ISBN: 978-84-691-1902-0/DL: T-355-2008

Se colocaba el tubo en la unidad de destilación y se procedía a dispensar 50 mL de NaOH. A continuación se destilaba el nitrógeno sobre 50 mL de una solución de ácido bórico al 4%. Se destilaban un total de 100 mL (150 mL de volumen final) puesto que no se observó ningún incremento en la concentración de nitrógeno cuando se destilaba un volumen mayor. Finalizada la destilación, se retiraba el erlenmeyer de la unidad Pro-Nitro y se valoraba con una solución de HCl 0,1N hasta observar el viraje del color verde inicial hasta el rojo burdeos final.

Cálculos:

La cantidad de nitrógeno de la muestra se calculó según la siguiente fórmula:

$$\text{Nitrógeno en g/24h} = (V*N*0{,}014) * V_{24}/Vo$$

Donde,

V= volumen de HCl gastado para la valoración; N= normalidad del HCl; Vo= volumen de orina utilizado para la valoración y V_{24}= volumen de orina recogido en 24 horas.

Para obtener la normalidad de la solución de ácido clorhídrico ésta se valoraba con una solución de carbonato sódico.

5.5. Evaluación de la composición corporal

5.5.1. Evaluación de la composición corporal mediante impedancia bioeléctrica tetrapolar

Para la estimación del porcentaje de grasa del organismo se utilizó el método de la impedancia bioeléctrica tetrapolar con un aparato multifrecuencia (Human-Im-Scam, Dietosystem, España). Esta técnica se basa en la resistencia que ofrece el organismo al paso de una corriente eléctrica alterna de intensidad constante (800 mA) cuando ésta se aplica en dos puntos distantes del organismo. A diferencia de los aparatos tradicionales, el Human-Im-Scan analiza el comportamiento bioeléctrico del organismo a 1, 5, 10, 50 y 100 KHz.

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

A todas las pacientes se les realizaron las medidas de impedancia tras un periodo de ayuno de 12 horas, sin previa realización de ejercicio intenso que pudiera provocar la pérdida de líquido corporal, y con la previa evacuación de la vejiga urinaria. Durante la prueba la paciente fue despojada de todos los objetos metálicos que estuvieran en contacto directo con el cuerpo y que por tanto pudieran alterar la resistencia corporal.

Las pacientes se colocaban en posición de decúbito supino con una separación en relación al eje del tronco de 30º para las extremidades superiores y de 45º para las inferiores. Se consensuó el hemicuerpo izquierdo para la colocación de los electrodos autoadhesivos, dos en el anverso de la muñeca y dos más en la cara anterior del tobillo. El electrodo proximal de la mano se situó en el punto medio que une las epífisis radial y cubital y el electrodo distal se situó a 5 cm por debajo del electrodo proximal, a nivel de la epífisis del tercer metacarpiano. El electrodo proximal del pie se situó en el punto medio de la línea que une los dos maléolos y el electrodo distal se colocó sobre la epífisis el tercer metatarsiano, a 6 cm de distancia del electrodo proximal.

Con estas determinaciones y aplicando las ecuaciones de predicción de Segal (Segal 1988) que corresponden a las características de la población de estudio se obtiene la estimación de la masa libre de grasa (MLG):

Ecuación de Segal específica por sexo y grado de adiposidad para la predicción de la cantidad de grasa corporal en mujeres normopeso:

$$MLG = (0{,}00064602 * talla^2) - (0{,}01397 * khz50) + (0{,}42087 * peso) + 10{,}43485$$

Ecuación de Segal específica por sexo y grado de adiposidad para la predicción de la cantidad de grasa corporal en mujeres con obesidad:

ISBN: 978-84-691-1902-0/DL: T-355-2008

$$MLG=(0,00091186*talla^2)-(0,01466*khz50)+(0,29990\ peso)-(0,07012*edad)+9,37938$$

Finalmente se obtiene el porcentaje de grasa corporal según la ecuación:

$$\%\ grasa=(peso\ total - MLG)*100$$

5.5.2. Evaluación de la composición corporal mediante antropometría

El peso de las pacientes se determinó mediante una balanza digital con una precisión de ± 100 gr. La altura se midió con un tallímetro fijo con precisión de 1 mm con las pacientes en posición ortostática.

Los pliegues de grasa subcutánea bicipital, tricipital, subescapular y suprailíaco se determinaron con un plicómetro de presión constante (Holtain LTD, Crymic, UK) con una precisión de 2 mm. Estos pliegues se midieron en todas aquellas pacientes en las cuales la apertura del plicómetro lo permitía.

El pliegue bicipital se determinó en la cara anterior del brazo no dominante, a nivel del bíceps, en el punto medio entre el acrómion y el olécranon en posición supina. El pliegue tricipital se midió en el mismo punto anterior y en el mismo brazo, también en posición supina pero en su cara posterior. El pliegue subescapular se determinó en el vértice inferior de la escápula izquierda y el pliegue suprailíaco a nivel de la cresta ilíaca, en la línea axilar anterior del lado izquierdo. Cada una de estas determinaciones se realizó por triplicado y consecutivamente por el mismo investigador, dándose como valor válido el resultante de la media de las tres medidas.

