RFS: no changes were found in the thyroid hormones of T4 and T3 in blood, but a hypothyroid-like state was detected in the liver before food presentation (11:00 h), whereas a hyperthyroid-like state was identified after food intake (14:00 h) (Aceves et al., 2003). Our data showing no changes in systemic cytokines, but an increased NF-κB signal in the liver of rats with restricted feeding after food intake (see Figure 4, panels A–C), suggest that the liver could be mounting a metabolic response to a local stressor. NF-κB is a pleiotropic transcription factor that regulates or influences several physiological processes (cellular proliferation, apoptosis, and calcium homeostasis) and pathological conditions (inflammation, cancer, and some inherited diseases) (Yates & Górecki, 2006). The hepatic activation of NF-κB under RFS is supported by the finding that the augmented level of NF-κB and number of positive nuclei in the hepatocytes at the 14:00 h peak were very similar to the values detected during the LPS treatment (see Figure 4, panels B & C). It is not clear what factor(s) could be promoting the putative stress-response in the liver after the food intake in the rats under RFS, but some possibilities are: diminution in ATP levels and shift in cytoplasmic and mitochondrial redox states (Díaz-Muñoz et al., 2000); augmented pro-oxidant reactions in the microsomal fraction (Luna-Moreno et al., 2006); and changes in hepatic hemodynamic properties exacerbated by the hyperphagia (Madsen et al., 2006).

Another finding of these experiments was the detection of a 24 h rhythm in the liver NF-κB-associated signal in the control group fed ad libitum (see Figure 4, panel A). The signal was higher during the dark period when the animals were active and feeding. At the beginning of the light period, the signal gradually decreased, reaching a minimum at the transition from the light to dark period. The pattern in the group under RFS was similar, except for the large increase in the NF-κB signal

observed after feeding (14:00 h). However, rats under RFS showed a ~40% greater NF-κB signal during the 24 h cycle. These findings support the notion advanced by others (Marpegan et al., 2004; Mendoza, 2007) that NF-κB and clock genes are related, suggesting that the NF-κB signaling cascade could be regulated by central as well as peripheral oscillators. They also suggest that restricted feeding could influence the relationship between NF-κB and clock genes in the liver.

Diurnal Variations and Feeding Regulation of Cytokines, Fibrinogen, and NF-κB during Restricted Feeding Schedules

Cytokines are well-recognized mediators of pro-inflammatory states. However, IL-1α, IL-6, and TNF-α also play a "non-pathological" role regulating various biological processes, such as sleep and food intake (Krueger et al., 2001; Plata-Salamán, 2001). In addition, a bi-directional communication between the neuroimmunological system and circadian clock has been postulated (Coogan & Wyse, 2008). Hence, the signaling associated with cytokines can be considered to be part of the organism's timing system. Several reports exist about circadian variations of IL-1α, IL-6, TNF-α, and fibrinogen in different tissues and species. Most of these rhythms show ultradian variations that fluctuate inversely with those of glucocorticoids (Petrovsky & Harrison, 1998). Our data showed complex rhythms for the cytokines studied, with more than one peak for both the control and RFS groups (see Figure 3). However, some differences in the 24 h patterns of circulating cytokines were detected in rats expressing the FEO:

- there were three peaks, whereas rats fed ad libitum showed only two peaks;
- no differences between dark and light periods were observed in the levels of IL-1α and IL-6, whereas the control group showed increased levels of both cytokines during the light period (see Table 1);
- levels of TNF-α were increased in the light period, while control rats showed TNF-α levels to be equal in both periods (see Table 1); and
- the last point could imply that the somnogenic role suggested for cytokines (Marshall & Born, 2002) might be altered in rats expressing the FEO.

We did not identify the cellular sources responsible for the circulating cytokines quantified in this study. Although endo/epithelial cells and resident macrophages (many near the interface with the external environment) are well-known potent producers of IL-1α, IL-6, and TNF-α, the situation is quite complex, as virtually all nucleated cells can also synthesize these cytokines. Independent of these considerations, our data strongly

indicate that the equilibrium between cytokine release to and removal from blood is modified during the expression of the FEO.

Fibrinogen is an APR protein produced by the liver, but it is also a precursor of the coagulation cascade. A two-peaked rhythm, dependent on the timing system, was reported for the fibrinogen gene cluster (Sakao et al., 2003). Our experiments also found a complex rhythm for circulating fibrinogen in control rats fed ad libitum that was greatly modified by the RFS (see Figure 3). Certainly the expression of the FEO did not increase fibrinogen levels, confirming that a generalized inflammatory response was not mounted. As a matter of fact, the FEO decreased the average circulating fibrinogen level by ∼15% compared to the control group (see Table 1). Unlike the phase shift observed in the corticosterone and Per1 rhythms (see Figure 1, panels A and B), the diurnal variations of fibrinogen were changed by the RFS: the peak in the group fed ad libitum was not observed, and the trough time changed from the start of light period (08:00 h) to the middle of the dark period (23:00 h). These findings imply that the FEO can modulate the timing of hepatic fibrinogen production, perhaps by regulating the transcriptional feedback of clock genes in the liver.

It is well established that cytokines decrease food intake, an effect associated with the cachexia-anorexia syndrome seen in some chronic diseases (Buchanan & Johnson, 2007). It has also been reported that acute and repetitive fasting promotes the elevation of some cytokines (Nishio et al., 2002). Three of the parameters studied in these experiments (IL-1α, IL-6, and fibrinogen) showed a significant elevation during fasting in comparison to the refeeding state in rats with one cycle of fasting-refeeding (the exceptions were TNF-α and hepatic NF-κB). RFS severely modified this response: no effect of fasting-refeeding was observed in the IL-1α, IL-6, and fibrinogen blood levels (see Figure 5). In the case of liver NF-κB, refeeding had an opposite effect. A significant enhancement in the signal was observed in response to food intake after 22 h of fasting. All these data support the notion that the responses to the RFS protocol (successive alternation of 2 h food access and 22 h of forced starvation during three weeks) are clearly different from those present during a simple fasting-refeeding cycle, suggesting a rheostatic adaptation.

Restricted Feeding Schedule and Peripheral Organs

Although molecular rhythms in peripheral organs, especially digestive tissues, are very sensitive to entrainment by food, the system able to measure time under RFS is probably localized in the brain (Davidson, 2006). However, characterization of the metabolic and physiological events that take place in organs outside the nervous system is fundamental

to understanding the extensive network of chronobiological adaptations underlying the physiological responses associated with food restriction.

It is apparent from the comparison between the 24 h rhythms of corticosterone and cytokines in control and experimental groups that under our RFS protocol, the light-entrained oscillator (LEO) in the SCN continues to function (see Figures 1 and 3). Especially in the daily corticosterone fluctuations, the pattern of the rats under RFS showed two peaks: one corresponding to the time of food presentation and the other corresponding to the circadian time prior to nocturnal activity. The most likely explanation for this observation is that peripheral organ rhythmicity in coincident LD periods and RFS are influenced not only by the LEO.

RFS involves successive episodes of 22 h of fasting with 2 h of food access for at least 21 days. This protocol could be considered as semi-chronic or chronic. The comparison done in Figure 5 involving rats subjected to an acute/single episode of fasting-refeeding served as a control for the feeding condition. This is pertinent because parameters such as the activation of FOS in brain stem nuclei respond mainly to food ingestion (with the exception of the nucleus of the solitary tract and parabrachial nucleus), independent of the RFS protocol (Ángeles-Castellanos et al., 2005).

In conclusion, the present results provide evidence that blood cytokines, such as IL-1α, IL-6, and TNF-α, as well as the APR protein fibrinogen change their diurnal variation in animals under RFS. Moreover, the stress-response marker NF-κB in the liver of these animals exhibited a significant increase at mealtime. None of the data supported the establishment of a systemic pro-inflammatory response reminiscent of an APR-like condition.

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Daytime restricted feeding modifies 24-h rhythmicity and subcellular distribution of liver glucocorticoid receptor and the urea cycle in rat liver

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ABSTRACT

The timing system in mammals is formed by a set of peripheral biological clocks coordinated by a light-entrainable pacemaker located in the suprachiasmatic nucleus. Daytime restricted feeding (DRF) modifies the circadian control and uncouples the light-dependent physiological rhythmicity, food access becoming the principal external time cue. In these conditions, an alternative biological clock is expressed, the food entrainable oscillator (FEO). Glucocorticoid hormones are an important part of the humoral mechanisms in the daily synchronization of the metabolic response of peripheral oscillators by the timing system. A peak of circulating corticosterone has been reported prior to food access in DRF protocols. In the present study we explored in the liver the 24 h-variations of: 1) the subcellular distribution of glucocorticoid receptor (GCR), 2) the activities of the corticosterone-forming and NADPH-generating enzymes (11 β–hydroxysteroid dehydrogenase type 1, and hexose-6-phosphate dehydrogenase, respectively), and 3) parameters related with the urea cycle (circulating urea and activities of carbamoyl phosphate synthetase and ornithine transcarbamylase) elicited by DRF. The results showed that DRF promoted an increase of more than 2 times of the hepatic GCR, but exclusively in the cytosolic compartment, since the GCR in the nuclear fraction showed a reduction. No changes were observed in the activities of 11 β-HSD 1 and H6PDH, but the rhythmicity of all of the urea cycle-related parameters were modified.

