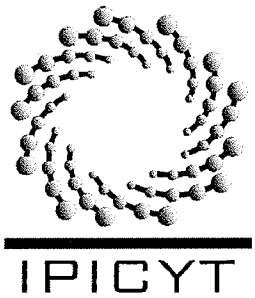

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

"MECANISMOS MOLECULARES DE LA MODULACIÓN DEL METABOLISMO Y LA DIFERENCIACIÓN ADIPOSA POR ISOORIENTINA"

Tesis que presenta

M. en C. María Guadalupe Gómez Espinoza

Para obtener el grado de

Doctor(a) en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Luis Antonio Salazar Olivo

San Luis Potosí, S.L.P., septiembre del 2013

IPICYT

Constancia de aprobación de la tesis

La tesis ***"Mecanismos moleculares de la modulación del metabolismo y la diferenciación adiposa por isoorientina"*** presentada para obtener el Grado de Doctora en Ciencias en Biología Molecular fue elaborada por **María Guadalupe Gómez Espinosa** y aprobada el **cuatro de septiembre del dos mil trece** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

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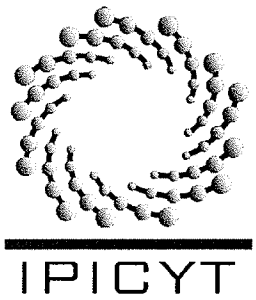

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a fin de efectuar el examen, que para obtener el Grado de:

DOCTORA EN CIENCIAS EN BIOLOGÍA MOLECULAR

sustentó la C.

María Guadalupe Gómez Espinoza

sobre la Tesis intitulada:

Mecanismos moleculares de la modulación del metabolismo y la diferenciación adiposa por isoorientina

que se desarrolló bajo la dirección de

Dr. Luis Antonio Salazar Olivo

El Jurado, después de deliberar, determinó

APROBARLA

Dándose por terminado el acto a las 14:00 horas, procediendo a la firma del Acta los integrantes del Jurado. Dando fe el Secretario Académico del Instituto.

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Al Sr Ignacio Gómez Velazquez y la Sra.

Ma. Guadalupe Espinoza Rivera por ser los mejores padres que se

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RESUMEN

MECANISMOS MOLECULARES DE LA MODULACIÓN DEL METABOLISMO Y LA DIFERENCIACIÓN ADIPOSA POR ISOORIENTINA

La Isoorientina (ISO), una isoflavona presente en numerosas plantas empleadas por la medicina tradicional, posee probados efectos hipoglucemiantes pero sus mecanismos de acción son aún poco conocidos. Al analizar los mecanismos moleculares de ISO encontramos que estimula la captación de glucosa en adipocitos múridos y humanos, tanto sensibles como resistentes a insulina. ISO ejerce este efecto mediante la inducción de la fosforilación apropiada de las proteínas de la vía de señalización canónica de la insulina (PI3K) y estimula la transcripción de genes que codifican para esta vía de señalización. Asimismo, encontramos que ISO bloquea la adipogénesis múrida y humana mediante la inhibición de la expresión de los genes proadipogénicos PPARγ, C/EBPα y KLF4 e induce la de los genes antiadipogénicos GATA2 y WNT10B. Nuestros resultados sugieren que ISO modula la expresión de estos genes por medio de la translocación al núcleo del factor de transcripción Nrf2. Por otra parte dado que WNT10B induce la osteogénesis de células troncales mesenquimales, analizamos el efecto de ISO sobre la actividad de la fosfatasa alcalina y la deposición extracelular de calcio en preadipocitos 3T3-F442A. Encontramos que ISO induce la expresión de ambos marcadores en preadipocitos múridos. En resumen, ISO induce la captación de glucosa por adipocitos activando las vía de señalización de insulina, bloquea la formación de nuevos adipocitos e induce la expresión de marcadores osteogénicos en preadipocitos 3T3-F442A. Estos resultados sugieren que ISO tiene un alto potencial para el desarrollo de nuevos tratamientos para el síndrome metabólico.

Palabras clave: isoflavonoides, adipogénesis, insulina, osteogénesis, diabetes.

Abstract

MOLECULAR MECHANISMS OF METABOLISM MODULATION AND ADIPOSE DIFFERENTIATION BY ISOORIENTIN

Isoorientin, an isoflavonoid widely present in the plants, is commonly used in traditional medicine, and has shown hypoglycemic effects, but its mode of action is not well understood. In this study we found that ISO induced glucose uptake in both insulin-senstive and insulin resistant murine and human adipocytes by stimulating the proper phosphorylation of proteins of the insulin canonic signaling pathway (PI3K) and the transcription of the genes encoding its proteins, was also induced. In addition, ISO inhibited the murine and human adipogenesis by inhibiting the expression of proadipogenic genes PPARγ, C/EBPα and KLF4 and by inducing the transcription of the antiadipogenic genes GATA2 and WNT10B. Our results suggest that ISO modulates gene expression by promoting the nuclear translocation of the transcription factor Nrf2. Given that it has been shown that WNT10B transcription factor induces osteogenesis in mesenchymal stem cells, we analyzed the effect of ISO on the activity of alkaline phosphatase and extracellular calcium deposition in 3T3-F442A preadipocytes and found that ISO increased the expression of both markers in murine preadipocytes. In summary, ISO induces glucose uptake in adipocytes through activation of the insulin signaling pathways, the inhibition of adipogenesis and induction of osteogenic markers in 3T3-F442A preadipocytes. These properties confers ISO a therapeutic potential to develop new drugs for metabolic syndrome treatment.

Key words: isoflavonoids, adipogenesis, insulin, osteogenesis, diabetes

INTRODUCCIÓN

Obesidad

La obesidad es un serio problema de salud que causa o exacerba múltiples enfermedades crónicas, incluyendo la diabetes tipo 2 (DT2), la hipertensión arterial, las enfermedades coronarias y algunos tipos de cáncer (Kopelman 2000). La obesidad se define como una acumulación anormal o excesiva de grasa, y es, junto con el sobrepeso, el quinto factor de riesgo de muerte en el mundo. Cada año fallecen por lo menos 2.8 millones de personas adultas como consecuencia del sobrepeso o la obesidad (OMS 2012). En México, de acuerdo a la Encuesta Nacional de Salud y Nutrición 2012 (ENSANUT 2012), el sobrepeso y la obesidad afectan a siete de cada 10 adultos. La prevalencia combinada de sobrepeso y obesidad es de 73 % para las mujeres y 69.4 % para los hombres de edad adulta. Los niños en edad escolar (ambos sexos), de 5 a 11 años, presentaron una prevalencia en 2012 de 19.8 %para sobrepeso y 14.6 % para obesidad. La prevalencia nacional combinada de sobrepeso y obesidad en adolescentes fue de alrededor de 35.8 % en mujeres y 34.1 % en hombres. En menores de cinco años la obesidad ha aumentado entre 1998 y 2012, de 7.8 % a 9.7 %, respectivamente (ENSANUT 2012).

Estrategias para el tratamiento de la obesidad

Las estrategias para la reducción de peso en individuos obesos incluyen cambios en el estilo de vida como ejercicio y dieta, terapias de comportamiento, tratamientos farmacológicos y, en casos extremos, intervenciones quirúrgicas. El

ejercicio y la dieta son las mejores estrategias para la prevención y el tratamiento, pero ambos requieren disciplina y son difíciles de mantener. Los fármacos autorizados actualmente por la Administración de Alimentos y Medicamentos en USA (Food and Drug Administration, FDA por sus siglas en ingles) para inducir la pérdida de peso son el Orlistat, aprobado en 1999, la Lorcaserina y la Fentermina-Topiramato, autorizadas en 2012.

El Orlistat disminuye la absorción de grasa al inhibir a la lipasa pancreática, la enzima principal de la hidrólisis de los triglicéridos. La eficacia del Orlistat (120 mg tres veces por día) para la pérdida de peso se demostró en varios ensayos aleatorios controlados de 2-4 años de terapia en los cuales se registró una pérdida de peso promedio de 6.2 kg con respecto al placebo. Además el Orlistat también induce mejoras en la presión sanguínea, en la resistencia a la insulina y en los niveles de lípidos en el suero. Este fármaco sin embargo ocasiona efectos secundarios como diarrea, flatulencia, inflamación, dolor abdominal, dispepsia y daño hepático (Ioannides-Demos *et al*, 2011).

La Lorcaserina es un supresor del apetito, de esta aún no esta claramente comprendido el mecanismo de acción, pero se cree que actúa como un agonista de los receptores de la serotonina subtipo 2C (5-HT_{2c}) localizados en las neuronas opio melanocortinas del hipotálamo. La Lorcaserina es administrada oralmente, su vida media en la sagre es de 1.5-2 h y su tiempo de eliminacion es de 11 h. La Lorcaserina sin embargo, ocasiona efectos adversos cardiovasculares, cerebrovasculares, hipertensión pulmonar y hemorragias, además de provocar dependencia (Taylor *et al.*, 2013).

La Fentermina-Topiramato ejerce efectos anoréxicos, y su mecanismo de acción es actuando como agonista del receptor adrenérgico, estimulando el sistema nervioso central, además como agonista del receptor GABA, estimulando los neurotransmisores y actuando como agonista de los canales de sodio. Sin embargo presenta efectos adversos como parestesia, xerostomía, constipación, infección de las vías respiratorias, insomnio y depresión (Cameron *et al.*, 2012).

Diabetes mellitus tipo 2

La diabetes mellitus tipo 2 (DT2) se caracteriza por la incapacidad de los tejidos para responder a la insulina. Se estima que alrededor del 90-95% de todos los casos de diabetes corresponden a DT2. La Federación Internacional de la Diabetes estima que en el año 2012 el número de adultos con diabetes se elevó a 371 millones, lo que representa el 8.3 % de la población mundial (IDF, 2012). Se estima que este número se incrementará a 552 millones de personas para el 2030, lo que equivaldrá al 9.9 % de la población adulta mundial. El primer lugar en prevalencia de diabetes lo tiene China con 90 millones de personas y México se ubica en el séptimo lugar con 10.3 millones de personas afectadas. La Tabla 1 enumera los países con mayor prevalencia de diabetes y la proyección para el 2030 (IDF 2012).

La diabetes se ha consolidado como la primera causa de muerte en nuestro país. En los últimos años, la frecuencia de la enfermedad aumentó de 5.7 % en el 2000 a 9.1% en 2012. Las mayores prevalencias del padecimiento están en siete entidades: Distrito Federal (12.3%), Nuevo León (11.4%), Veracruz (10.6%), Estado de México (10.5%), Tamaulipas (10.3%), Durango (10.2%) y San Luis

Potosí (10.0%), los cuales rebasan el promedio nacional de 9.1 % (ENSANUT 2012).

Tabla 1. Población de adultos (20-79) con diabetes durante el año 2011 en los países con mayor prevalencia y estimación de la misma para el año 2030 (IDF 2012).

País	Millones 2011	País	Millones 2030
1 China	90.0	**1 China**	129.7
2. India	61.3	**2. India**	101.2
3. USA	23.7	**3. USA**	29.6
4. Federación Rusa	12.6	**4. Brasil**	19.6
5. Brasil	12.4	**5. Bangladesh**	16.8
6. Japón	10.7	**6. México**	16.4
7. México	10.3	**7. Federación Rusa**	14.1
8.Bangladesh	8.4	**8. Egipto**	12.4
9. Egipto	7.3	**9. Indonesia**	11.8
10. Indonesia	7.3	**10. Pakistán**	11.4

Estrategias para el tratamiento de la diabetes

Los hipoglucemiantes orales, los fármacos más usados en el tratamiento de DT2, actúan por alguna de las siguientes vías: 1) limitan la absorción intestinal de glucosa, 2) estimulan la síntesis y/o secreción de insulina por las células β-pancreáticas, 3) reducen la producción hepática de glucosa por inhibición de la gluconeogénesis o 4) estimulan la incorporación de glucosa en los tejidos blanco de la insulina como el tejido adiposo o el muscular. Esta última vía representa el blanco terapéutico óptimo para el tratamiento de la DT2, debido a que el problema

en la DT2 es la incapacidad de los tejidos blancos a responder a la insulina. No obstante que existe una gran variedad de medicamentos para el tratamiento de la DT2, todos tienen efectos secundarios indeseables.

Los inhibidores de las alfa-glucosidasas que modulan la absorción intestinal de glucosa provocan fallas renales, hepatopatías y daños intestinales. Las sulfonilúreas y las meglitinidas que estimulan la síntesis y/o secreción de insulina por las células β-pancreáticas inducen fallas hepáticas y renales. Las biguanidas, que inhiben la producción hepática de glucosa tienen efectos adversos tales como anorexia, molestias abdominales, diarreas y mala absorción de la vitamina B_{12}. Finalmente, las tiazolidinedionas, que estimulan la incorporación de glucosa en los tejidos blanco de la insulina, provocan daños hepáticos, fallas cardiacas, e inducen obesidad y osteoporosis (Rendell y Kirchain, 2000; Philippe y Raccah, 2009).

Debido a la alta relación entre la obesidad y la diabetes, las dos afecciones metabólicas más importantes a escala mundial, se ha acuñado el término de diabesidad. Dado que los tratamientos farmacológicos para ambas afecciones tienen efectos secundarios indeseables, se ha propuesto la búsqueda de nuevas alternativas terapéuticas más eficientes. Estudios recientes en nuestro laboratorio muestran que la Isoorientina (ISO) puede ser un compuesto alternativo para el tratamiento de la diabesidad.

Isoorientina: naturaleza química, distribución y actividad biológica endógena

ISO es un isoflavonoide, que forma parte de una familia de compuestos de bajo peso molecular con estructuras basadas en un núcleo común de tres anillos que pueden ser o no glucosilados. Estos compuestos están presentes en todas las

estructuras de las plantas vasculares: hojas, flores, frutos y semillas, así como en productos elaborados a partir de ellas como el té y el vino tinto.

ISO, antes llamado homo-orientina, es un isómero de la C-glucosil luteolina. Koeppen determinó a ISO como 6-C-β-D-glucopiranosil-luteolina (Figura 1), la cual es una forma glucosilada de la luteolina. Este estudio se basó en evidencia química y análisis con el espectro de los compuestos en solución de etóxido de sodio (los cuales son típicamente flavonas que contienen libre únicamente el grupo hidroxilo en la posición 5) (Koeppen, 1965). ISO se encuentra ampliamente distribuido en el reino vegetal y es un flavonoide presente en plantas comestibles como el trigo sarraceno (*Fagopyrum esculentum)*, una herbácea originaria de Asia central, cultivada para consumo humano y animal (Dietrych-Szostak *et al.,* 1999; Kim *et al.,* 2011; Lim *et al.*, 2012). ISO se encuentra también en el pepino (*Cucumis sativus*), una planta herbácea anual de la familia de las cucurbitáceas con usos culinario, cosmético y medicinal (McNally *et al,.* 2003) y en el guaje (*Lagenaria siceraria)*, una cucurbitácea trepadora con usos gastronómico, medicinal y artesanal (Mali y Bodhankar, 2012), entre otros.

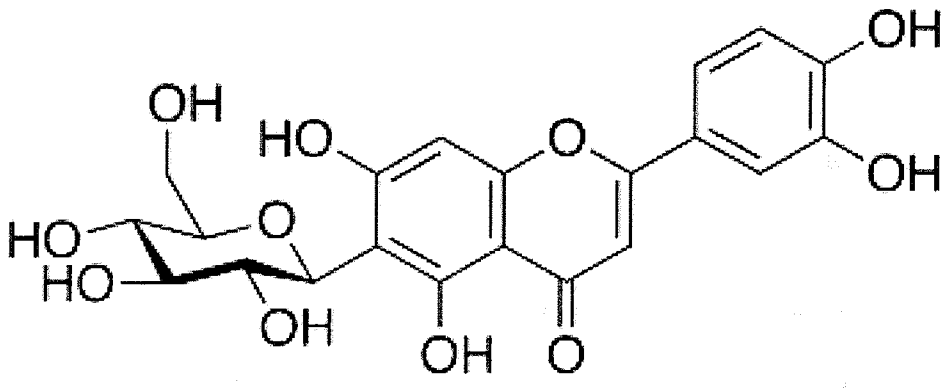

Figura 1. Estructura química de la Isoorientina

McMullen y colaboradores (2004) describieron la ruta biosintética para ISO cuando trataban de dilucidar la ruta biosintética para la maizina, un flavonoide que confiere resistencia natural al maíz contra larvas de lepidópteros. En este estudio se emplearon variedades de maíz azul y negro, donde a partir de la estructura básica de las flavonas mediante la acción de la Pr1 flavona-3'-hidroxilasa se obtiene la di-hidroxil-flavona, la cual se convierten luteolina por la flavona sintasa. ISO se sintetiza finalmente por acción de la C-glucosil transferasa (Figura 2) (McMullen *et al.*, 2004).