INTRODUCTION

The timing system in mammals is assembled by a set of coordinated oscillators: a pacemaker in the suprachiasmatic nucleus (SCN) and a variety of clocks localized in several brain areas and peripheral organs [(1)]. Light entrains SCN via the retino-hypothalamic tract, and then its rhythmicity is communicated to the rest of the organism by means of neural and blood-borne signals [(2)]. Hence, light coordinates most of the physiological responses by sustaining the daily cycles of rest–activity, fasting–feeding, and hormonal secretion [(3)]. In this context, daily glucocorticoid secretion in most mammals occurs at the end of the resting period as metabolic preparation for wakefulness, orchestrating the fasting response and promoting the search for food [(4)].

Daytime restricted feeding in nocturnal animals uncouples the SCN from the peripheral oscillators, turning feeding as the principal synchronizer. The daily expression of molecular clock genes from peripheral organs (liver, pancreas, kidney and lung), shift their phases in response to the imposed meal time [(5)]. This protocol does not affect the circadian rhythmicity of the SCN [(6)]. Restricted feeding is accompanied by metabolic and physiological adaptations in the peripheral organs, including the onset of an arousal behavior previous to food access known as the food anticipatory activity (FAA) [(7)]. Because these changes remain even in the absence of a functional SCN, the existence of an alternative oscillator known as food entrained oscillator (FEO) has been postulated [(8, 9)]. The peak of serum glucocorticoid is coincident with the FAA during restricted feeding at daytime in rats [(10, 11)], and is independent of the SCN activity [(9)].

Glucocorticoid secretion is under circadian modulation, and relies on an ACTH-dependent process. However, an alternative pathway involving the autonomic nervous system has also been reported [(12)]. Cellular signaling of these hormones involves ubiquitous genomic receptors (with the exception of the adult SCN) known as NR3C1, as well as membrane-located receptors associated to second messenger systems [(13)]. Glucocorticoid action is associated to the induction of metabolic pathways characteristic of fasting, such as gluconeogenesis and the urea cycle, and is dependent on the combination of a variety of co-activators and co-repressors [(14)].

Another point of regulation of glucocorticoid signaling is the intracellular conversion between the active hormone corticosterone and the inactive metabolite cortisone by the activity of the enzyme 11 β hydroxysteroid dehydrogenase (11 β-HSD). In the liver, the 11 β-HSD type 1 catalyzes the NADPH-dependent reduction of

cortisone to form corticosterone. The NADPH is supplied by the microsomal enzyme hexose 6 phosphate dehydrogenase (H6PD) [15].

Glucocorticoids are candidates to link the SCN signaling to the rhythmicity of the peripheral oscillators, based on the ability of dexamethasone to induce circadian gene expression in cultured rat-1 fibroblasts, and transiently change the phase of gene expression in liver, kidney, and heart [16]. It was also postulated that glucocorticoid response in peripheral oscillators made slower the daytime food-induced phase-shifting in liver and kidney [17]. Reddy et al. reported that glucocorticoids entrain nearly 60% of the liver circadian transcriptome [18]. This action is accomplished by acting directly in the glucocorticoid response elements (GRE) of target genes, or indirectly by regulating elements of the molecular clock. Thus, a positive GRE element appears to mediate *per1* induction by glucocorticoids [19], whereas the promoter of *rev-erbα* has been proposed to contain a negative GRE that mediates glucocorticoid induced repression [20].

Glucocorticoid are not the exclusive synchronizer of peripheral tissues since hepatocytes of mice lacking GCR showed no alteration in circadian gene expression [17]. However, it is highly plausible that glucocorticoids play a strategic role in the coordination between SCN and peripheral clocks, and in the interplay linking metabolism and circadian oscillators. Nevertheless, it remains to be determined the adaptations elicited by daytime restricted feeding in the status of the intracellular GCR, the equilibrium between corticosterone and cortisone, and the activity of the urea cycle.

Hence, in order to gain more understanding regarding the physiological adjustments in the liver of rats under restricted feeding schedules and expressing the FEO, the aim of this project was to evaluate the 24 h rhythmicity of the cytoplasmic and nuclear localization of the glucocorticoid receptor, the enzymatic activity and presence of the 11 β-HSD type 1 isoform, and the production of urea, including the activity of 2 enzymes of the urea cycle: carbamoyl phosphate synthetase-1 (CPS1) and ornithine transcarbamylase (OTC) in the liver.

EXPERIMENTAL METHODS

Animals and Housing. Adult male Wistar rats weighing 120-150 g at the beginning of the experiment were maintained in a 12:12 h light-dark cycle (lights on at 08:00 h) and constant temperature (22 ± 1 °C). The light intensity at the surface of the cages averaged 350 lux. Animals were kept in groups of 5 in transparent acrylic cages

(40 X 50 X 20 cm) with free access to water and food unless stated otherwise. All experimental procedures were approved and conducted according to the institutional guide for care and use of animal under biomedical experimentation (Universidad Nacional Autónoma de México).

Experimental Design. Control and experimental groups were similar to those reported by Luna-Moreno et al. [11]. To determine daily and food-entrained rhythmicity, rats were randomly assigned to one of the following feeding conditions for 3 weeks:

1. Control animals fed *ad-libitum* with free access to food and water throughout the 24-h period; and
2. Experimental group with restricted feeding, in which food availability was limited manually to 2 h daily, from 12:00 to 14:00 h.

At the end of the feeding protocol, different subgroups of animals were sacrificed at 3-h intervals over a 24-h period, starting at 08:00 h.

In addition, to establish a comparison of the fasting and subsequent re-feeding response in the RF group, 2 additional control groups were included:

1. Animals fed *ad-libitum* were maintained with free food access for 3 weeks; on the last day, food was removed at 14:00 h, and they were sacrificed (at 11:00 h) after 21 and 45 h (~1 and 2 days) of deprivation.
2. A second group of rats was similarly deprived of food for 21 and 45 h and then re-fed for 2 h (from 12:00 to 14:00) sacrificed at 14:00 h before tissue sampling.

Blood and Liver Sampling. Rats were sacrificed and then beheaded for trunk blood collection. Blood was collected and centrifuged at 5,000 rpm for 5 min to obtain serum. A liver section (~5 g) was processed for homogenate and subcellular fractionation (nucleus, mitochondria, microsomes and cytosol). The subcellular fractionation was done according to the report of Aguilar-Delfín et al. [21]. Briefly, liver was homogenized in 10 mM Tris-HCl (pH 7.4; 1:10 w/v), the homogenate was centrifuged at 1,500 g for 15 min, and the pellet was kept for further isolation of the nuclear fraction. The supernatant was centrifuged at 10,000 g for 20 min to sediment the mitochondrial fraction. The new supernatant was ultracentrifuged at 100,000 g for 60 min yielding the microsomal (pellet) and the cytosolic fractions (supernatant). The nuclear fraction was prepared from the first pellet using the citric acid method reported by Reiners and Busch [22].

Western blotting for glucocorticoid receptor and 11 β-HSD type 1. GCR and 11 β-HSD type 1 were assayed by Western blotting according to procedure reported by

Chilov et al. [23]. Total protein was measured using the Folin phenol reagent [24]. Equal amounts of proteins were mixed with 2x Laemmli sample buffer (Bio-Rad) and incubated at 80 °C for 10 min. The homogenate, the nuclear and the cytosolic samples were separated on a 10 % polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and then incubated overnight with primary rabbit polyclonal anti-GCR antibody (Sc-1004, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 1:250 dilution. The microsomal protein was separated on a 10% polyacrylamide gel, electroblotted and incubated overnight with primary rabbit polyclonal anti *11 β-HSD type 1* antibody (Sc- 20175, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 1:500 dilution. Membranes were washed and incubated 2-h with alkaline phosphatase (AP)-conjugated secondary donkey anti-rabbit antibody at 1:5000 dilution (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), and bands were visualized using the AP conjugate substrate kit (Bio-Rad) according to the manufacturer´s instructions. β-Actin and tubulin were used as loading controls, the first for cytoplasmic and microsomal fractions, the second for nuclear fraction.