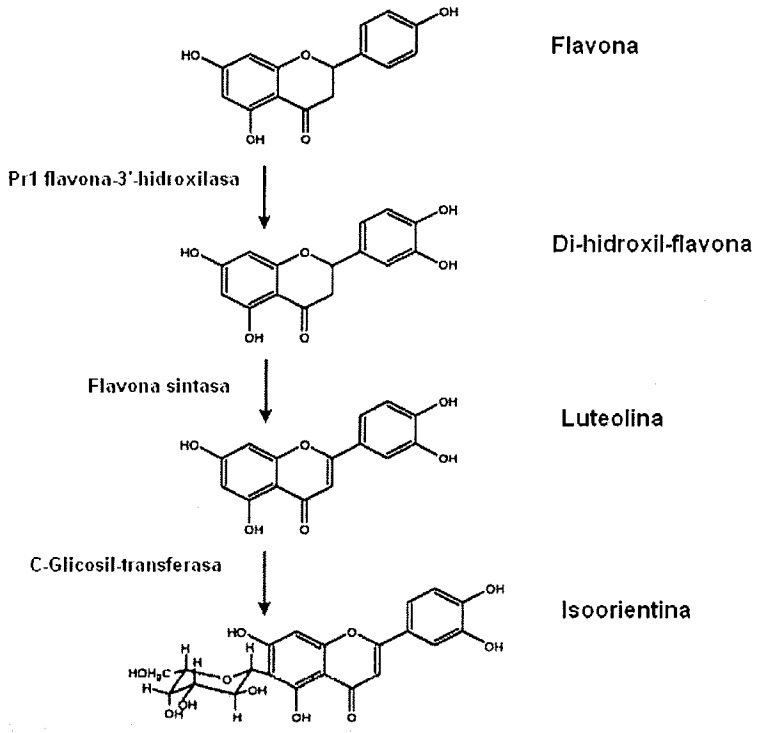

Figura 2. Biosíntesis de Isoorientina (tomado de McMullen *et al.*, 2004)

En el maíz, ISO es un metabolito secundario que se encuentra en los estigmas, donde ayuda a la defensa de la planta. El híbrido T218 muestra un

contenido de ISO mayor al 2.0% en peso seco en los estigmas, suficiente para protegerlo del gusano elotero (*Helicoverpa zea*) (Widstrom y Snook, 1998). ISO también inhibe el crecimiento del gusano cogollero del maíz (*Spodoptera frugiperda)*, plaga que afecta el maíz y el algodón entre otros (Johnson *et al.,* 2002). ISO se presenta de igual manera en las hojas del pepino (*Cucumis sativus)*, donde participa en el control de infecciones (McNally *et al.,* 2003); éstas estimulan la producción de ISO y otros flavonoides como orientina, Isovitexina y vitexina (Amany y Aboellil, 2007).

Existen factores abióticos que influyen en la concentración de ISO como la senescencia de las hojas, la cual provoca una disminución de la concentración, mientras que altas concentraciones de CO_2 la incrementan en trigo (*Triticum aestivum*) (Peñuelas *et al.,* 2000). Al tratar con metil jasmonato o inducir estrés salino en el trigo sarraceno o alforfón (*Fagopyrum esculentum*) se incrementa la concentración de ISO en el grano (Kim *et al.,* 2011; Lim *et al.,* 2012). La época de colecta es otro factor que regula la concentración de ISO; en las hojas del bambú (*Sasa argenteastriatus)* la mayor concentración del flavonoide se presenta entre otoño e invierno (Ni *et al.,* 2012). Los ciclos de luz y obscuridad también afectan la producción de ISO. En la planta *Fagopyrum tataricum* ciclos de 8 h de luz/oscuridad inducen una producción de ISO de 0.25 mg/g de peso seco, por encima de la concentración alcanzada con ciclos de 10 h, donde el rendimiento es de 0.1 mg/g de peso seco (Li *et al.,* 2012).

Efectos biológicos de ISO

ISO se encuentra presente en plantas usadas por la medicina tradicional para tratar una amplia variedad de afecciones (López-Lázaro, 2009). Las actividades biológicas que ISO ejerce sobre células o tejidos animales son diversas; la más reportada es su actividad antioxidante, la cual se ha documentado en extractos de flores y hojas de *Aloe vera* (Keyhanian *et al.,* 2007; Ranghoo-Sanmukhiya *et al.,* 2010), las partes aéreas de *Commelina communis* (Shibano *et al.*, 2008) y las hojas de *Gentiana piasezkii* (Wu *et al.*, 2006), entre otros. ISO mostró también un efecto inhibitorio de la amplitud de contracciones espontáneas en útero de rata y cobayo dependiente de la concentración, sugiriendo que ISO tiene efecto miolitico a bajas concentraciones (Afifi *et al.,* 1999). Por otro lado, ISO presentó actividad antiviral en embriones de pollos infectados con el virus APMV-10, causante de la enfermedad de Newcastle (Abd-Alla *et al.,* 2012). También presenta efecto ansiolítico en ratones Swiss albinos (Okuyama *et al.,*1996), y reduce la actividad proliferativa de las células HepG2 (células de hepatoblastoma) (Pacifico *et al.,* 2010). De igual manera ISO presenta actividad antihipertensiva y cardioprotectora en ratas Wistar macho (Mali *et al.,* 2012).

Extractos de diferentes plantas, en los cuales se mostró la presencia de ISO, presentan diferentes actividades biológicas. Los extractos de los frutos de *Anthopterus wardii* disminuyen la inflamación inducida por la interleucina 8, e inhiben la expresión de la metaloproteinasa de la matriz tipo 1 con una actividad máxima inhibitoria de 100 µg/ml en células epiteliales pequeñas de la vía respiratoria (Dastmalchi *et al.,* 2011; Flores *et al.,* 2012), esta actividad también la

presentan los extractos etanólico y acuoso de *Biophytum sensitivum* en macrófagos (Qiao *et al.,* 2012). Otra actividad reportada para extractos obtenidos de *Eminium spiculatum* es la actividad antibacterial contra *Escherichia coli* con una concentración de 1 µg/mL (Afifi *et al.,* 2011), los extractos de la melaza de la caña de azúcar contra *Streptococcus sobrinus* con una concentración mínima inhibitoria CMI[1]= 4 mg/mL (Takara *et al.,* 2007), los extractos de hojas de *Aloe vera* contra *Klebsiella pneumonine* con una CMI=12.8 mg/mL y contra *staphylococcus aurous* con una CMI = 29.8 mg/mL (Ranghoo-Sanmukhiya *et al,* 2010). El extracto etanólico de las hojas de *Echinodorus grandiflorus* presenta actividad anti edematogénica en ratones Swiss hembra (Garcia *et al.,* 2010). Además, el extracto proveniente de *Gentiana asclepiadea*, previene el daño del ADN por oxidación en células HEK293 de riñón humano (Hudecová *et al,.* 2012). El extracto butanólico de *Pasiflora edulis* presenta efecto sedativo a dosis de 20 mg/kg (Deng *et al.,* 2010). Los extractos de flores de *Gentiana oliveri* y de las hojas de *Cecropia pachystachya* inducen efecto hipoglicémico en ratas wistar diabéticas inducidas con aloxan. De igual manera el extracto de las partes aéreas de *Biophytum sensitivum* presenta efecto hipoglucémico en conejos diabéticos inducidos con aloxan (Sezik *et al,* 2005; Aragão *et al,.* 2010; Qiao *et al.*, 2012). Los extractos acuosos de *Cecropia obtusifolia* y *Cecropia peltata* inhiben la gluconeogénesis con una IC_{50}=224 µg/mL e IC_{50}=160 µg/mL respectivamente en ratas Wistar diabéticas inducidas con estreptozotocina (Andrade-Cetto *et al.,* 2010).

Debido a los múltiples efectos biológicos de ISO y al bajo rendimiento obtenido en la naturaleza, algunos trabajos han explorado la síntesis química de

este isoflavonoide para producir cantidades suficientes del compuesto con miras a su posible uso terapéutico. La figura 3 esquematiza las reacciones químicas de la síntesis de ISO, en la cual a partir de floroacetofnona se obtiene un rendimiento total del 15% en 10 pasos. La C-glucosil fluoroacetofenona derivada de un intermediario de síntesis contiene un grupo hidroxilo libre obtenido por la hidrogenólisis, para tomar las ventajas de las diferentes velocidades de hidrogenólisis protegiendo al grupo bencilo y al 2-metilbencilo. La condensación del derivado del aldol de la C-glucosil fluoroacetofenona deriva con 3,4-bis-benzilloxibenzaldehido que ofrece a la chalcona correspondiente como un precursor de la 6-C-glucosil flavona (Kumazawa *et al,*. 2000).

Farmacocinética de ISO

Es poco lo que se conoce sobre la farmacocinética de ISO. La administración del Isoflavonoide a ratas por vía intravenosa permite que el compuesto llegue a riñón, pulmón y corazón, y su tiempo de excreción es corto (Huang *et al.,* 2011). Sí la vía de administración es oral, ISO puede ser transformado en el intestino, debido a que se han encontrado bacterias de la flora intestinal humana que modifican su estructura. La bacteria CG19-1, una nueva especie de *Lachnosporaceae* degrada ISO a 3-(3,4-dihidroxifenil) ácido propiónico vía luteolina (Braune *et al.,* 2011). Otra bacteria que interviene en el metabolismo de ISO es *Eubacterium cellulosolvens,* la cual convierte a ISO en orientina (Braune y Blaut, 2012).

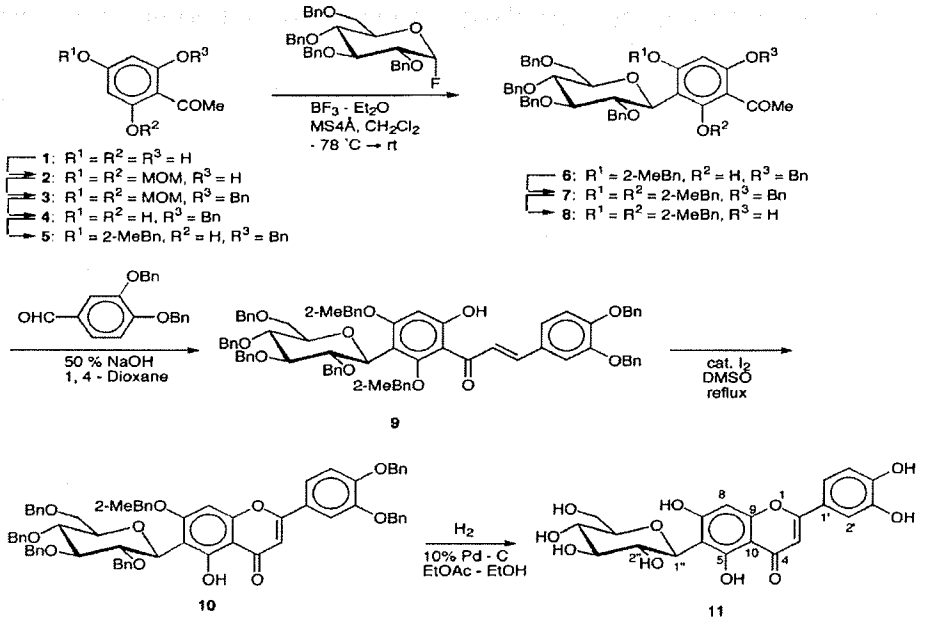

Figura 3. Síntesis química de Isoorientina (tomado de Kumazawa *et al.*, 2000)

ISO induce la captación de glucosa en adipocitos múridos y humanos e inhibe la adipogénesis en preadipocitos de ambos linajes celulares

ISO está presente en numerosas plantas empleadas como antidiabéticos por distintos sistemas de medicina tradicional. Dado que se desconocen los mecanismos de su efecto, nosotros estudiamos dichos mecanismos. Nuestros resultados muestran que ISO estimula la captación de glucosa por adipocitos múridos y humanos tanto sensibles como resistentes a insulina. Los adipocitos sensibles a insulina incorporaron marcadamente 2-NBDG en presencia de la hormona, mientras que las células resistentes a insulina no incorporaron el análogo. Sin embargo en los cultivos tratados con ISO se obtuvo un incremento robusto en la incorporación del 2-NBDG tanto en células sensibles como en

células resistentes a insulina. El efecto estimulante de ISO fue dependiente de la concentración y se presentó tanto en células múridas como humanas. La inducción de la captación de glucosa por adipocitos fue suprimida por los inhibidores de la vía de señalización de la insulina, HNMPA-$(AM)_3$ (inhibidor del receptor de insulina), wortmanina (inhibidor de PI3K), inhibidor de las isoformas a y b de AKT (AKT 1/2) e indinavir (inhibidor de GLUT4). Estos inhibidores también bloquearon la fosforilación correcta de los componentes de la vía de señalización de la insulina inducida por ISO en adipocitos sensibles y resistentes a la hormona. Adicionalmente, ISO estimuló la transcripción de genes que codifican los componentes de la vía de señalización de la insulina en adipocitos múridos sensibles y resistentes a la hormona. En células sensibles a insulina ISO incrementó la expresión de IR, IRS1, IRS2 y PI3K a niveles incluso mayores que los inducidos por insulina, mientras que los genes AKT1, AKT2, GLUT4 y GLUT1 mantuvieron un nivel de expresión similar sin importar si las células son inducidas con ISO o insulina. En células resistentes, tanto ISO como insulina indujeron la expresión de los genes al mismo nivel excepto por IRS2, el cual mostró una mayor expresión con insulina. En resumen nuestros resultados indican que ISO ejerce sus efectos antidiabéticos através de la vía de señalización de la insulina en adipocitos, revierte la resistencia a insulina causada en estas células por estimulación con el factor de necrosis tumoral tipo alfa (TNF-α) mediante la estimulación apropiada de las proteínas en esta vía de señalización, e induce la expresión de los genes que codifican para estas proteínas (Alonso-Castro *et al.*, 2012).

Otros compuestos conocidos por su capacidad de incrementar la captación de glucosa como las tiazolidinedionas, e.g. rosiglitazona, actúan uniéndose al tipo gamma del receptor activado por inductores de la proliferación peroxisomal (PPARγ), promoviendo el efecto proadipogénico de dichos compuestos. Por ello, en otra serie de experimentos mostramos que ISO afecta marcadamente la adipogénesis múrida y humana. Concentraciones inocuas del compuesto (1-50 μM) inhibieron la acumulación lipídica en preadipocitos 3T3-F442A en función de la concentración. Asimismo, ISO 50 μM disminuyó la acumulación lipídica en preadipocitos humanos subcutáneos en un 80% lo que nos permite concluir que ISO bloquea la adipogénesis múrida y humana. Por el contrario, ISO no afectó la acumulación lípidica en adipocitos terminalmente diferenciados de ninguno de los dos linajes, lo que sugiere que ISO carece de efectos lipoliticos. La expresión de los genes que codifican para los factores proadipogénicos PPARγ, la isoforma alfa de la proteína de unión a secuencias CCAAT (C/EBPα) y el factor tipo kruppel número 4 (KLF4) se redujo después de 48 h de tratamiento con ISO, mientras que se incrementó la expresión de los genes que codifican para los factores antiadipogénicos proteína 2 de unión a secuencias GATA (GATA2) y WNT10B. Se sabe que la actividad de ISO sobre células HepG2 es mediada por el factor de transcripción Nrf2 (Lim *et al.*, 2007). Para determinar si Nrf2 media también los efectos de ISO sobre células adiposas analizamos su expresión génica y su localización celular de la proteína en preadipocitos 3T3-F442A. Encontramos que en los cultivos tratados con ISO la expresión de la proteína Nrf2 así como su acumulación en núcleo se incrementó, a partir de las 48 h, mientras que en las

células tratadas con medio adipogénico la acumulación de la proteína disminuyó respecto a la condición inicial. En resumen, nuestros resultados muestran que ISO inhibe reversiblemente la adipogénesis por modulación de la expresión de factores de transcripción proadipogénicos y antiadipogénicos a través de la vía de señalización Nrf2. Esto sugiere que la isoflavona puede ser un candidato promisorio para el desarrollo de nuevos tratamientos antiobesidad (Articulo en preparación).

En las células troncales mesenquimales el incremento en la expresión de WNT10b inhibe la adipogénesis y además estimula la osteogénesis (Lefterova y Lazar, 2009). Por ello analizamos el efecto de ISO sobre la expresión de los marcadores de osteogénesis, tales como la actividad de fosfatasa alcalina y la deposición extracelular de calcio y encontramos que ISO es capaz de inducir ambos marcadores en preadipocitos 3T3-F442A (figuras 4 y 5). Adicionalmente, para evaluar los efectos osteogénicos de ISO sobre un linaje osteoblástico, obtuvimos cuatro poblaciones de osteoblastos de rata, de tres días de nacidas, por digestión con colagenasa o explantes (figura 6). Nuestros resultados muestran que las cuatro poblaciones de osteoblastos de rata presentan la apariencia celular típica del linaje durante la fase de proliferación. Cultivadas en condiciones osteogénicas, las poblaciones de osteoblastos de rata mostraron diferente grado de actividad de fosfatasa alcalina y todas expresan los marcadores osteogénicos ATF4 y RUNX2 (figura 6). Hasta el momento hemos determinado las concentraciones de ISO no citotóxicas para la población de osteoblastos de las parietales de rata que mostró la mayor actividad de la fosfatasa alcalina. Concentraciones de ISO de 25 μM o menores no afectaron significativamente la

viabilidad celular, en tanto que ISO 50 y 75 µM redujeron el crecimiento celular en 21% y 35 % respectivamente, comparada con las células control (figura 7). Experimentos futuros con estas poblaciones de osteoblastos de rata y poblaciones de osteoblastos humanos normales que recientemente establecimos (datos no mostrados) nos permitirán estudiar los mecanismos moleculares a través de los cuales ISO modula efectos osteogénicos.