11 β-HSD type 1 Activity. This enzymatic activity was determined using the assay described by Thurston et al. [25]. 30 mg of microsomal protein were transferred to glass tubes, containing 700 µl of PBS (phosphate buffered saline). Blanks contained 100 µl BSA (bovine serum albumin) solution (1 mg/ml prepared in PBS). Each triplicate set of tubes was pre-incubated for 30 min at 37° C in a water bath. To initiate the assay, each tube received NADPH 4 mM (Sigma, USA) and 100 µl PBS containing 0.1 µCi [1,2-^{3}H] cortisone (Perkin Elmer, Rodgau-Jugesheim, Germany), and unlabelled cortisone (Sigma, USA) each to a final steroid concentration of 100 nM. The tubes were then returned to the water bath for 60 min, end the reactions were terminated by the addition of 2 ml ice-cold chloroform (J.T. Baker, Mexico) to each tube. To partition the organic and aqueous phases, these tubes were centrifuged at 1,000 g for 30 min. After aspirating the aqueous supernatant, the organic extracts were evaporated over night at room temperature. The steroid residues were re-suspended in 20 µl ethyl acetate containing either corticoesterone or cortisone 1 mM (Sigma) and resolved by thin layer chromatography (TLC), using Silica 60 TLC plates (Merck) in an atmosphere of 92:8 (v/v) chloroform:95% (v/v) ethanol (Merck). The spots corresponding to corticosterone were scraped and the [^{3}H]-corticosterone was quantified using a Bioscan 200 TLC

radiochromatogram scanner (LabLogic, Sheffield, UK). Protein concentration was measured using the Lowry method [24].

Hexose 6 Phosphate Dehydrogenase Activity. It was measured after disrupting the microsomal membranes by preincubation at 4 °C with RIPA buffer. The incubation mixture contained 0.3 mM glucosamine-6-phosphate, 100 mM glycine-NaOH buffer (pH 10), 1 mM NADP+, 1% BSA, in a total volume of 1 ml. Reactions were started by the addition of tissue homogenate or microsomes (~10 and 3 mg of protein, respectively) to 900 µl of the reaction mixture at room temperature. The increase in absorbance at 340 nm was monitored during the first 5 min of incubation using a spectrophotometer (Ultrospec 3,300; Pharmacia Biotech). The amount of NADPH produced was calculated using 6.22 as extinction coefficient. Specific activities were expressed as µmoles of NADPH formed per min per mg of protein [26].

Urea cycle parameters. Urea was determined by a standard enzymatic method (ELITech, France) in which urea is first cleaved by urease into CO_2 and NH_3. In a second step, the NH_3 reacts with phenol and hypochlorite under alkaline conditions to produce a blue compound that is determined colorimetrically at 340 nm. The carbamoyl phosphate synthetase-1 (CPS1) activity was determined according to the method reported by Pierson [27]. The reaction mixture consisted of NH_4HCO_3 5 mM, ATP 5 mM, magnesium acetate 10 mM, N-acetyl glutamate 5 mM, dithiothreitol 1 mM, triethanolamine 50 mM (pH 8.0) and 200 µg of mitochondrial protein in a final volume of 0.6 ml. The reaction was run 10 min at 37 °C and the carbamoyl phosphate was converted to hydroxy-urea by the addition of 30 µl of hydroxylamine 2 M, and incubated for 10 min at 95 °C. To quantify the hydroxy-urea, 2.4 ml of chromogenic reagent was added followed by an incubation of 15 min at 95 °C. After cooling the samples at room temperature, absorbance was measured at 458 nm in a spectrophotometer. The ornithine transcarbamylase (OTC) activity was measured following the technique reported by Bagrel et al. [28]. The reaction mixture consisted of 400 µl of ornithine 20 mM/urease solution and 100 µg of mitochondrial protein. The mixture was incubated at 37 ºC for 5 min and 400 µl of carbamoyl phosphate 50 mM solution was added, and incubated other 20 min. The reaction was stopped with 1 ml of trichloroacetic acid (100 g/l). Subsequently, the tubes were centrifuged at 5,000 rpm for 10 min, and 1 ml of the supernatant was added to diacetyl monoxime solution (100 mM) and 4 ml of phosphoferric-antipyrine reagent (antipyrine 65 mmol, antipyrine 12.5

mmol, $FeCl_3$ 12.5 mmol, 625 ml H_3PO_4 and 375 ml distilled water) to detect the citrulline formed by the action of the OTC. The mixture was incubated in a boiling water bath for 20 min followed by 10 min at room temperature. Finally, the color was read in a spectrophotometer at 460 nm.

Data Analysis. Data were grouped for experimental condition and time, and are presented as mean ± standard error of the mean (S.E.M.). They were compared with a two-way ANOVA for independent measures with a factor for group (two levels) and a factor for time (eight levels). In order to determine significant time effects for each curve, a one-way ANOVA was performed for individual groups. The one- and two-way ANOVAs were followed by a Tukey post hoc test with significant values set at $p<0.05$. Statistical analysis was performed with the program GraphPad Prism version 5.0 for Windows (GraphPad Software, USA). All graphs were drawn using the Sigmaplot curve-fitting program (Jandel Scientific, USA).

RESULTS

24-h rhythmicity of subcellular distribution of liver glucocorticoid receptor. Figure 1 shows the daily pattern of the liver α subunit of the GCR in rats fed *ad-libitum* (AL) and under daytime restricted feeding (DRF). AL group showed a peak in GCR presence in nuclear and cytosolic fractions in the transition between light and dark periods (upper and lower panels, respectively), as it was expected in *ad-libitum* fed conditions. This 24-h pattern is analogous to the pattern of circulating corticosterone reported elsewhere [(11)]. In contrast, GCR pattern in DRF group showed significant differences in both fractions: It was not observed any fluctuation in the nuclear fraction, and the values were lower than the AL group in the light and dark periods (upper panel and Table 1). By the contrary, DRF promoted a marked increased in the cytosolic presence of the GCR (~159%), with 2 peaks, one at the times before and after food access (11:00 and 14:00 h), and the other in the transition between light and dark periods (lower panel and Table 1). This 24-h pattern was similar to the one described for circulating corticosterone in rats under DRF protocol [(11)]. No differences were observed in the effect of DRF promoting the higher levels of liver GCR when light and dark periods were compared (Table 1).

As to the control of feeding conditions, the effect of fasting – refeeding on the presence of liver GCR is shown in Figure 2. One day of fasting followed by 2 h of food access did not affect the presence of GCR in liver homogenate. However, 2 days of

fasting promoted a significant increased that was reverted after meal time. In comparison, DRF showed higher levels of GCR than the groups of one day fasting – refeeding, but without differences with the rats with the fasting – refeeding of 2 days (upper panel). In the nuclear fraction was evident an effect of refeeding decreasing the levels of GCR after one day of fasting, effect that was not observed in the groups of 2 days fasting – refeeding. A significant reduction of GCR was shown in the rats under DRF (~85%), in comparison with both controls of feeding condition (middle panel). In contrast, in the cytoplasmic fraction the presence of GCR in the DRF groups was increased ~4 times in comparison to both controls of feeding condition. A discrete but significant diminution was observed after 2 days of fasting that was partially reverted by meal access (lower panel).

The occurrence of corticosterone, the active glucocorticoid in rodents, within the hepatic organ depends on the activity of a system formed by 2 enzymes: the NADPH-dependent 11 β-HSD 1 and the NADPH-regenerating H6PDH. Figure 3 depicts the effect of DRF on the presence and activity of 11 β-HSD 1 (upper and middle panels, respectively), as well as the activity of H6PDH (lower panel). In spite of some tendencies, no significant changes were observed: None of these parameters showed a rhythmic pattern, and neither there was any significant difference between the temporal patterns of control AL group and the DRF-treated rats. In contrast, acute fasting – refeeding of 1 and 2 days showed significant changes in presence and activity of 11 β-HSD 1 in comparison with DRF group (Figure 4). It can be seen in the upper panel that the presence of 11 β- HSD 1 was responsive to the feeding condition since there was a discrete but significant reduction caused by refeeding in the group of 1 day of acute treatment as well as in the DRF rats. However, the most conspicuous difference was in the higher levels of this enzyme promoted by the DRF protocol: It increased ~95% in fasting and ~70% in the fed state (including 1 and 2 days of acute treatment). The activity of 11 β- HSD 1 was also sensitive to the fasting- refeeding protocol (middle panel). This time the activity was higher after refeeding in both acute treatments (1 and 2 days), but not in the DRF groups. In accordance with the western blot results, the activity of 11 β- HSD 1 was 2 – 4 times significantly higher in the rats under the DRF protocol. Hence, the data indicated an up-regulation of 11 β-HSD 1 promoted by restricted food access. Fasting – refeeding condition did not affect the activity of H6PD in any of the experimental groups (lower panel).

Glucocorticoid hormones are some of the principal endocrine regulators of the induction and activity of the enzymes underlying the urea cycle [29]. Figure 5 shows the effect of DRF protocol on the 24 h rhythmicity of circulating urea (upper panel) and the activities of the liver mitochondrial enzymes of the urea cycle, carbamoyl phosphate synthetase 1 (CPS1, middle panel), and ornithine transcarbamylase (OTC, lower panel). In agreement with the temporal pattern of circulating corticosterone [11] and nuclear and cytoplasmic liver GCR (Figure 1), circulating urea in AL rats depicted a peak in the transition between the light and the dark periods (upper panel). Differently, DRF promoted high levels of urea previous to food access (at 11:00 h) which decreased to very low levels in response to meal time (14:00 h). After 17:00 h, the circulating urea was enhanced to reach a peak (08:00 and 11:00 h) just before feeding (upper panel). In spite of the dissimilar rhythmicity, there was no change in the average values of blood urea between AL and DRF groups, neither in the values measured in the light and in the dark periods (Table 2). CPS1 is the rate-limiting enzyme of the urea cycle. It depicted a rhythm in the AL group with higher values (~44%) in the darkness (middle panel and Table 2). CPS1 rhythm was lost in the DRF group, but food restriction caused significant higher CPS1 activity during the light period (~72%) (middle panel and Table 2). Diurnal variation of OTC showed constant values in the AL rats with the exception of a significant diminution at 05:00 h (lower panel). In contrast, DRF promoted a very different rhythmicity in the OTC activity, with low values in the light period and significant increment in the darkness (~80%) (lower panel and Table 2).