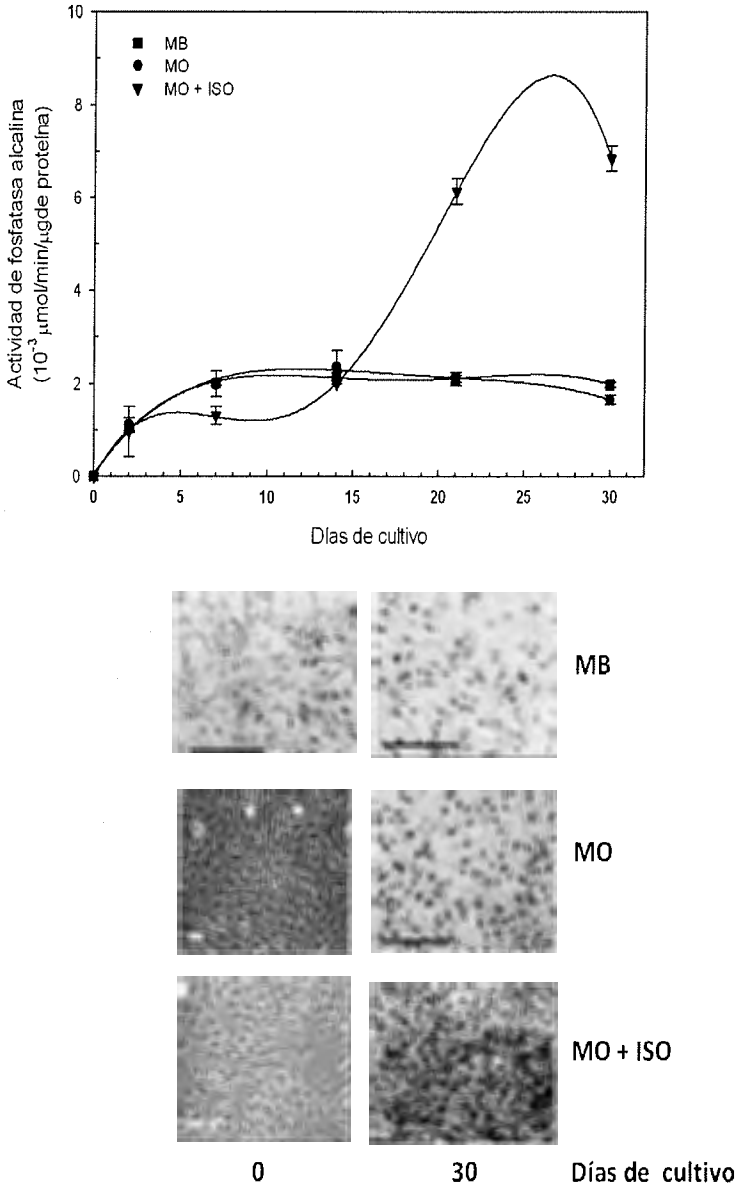

Figura 4. ISO induce la actividad de la fosfatasa alcalina en preadipocitos 3T3-F442A. Cultivos confluentes de preadipocitos 3T3-F442A se cultivaron por 31 días en medio basal (MB), medio osteogénico (MO), medio osteogénico suplementado con ISO 50 µM (MO + ISO). En los días señalados se extrajo la proteína total de cultivos bajo los distintos tratamientos para analizar la actividad de la fosfatasa alcalina mediante la hidrólisis del 4-nitrofenil fosfato.

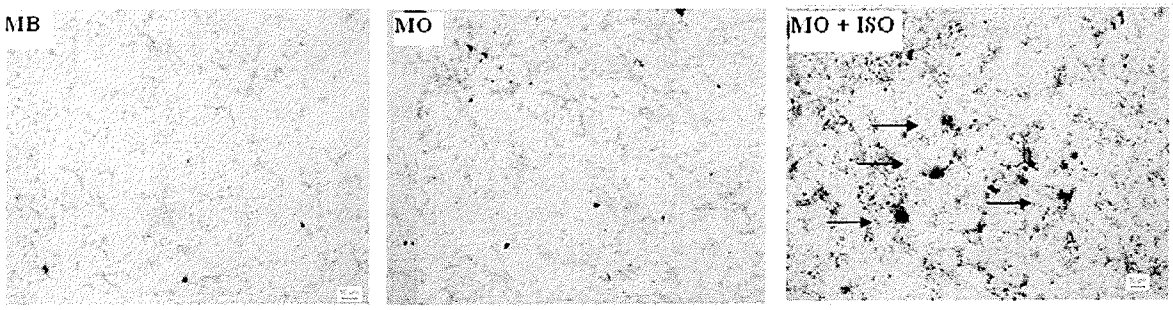

Figura 5. ISO induce la formación de depósitos de calcio en preadipocitos 3T3-F442A. Cultivos confluentes de preadipocitos 3T3-F442A se mantuvieron por 43 días en medio basal (MB), medio osteogénico (MO) y medio osteogénico suplementado con ISO 50 μM (MO + ISO). Al cabo del tratamiento las células fijadas se tiñeron por el método de von Kossa para observar los depósitos de calcio. Las flechas indican la formación de tales depósitos.

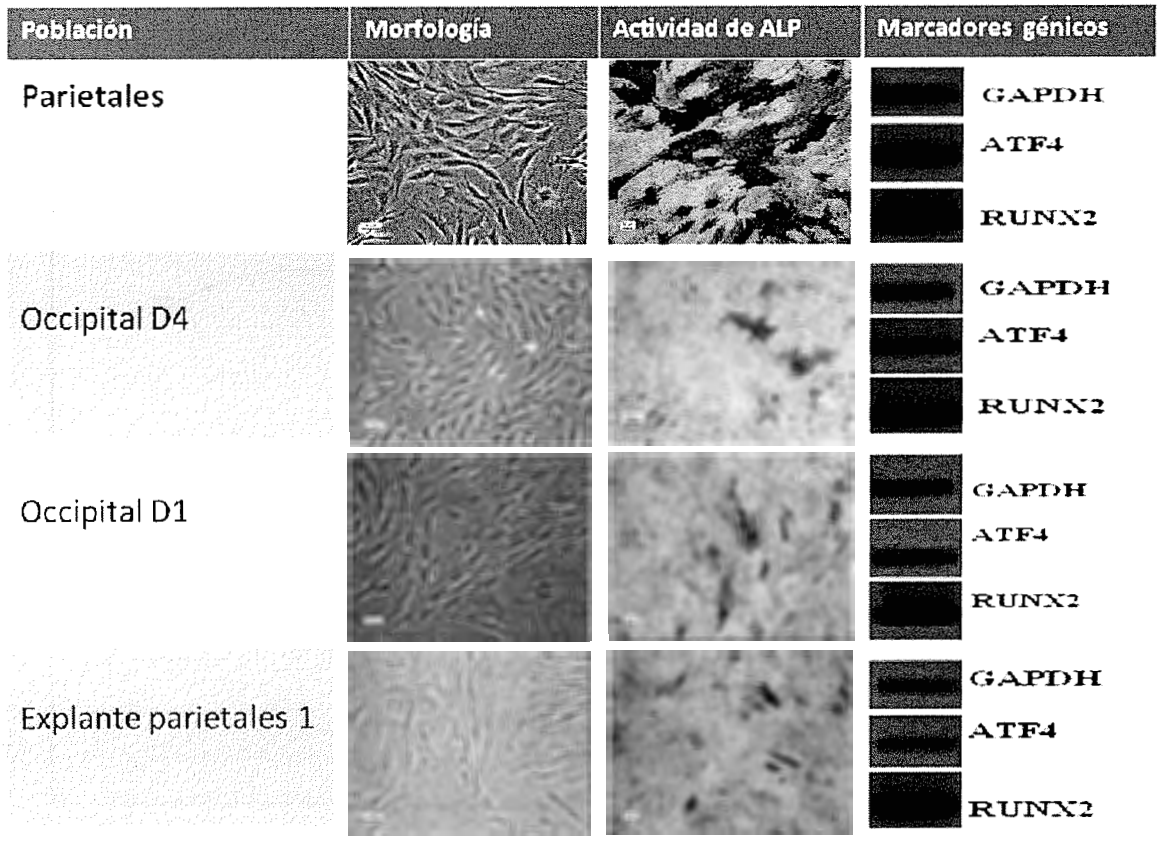

Figura 6. Caracterización de poblaciones de osteoblastos primarios de rata. Osteoblastos de rata de tres días de nacidas se aislaron del hueso señalado por digestión con colagenasa o mediante explante. Células de las poblaciones obtenidas se sembraron en cajas de 35 mm (2 X 10^4 células por caja) con medio basal (MB). Cuatro días después, al llegar a confluencia, se evaluó la morfología de cada la población y los cultivos se realimentaron con medio osteogénico (MO). Dos días después se extrajo el RNA total para analizar la expresión génica de GAPDH, ATF4 y RUNX2. La actividad de la fosfatasa alcalina se evaluó luego de 15 días de cultivo.

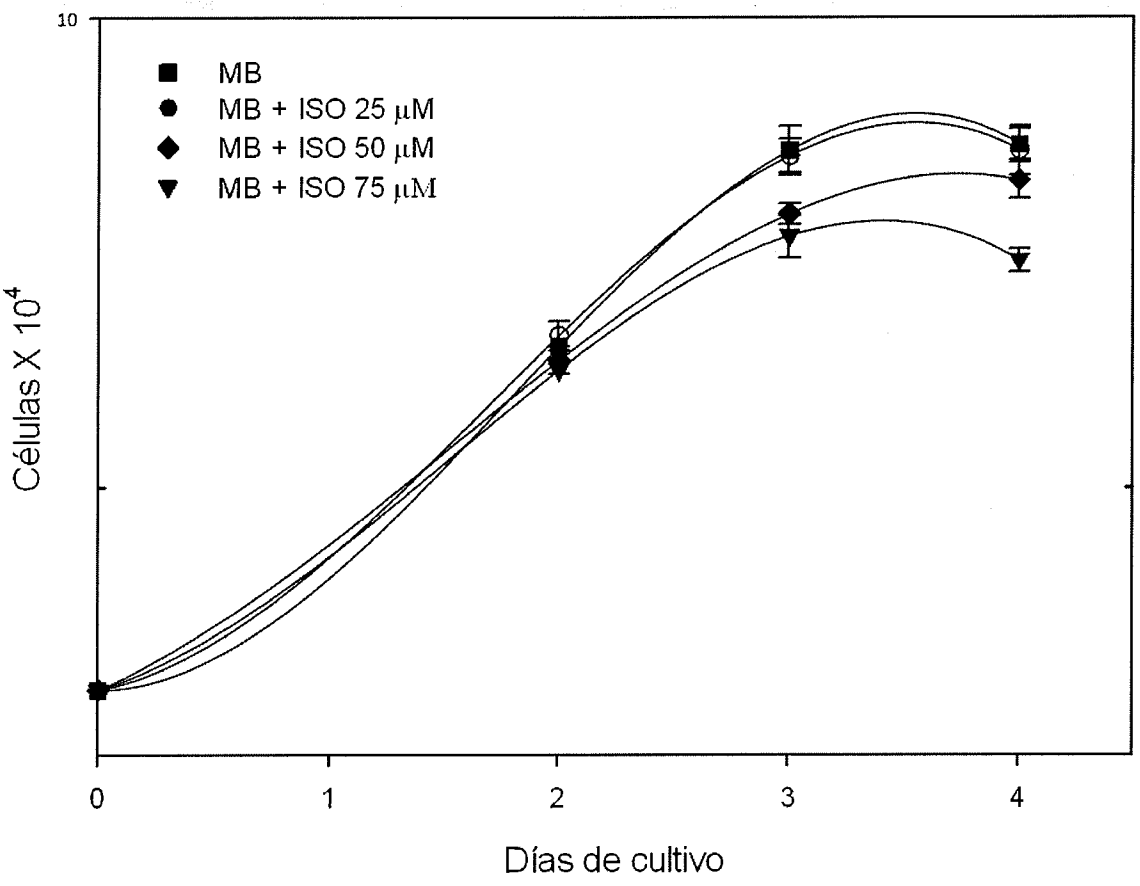

Figura 7. Determinación de las concentraciones inocuas de ISO para osteoblastos de rata. Osteoblastos de las parietales de rata provenientes del tercer subcultivo se sembraron en placas de 24 pozos (1 X 10^4 células por pozo) con medio basal (MB). Dos días después los cultivos se realimentaron con MB o MB adicionado con ISO a las concentraciones señaladas. El número de células de cada tratamiento se determinó a los días indicados por recuento celular con hematímetro. La figura representa la media de dos experimentos independientes con tratamientos por duplicado ±SD

Discusión

ISO es un C-glucosilflavonoide que se encuentra en muchas plantas empleadas por la medicina tradicional como antidiabéticas (López-Lázaro, 2009). Se considera que ISO es uno de los compuestos responsable del efecto hipoglicémico en algunas de estas plantas (Sezik *et al.,* 2005; Aragão *et al.,* 2010; Qiao *et al.,* 2012). Sin embargo poco se sabe sobre los mecanismos moleculares mediante los cuales ejerce su efecto antidiabético. Hasta la fecha solo se ha reportado que ISO inhibe la gluconeogénesis (Andrade-Cetto *et al.,* 2010).

Se ha mostrado que otros compuestos aislados de plantas usadas como antidiabéticas por la medicina alternativa también son capaces de inducir la captación de glucosa. Shikonin, un derivado de naftoquinona, estimula la captación de glucosa en adipocitos 3T3-L1 (Kamei *et al.,* 2002). El gensinosido Rg1 promueve la captación de glucosa en la línea celular de mioblastos de ratón (C2C12) resistentes a la insulina (Lee *et al.,* 2012). Sin embargo, el efecto de estos compuestos en células humanas es aún desconocido. Otro trabajo mostró que el ácido clorogénico, un ester de ácido cafeico estímulo la captación de glucosa en adipocitos 3T3-F442A sensibles y resistentes a insulina, el efecto mostrado es comparable al obtenido con insulina o roziglitazona (Alonso-Castro *et al.* 2008). Otros compuestos conocidos por su capacidad para incrementar la captación de glucosa como las tiazolidinedionas actúan uniéndose a PPARγ promoviendo la adipogénesis. Para analizar si ISO presenta efecto antiadipogénico y alguno de los probables mecanismos mediante los cuales actúa, nosotros evaluamos el efecto de ISO sobre la adipogénesis en cultivos de

preadipocitos 3T3-F442A y humanos normales. Encontramos que ISO afecta marcadamente la adipogénesis múrida, dependiendo de la concentración (1-50 μM) y en Preadipocitos humanos subcutáneos observamos que ISO 50 μM disminuyó 80% la acumulación lipídica ($P \leq 0.05$). Las diferencias para bloquear la adipogénesis en células grasas múridas y humanas en respuesta a ISO podría ser el distinto grado de homogeneidad en las poblaciones. En ambos casos nuestros resultados muestran un claro efecto de ISO sobre el bloqueo de la adipogénesis, estos resultados sugieren que ISO podría ser responsable del efecto antiadipogénico que muestran plantas empleadas en la medicina tradicional contra la obesidad.

El bloqueo de la adipogénesis por ISO en preadipocitos 3T3-F442A ocurrió durante la etapa del compromiso de las células a la diferenciación terminal. La expresión de los genes antiadipogénicos se vio disminuida en el medio adipogénico (MA), condiciones optimas para permitir la diferenciación adipogénica, esto concuerda con lo reportado en la literatura (Lefterova *et a.,* 2009). Se ha reportado que diversos compuestos son capaces de inhibir la adipogénesis, por disminución de la expresión de los genes proadipogénicos (CEBPα y PPARγ) como la lactoferrina en células MC3T3-G2/PA6 (Yagi *et al.,* 2008). De igual manera encontramos aquellos compuestos que suprimen la expresión de los genes proadipogénicos vía WNT como la genisteína en células 3T3-L1(Harmon *et al.* 2002).

Se sabe que la actividad de ISO sobre células HepG2 es mediada por el factor de transcripción Nrf2 (Lim *et al.*, 2007). Se ha reportado que la deficiencia

de Nrf2 deteriora la adipogénesis y protege de la obesidad inducida por la dieta en ratones (Pi *et al.,* 2010). Resultados que obtuvimos mediante bioinformatica muestran que Nrf2 tiene probables genes blanco implicados en la adipogénesis temprana; PPARγ, CEBPα, CEBPβ, CEBPδ y KLF4. De los cuales se ha demostrado mediante ensayos GST pull-down la interacción directa entre PPARγ - Nrf2 en macrófagos (Ikeda *et al.,* 2000) y ensayos de inmunoprecipitación de cromatina indican la interacción entre Nrf2-CEBPβ en adipocitos 3T3-L1 (Hou *et al.,* 2012). Nuestros análisis de expresión génica muestran que ISO incrementa la expresión de Nrf2, al igual que la acumulación de la proteína Nrf2 en núcleo a partir de las 48 h. Resultados reportados para células de linaje adiposo (3T3-L1) muestran que la disminución de la expresión de Nrf2 disminuye la adipogénesis (Pi *et al.,* 2010; Hou *et al.* 2012). Sin embargo en células ST2 y MEFS la disminución en la expresión de Nrf2 promueve la adipogénesis (Shin *et al.,* 2007; Chartoumpekis *et al.,* 2011). Estos resultados son notorios debido que a pesar de su cercanía del linaje celular 3T3-F442A con el 3T3-L1, las células responden al igual que las células ST2 y MEFS.