Figure 6 shows the effects of fasting – refeeding on the levels of circulating urea and the activities of CPS1 and OTC. Urea in blood was responsive to the feeding condition decreasing ~28% and ~44% by refeeding after 1 and 2 of fasting, respectively. The effect associated to the food access showed a more accentuated response in the groups under DRF protocol (reduction of ~62%) (upper panel). The activity of CPS1 did not show any change comparing the fasted and the fed states in all conditions (1 and 2 days of fasting – refeeding, and DRF at 11:00 h and 14:00 h). However, it was evident that the activity of CPS1 in the groups under DRF protocol depicted a diminution of more than 50% in comparison to their control groups of feeding condition (middle panel). In contrast, OTC activity was sensitive to fasting – refeeding in a complex way: after 1 day of fasting, refeeding increased OTC activity ~50%, but after 2 days of fasting, refeeding reduced OTC activity ~40%. Under the protocol of DRF OTC activity

was more similar to the 2 days of fasting – refeeding response since it was decreased after mealtime by ~29% (lower panel).

DISCUSSION

Restricted feeding during daytime for nocturnal animals is an efficient zeitgeber capable of influencing most of the aspects of the circadian rhythmicity [30]. Glucocorticoids are endocrine messengers that are secreted rhythmically and contribute to the diurnal entrainment of peripheral oscillators [12], including a diverse range of functionally important circadian genes [18]. Because the 24-h rhythmicity of circulating corticosterone changes in association to DRF [11], and the metabolic activity of the liver is notoriously modified [10, 31], the aim of this project was to explore features of glucocorticoid´s signaling: 1) subcellular distribution of GCR, 2) availability of corticosterone within the liver, and 3) parameters related to urea cycle (considered as one of the liver metabolic output of glucocorticoid action).

Glucocorticoid signaling and restricted feeding schedules. An accepted marker in the DRF protocol is the appearance of a peak of circulating corticosterone during the time of FAA [10, 11]. Additional information is obtained when 24-h rhythm is considered: rats with restricted food access show an increase of ~23% in the daily level of blood corticosterone, and a second peak is seen in the transition between the light and dark periods [11]. The second peak of circulating glucocorticoid is similar to the acrophase shown by the control group fed ad-libitum, in amplitude and in time [11]. Interestingly, the temporal pattern of GCR in Figure 1 is analogous to the diurnal variations of circulating glucocorticoids: two peaks in the DRF group (only in cytosolic fraction) and one peak in the group fed ad-libitum (in both, cytoplasmic and nuclear fractions). These data indicate that the 24-h rhythmicity of GCR under DRF is controlled by an oscillatory mechanism different from the one regulating diurnal variations of GCR in ad-libitum conditions. One possibility is that this oscillator could be the FEO, but more experiments are needed to test the feasibility of this notion.

An outstanding result of the present study is the significant increase (~150%) in the presence of liver GCR in the DRF protocol, mainly in the cytoplasmic fraction (Figure 1 and Table 1). Dutta and Sharma [32] also reported an enhanced level (~41%) of GCR in hepatic cytosolic fraction of Balb/c mice fed on alternate days for a period of 3 months. The feeding protocol and the rodent species used by these authors were different from the experimental conditions in the present work, opening the possibility

to explain the evident difference in the magnitude in the increment of GCR observed in the 2 reports. It has been reported that GCR are up-regulated by the presence of glucocorticoid ligands, and in response of the transcriptional activity of factors such as c-myb, c-Ets and HNF-1α [33, 34]. In our experimental protocol, the first condition is accomplished: corticosterone is increased ~23% under DRF [11], but the participation of the other factors remains to be explored.

Besides the increment of liver GCR, another aspect that changed noticeably in the rats under DRF was their subcellular distribution: most of the receptors were located in the cytosol, and only a small proportion within the nucleus (Figure 1). It is unlikely that this finding is due to modifications in the sedimentary properties of the hepatic cellular fractions, since we have observed no changes in the yield and the characteristics of the mitochondrial and microsomal fractions under the DRF protocol [35] and data not published, respectively. In the cytoplasm, the GCR interact with heat shock proteins (HSPs) forming dynamic complexes that are subject to circadian regulation [36]. Upon ligand contact, the HSPs dissociate and the activated GCR is translocated into the nucleus to exert a transcriptional role. However, recent reports also indicate a signaling activity of the complex glucocorticoid – GCR in the cytosol, in complement to the nuclear transcriptional activity. These actions may involve the modulation of ligand- and voltage-dependent ion channels, and the activation of G proteins and ERK1/2 related pathways [13]. The possibility that these actions could be taken place in the liver of rats under DRF is high because of the elevated ratio of cytosolic / nuclear GCR, but additional experiments should be done.

Within the liver of rodents, the active ligand corticosterone is in redox equilibrium with the inactive metabolite cortisone. The formation of corticosterone requires the activity of the NADPH-dependent enzyme 11 β-HSD. The NADPH is supplied by the microsomal enzyme H6PD. Both enzymes have been considered as diabetogenic factors and are involved in obesity as well as in the generation of the metabolic syndrome [37]. Our results did not show changes in the rhythmicity of both enzymes, and neither an effect by feeding condition. However, it should be considered that the enzymatic assays were done in *in vitro* conditions and possibly far from the biochemical circumstances from the intracellular milieu.

Urea cycle parameters and restricted feeding schedules. GCR output varies according to the metabolic and physiological condition of each tissue depending on a complex set of transcriptional co-activators and co-inhibitors. Not only the presence but

also covalent modifications can modulate the activity of GCR [38]. In addition, GCR can initiate a cascade of gene activation and act through intermediate factors to finally modulate the output of metabolic pathways. Reddy et al. [18] reported that only ~24% of the circadian genes regulated by dexamethasone contain glucocorticoid response elements. Glucocorticoids fulfill many different functions in body homeostasis and stress responses [39]. Among the principal targets of these hormones is the regulation of nitrogen metabolism, which in ureotelic organisms is accomplished by the urea/ornithine cycle which is an enzymatic system that converts ammonia into urea in the periportal section of the liver [40]. The urea cycle is controlled by both a positive control by nutritional (fasting and high protein diet) and hormonal (glucagon and glucocorticoids) factors, whereas insulin acts as an inhibitory signal [41].

So far, no reports have been published exploring the rhythmicity of urea cycle-related parameters in protocols of DRF. Our findings showed an evident change in the 24-h rhythmicity of circulating urea and 2 of the most important enzymes in the urea cycle, CPS1 and OTC (Figure 5). The temporal pattern of urea and corticosterone in blood, as well as liver GCR, showed a good coincidence in the control and experimental groups: in AL rats a single peak in the transition between light and darkness period, and 2 peaks (during FAA and also in the transition between light and darkness period) in the rats under DRF protocol. A distinctive feature is the marked decreased in circulating urea and corticosterone after feeding [11], maybe a response to an intense insulin signaling. Micro-arrays data indicated a down-regulation of some genes related to the urea cycle at this time: at 11:00 h, CPS-1 and argininosucinate synthetase 1; at 14:00 h, arginosuccinate synthetase 1 and arginosuccinate lyase (Data not shown). Transcriptional regulation of urea cycle involves a variety of factors such as C/EBPs, Sp1, NF-1, NF-Y and HNF-4α. The action exerted by glucocorticoids signaling on urea cycle is mediated mainly by HNF-4α [18, 42]. It remains to be explored if HNF-4α is also modulated during DRF.

CPS1 is an abundant mitochondrial enzyme that initiates the set of reactions in the urea cycle. Its activity is allosterically dependent of N-acetylglutamate [43]. The average of the 24-h levels was higher in the DRF group, mainly because an increment in the CPS1 activity during the light period (Figure 5 and Table 2). This difference is surely due to an augmentation in the presence of the enzyme. It remains to be explored if the availability of N-acetylglutamate is regulated by food restriction. It was reported that sirtuin 5, a deacetylase mitochondrial enzyme, recognizes CPS1 as substrate,

resulting in an enhancement of its activity [44]. This fact relates CPS1 to the nutritional state of the organism, since sirtuins is a family of NAD^+-dependent proteins that are responsive to the metabolic status of the cell [45].

OTC is the second enzyme of the urea cycle and also located within the liver mitochondria. Its activity is little responsive to hormonal stimulation (glucagon and glucocorticoids), but it shows a very clear diurnal rhythmicity [18, 46]. DRF protocol promoted a substantial change in the 24-h rhythmicity of OTC in comparison to control group fed *ad-libitum* (Figure 5, lower panel). OTC is not an allosteric enzyme, but recently it was demonstrated that is also regulated by acetylation-deacetylation, not by sirtuin 5, but by sirtuin 3. As with CPS1, deacetylation of OTC increases its activity [47].

Circadian adaptations of liver metabolism during restricted feeding. After 3 weeks, daily rhythmicity is greatly modified by 2 h of daytime restricted food access. The adaptations occur at several levels: 1) A great hyperphagia after mealtime alters the process of nutrient assimilation [48]; in consequence, the content of food in the gastric chamber last ~20 h and promotes changes in the rhythm of circulating ghrelin (data not shown); 2) 24-h fluctuations of a number of endocrine signals are largely modified, including thyroid hormones [49], growth hormone and insulin-like growth factor-1 (data not shown), cytokines [11, 48]; 3) an enhancement in lipid mobilization from the adipose tissue and reduction of hepatic glycogen degradation [31, 50]; 4) change in the set point of regulatory parameters in the metabolic control of the liver such as cytoplasmic and mitochondrial redox state as well as adenine nucleotides-related energy charge [10]. The modifications include not only phase shifts of 24-h rhythmicity, e.g. expression of liver PER1 [11], but also significant increment (e.g. liver peroxisomal markers, [51]) or reduction (e.g. activity of liver phosphenolpyruvate carboxycinase, data not shown) in the amplitude of diurnal rhythms. Besides, the mesor of some parameters show a significant increment (e.g. mitochondrial proton-motive electrochemical force, [35]) whereas in others get reduced (e.g. leptin, [48]). One possible interpretation of all these modifications underlying circadian entrainment to food is the emergence of an alternative distributed oscillator different from the SCN, known as the FEO that integrates metabolic, physiological and behavioral responses [52, 53].

It is in this context in which the findings of the present research should be considered: upon DRF protocol / expression of the FEO, glucocorticoid signaling adopts a new category of rheostatic equilibrium in which there is an up-regulation of GCR as well as a modification in the rhythmicity of the urea cycle.

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CONFLICT OF INTEREST STATEMENT

DLM and MDM designed the study and wrote the manuscript. DLM and BGA carried out the experimental work. BGA is an undergraduate student (Biology School, University of Querétaro) and DLM is a PhD student in the program of Biomedical Sciences (UNAM). MDM received salary from the UNAM, which is a public university funded by the Mexican government. There is no conflict of interest.

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FIGURE LEGENDS

Figure 1. Daily variations of glucocorticoid receptor in the liver during the protocol of daytime restricted feeding. Western blotting of 24-h cycle for glucocorticoid receptor (GCR) in rats fed *ad-libitum* (AL) and under daytime restricted feeding (DRF) in nuclear (upper panel) and cytosolic (lower panel) fractions. Open symbols, AL group; filled symbols, DRF group. Light on at 08:00 h and off at 20:00 h. Dark box indicates mealtime (12:00 – 14:00 h). Tubulin and β-actin were used as loading controls for nuclear and cytosolic fractions, respectively. Data are expressed as mean ± SEM of at least 8 independent observations. Representative western blot experiments are shown. *****Time points with a significant difference between AL and DRF groups (Tukey post hoc test, $p< 0.05$).

Figure 2. Comparison between daytime restricted feeding protocol and fasting and re-feeding (~1 and 2 days) on the levels of glucocorticoid receptor in the liver. Western blotting of glucocorticoid receptor in groups of fasting and re-feeding (Re-f) for ~1 and 2 days, as well as rats under daytime restricted feeding (DRF). Experiments were done in liver homogenate (upper panel), nuclear fraction (middle panel) and cytosol (lower panel). Fasting ~1 and 2 days, white bars with diagonal pattern; fasting ~1 and 2 days plus 2 h re-feeding, white bars with punctuated pattern, DRF before (at 11:00 h) and after (14:00 h) food access, black bars. Tubulin (for nucleus) and β-actin (for homogenate and cytosol) were used as loading controls. Data are expressed as mean ± SEM of at least 8 independent observations. **a** Significant difference for Fasting vs Re-fed, **b** for fasting 2 days vs Re-fed 2 days, **c** for fasting vs DRF 11 h, **d** for Re-fed vs DRF 14 h, **e** for fasting 1 day vs fasting 2 days, **f** for Re-fed 1 day vs Re-fed 2 days, **g** for DRF 11 h vs DRF 14 h (Tukey post hoc test, $p< 0.05$).

Figure 3. Daily variations of 11 β–hydroxysteroid dehydrogenase type 1 (presence and activity) and hexose-6-phosphate dehydrogenase (activity) in the liver during the protocol of daytime restricted feeding. Rats fed *ad-libitum* (AL, open symbols, panels at left) and under daytime restricted feeding (DRF, filled symbols, panels at right). Upper panel: Western blotting of 24-h cycle for 11 β–hydroxysteroid dehydrogenase type 1 (11 β HSD 1); β-actin was used as loading control. Middle panel: 24-h cycle of 11 β HSD 1 activity (fraction). Lower panel: 24-h cycle of hexose-6-phosphate dehydrogenase (H6PD) activity. Experiments were done in liver microsomal fraction. Light on at 08:00 h and off at 20:00 h. Dark box indicates mealtime (12:00 –

14:00 h). Data are expressed as mean ± SEM of at least 8 independent observations. *****Time points with a significant difference between AL and DRF groups (Tukey post hoc test, $p < 0.05$).

Figure 4. Comparison between daytime restricted feeding protocol and fasting and re-feeding (~1 and 2 days) on the presence and activity of 11 β–hydroxysteroid dehydrogenase type 1, and the activity of hexose-6-phosphate dehydrogenase in the liver. Western blotting (upper panel) and activity (middle panel) of 11 β–hydroxysteroid dehydrogenase type 1 (11 β HSD 1) and activity of hexose-6-phosphate dehydrogenase (H6PD) (lower panel) in groups of fasting and re-feeding (Re-f) for ~1 and 2 days, as well as rats under daytime restricted feeding (DRF). Experiments were done in liver microsomal fraction; for western blot experiments,β-actin was used as a loading control. Fasting ~1 and 2 days, white bars with diagonal pattern; fasting ~1 and 2 days plus 2 h re-feeding, white bars with punctuated pattern, DRF before (at 11:00 h) and after (14:00 h) food access, black bars. Data are expressed as mean ± SEM of at least 8 independent observations. **a** Significant difference for Fasting vs Re-fed, **b** for fasting 2 days vs Re-fed 2 days, **c** for fasting vs DRF 11 h, **d** for Re-fed vs DRF 14 h, **e** for fasting 1 day vs fasting 2 days, **f** for Re-fed 1 day vs Re-fed 2 days, **g** for DRF 11 h vs DRF 14 h (Tukey post hoc test, $p < 0.05$).

Figure 5. Daily variations of circulating urea and activities of carbamoyl phosphate synthetase 1 and ornithine transcarbamylase in the liver during the protocol of daytime restricted feeding. Rats fed *ad-libitum* (AL, open symbols) and under daytime restricted feeding (DRF, filled symbols). Upper panel: 24-h cycle of circulating urea; middle panel: 24-h cycle of carbamoyl phosphate synthetase 1 (CPS 1) activity; lower panel: 24-h cycle of ornithine transcarbamylase (OTC) activity. Enzymatic activities were measured in the liver mitochondrial fraction. Light on at 08:00 h and off at 20:00 h. Box with arrowheads indicates mealtime (12:00 – 14:00 h). Data are expressed as mean ± SEM of at least 8 independent observations. *****Time points with a significant difference between AL and DRF groups (Tukey post hoc test, $p < 0.05$).

Figure 6. Comparison between daytime restricted feeding protocol and fasting and re-feeding (~1 and 2 days) on the levels of circulating urea and activities of carbamoyl phosphate synthetase 1 and ornithine transcarbamylase in the liver. Circulating urea levels (upper panel), activity of carbamoyl phosphate synthetase 1 (CPS 1) (middle panel), and activity of ornithine transcarbamylase (OTC) (lower panel)

in groups of fasting and re-feeding (Re-f) for ~1 and 2 days, as well as rats under daytime restricted feeding (DRF). Data are expressed as mean ± SEM of at least 8 independent observations. **a** Significant difference for Fasting vs Re-fed, **b** for fasting 2 days vs Re-fed 2 days, **c** for fasting vs DRF 11 h, **d** for Re-fed vs DRF 14 h, **e** for fasting 1 day vs fasting 2 days, **f** for Re-fed 1 day vs Re-fed 2 days, **g** for DRF 11 h vs DRF 14 h (Tukey post hoc test, $p < 0.05$).