Los reportes sobre los mecanismos moleculares de la acción de ISO se han descrito en células HepG2, donde la actividad antioxidativa es mediada por la inducción de Nrf2 en respuesta a la señalización de PI3K (Lim *et al.*, 2007). A su vez induce apoptosis la cual es mediada a través de la disfunción mitocondrial vía PI3K/AKT (Yuan *et al.,* 2012), mientras inactiva ERK1/2 y activa a las cinasas JNK y p38 y simultáneamente estimula a ROS, lo que activa la vía de señalización MAPK cinasas (Yuan *et al.*, 2013). Sin embargo ISO no tiene efecto tóxico sobre

células normales de hígado (Yuan *et al.,* 2012). El bloqueo de la adipogénesis puede dar lugar a re direccionar otra vía de diferenciación, en las células troncales mesenquimales el incremento en la expresión de WNT10b inhibe la adipogénesis al tiempo que estimula la osteogénesis (Lefterova y Lazar, 2009). Esto concuerda con nuestros resultados. Como se puede observar es poco lo reportado sobre mecanismos moleculares de ISO, por ello nuestros resultados resultan un aporte al conocimiento de dichos mecanismos.

CONCLUSIÓN

ISO estimuló la captación de glucosa epigenética y géneticamente. A nivel epigénetico el compuesto induce la correcta fosforilación de las proteínas de la vía de señalización de la insulina y a nivel génico, induce la expresión de los genes que codifican dichas proteínas tanto en células sensibles como resistentes a insulina.

ISO tiene un claro efecto antiadipogénico que actúao durante el periodo del compromiso a la diferenciación. Inhibe la expresión de factores de transcripción permisivos de la adipogénesis e induce la expresión de genes antiadipogénicos. Estos cambios en la expresión génica son mediados por la vía de señalización Nrf2.

PERSPECTIVAS

La capacidad de ISO para estimular la captación de glucosa por células sensibles y resistentes a insulina, su claro efecto antiadipogénico y la posibilidad de que estimule la osteogénesis, confieren al compuesto potencial antidiabético, antiobesidad y antiosteoporótico. La validación de este potencial demanda extender el conocimiento acerca de la farmacocinética y la farmacodinámica del compuesto, así como de sus posibles efectos adversos.

Estos estudios, y la confirmación *in vivo* de sus efectos biológicos deseables, son indispensables para determinar la utilidad del compuesto para el desarrollo de nuevos tratamientos contra la obesidad, la diabetes y la osteoporosis.

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Anexo

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Isoorientin Reverts TNF-α-Induced Insulin Resistance in Adipocytes Activating the Insulin Signaling Pathway

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Isoorientin (ISO) is a plant C-glycosylflavonoid with purported antidiabetic effects but unexplored mechanisms of action. To gain insight into its antidiabetic mechanisms, we assayed nontoxic ISO concentrations on the 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxy-D-glucose (2-NBDG) uptake by murine 3T3-F442A and human sc adipocytes. In insulin-sensitive adipocytes, ISO stimulated the 2-NBDG uptake by 210% (murine) and 67% (human), compared with insulin treatment. Notably, ISO also induced 2-NBDG uptake in murine (139%) and human (60%) adipocytes made resistant to insulin by treatment with TNF-α, compared with the incorporation induced in these cells by rosiglitazone. ISO induction of glucose uptake in adipocytes was abolished by inhibitors of the insulin signaling pathway. These inhibitors also blocked the proper phosphorylation of insulin signaling pathway components induced by ISO in both insulin-sensitive and insulin-resistant adipocytes. Additionally, ISO stimulated the transcription of genes encoding components of insulin signaling pathway in murine insulin-sensitive and insulin-resistant adipocytes. In summary, we show here that ISO exerts its antidiabetic effects by activating the insulin signaling pathway in adipocytes, reverts the insulin resistance caused in these cells by TNF-α by stimulating the proper phosphorylation of proteins in this signaling pathway, and induces the expression of genes encoding these proteins. (*Endocrinology* 153: 0000–0000, 2012)

Diabetes mellitus is a heterogeneous complex of metabolic disorders with diverse etiopathogenesis, characterized by the common trait of chronic hyperglycemia (1). Type 2 diabetes, resulting from insulin resistance and a progressive deficiency in the secretion of this hormone (2), accounts for more than 90% of diabetes cases worldwide (3), and its incidence is growing at alarming rates.

The insulin resistance that characterizes type 2 diabetes results from dysfunctions in the insulin signaling pathway. Induction of glucose uptake by insulin on its target cells, *i.e.* adipocytes and muscle cells, is initiated by its binding to insulin receptor (IR), which consist of disulfide-linked subunits in a β-α-α-β configuration. The α-subunit is completely extracellular, whereas the β-subunit possesses a single transmembrane domain with tyrosine kinase activity. The binding of insulin to the α-subunit leads to autophosphorylation of the β-subunits of receptor and activate IR substrates (IRS) by phosphorylation, which in turn activate p85, the regulatory subunit of phosphatidylinositol-3-kinase (PI3K). When PI3K phosphorylates the two key residues of protein kinase B (AKT), T308 and S473, the translocation of glucose transporter (GLUT)4 vesicles from their intracellular pool to the plasma membrane is stimulated, and glucose uptake is initiated (4).

Thiazolidinediones (TZD) and other drugs are used to restore insulin responsiveness in type 2 diabetic patients (5). However, TZD does not restore a proper glycemic control, and all of them have undesirable side effects (5).

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Abbreviations: AKT, Protein kinase B; BSA, basal medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; HNMPA-(AM)$_3$, hydroxy-2-naphthalenylmethylphosphonic acid tris-acetoxy-methyl ester; IR, insulin receptor; IRS, IR substrate; ISO, isoorientin; L15, Leibovitz L15; 2-NBDG, 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxy-D-glucose; PI3K, phosphatidylinositol-3-kinase; qRT-PCR, quantitative RT-PCR; ROZ, rosiglitazone; TZD, thiazolidinedione.

In particular, TZD inhibits hepatic regeneration (6), induces obesity (7), and causes osteoporosis (8). Such side effects have greatly stimulated the search for new compounds that can restore the insulin sensitivity in diabetic tissues but without the undesirable effects of drugs currently in use (9, 10).

A number of plant compounds have been documented to exert antidiabetic properties (11, 12). Among these active principles, shikonin (13, 14), tannic acid (15), 1,2,3,4,6-penta-O-galloyl-α-D-glucopyranose (16), kaempferitrin (17), diverse ginsenosides (18–20), and luteolin (21) have been shown to induce glucose uptake mediated by insulin signaling pathway in adipocytes or to improve insulin sensitivity in other cell lineages. However, most of these studies were restricted to analyze the effect of the compounds on insulin-sensitive cells without addressing their effect on diabetic-like adipocytes.

Isoorientin (ISO) (Fig. 1A) is a C-glycosylflavonoid present in many plants employed by diverse complementary and alternative medicine systems for the treatment of type 2 diabetes (22). ISO was shown to reduce plasmatic glucose, cholesterol, and triglyceride concentrations in streptozotocin-induced diabetic rats (23). The mechanisms mediating the antidiabetic properties of ISO are still unknown, although results obtained with animal models and clinical trials rule out the possibility that these properties may be due to insulin-secreting effects (23–25) or modulation of the intestinal absorption of glucose (26). In this work, we used *in vitro* cultures of murine and human adipocytes to investigate the antidiabetic mechanisms of ISO. Our results show that ISO stimulates the glucose uptake in both insulin-sensitive and insulin-resistant adipocytes through an insulin-like signaling pathway, inducing the phosphorylation of IR, PI3K, and AKT in insulin-sensitive and diabetic-like adipocytes and stimulating the gene expression of components of the insulin signaling pathway.

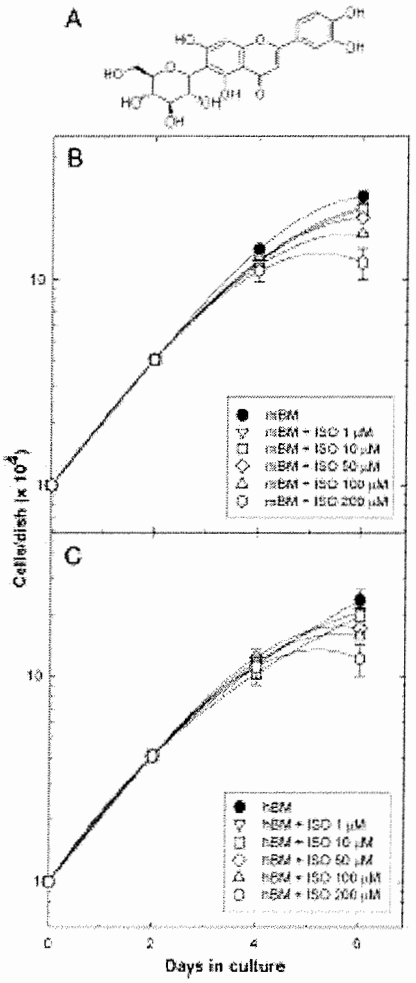

FIG. 1. Determination of innocuous ISO concentrations for murine and human preadipocytes. Murine 3T3-F442A (B) and normal human sc preadipocytes (C) were seeded in 24-well culture plates (1×10^4 cells/well) with BM. Two days later, cultures were fed with BM added with the indicated ISO (A) concentrations, and cell number in each treatment was determined at the signaled days by direct cell counting in a hematocytometer. Figures represent the mean value of two independent experiments in triplicate ± SD. mBM, Murine BM; hBM, human BM.

Materials and Methods

Reagents and cell lines

Murine 3T3-F442A preadipose cell line was a gift from W. Kuri-Harcuch (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico City, Mexico). Human preadipocytes were isolated by collagenase digestion (27) of sc adipose tissue samples obtained from nondiabetic patients undergoing elective cosmetic surgery, who gave their informed consent for the use of discarded tissues and with the approval of the corresponding Research Ethic Committee. DMEM, Leibovitz L15 (L15) medium and fetal bovine serum (FBS) were from GIBCO BRL (Grand Island, NY), whereas calf serum was from HyClone (Logan, UT). ISO, purchased from Chromadex (Santa Ana, CA), was 95% purity according to the manufacturer. Human TNF-α and 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxy-D-glucose (2-NBDG) were obtained from Peprotech (London, UK) and Invitrogen (Carlsbad, CA), respectively. Rosiglitazone (RGZ), from Cayman Chemical Co. (Ann Arbor, MI), was 98% purity according to the manufacturer. Hydroxy-2-2naphthalenylmethylphosphonic acid tris-acetoxy-methyl ester [HNMPA-(AM)$_3$], Wortmannin, and AKT 1/2 kinase inhibitor were from Calbiochem (San Diego, CA). Indinavir was a gift from Merck Sharp & Dohme (Mexico City, Mexico).

Cell viability assays

Murine and human preadipocytes were seeded in murine basal medium (BM) (DMEM added with 7% calf serum) or human BM (L15 added with 5% FBS) in 24-well plates at a density of 1×10^4 cells/well. Two days later, cultures were fed with BM added with concentrations of ISO ranging from 1 to 200 μM. Cell cultures were maintained with change of medium every other day at 37 C in humidified atmospheres containing (DMEM) or not (L15) 5% CO_2. Cell viability was monitored for several days by direct cell counting in a hemacytometer.

2-NBDG uptake assays

Murine 3T3-F442A and human adipocytes were differentiated on 96-well fluorescence plates as described previously (28). Briefly, 3T3-F442A preadipocytes were induced to adipogenesis with DMEM containing 10% FBS, insulin 5 μg/ml, and d-biotin 1 μM, whereas human preadipocytes were differentiated into adipocytes with L15 medium containing 5% FBS, 25 μM 3-isobutyl-1-methylxanthine, 100 nM dexamethasone, 1 μM RGZ, 100 nM insulin, 0.2 nM and triiodothyronine for 3 d and thereafter maintained in L15 medium supplemented with 5% FBS, 100 nM insulin, 0.2 nM and triiodothyronine. Mature adipocytes were incubated for 60 min with PBS containing 1 mg/ml BSA and 80 μM fluorescent glucose analog 2-NBDG (29) in the presence of nontoxic concentrations of ISO. Control cultures were treated with 100 nM insulin or 10 μM RGZ. After incubation, free 2-NBDG was washed out from cultures, and fluorescence retained in cell monolayers was measured with a Tecan-GENios fluorescence microplate reader (Tecan, Salzburg, Austria). Values of 2-NBDG uptake in the absence of insulin were subtracted from those obtained with 100 nM insulin to establish 100% of specific 2-NBDG incorporation. ISO effects on 2-NBDG uptake were also evaluated on 3T3-F442A and human adipocytes preincubated with 10 ng/ml TNF-α for 7 d to induce insulin resistance (30).

The effect of ISO on 2-NBDG uptake was also assayed in the presence of insulin signaling pathway inhibitors. Mature murine and human adipocytes were washed from adipogenic medium and incubated with PBS/BSA for 2 h. Inhibitors for IR tyrosine kinase activity [HNMPA-$(AM)_3$, 200 μM] (31), PI3K (Wortmannin, 200 nM) (32), AKT 1/2 (AKT Inhibitor VIII trifluoroacetate salt hydrate, 420 nM) (33), and GLUT4 (Indinavir, 100 μM) (34) were individually added in PBS/BSA to cell cultures for 1 h. After incubation, 80 μM 2-NBDG and nontoxic concentrations of ISO were added to cell cultures. Control cultures were treated with 100 nM insulin. After incubation, cells were washout with PBS/BSA, and the glucose uptake was quantified as described above.

Phosphorylation of insulin signaling pathway elements

The total content of phosphorylated IR (β-subunit) in insulin-sensitive and insulin-resistant 3T3-F442A and human adipocytes was analyzed using IR phosphotyrosines 1162 and 1163 [IR (pYpY1162/1163)] ELISA kit (Biosource, Camarillo, CA). The amounts of phosphorylated p85 of PI3K and T308 and S473 residues of AKT were measured in insulin-sensitive and insulin-resistant adipocytes using the Cellular Activation of Signaling ELISA kit following the manufacturer's instructions (SuperArray Biosciences, Frederick, MD). Studies were carried out as the same way as for the inhibitor assays with minimal modifications. The time of incubation was 20 min and D-glucose (Invitrogen) was used instead of 2-NBDG. Plates were read at 450 nm in an ELISA reader (Bio-Rad Laboratories, Hercules, CA). The relative extent of target protein phosphorylation was determined normalizing the phosphoprotein ratio to the total protein for the same experimental condition. The values of phosphorylation obtained in the presence of 100 nM insulin were subtracted to those obtained with each inhibitor concentration to establish the 100% of specific phosphorylation.

Gene expression analysis of insulin signaling pathway elements

Mature insulin-sensitive and insulin-resistant murine 3T3-F442A adipocytes were starved for 22 h in PBS and then fed with PBS added with 100 nM insulin or 50 μM ISO for 1 h at 37 C. Cell monolayers were then lysed directly with TRIzol (Invitrogen), and total RNA was isolated according to the manufacture's instructions. RNA integrity in the samples was confirmed using 8S and 28S rRNA, and RNA concentration was determined by spectroscopy at 260 nm. After deoxyribonuclease I digestion (Fermentas, Hunover, MD), 6 μg of total RNA were RT using Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Gene expression of IR, IRS1, IR52, PI3K, AKT1 AKT2, GLUT1, GLUT4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using probes designed with IDT Scitools RealTime PCR (Integrated DNA Technologies, Coralville, IA) (Table 1) and employing PrimeTime qPCR assays (Integrated DNA Technologies), QuantitectProbe PCR

TABLE 1. Primer pair sequences used for RT-PCR

Name	Accession no.	Sense primer	Antisense primer
IR	NM_010568.2	AATAATGAGCTCTGCTACCTGG	TCTGACAATGACTGTGTGTCCA
IRS1	NM_010570.4	CCAGAGTCAAGCCTCACACA	ATTCTCCTGGGCTTTTGGG
IRS2	NM_001081212.1	GCCACTGCTACAGCTCTTTGCC	GCACTGCTGAGTGATGAGCTG
PI3K	NM_008839.2	TGGGTACTGCGTGGCAACCTTT	TGGTGTACTCTTGTGCTCCCT
AKT isoform a (AKT1)	NM_009652.3	AAGGCCACAGGCCGCTACTA	AAGAAGAGCTCGCCCCCGTT
AKT isoform b (AKT2)	NM_007434.3	ACAAGTGTGGCTCCCCCAGTGA	TGCAATGATGACCTCCTTCCGC
GLUT4	NM_009204.2	AACCAGCATCTTCGAGTCGGCT	AGCTCATGGCTGGAACCCGT
GLUT1	NM_011400.3	AGCAGCCTGTGTACGCCACCAT	GACATCCAAGGCAGCCGTTCCA
GAPDH	NM_008084.2	TCAAATGGGGTGAGGCCGGT	TGGTGCAGGATGCATTGCTGA

The name of the analyzed genes, their GenBank accession no., and 5′–3′ nucleotide sequences of the sense and antisense primers are presented.

kit (QIAGEN, Hilden, Germany), and LightCycler 2.0 (Roche, Mannheim, Germany). Results were analyzed using the comparative threshold cycle method.

Statistical analysis

Experimental values are expressed as mean ± SD of at least two experiments in triplicate. Data were analyzed by using one-way ANOVA. The level of $P \leq 0.05$ was used as criterion of statistical significance. All calculations were done employing the JMP 5.1 program (SAS Institute, Inc., Cary, NC).

Results

Determination of ISO innocuous concentrations for preadipose cells

To confidently assay the effects of ISO on the adipose metabolism, we first determined ISO concentrations not affecting the preadipose cell viability. ISO at concentrations ranging from 0.01 to 200 μM did not significantly affect the murine or human preadipose cell viability, albeit 100 and 200 μM ISO reduced the murine cell growth by 35 and 51% (Fig. 1B) and human cell growth by 34 and 47% (Fig. 1C), respectively, compared with untreated cells. Lower concentrations of ISO not appreciably affected the cell growth. Thus, the effects of ISO on 2-NBDG uptake were evaluated at concentrations ranging from 0.01 to 50 μM.

ISO stimulates 2-NBDG uptake in insulin-sensitive and insulin-resistant 3T3-F442A and human adipocytes

To determine whether ISO stimulates the glucose uptake by adipose cells, the 2-NBDG uptake by terminally differentiated murine 3T3-F442A and human sc adipocytes was assayed. ISO stimulated 2-NBDG uptake in both cell types in a concentration-dependent manner. In insulin-sensitive 3T3-F442A adipocytes, ISO stimulated 2-NBDG incorporation by 100% (0.1 μM), 182% (10 μM), and 210% (50 μM) respect to 100 nM insulin (Fig. 2A). Tested at the same concentrations on insulin-sensitive human adipocytes, ISO promoted 2-NBDG uptake by 30, 47, and 67%, respectively, compared with insulin treatment (Fig. 2B).

The effects of ISO were also assayed on murine and human adipocytes made insulin resistant by TNF-α treatment to further evaluate its antidiabetic potential. In murine diabetic-like adipocytes, ISO induced 2-NBDG uptake by 60% (0.1 μM), 125% (10 μM), and 139% (50 μM), respect to 10 μM RGZ (Fig. 2A), whereas in insulin-resistant human adipocytes did it by 30% (0.1 μM), 49% (10 μM), and 60% (50 μM) compared with RGZ control (Fig. 2B).

ISO stimulates adipose glucose uptake through an insulin-like signaling pathway

To identify the signaling pathway by which ISO stimulates the glucose uptake in adipose cells, we assayed the effect of ISO on glucose uptake by murine and human cells in the presence of insulin signaling pathway inhibitors HNMPA-$(AM)_3$ for IR, Wortmannin for PI3K, AKT Inhibitor VIII for AKT 1/2, and Indinavir for GLUT4. All the assayed inhibitors abolished ISO-induced glucose transport in 3T3-F442A and human adipocytes, because the glucose uptake reached in presence of blockers was indistinguishable of the low glucose uptake observed in cells incubated with PBS alone (Fig. 3).

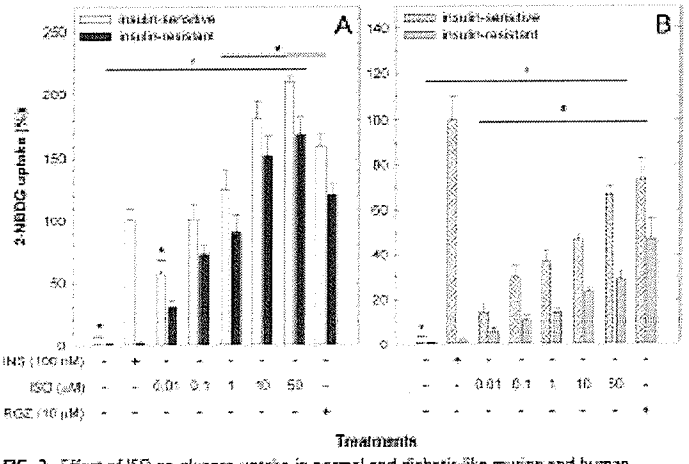

FIG. 2. Effect of ISO on glucose uptake in normal and diabetic-like murine and human adipocytes. Insulin-sensitive and insulin-resistant 3T3-F442A (A) and human (B) adipocytes were incubated for 60 min with PBS/BSA containing 2-NBDG 80 μM and the indicated concentrations of ISO. Control treatments received 100 nM insulin (INS) or 10 μM RGZ. After incubation, free 2-NBDG was cleared from cultures, and fluorescence retained in cell monolayers was measured in a fluorescence reader. The results are presented as the mean ± SD of three independent experiments in triplicate. *, Statistically significant from INS ($P \leq 0.05$); ^, statistically significant from RZG ($P \leq 0.05$).

ISO induces phosphorylation of IR, PI3K, and AKT in insulin-sensitive and insulin-resistant murine and human adipocytes

The effect of ISO on phosphorylation of IR, PI3K, and AKT was evaluated using insulin-sensitive 3T3-F442A and human adipocytes. Our results showed that ISO stimulated the phosphorylation of IR in murine adipocytes

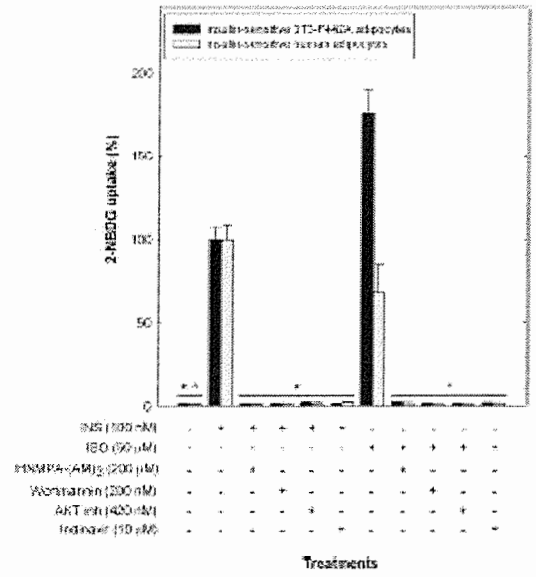

FIG. 3. Signaling pathway for ISO induction of glucose uptake in murine and human adipocytes. The effect of ISO on 2-NBDG uptake in insulin-sensitive 3T3-F442A and human adipocytes was assayed in the presence of the insulin pathway inhibitors $HNMPA-(AM)_3$ (IR inhibitor), Wortmannin (PI3K inhibitor), AKT isoforms a and b (AKT 1/2) inhibitor (inh) VIII, and Indinavir (GLUT4 inhibitor). Control treatments were incubated with 100 nM insulin (INS). Results represent the mean ± SD of three independent experiments in triplicate. *, Statistically significant from INS ($P \leq 0.05$); ^, statistically significant from ISO ($P \leq 0.05$).

by 70% (Fig. 4A) and by 49% in human cells (Fig. 4B) compared with 100 nM insulin. Also, ISO induced the phosphorylation of PI3K by 85% (murine adipocytes) (Fig. 4C) and 80% (human adipocytes) (Fig. 4D), whereas AKT was phosphorylated after treatment with ISO by 58 and 56% in 3T3-F442A and human adipocytes, respectively (Fig. 4, E and F).

To further evaluate the mechanism by which ISO induces glucose uptake in insulin-resistant adipocytes, the effect of ISO on phosphorylation of IR, PI3K, and AKT was also evaluated using 3T3-F442A and human diabetic-like adipocytes. The results demonstrate that ISO stimulated the phosphorylation of IR in these adipocytes by 41% (murine) (Fig. 4A) and 39% (human) (Fig. 4B) compared with the phosphorylation showed in insulin-sensitive adipocytes. ISO also phosphorylated PI3K in diabetic-like adipocytes by 59% (murine) (Fig. 4C) and 65% (human adipocytes) (Fig. 4D) and AKT by 52% (murine) (Fig. 4E) and 54% (human) (Fig. 4F). The phosphorylation of the insulin signaling pathway elements induced by ISO, insulin, or RGZ was abolished in both cell lines by the respective inhibitors (Fig. 4), confirming the results obtained with insulin-sensitive adipocytes (Fig. 3) and extending them to insulin-resistant cells.

ISO induces the gene expression of insulin signaling pathway elements in 3T3-F442A adipocytes

The effect of ISO on the expression of genes encoding proteins of insulin signaling pathway was evaluated by quantitative RT-PCR (qRT-PCR) real time using specific probes for IR, IRS1, IRS2, PI3K, AKT1, AKT2, GLUT1, and GLUT4. Insulin-sensitive and insulin-resistant mature 3T3-F442A adipocytes were incubated for 22 h with PBS to deplete their mRNA pools. Then, cultures were treated with PBS containing 10 nM insulin or 50 μM ISO, and total RNA was recovered for each treatment for qRT-PCR real-time analyses. The expression of all analyzed genes was completely reduced by fasting adipocytes, except for GAPDH, the housekeeping gene. After treatment with insulin or ISO, gene expression was reactivated in both insulin-sensitive and insulin-resistant adipocytes. In insulin-sensitive cells, ISO induced a higher expression of IR, IRS1, IRS2, and PI3K genes than insulin (Fig. 5), whereas AKT1, AKT2, GLUT4, and GLUT1 genes were similarly induced by insulin or ISO. In insulin-resistant cells, insulin and ISO induced gene expression at the same extent, except for IRS2, which showed higher expression with insulin. Notoriously, the expression of the insulin-independent GLUT1 was poorly induced in insulin-sensitive cells but robustly induced by both compounds in insulin-resistant adipocytes (Fig. 5).

Discussion

ISO is a C-glycosylflavonoid found in many plants reputed as antidiabetic (22) and a proven responsible for the hypoglycemic effects in some of these plants (23), although the mechanisms mediating the antidiabetic properties of this compound are still unknown. To gain insight into the ISO antidiabetic mechanisms, we evaluated its ability to induce the 2-NBDG uptake in cultured 3T3-F442A and human normal sc adipocytes.

ISO induced 2-NBDG uptake in a concentration-dependent manner in both cell types, with greater potency than 100 nM insulin or 10 μM RGZ ($P \leq 0.05$) in murine adipocytes and with lower potency than approved drugs on human cells. Such differences in murine and human fat cells to incorporate glucose in response to ISO could be due to the distinct homogeneity degree in these two cell populations. 3T3-F442A is an established clonal cell line with high adipogenic ability, whereas human adipocytes used in this work correspond to a human strain of normal

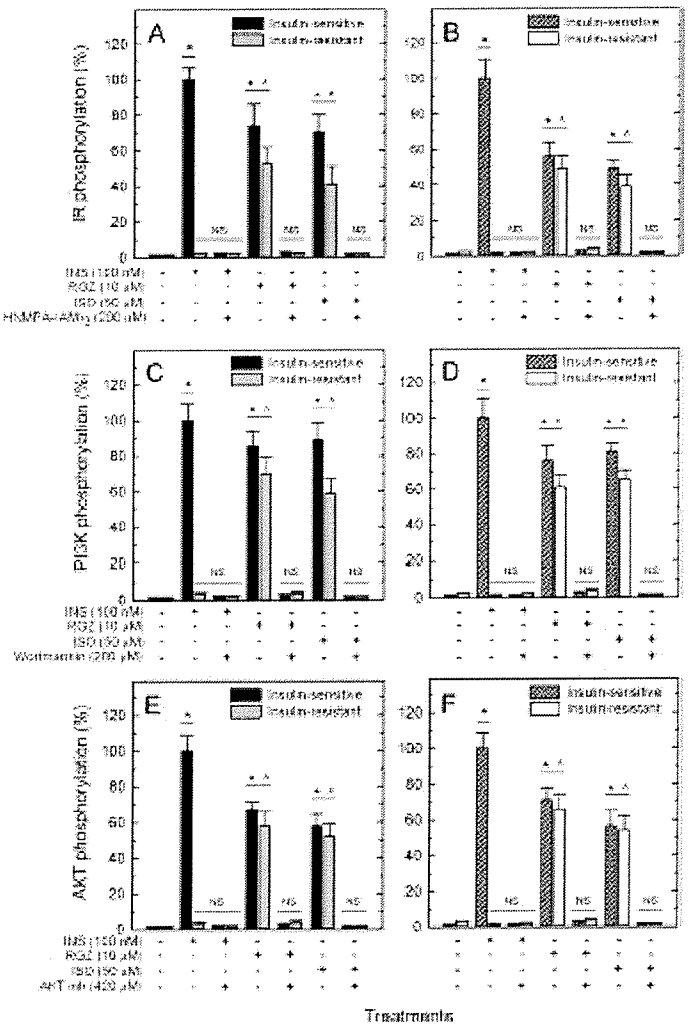

FIG. 4. Effect of ISO on IR, PI3K, and isoforms a and b of AKT phosphorylation in mature adipocytes. The total content of phosphorylated IR (A and D), p85 of PI3K (B and E), and T308 and S473 residues of AKT (C and F) were measured in insulin (INS)-sensitive and insulin-resistant 3T3-F442A (A, C, and E) and human (B, D, and F) adipocytes using the Cellular Activation of Signaling ELISA kit. Assays were done in three independent experiments in triplicate following the manufacturer's instructions. Plates were read at 450 nm in an ELISA reader. *, Statistically significant from depleted insulin-sensitive cells ($P \leq 0.05$); ^, statistically significant from depleted insulin-resistant cells ($P \leq 0.05$). NS, Not significant. AKT inh, Inhibitor for AKT.

stromal fat cells with a higher heterogeneity and lower adipogenic ability. In any case, our results clearly demonstrate the ability of ISO to stimulate glucose uptake in murine and human cultured adipocytes and suggest that this is a mechanism by which ISO exerts its antidiabetic properties.

3T3-F442A and human normal adipocytes are both insulin-sensitive cells, and they represent experimental models for normal adipose tissue instead of a type 2 diabetes model systems. We therefore also assayed ISO effects on murine and human adipocytes treated with TNF-α to induce insulin resistance (30), a prominent trait of type 2 diabetes. In such assays, TNF-α-treated murine and human cells failed to incorporate 2-NBDG in response to insulin, whereas their normal counterparts incorporated the glucose analog at high rates under insulin stimulus. By contrast, 10 or 50 μM ISO strongly induced glucose uptake in murine adipocytes resistant to insulin with greater potency than 10 μM RGZ ($P \leq 0.05$) and also stimulated glucose uptake by insulin-resistant human adipocytes although with a lower potency than RGZ.

The ISO stimulation of glucose uptake in insulin-sensitive cells was abolished by HNMPA-$(AM)_3$, an IR inhibitor, Wortmannin, which inhibits PI3K, AKT 1/2 inhibitor, and Indinavir inhibiting GLUT4. These results suggest that ISO uses the insulin signaling pathway to stimulate glucose uptake by murine or human adipose cells. This view on the ISO mechanism was reinforced by our results showing that ISO treatment of murine and human adipocytes resulted in tyrosine phosphorylation of IR β-subunit (Y1162 and Y1163 residues) and p85 subunit of PI3K, as well as in the phosphorylation of two key residues in AKT, T308 and S473. The ability of ISO to induce proper phosphorylation patterns of insulin signaling pathway elements was comparable in strength with those of 10 μM RGZ ($P \leq 0.05$) in both murine and human adipocytes. Moreover, the phosphorylation of insulin signaling pathway components was induced by ISO also on insulin-resistant cells. This result and the previously showed ISO induction of glucose uptake in insulin-resistant adipocytes demonstrate that ISO is able to revert insulin resistance, the outstanding trait of type 2 diabetes, and does so by inducing the

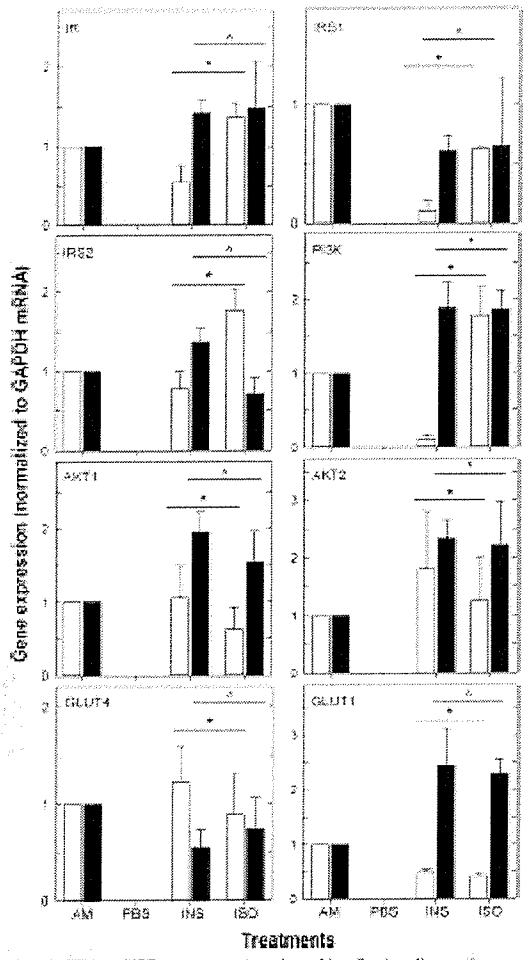

FIG. 5. Effect of ISO on gene expression of insulin signaling pathway elements. Insulin (INS)-sensitive (□) or insulin-resistant (■) 3T3-F442A adipocytes terminally differentiated in adipogenic medium (AM) were incubated with PBS for 22 h to deplete mRNA pools. Then, cultures were subjected to the indicated treatments for 1 h, and total RNA from each treatment was recovered and analyzed by qRT-PCR for the expression of insulin signaling pathway genes. The threshold cycle method was employed for the analysis of gene expression, and GAPDH was used as housekeeping gene. *, Statistically significant from depleted insulin-sensitive cells ($P \leq 0.05$); ^, statistically significant from depleted insulin-resistant cells ($P \leq 0.05$).

proper protein phosphorylation in the signaling pathway of insulin.