Table 1

Liver glucocorticoid receptor in liver homogenate, nucleus and cytosol in rats under a protocol of daytime food restriction

GLUCOCORTICOID RECEPTOR/ β-ACTIN OR TUBULIN (Relative Concentration)						
	Homogenate		Nucleus		Cytosol	
	Control	DRF	Control	DRF	Control	DRF
Mean	0.61±0.12	1.49±0.28 *	0.46±0.20	0.21±0.05	1.43±0.37	3.70±0.89 *
Light	0.63±0.11	1.54±0.21 *	0.39±0.17	0.20±0.04	1.21±0.29	3.90±0.96 *
Dark	0.59±0.12	1.43±0.35 *	0.54±0.24	0.22±0.06	1.65±0.45	3.48±0.80 *

Comparison between groups fed *ad-libitum* (AL) and under restricted daytime restricted feeding (DRF) of average values of 24-h cycle (mean), light period (08:00, 11:00 h, 14:00 and 17:00 h) and dark period (20:00, 23:00, 02:00 and 05:00 h). Values were calculated from data in Figure 1. Data are expressed as mean ± SEM of at least 8 independent observations. ***** Significant difference for AL vs DRF and ** for Light vs Dark periods (Tukey post hoc test, $p< 0.05$).

Table 2

Urea cycle-related parameters in rats under a protocol of daytime food restriction

	UREA (mg/ dL)		CPS1 (nmol hydroxy urea/ µg protein/ min)		OTC (µg citrulline/ µg protein/ min)	
	Control	DRF	Control	DRF	Control	DRF
Mean	41.20±3.40	38.60±3.80	0.22±0.02	0.29±0.03 *	0.68±0.05	0.50±0.06*
Light	43.10±3.90	40.00±3.70	0.18±0.02	0.31±0.03 *	0.72±0.04	0.36±0.03*
Dark	39.30±3.03	37.10±3.80	0.26±0.02 **	0.28±0.03	0.64±0.06	0.65±0.09**

Comparison between groups fed *ad-libitum* (AL) and under restricted daytime restricted feeding (DRF) of average values of 24-h cycle (mean), light period (08:00, 11:00 h, 14:00 and 17:00 h) and dark period (20:00, 23:00, 02:00 and 05:00 h). Values were calculated from data in Figure 5. CPS1, carbamoyl phosphate synthetase 1; OTC, ornithine transcarbamylase. Data are expressed as mean ± SEM of at least 8 independent observations. ***** Significant difference for AL vs DRF and ** for Light vs Dark periods (Tukey post hoc test, $p< 0.05$).

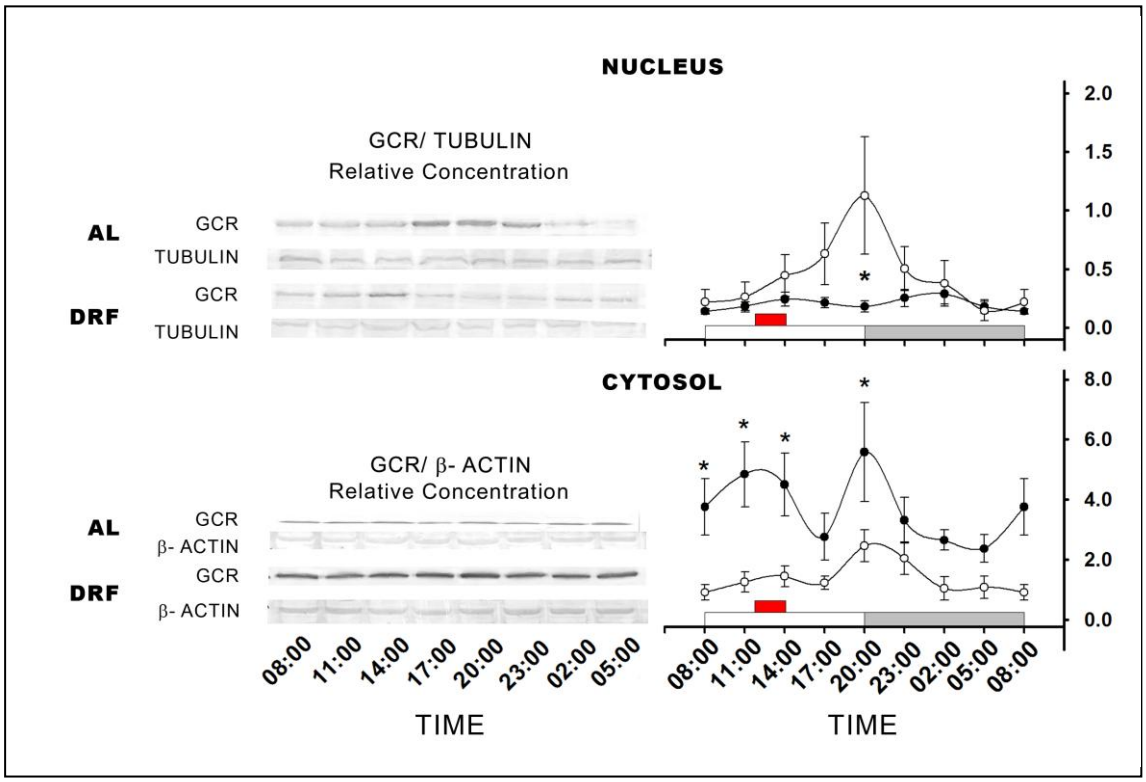
NUCLEUS
GCR/ TUBULIN
Relative Concentration
AL
GCR
TUBULIN
DRF
GCR
TUBULIN
2.0
1.5
1.0
0.5
0.0
CYTOSOL
GCR/ β- ACTIN
Relative Concentration
AL
GCR
β- ACTIN
DRF
GCR
β- ACTIN
8.0
6.0
4.0
2.0
0.0
08:00
11:00
14:00
17:00
20:00
23:00
02:00
05:00
08:00
TIME
TIME

Figure 1

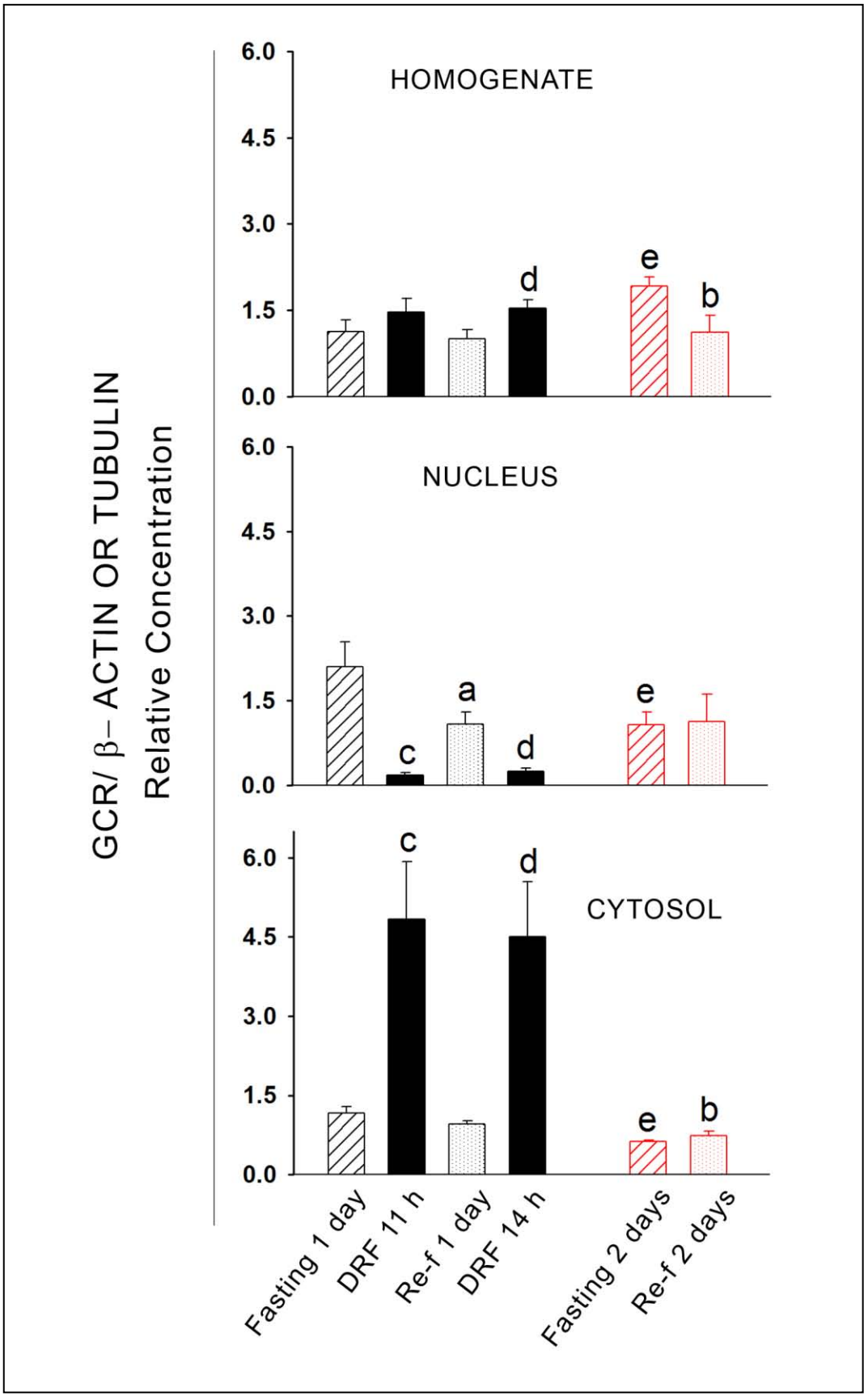
GCR/ β– ACTIN OR TUBULIN
Relative Concentration
HOMOGENATE
NUCLEUS
CYTOSOL
6.0
4.5
3.0
1.5
0.0
a
b
c
d
e
Fasting 1 day
DRF 11 h
Re-f 1 day
DRF 14 h
Fasting 2 days
Re-f 2 days