In murine adipocytes, glucose uptake was stimulated more efficiently by ISO than by insulin, although ISO was less efficient than insulin to stimulate IR and AKT phosphorylation. These contradictory data suggest that, at least in this cell line, ISO also acts by mechanisms additional to the canonical insulin signaling pathway. Preliminary experiments in our laboratory suggest that the activation of Rho family GTPase TC10 signaling pathway (35) could be one such mechanism (data not shown).

As an additional way for ISO antidiabetic mechanisms, we also explored its effect on the expression of genes related to insulin signaling and glucose uptake in adipocytes. qRT-PCR analyses showed that in insulin-sensitive adipocytes, ISO induced the expression of IR, IRS1, IRS2, and PI3K that were increased when compared with the effect of insulin. However, comparable levels of gene expressions were found after insulin and ISO stimulation in insulin-resistant models. The mechanisms by which ISO induce the expression of these genes and its physiological significance are now under study in our laboratory.

Our results reinforce the evidence that ISO stimulates glucose uptake in murine and human adipocytes that are sensitive or resistant to insulin, and the effects are concentration dependent. Although 50 μM, the most active ISO concentration, seems to be a very high concentration for a polyphenol, lower ISO concentrations also exert significant effects on the glucose uptake by both cell lineages and under both conditions of insulin responsiveness. Although no data exist for plasma concentrations and bioavailability of ISO, it has been shown that isoflavones, such as daidzein and genistein, reached plasma concentrations of 3.14 and 4.09 μmol/liter in healthy male volunteers fed with only 0.84 g of soybean flour/kg body weight 7–8 h after ingestion (36). These results suggest that plasma concentrations of ISO physiologically relevant could be achieved by eating ISO-enriched foods or by pharmaceutical formulations of the compound. Other studies have shown that ingestion of 132 mg of isoflavones/day favorably alters insulin resistance, glycemic control, and serum lipoproteins in postmenopausal women with type 2 diabetes (37). A recent work has shown that micromolar concentrations of luteolin, the aglycone parent of ISO, induced the phosphorylation of elements in the insulin signaling pathway and prevent the palmitate-evoked inflammatory response in human enothelial cells (21).

Other natural compounds isolated from plants used as antidiabetics by diverse complementary and alternative medicine systems have been shown to induce glucose uptake in insulin-sensitive cultured cells. Shikonin, a naphthoquinone derivative, stimulated glucose uptake in 3T3-L1 adipocytes (13) and in L6 skeletal muscle myotubes via an insulin-independent pathway (14). Ginsenosides Rb(1), Re, Ck, Rg1, and Rg3, a group of triterpenoid saponins found nearly exclusively in *Panax* species (38), stimulate glucose transport in 3T3-L1 adipocytes and C2C12 or L6 myotubes, mainly activating the insulin

signaling pathway (18–20). Recently, it was shown that ginsenoside Rg1 also promotes glucose uptake in insulin-resistant C2C12 cells (39), suggesting that remaining ginsenosides could be active on insulin-resistant cells. However, the effect of these compounds on human cells, sensitive or resistant to insulin, is yet unknown.

We previously showed that chlorogenic acid, an ester of caffeic acid, stimulated glucose uptake in insulin-sensitive and insulin-resistant 3T3-F442A adipocytes with a comparable potency with insulin or RGZ (28). We also showed that rexinoids honokiol and magnolol, isolated from *Magnolia dealbata* seeds, stimulated glucose uptake in insulin-sensitive and insulin-resistant murine and human adipocytes using the insulin signaling pathway (40). Our present results show that ISO is an additional natural compound with high capacity to induce the glucose uptake by insulin-targeted mammalian cells in spite of their responsiveness to this hormone.

In summary, our study provides evidence for the first time that ISO stimulates the uptake of glucose in both insulin-sensitive and insulin-resistant murine 3T3-F442A and human sc adipocytes through the insulin signaling pathway, inducing the proper phosphorylation of proteins in this signaling pathway, as well as stimulating the transcription of their codifying genes. The strong induction of glucose uptake exerted by ISO on both insulin responsive or insensitive adipose cells, coupled with its broad distribution in plants, notably in food plants (41, 42), and its proved *in vitro* production (43) makes this natural compound a promissory candidate to "mow the GRAS" (10) in the two main types of diabetes mellitus.

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Isoorientin blocks adipogenesis by inhibiting the commitment to terminal differentiation through the Nrf2 signaling pathway

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L. A. S.-O. designed, supervised and analyzed the experiments and wrote the manuscript. G. G.-E. and A. J. A.-C. conducted the experiments and contributed to their analysis. All authors reviewed, edited and approved the manuscript.

Conflict of Interest:

Authors declare no conflict of interest.

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Abstract

Aim: The C-glycosylflavone isoorientin (ISO) stimulates the glucose uptake into adipocytes by activating and upregulating the insulin signaling pathway, but its effects on adipogenesis are unknown. Thus, we tested ISO actions on in vitro adipogenesis of murine and normal human subcutaneous preadipocytes.

Methods: The effect of ISO on adipogenesis was evaluated by quantifying lipid accumulation, glycerol-3-phosphate dehydrogenase activity and percent of adipocytes per dish. The expression of proadipogenic and antiadipogenic transcription factors in the presence or the absence of ISO was evaluated by qRT-PCR. The cellular location of Nrf2 protein was determined by Western blot.

Results: Applied to preadipocytes, ISO inhibited the lipid accumulation in a dose-dependent manner; ISO 50 μM blocked 3T3 and human adipogenesis by 96% and 93%, respectively. However, ISO did not exert lipolytic effects on terminally differentiated murine or human adipocytes. ISO reversibly blocked the commitment to adipogenesis in 3T3-F442A cells by downregulating the expression of proadipogenic transcription factors KLF4, PPAR-γ and C/EBP-α, and upregulating the expression of antiadipogenic transcription factors GATA2 and Wnt10B. Since some of these transcription factors contain Nrf2-responsive ARE sequences, we evaluated the effect of ISO on the Nrf2 expression and found that ISO increased the expression de Nrf2 mRNA and the nuclear accumulation Nrf2 protein.

Conclusions: ISO reversibly inhibits adipogenesis by modulating the expression of proadipogenic and antiadipogenic transcription factors through the Nrf2 signaling pathway.

Our results suggest isoorientin could be a promissory candidate to develop new antiobesity treatments.

Keywords: Isoorientin, antiadipogenesis, 3T3-F442A adipocytes, human adipocytes, Nrf2

Introduction

Obesity is a serious health problem as it causes or exacerbates multiple chronic diseases, including type II diabetes, hypertension, coronary heart disease, and some types of cancer [1]. In addition, obesity and type 2 diabetes have become the most prevalent metabolic disorders nowadays. Their increasing incidence constitutes a serious threat for health systems worldwide [2,3] and suggests that current strategies to combat these affections are far from being efficient.

Obesity is characterized at the cellular level by increases in the number and/or size of adipocytes. The increase in adipocyte number occurs through the triggering of adipocyte differentiation by a two-step process: commitment of precursor stem cells to an adipose fate and terminal differentiation of these cells into mature adipocytes [4,5]. Adipose differentiation is regulated by changes in gene expression and accompanied by coordinated changes in cell morphology and hormone sensitivity. These changes are regulated by proadipogenic transcription factors, including Kruppel-like factor 4 (KLF4), peroxisome proliferator-activated receptor γ (PPAR-γ) and CCAAT/enhancer-binding proteins (C/EBPs), and prevented by antiadipogenic transcription factors such as GATA binding protein 2 (GATA2) and Wingless-type MMTV integration site family member 10B (Wnt10B) [4-6].

In spite of our growing knowledge on adipose biology, contemporary approved antiobesity drugs are directed to non-adipose targets. These include Orlistat, a potent inhibitor of pancreatic lipase that reduces intestinal fat absorption, Lorcaserin, a selective agonist of hypothalamic subtype 2c (5-HT_{2C}) serotonin receptors, and Phentermine-topiramate that exerts anorexic effects by yet unclear mechanisms. All these drugs present

diverse adverse effects and high costs that will likely prevent their use as first-line antiobesity agents [7,8]. Therefore, the development of new and more efficient anti-obesity drugs is a high priority for health systems.

A number of plant compounds have been shown to present antiadipogenic activity and their mechanisms of action are being studied [9-11]. Isoorientin (ISO), a C-glycosylflavonoid found in many plants used by diverse complementary and alternative medicine systems for the treatment of type 2 diabetes [12], has been shown to reduce plasmatic glucose, cholesterol, and triacylglyceride concentrations in streptozotocin-induced diabetic rats [13]. Recently, we reported that ISO stimulates the glucose uptake in both insulin-sensitive and insulin-resistant murine 3T3-F442A and human subcutaneous adipocytes by inducing the proper phosphorylation of proteins in the insulin signaling pathway and by stimulating the transcription of their coding genes [14]. Other natural or synthetic compounds stimulating the glucose uptake in adipocytes also induce the expression of PPAR-γ [15-17], which leads to development of new adipocytes with the concomitant risk of obesity [18,19]. Thus, the possibility exists that antidiabetic-like properties of ISO are accompanied with proadipogenic effects.

Here we show that ISO reversibly blocks the in vitro murine and human adipogenesis by modulating the expression of proadipogenic and antiadipogenic transcription factors. ISO exerts such antiadipogenic effects activating and upregulating the Nrf2 signaling pathway and without exerting lipolytic effects.

2 Material and methods

2.1 Materials

Murine 3T3-F442A preadipocytes and adult cat serum were kindly provided by Dr. W. Kuri-Harcuch (CINVESTAV, México). Isolation of normal human subcutaneous preadipocytes from non-diabetic patients undergoing elective cosmetic surgery, who gave their informed consent for the use of discarded tissues and with the approval of corresponding Research Ethic Committee, was previously described [20]. Dulbecco's Modified Eagle medium (DMEM) and Leibovitz L15 medium (L15) were from GIBCO BRL (Grand Island, NY, USA) whereas fetal bovine serum (FBS) and calf serum (CS) were from HyClone (Logan, UT, USA). ISO, purchased from Chromadex (Santa Ana, CA, USA), and Rosiglitazone (RGZ), from Cayman Chemical Co. (Ann Arbor, MI), were 95% and 98% purity according to the manufacturers. All other chemicals were from Sigma Chemical (St. Louis, MO, USA).

2.2 Effects of ISO on murine and human adipogenesis

Preconfluent 3T3-F442A preadipocytes were treated with murine adipogenic medium (mAM; DMEM containing 10% FBS, 5 μg/ml insulin and 1 μM d-biotin) added with non-toxic concentrations of ISO [14]. Parallel cultures were fed with non-adipogenic medium (NAM; DMEM containing 4% adult cat serum, 5 μg/ml insulin, and 1 μM d-biotin) [21] as an adipogenic negative control. After seven days, the extent of lipid accumulation was estimated by staining intracellular triacylglycerides with oil red O [22]. Normal human

preadipocytes were induced to adipogenesis with human adipogenic medium (hAM; L15 medium containing 5% FBS, 25 μM 3-isobutyl-1-methylxanthine, 100 nM dexamethasone, 1 μM RGZ, 100 nM insulin and 0.2 nM triiodothyronine) [20] or hAM added with ISO 50 μM. Parallel cultures were fed with basal medium (BM; L15 added with 5% FBS) as an adipogenic negative control. After thirty days, lipid accumulation was estimated by staining intracellular triacylglycerides with oil red O as described above.

2.3 Evaluation of lipolytic effects of ISO on 3T3 and human adipocytes

Terminally differentiated 3T3-F442A or human adipocytes were treated with BM or BM added with ISO 50 μM for seven (3T3 cells) or fifteen (human cells) days. Then, lipid accumulation was quantified by staining intracellular triacylglycerides with oil red O as described previously.

2.4 Characterization of the anti-adipogenic effect of ISO

Preconfluent 3T3-F442A preadipocytes were induced to adipogenesis with mAM, mAM added with ISO 50 μM or NAM. Seven days later, some ISO-treated cultures were re-fed with mAM or NAM. Lipid accumulation [22], glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) activity [23], and percent of adipocytes per well were estimated for each treatment at the fourteenth day of culture.

2.5 Gene expression analysis

Total RNA from 3T3-F442A cells induced to differentiation with mAM for 48 h was obtained with TRIzol (Invitrogen) according to the manufacturer's instructions. RNA

integrity in the samples was confirmed using 8S and 28S rRNA, and RNA concentration was determined by spectroscopy at 260 nm. After DNase I digestion (Fermentas, Hunover, MD, USA), 6 μg of total RNA were retrotranscribed using Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Gene expression of KLF-4, PPAR-γ, C/EBPα, GATA2, Wnt10B, Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using probes designed with IDT Scitools Real Time PCR (Integrate DNA Technologies, Coralville, IA, USA) (Table 1) and employing Prime Time qPCR assays (Integrated DNA technologies), QuantitectProbe PCR Kit (Qiagen, Hilden, Germany), and LightCycler 2.0 (Roche, Mannheim, Germany). Results were analyzed using the comparative threshold cycle method [24].

2.6 Western blot analysis

Cytosolic and nuclear extracts of 3T3-F442A cells were obtained 5 h and 48 h after induction to differentiation using protocols previously described [25]. Proteins in the extracts were separated using 10%, 6% and 5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked (Tris-buffered saline, 0.1% Tween 20 and non-fat dry milk) and incubated with antibodies against GAPDH, Nrf2 and Lamin A (Santa Cruz Biotechnology, Dallas, TX, USA). After incubation with rabbit IgG antibody to detect primary antibodies, proteins were visualized using enhanced chemiluminescence (Amersham; Buckinghamshire, UK).

2.7 Statistical analysis

Experimental values are expressed as mean ± standard deviation of at least two experiments in triplicate. Data were analyzed by using one-way ANOVA. The level of $P \leq 0.05$ was used as criterion of statistical significance. All calculations were done employing the JMP 5.1 program (SAS Institute Inc, Cary, NC, USA).

3 Results

3.1 ISO inhibits murine and human adipogenesis but lacks of lipolytic effects

Several compounds, either natural or synthetic, that stimulate the glucose uptake into adipose cells also induce the transcription of the adipogenic master gen PPAR-γ [15-17], which leads to the development of new adipocytes with the concomitant risk of obesity. To determine whether ISO affects the development of adipose tissue, we evaluated its effect on murine and human adipogenesis. As shown in figure 1A, ISO decreased the lipid accumulation by 3T3-F442A cells in a concentration-dependent manner. Added to murine adipogenic medium (mAM), ISO 0.01 μM decreased lipid accumulation by 20% compared to mAM control whereas ISO concentrations of 1 μM and 50 μM reduced triacylglycerol cell content by 40% and 90%, respectively. ISO also exerted a concentration-dependent antiadipogenic effect on normal human subcutaneous preadipocytes. In these cells, ISO 50 μM blocked lipid accumulation by 93% (figure 1B).

The diminished lipid accumulation induced by ISO could be the result of a lipolytic property of the compound or from its capacity to block the commitment to terminal adipogenesis. To evaluate whether ISO exerts lipolytic action, we assayed its effect on the lipid accumulation of terminally differentiated 3T3-F442A and human adipocytes. Our results showed that ISO lacks of lipolytic effects on murine or normal human mature adipocytes, since mature adipose cells of both lineages retain their intracellular content of triacylglycerols after seven (3T3 cells) or fifteen (human cells) days of incubation with basal medium added with ISO 50 μM, the maximal antiadipogenic ISO concentration tested (figure 2).

3.2 ISO reversibly blocks the commitment to adipogenesis in 3T3-F442A cells

We performed additional experiments to confirm that ISO blocks adipogenesis by preventing the establishment of commitment to the terminal adipose differentiation and to determine whether ISO antiadipogenesis is or not reversible. 3T3-F442A preadipocytes were induced to terminal differentiation with adipogenic medium (mAM) or mAM added with ISO 50 μM (mAM + ISO). Parallel cultures were maintained at undifferentiated stage with non-adipogenic medium (NAM) [21]. Seven days later, some ISO-treated cultures were refed with mAM or NAM and maintained under such conditions for additional seven days. The adipose differentiation of ISO-treated cultures (mAM + ISO) was blocked according to their low levels in triacylglyceride accumulation (5%), percentage of adipocytes per culture (10%), and GPDH activity (5%) compared to mAM control. Cultures treated with mAM + ISO and subsequently refed with mAM (AM+ISO⟹AM) reverted the blocking effects of ISO as judging by their increased levels of lipid accumulation (43%), percentage of adipocytes (40%) and GPDH activity (36%) respect to the mAM + ISO treatment (figure 3). Such increases were not observed in ISO-treated cultures re-fed with NAM (AM+ISO ⟹ NAM), which retained the low values in the three evaluated adipose parameters (figure 3).

3.3 ISO modulates the expression of transcription factors during commitment stage in 3T3-F442A preadipocytes

To further characterize the molecular mechanisms of ISO antiadipogenesis we evaluated by qRT-PCR the gene expression of proadipogenic and antiadipogenic

transcription factors in 3T3-F442A preadipocytes induced to terminal differentiation in the absence or the presence of ISO. The expression of proadipogenic transcription factors KLF4, PPAR-γ and C/EBP-α was increased 7.9-fold, 9.3-fold, and 1.6-fold in cells treated with murine adipogenic medium (mAM), respect to undifferentiated cells (NAM) (figure 4). These increases in gene expression were completely abrogated by the addition of ISO 50 μM to mAM (mAM + ISO) (figure 4). The gene expression of KLF4 in the presence of ISO was even lower than that achieved in undifferentiated cells, whereas gene expression of PPAR-γ and C/EBP-α in the presence of ISO remained at the low levels expressed in precommitted cells (NAM). On the other hand, ISO induced the overexpression of the antiadipogenic transcription factors GATA2 and Wnt10B 1.3-fold and 8.6-fold, respect to the level of expression of these genes in undifferentiated cells. ISO induced 1.6-fold (GATA2) and 4.5-fold (Wnt10B) the level of gene expression for these antiadipogenic transcription factors respect to levels attained under mAM (figure 4).