Figure 2

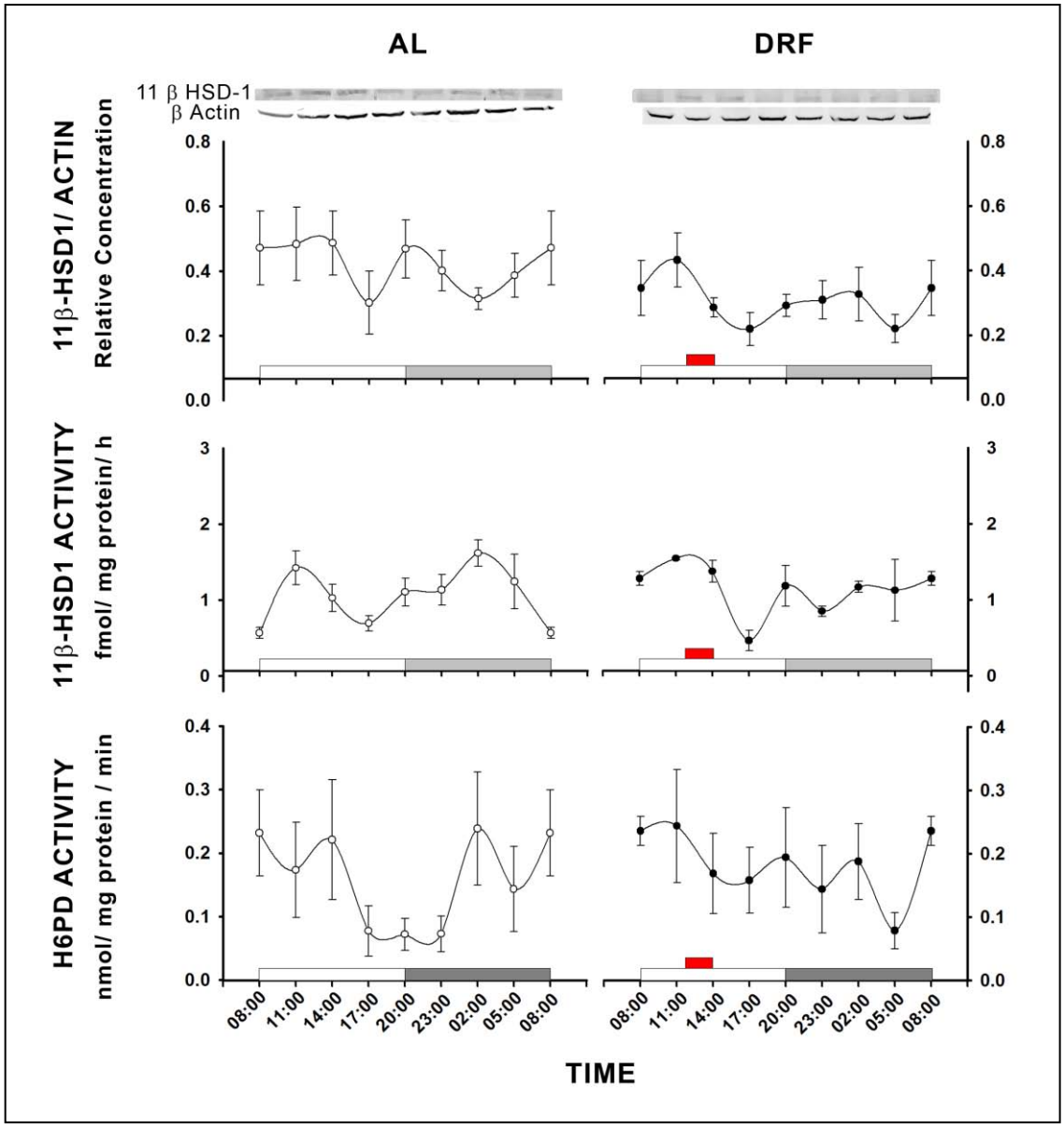
AL
DRF
11 β HSD-1
β Actin
11β-HSD1/ ACTIN
Relative Concentration
11β-HSD1 ACTIVITY
fmol/ mg protein/ h
H6PD ACTIVITY
nmol/ mg protein / min
0.0
0.2
0.4
0.6
0.8
0
1
2
3
0.1
0.3
08:00
11:00
14:00
17:00
20:00
23:00
02:00
05:00
TIME

Figure 3

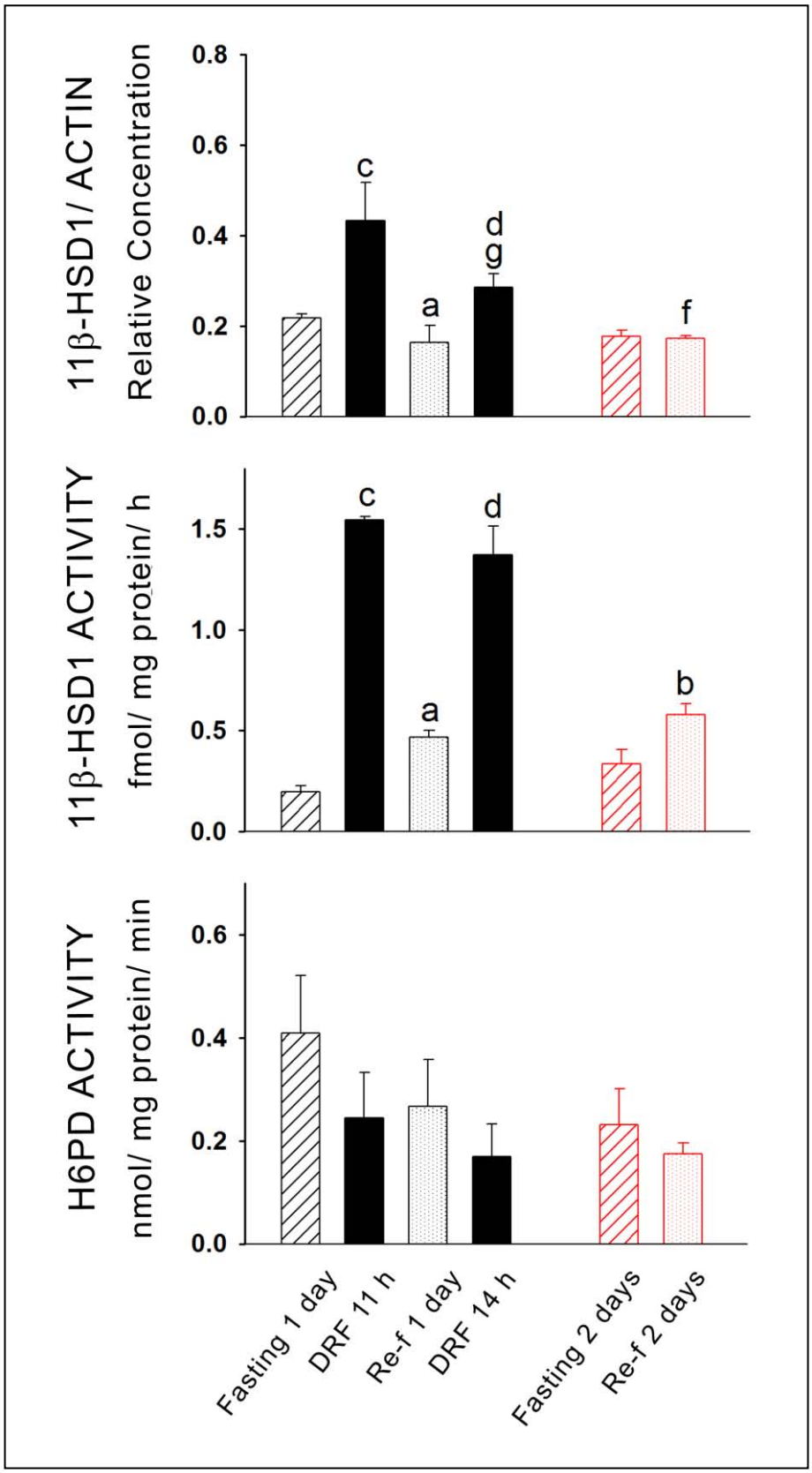
11β-HSD1/ ACTIN
Relative Concentration
0.8
0.6
0.4
0.2
0.0
c
a
d
g
f
11β-HSD1 ACTIVITY
fmol/ mg protein/ h
1.5
1.0
0.5
0.0
c
a
d
b
H6PD ACTIVITY
nmol/ mg protein/ min
0.6
0.4
0.2
0.0
Fasting 1 day
DRF 11 h
Re-f 1 day
DRF 14 h
Fasting 2 days
Re-f 2 days