3.4 ISO blocks adipogenesis through Nrf2 signaling pathway

We previously showed that ISO stimulates the glucose uptake in murine and human adipocytes by activating the insulin signaling pathway, including phosphatidylinositol 3-kinase (PI3K) [14]. It also been documented that ISO induces Nrf2 pathway-driven antioxidant response through PI3K signaling [26]. To determine whether ISO antiadipogenesis is mediated by Nrf2 signaling pathway we analyzed the gene expression and cellular localization of this transcription factor in 3T3-F442A cells treated with ISO.

The level of Nrf2 gene expression in 3T3-F442A preadipocytes was increased after only 5 h of induction with adipogenic medium (mAM), both in the absence (1.8-fold) and the presence of ISO 50 μM (2.0-fold), with no significant difference between these treatments. However, in 3T3-F442A preadipocytes treated with mAM the expression level of Nrf2 remained constant after 48 h of induction (1.5-fold) while in the presence of ISO Nrf2 expression level was increased 3.2-fold, respect to non-induced cells (0h, BM) (figure 5A). The induction of Nrf2 gene expression by ISO was accompanied by cell relocation of the respective protein. While in non-induced preadipocytes (0 h; BM) Nrf2 protein remained largely cytosolic, Nrf2 protein became predominantly nuclear after treatment of these cells with ISO 50 μM (figures 5B, 5C).

Discussion

Recently we showed that the C-glycosylflavone isoorientin (ISO) stimulates glucose uptake in human and murine adipocytes, both sensitive and resistant to insulin, by inducing the proper phosphorylation of proteins in the insulin signaling pathway and upregulating the transcription of their coding genes [14]. To determine whether ISO affects adipogenesis, we evaluated its effect on cultured murine and human subcutaneous preadipocytes induced to terminal differentiation. Our results show that ISO did not stimulate but rather decreased the intracellular lipid accumulation in a concentration-dependent manner when applied to differentiating preadipocytes of both adipose lineages. On the other hand, ISO did not affect the intracellular lipid content when applied to terminally differentiated adipocytes. This result proved that ISO lacks of lipolytic effects exerted by other natural preparations [27,28], and suggested that ISO antiadipogenic properties derivate from its capacity to block the commitment to terminal adipose differentiation.

This hypothesis was tested in two ways. First, confluent 3T3-F442A preadipocytes whose differentiation was blocked for seven days by incubating them with ISO-added adipogenic medium were refed with either fresh adipogenic or non-adipogenic medium, and maintained under such culture conditions for additional seven days. Cells refed with adipogenic medium reinitiated the terminal adipose differentiation and significantly increased their content of intracellular triacylglycerides, levels of GPDH activity and their percentage of mature adipocytes per dish respect to cultures maintained in ISO-containing adipogenic medium. On the contrary, ISO-treated cultures refed with non adipogenic

medium, a culture condition preventing the establishment of commitment to terminal differentiation [22], exhibited the same low levels in the three adipogenic parameters as cultures maintained in ISO-containing adipogenic medium.

The commitment to terminal adipose differentiation is driven by the activation of proadipogenic transcription factors and the turning off of antiadipogenic ones [4-6]. To confirm that ISO blocks the commitment to terminal adipogenesis we quantified by qRT-PCR the expression of transcription factors relevant to adipogenesis. Our results showed that the expression of proadipogenic transcription factors KLF4, PPAR-γ and C/EBP-α was drastically decreased in preadipocytes treated with ISO for 48 h while the expression of antiadipogenic transcription factors GATA2 and Wnt10B was significantly increased under the same condition. Therefore, our evidences at both cellular and molecular levels demonstrate that ISO reversibly interferes the establishment of commitment to terminal adipogenesis.

This result does not disagree from the previous report by Sezik et al. [13] showing that ISO induced body weight gain in streptozotocined-rats. Results for Sezik and coworkers did not establish that ISO-induced weight gain was due to increases in fat mass and therefore it might reflect a better nutrient use by streptozotocined-rats in response to the insulinomimetic ISO effects [14], rather than an obesogenic effect of ISO. On the other hand, the absence of lipolytic activity of ISO distinguish it from other antiadipogenic compounds, both endogenous, i.e. TNF- α, and exogenous, e.g. genistein or 2,4,5-TMBA [27,28], and suggests that ISO lacks of cachectic undesirable properties and therefore would be and advantageous candidate to develop new anti-obesity strategies.

We also evaluated the participation of Nrf2 on the modulation of gene expression by ISO. A previous work showed that ISO purified from leaves of *Sasa borealis* bamboo protects HepG2 cells against oxidative damage by upregulation and activation of Nrf2, dependent upon PI3K/AKT signaling [26]. Also, we previously showed that ISO stimulates the glucose uptake in adipocytes by both activating and upregulating PI3K/AKT signaling [14], and a bioinformatics screening showed us that ISO-downregulated KLF4, C/EBP-α and PPAR-γ genes all contain Nrf2-responsive ARE sequences. Our qRT-PCR results showed that ISO induces a two-fold expression of Nrf2 gene in 3T3-F442A preadipocytes after 48 h of treatment respect to the control maintained under adipogenic medium. Moreover, our western-blot assays demonstrated that ISO strongly stimulates the nuclear translocation of Nrf2 protein as early as 5 h of treatment. So, our results indicate that Nrf2 signaling mediates the antiadipogenic effect of ISO.

Nrf2 is abundantly expressed in adipose tissue, and its role in the regulation of adipogenesis has been previously documented, although the experimental results obtained are controversial. Shin and coworkers [29] showed that Nrf2-/- mouse embryonic fibroblasts (MEFs) exhibit markedly accelerated adipogenesis upon stimulation, while Keap1-/- MEFs (which exhibit higher Nrf2 signaling) differentiated slowly compared to their congenic wild-type MEFs. Since ectopic expression of aryl hydrocarbon receptor (AHR) and dominant-positive Nrf2 in Nrf2-/- MEFs also substantially delayed differentiation, they postulated that Nrf2 inhibits adipogenesis through the interaction with the AHR pathway. A similar antiadipogenic role of Nfr2 is suggested by Takahashi et al. [30] in the inhibition of 3T3-L1 adipogenesis by the rosemary-derived compounds carnosic

acid and carnosol derived. Similarly, the hormonal induction of adipogenesis in the mouse bone marrow-derived ST2 cell line was accompanied with a continuously decreased abundance of nuclear Nrf2 [31].

The antiadipogenic signaling of Nrf2 evidenced on in vitro systems has been confirmed on in vivo models. Shin et al. [32] showed that the synthetic oleanolic triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Imidazolide or CDDO-Im), an extremely potent activator of Nrf2 signaling, effectively prevented high-fat diet-induced increases in body weight, adipose mass, and hepatic lipid accumulation in wild-type C57BL/6J mice but not in Nrf2-disrupted mice. Moreover, it has been shown that the nuclear content of Nrf2 protein was reduced and Keap1 mRNA expression increased in adipose tissue of C57BL/6J mice fed with a high-fat diet. Administration of Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione], a Nrf2 activator, prevented or significantly attenuated the effect of HFD on glucose disposal, body weight and fat gain, while restoring the levels of nuclear of Nrf2 protein and Keap1 mRNA expression [33]. Although some works suggest a proadipogenic role for Nrf2 [34,35], this role is hard to conciliate with evidences showing that Nrf2 activators, such as arsenic or tert-Butylhydroquinone [36], also block in vitro adipogenesis [47] and reduce the lipid accumulation in vivo [38].

We propose an antiadipogenic mechanism for ISO which considerate the activation of PI3K by the isoflavone previously shown [14,26]. Once activated, PI3K will induce the dissociation of the cytosolic Nrf2-Keap1 complex and the phosphorylation and subsequent nuclear translocation of Nrf2. In the nucleus, Nrf2 will down-regulate proadipogenic transcription factors like Klf4, PPAR-γ and C/EBP-α and will stimulate the transcription of

antiadipogenic factors such as GATA2 and Wnt10B (figure 6).

In conclusion, our results demonstrate that ISO inhibits murine 3T3 and human adipogenesis by reversibly blocking the commitment to terminal differentiation of preadipocytes and without lipolytic effects on mature adipose cells. The reversible antiadipogenic effects of ISO and its lack of lipolytic effect, added to its previously reported insulinomimetic property [14], suggest this isoflavone could be a promissory candidate to develop new treatments for diabesity.

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Figure legends

Figure 1. Effect of isoorientin (ISO) on murine and human adipogenesis. Adipogenesis of murine 3T3-F442A (A) or normal human normal subcutaneous preadipocytes (B) was induced as described under Material and Methods in the absence or the presence of non-toxic concentrations of ISO. Control cultures received non adipogenic medium (NAM; 3T3 preadipocytes) or were maintained in basal medium (BM; human preadipocytes). After seven (3T3 preadipocytes) or 30 days (human preadipocytes), cultures were fixed and stained with oil red O and adipose conversion was quantified by intracytoplasmic lipid accumulation. The results are presented as the mean ± SD of three independent experiments in triplicate. Lowercase letters indicate significant differences according to ANOVA test ($P \leq 0.05$).

Figure 2. Effect of isoorientin (ISO) on lipid accumulation of mature murine and human adipocytes. Terminally differentiated murine 3T3-F442A or human normal subcutaneous adipocytes were incubated with BM in the absence or the presence of ISO 50 μM. After seven (3T3 preadipocytes) or 15 days (human preadipocytes) of treatment, lipid accumulation was estimated by staining intracellular triacylglycerides with oil red O. The results are presented as the mean ± SD of three independent experiments in triplicate. Lowercase letters indicate significant differences according to ANOVA test ($P \leq 0.05$).

Figure 3. Characterization of the anti-adipogenic effect of ISO. Preconfluent 3T3-F442A preadipocytes were induced to adipose differentiation with mAM. Parallel cultures

received mAM added with ISO 50 μM or NAM during the same time. Then, some ISO-treated cultures were re-fed with mAM or NAM. Lipid accumulation, the percent of adipocytes per well, and the activity of glycerol-3-phosphate dehydrogenase (GPDH) were estimated for each treatment after seven days of incubation. The results are presented as the mean ± SD of three independent experiments in triplicate. Lowercase letters indicate significant differences according to ANOVA test ($P \leq 0.05$).

Figure 4. Effect of ISO on gene expression of proadipogenic and antiadipogenic transcription factors. Preconfluent 3T3-F442A preadipocytes were induced to differentiation with adipogenic medium (AM), AM added with ISO 50 μM (MA + ISO) or maintained under non adipogenic medium (NAM). After 48 h of incubation total RNA was recovered from cultures and the gene expression quantified by real-time RT-PCR using GAPDH as a constitutive gene as described under Material and methods. Three independent experiments were performed and data from a representative experiment are shown. * denotes statistical significance at $P < 0.05$.

Figure 5. ISO upregulates and activates Nrf2 signaling pathway. Confluent 3T3-F442A preadipocytes maintained in basal medium (BM) were refed with adipogenic medium (mAM) or adipogenic medium added with ISO 50 μM (MA +ISO). After 5h or 48 h of incubation, total RNA and cytosolic and nuclear proteins were recovered from cultures. Nrf2 gene expression quantified by real-time RT-PCR using GAPDH as a constitutive gene (A) and cytosolic and nuclear Nrf2 proteins were analyzed by Western blot (B) and

quantified by densitometry using GAPDH and Lamin A proteins as cytosolic and nuclear controls. Data are representative of three independent experiments. * denotes statistical significance at $P < 0.05$.NS, not significant.

Figure 6. Proposed antiadipogenic mechanism of ISO. ISO activates PI3K, which induces the dissociation of the cytosolic Nrf2-Keap1 complex and the phosphorylation and subsequent nuclear translocation of Nrf2. In the cell nucleus, Nrf2 downregulates the transcription of proadipogenic transcription factors such as Klf4, PPAR-γ and C/EBP-α and stimulates the transcription of antiadipogenic factors such as GATA2 and Wnt10B.

TABLE 1. Primer par sequences used for RT-PCR

Name	Accession number	Sense primer	Antisense primer
Peroxisome proliferator-activated receptor gamma (PPAR-γ)	NM_011146.3	TCGGCATGTCGCACAACGCTAT	AGCGGTAGAACACGTGCACACT
CCAAT/enhancer-binding protein alpha (C/EBP-α)	NM_007678.3	GGCAAAGCCAAGAAGTCGGTGG	AGCGTGTCCAGTTCACGGCTCA
Kruppel-like factor 4 (KLF4)	NM_010637.3	TGCCAGAGGAGCCCAAGCCAAA	TGCCTGGTCAGTTCATCGGAGC
Wingless-type MMTV integration site family member 10B (Wnt10B)	NM_011718.2	TGGAACTGCTCGGCACTGGA	AGCAGCTTGGCTCTAAGCCGGT
GATA binding protein 2 (GATA2)	NM_008090.5	AGCCTGCAACACACCACCCGAT	TGCCATCTCGTCGCCAGAGA
Nuclear factor erythroid-derived 2-related factor 2 (Nrf2)	NM_010902.3	TGAGCTTAGGGCAAAAGCTCTCCA	AAGTGGCCCAAGTCTTGCTCCAG
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	NM_ 008084.2	TCAAATGGGGTGAGGCCGGT	TGGTGCAGGATGCATTGCTGA

The name of the analyzed genes, their GenBank accession number, and 5'- to –3' nucleotide sequences of the sense and antisense primers are presented.

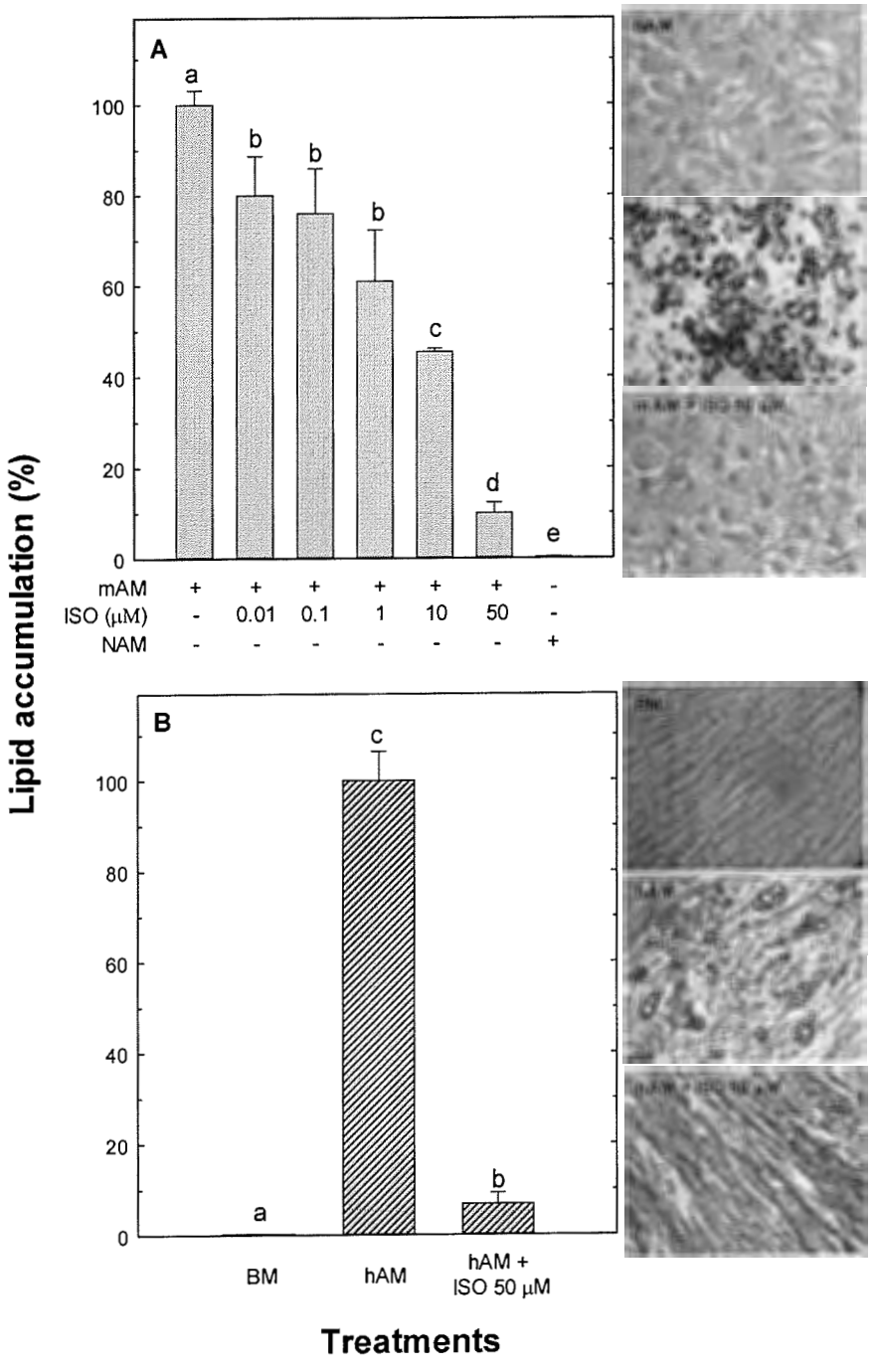
A
a
b
b
b
c
d
e
100
80
60
40
20
0
mAM + + + + + + -
ISO (μM) - 0.01 0.1 1 10 50 -
NAM - - - - - - +
B
c
b
a
100
80
60
40
20
0
BM
hAM
hAM + ISO 50 μM
Lipid accumulation (%)
Treatments