Figure 4

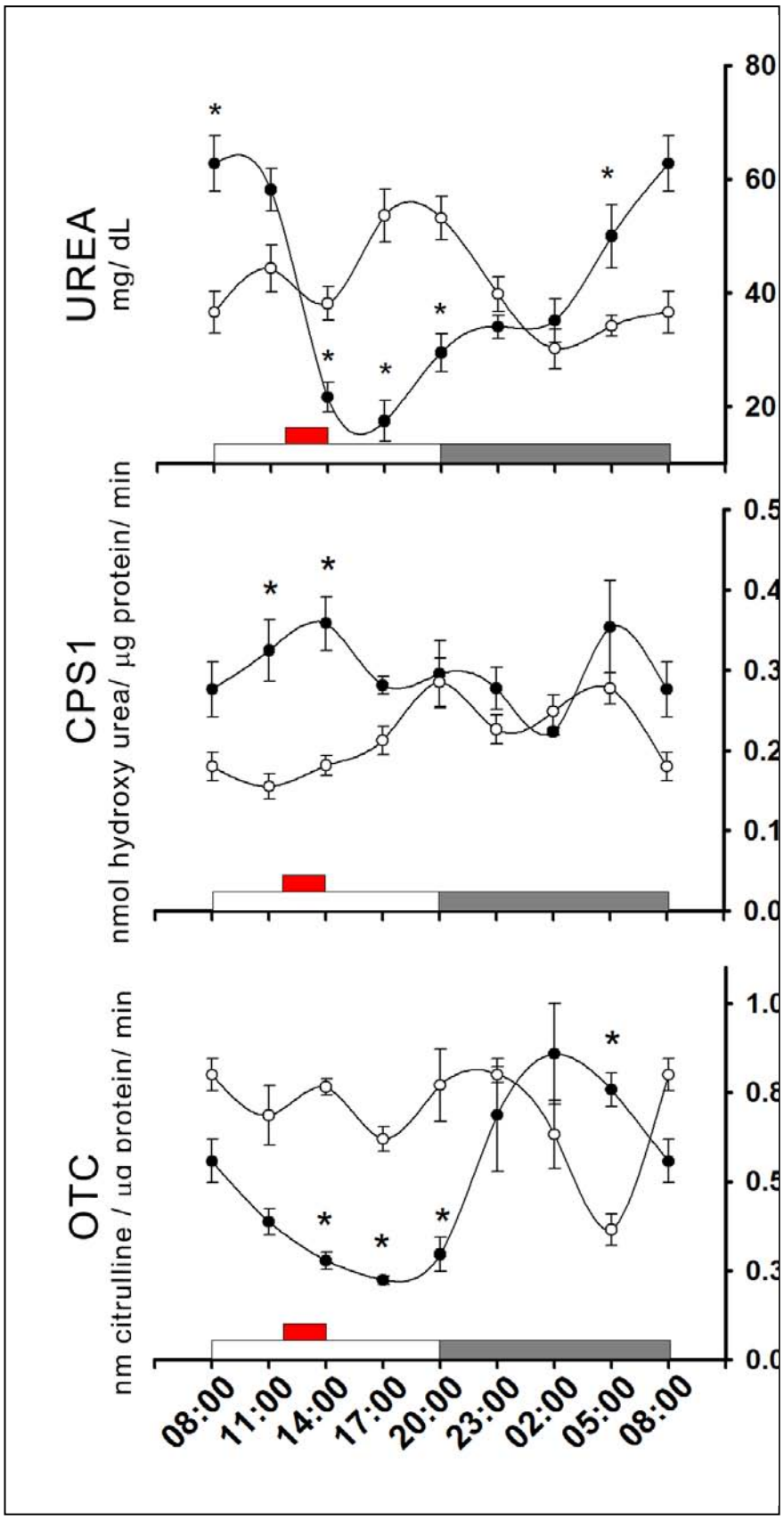
UREA
mg/ dL
80
60
40
20
CPS1
nmol hydroxy urea/ µg protein/ min
0.5
0.4
0.3
0.2
0.1
0.0
OTC
nm citrulline / µg protein/ min
1.0
0.8
0.5
0.3
0.0
08:00
11:00
14:00
17:00
20:00
23:00
02:00
05:00
08:00

Figure 5

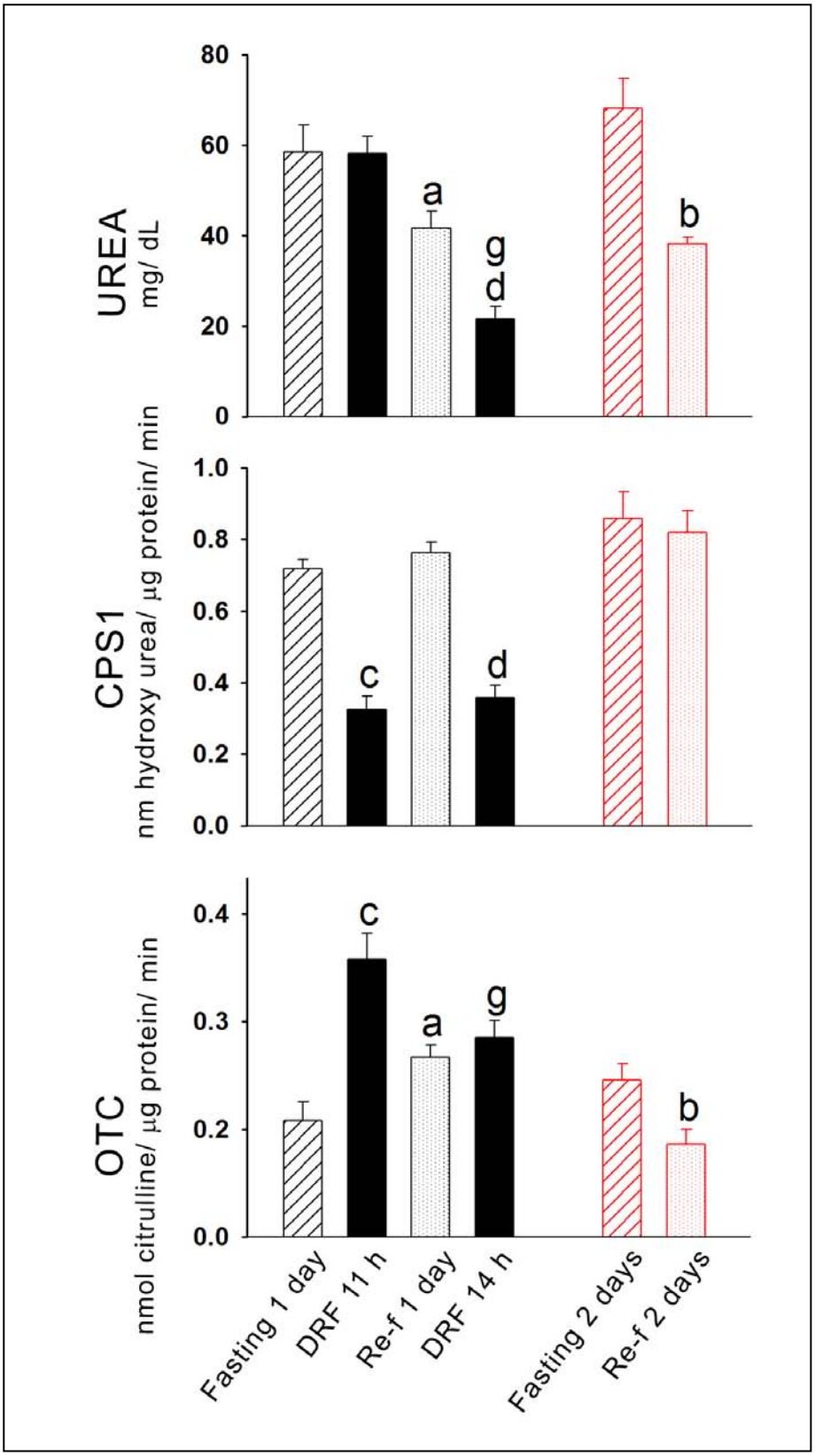
UREA
mg/ dL
80
60
40
20
0
a
g
d
b
CPS1
nm hydroxy urea/ µg protein/ min
1.0
0.8
0.6
0.4
0.2
0.0
c
d
OTC
nmol citrulline/ µg protein/ min
0.4
0.3
0.2
0.0
c
a
g
b
Fasting 1 day
DRF 11 h
Re-f 1 day
DRF 14 h
Fasting 2 days
Re-f 2 days

Figure 6