Figure 1

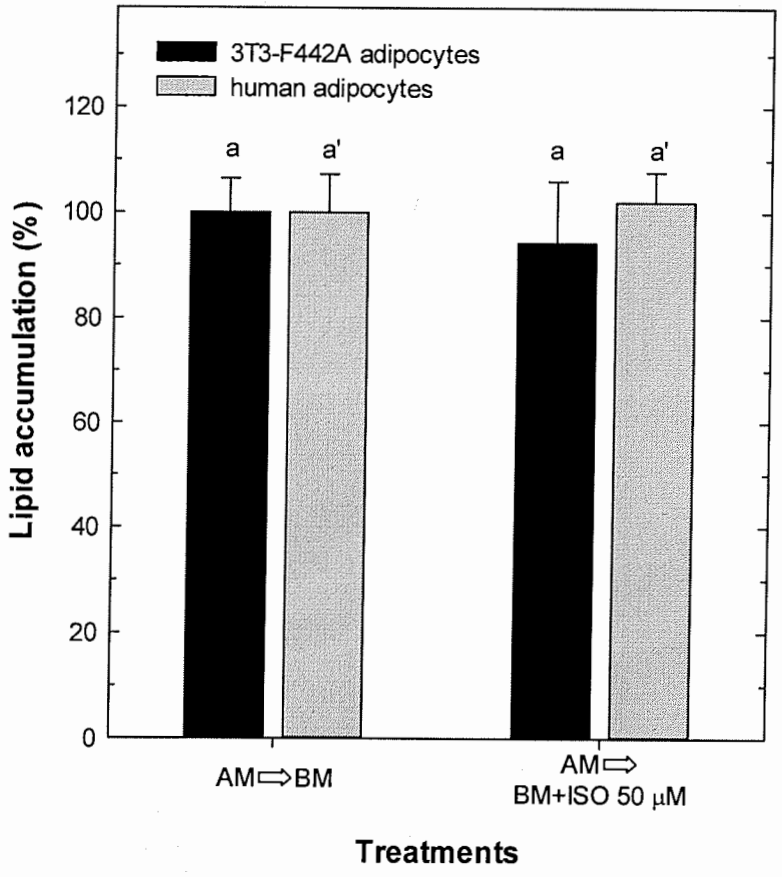
3T3-F442A adipocytes
human adipocytes
Lipid accumulation (%)
120
100
80
60
40
20
0
a
a'
a
a'
AM⇨BM
AM⇨
BM+ISO 50 μM
Treatments

Figure 2

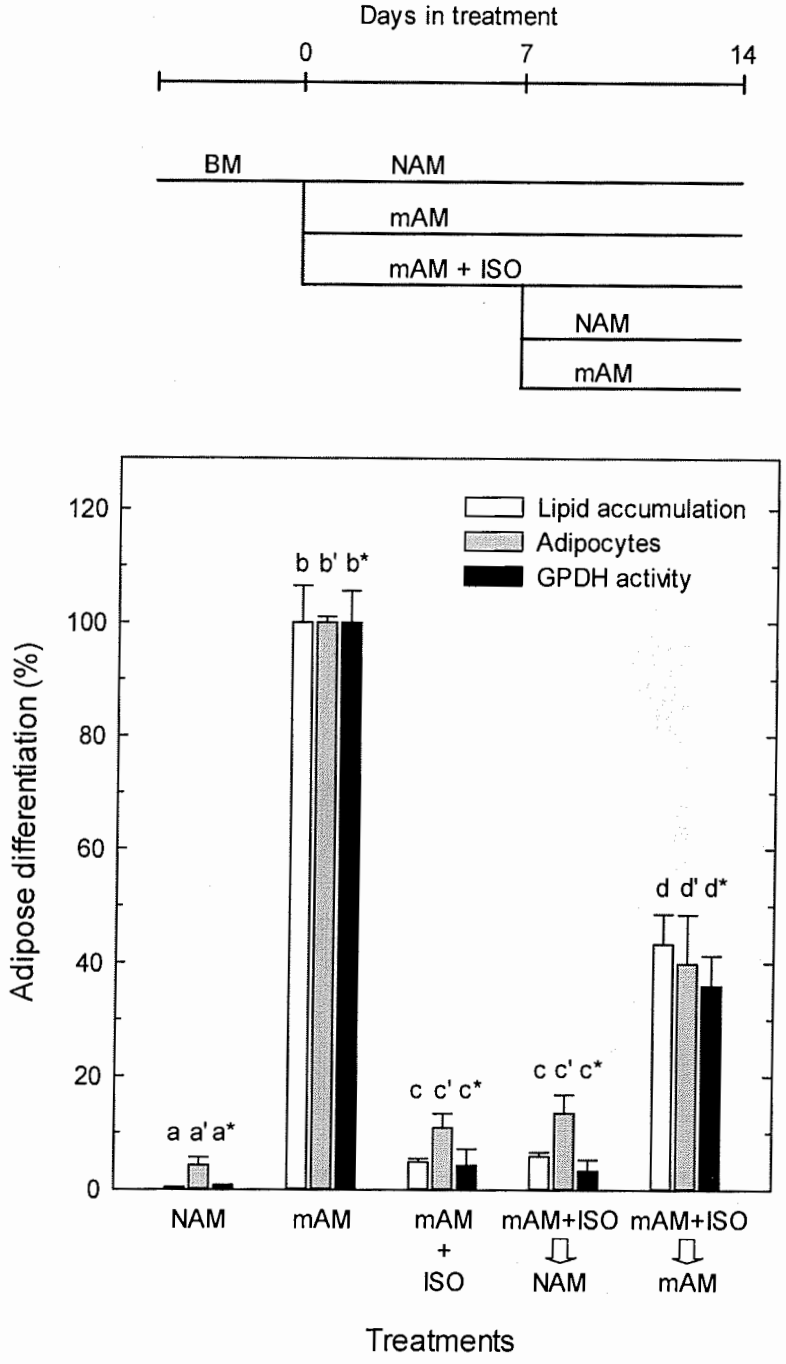
Days in treatment
0
7
14
BM
NAM
mAM
mAM + ISO
NAM
mAM
Lipid accumulation
Adipocytes
GPDH activity
Adipose differentiation (%)
120
100
80
60
40
20
0
a a' a*
b b' b*
c c' c*
c c' c*
d d' d*
NAM
mAM
mAM + ISO
mAM+ISO ⇩ NAM
mAM+ISO ⇩ mAM
Treatments

Figure 3.

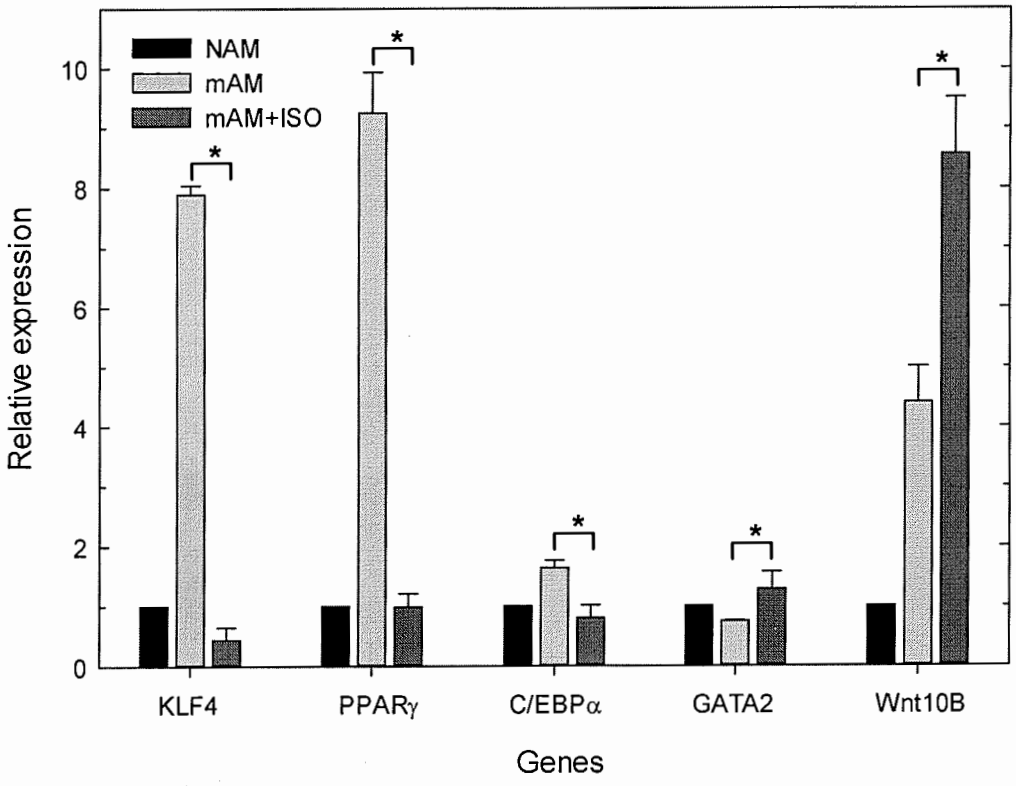
NAM
mAM
mAM+ISO
Relative expression
0
2
4
6
8
10
KLF4
PPARγ
C/EBPα
GATA2
Wnt10B
Genes
*

Figure 4.

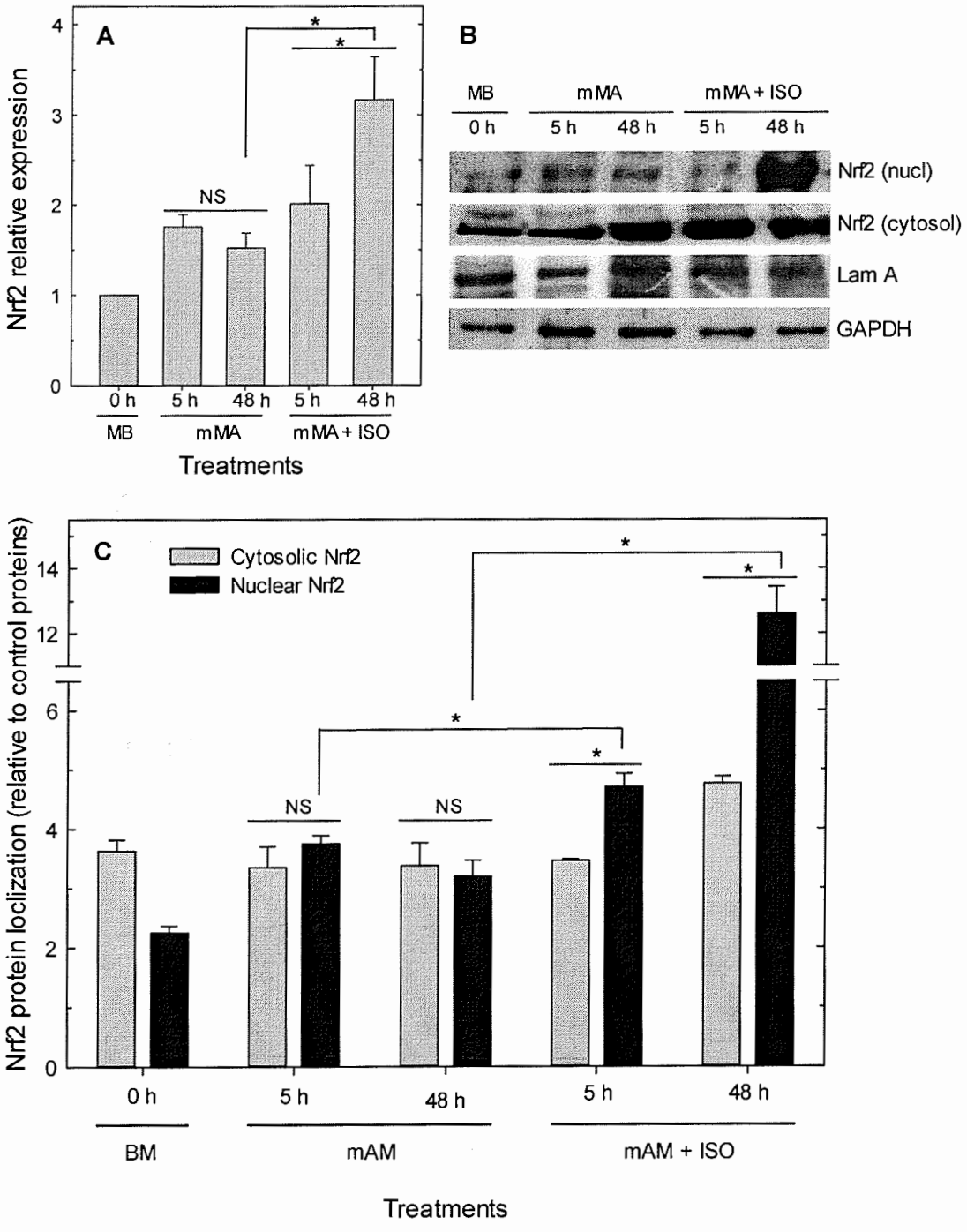
A
Nrf2 relative expression
NS
0 h
5 h
48 h
5 h
48 h
MB
mMA
mMA + ISO
Treatments
B
MB
mMA
mMA + ISO
0 h
5 h
48 h
5 h
48 h
Nrf2 (nucl)
Nrf2 (cytosol)
Lam A
GAPDH
C
Cytosolic Nrf2
Nuclear Nrf2
Nrf2 protein loclization (relative to control proteins)
NS
NS
0 h
5 h
48 h
5 h
48 h
BM
mAM
mAM + ISO
Treatments

Figure 5.

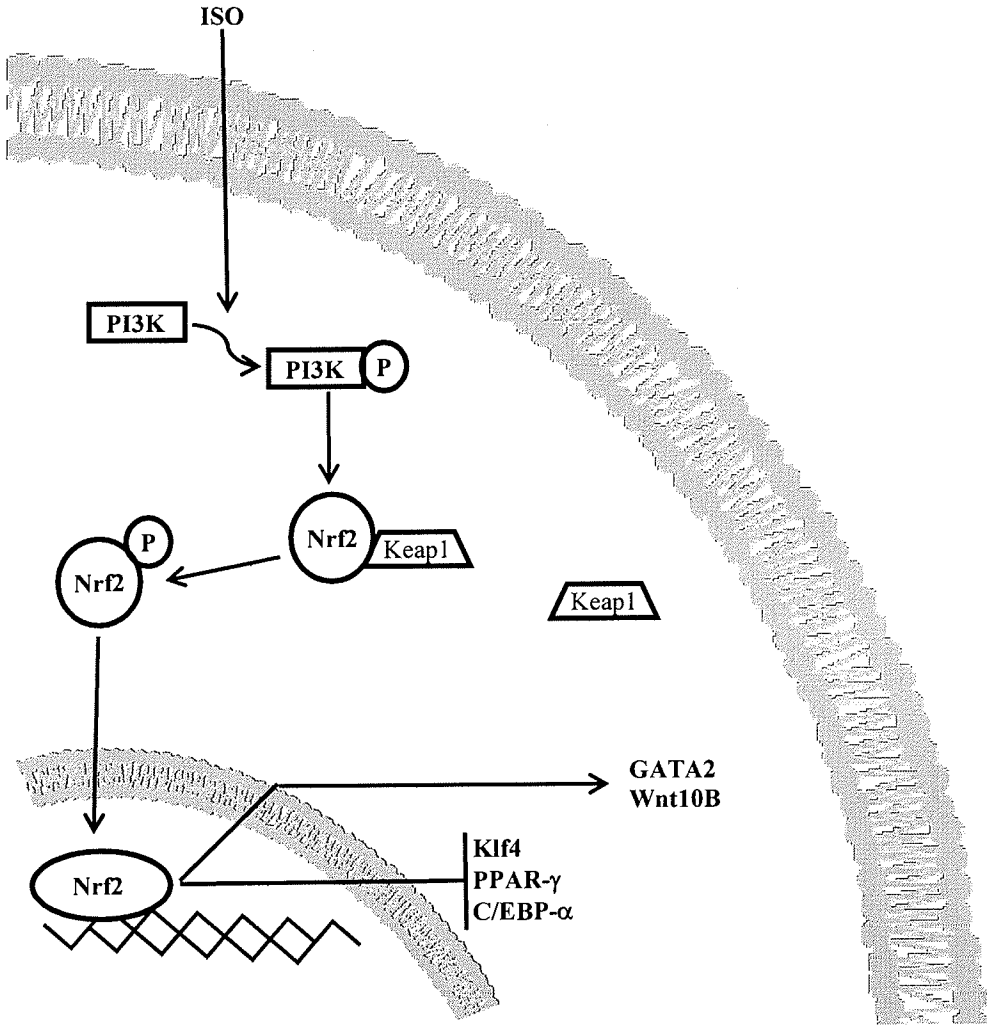
ISO
PI3K
PI3K
P
Nrf2
Keap1
P
Nrf2
Keap1
GATA2
Wnt10B
Klf4
PPAR-γ
C/EBP-α
Nrf2

Figure 6.