El perímetro braquial se determinó mediante una cinta métrica flexible milimetrada en el punto medio de la distancia entre el acromion y el olécranon del brazo no dominante.

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

5.6. EVALUACIÓN DE LA DISTRIBUCIÓN DE LA GRASA CORPORAL

La distribución de la grasa corporal se determinó a partir del índice cintura-cadera. La circunferencia de la cintura se midió con la paciente en bipedestación, con una cinta métrica milimetrada a la altura del punto medio entre la última costilla y la cresta ilíaca. El diámetro de la cadera se midió alrededor de las nalgas, a la altura de la sínfisis del pubis. El cociente cintura/cadera se utilizó para clasificar a las pacientes obesas en:

- obesidad tipo androide cuando el índice cintura/cadera era superior a 0,9.
- obesidad tipo ginoide cuando el índice cintura/cadera era inferior a 0,9.

5.7. DETERMINACIONES BIOQUÍMICAS

Para las determinaciones analíticas realizadas en el Hospital Sant Joan de Reus se emplearon las técnicas de rutina en el ámbito hospitalario: glucosa y hemoglobina glicosilada (método colorimétrico, ITC Diagnostics, IZASA SA, Barcelona), colesterol total y triglicéridos (método enzimático CHOD-POD), colesterol unido a fracciones lipoproteicas (método colorimétrico, ITC Diagnostics, IZASA SA, Barcelona), urea, ácido úrico y creatinina (método colorimétrico, ITC Diagnostics, IZASA SA, Barcelona), proteínas totales (método de GORNALL), albúmina (método verde del bromocresol), prealbúmina y transferrina (inmunoturbidimetría), hemograma (autoanalzador Coulter Max-M), VSG (eritrosedimentación), Tirotropina (método colorimétrico, ITC Diagnostics, IZASA SA, Barcelona).

La determinación de las concentraciones plasmáticas de ácidos grasos libres, 3-hidroxibutirato y beta-estradiol fueron realizadas en los laboratorios del Departamento de Bioquímica y Biología Molecular de la Facultad de Biología de la Universidad de Barcelona, bajo la dirección del Profesor Marià Alemany. Los ácidos grasos libres se determinaron mediante un método comercial (NEFAC kit, Wako Chem, Neuss, Alemania) basado en el método enzimático descrito por Gutman (Gutman 1974). Los niveles de beta-hidroxibutirato se determinaron por

ISBN: 978-84-691-1902-0/DL: T-355-2008

espectofotometría (30UV kit, Sigma, St Louis, MO USA) y el beta-estradiol se determinó mediante una ELISA comercial (Immunotech, Marsella, Francia).

5.8. DETERMINACIÓN DE ESTRONA LIBRE Y ÉSTERES DE ESTRONA EN PLASMA

La determinación del contenido plasmático de estrona libre y esterificada se realizó mediante cromatografía líquida (HPLC) siguiendo el protocolo descrito por Ardévol y colaboradores (Ardévol 1997). Estas determinaciones fueron realizadas por el equipo del Profesor Marià Alemany en la Unidad de Bioquímica y Biología molecular de la Facultad de Biología, Universidad de Barcelona.

5.9. DETERMINACIÓN DE LEPTINA PLASMÁTICA

La leptina plasmática fue determinada mediante un RIA comercial (Linco Research, St Louis, MO) con un coeficiente de variación inter e intraensayo de 3,4% y 8,3% respectivamente, y con una sensibilidad de 0,5 ng/mL. Esta técnica se basa en la competencia que se establece por la unión con un anticuerpo específico entre la leptina de la muestra y una leptina humana purificada marcada radiactivamente con I^{125} que se añade a una concentración constante y conocida en cada una de las muestras a valorar. Así pues, la leptina endógena de cada muestra desplazará de manera proporcional a su concentración la unión entre el anticuerpo específico y la leptina marcada, la cual puesto que queda en forma libre, será eliminada en los lavados sucesivos de la muestra.

Material

- Tubos de poliestireno de 5 mL
- Micropipetas de 200 μL y 1000 μL
- Puntas para micropipetas de 200 μL y 1000 μL
- Vórtex Heidolph (J.P. Selecta, Barcelona, España)

ISBN: 978-84-691-1902-0/DL: T-355-2008

- Centrífuga refrigerada a 5000 rpm (J.P. Selecta, Barcelona, España)
- Contador partículas gama (COBRA II)

Reactivos

- RIA kit (Linco Research, St Louis, MO)

Protocolo

A cada tubo se añadían 300μL de tampón de ensayo, 100μL de patrón o muestra, 100μL de leptina marcada con I^{125} y 100 μL de anticuerpo específico. Para facilitar las uniones se dejaban incubar las muestras toda la noche a 4ºC. Posteriormente se precipitaban los complejos antígeno-anticuerpo añadiendo 1 mL de agente precipitante, se agitaba en un vórtex y se dejaba incubar 20 minutos más a 4ºC. Posteriormente se centrifugaban las muestras a 5000 rpm durante 15 minutos y a 4 ºC, y se descartaba el sobrenadante, dejándose secar el precipitado a temperatura ambiente.

Las cuentas por minuto (cpm) se midieron en un contador de radiación gama. La curva estándar resultante fue linealizada mediante la transformación logarítmica y la concentración de leptina de las muestras, expresada en ng/mL, se obtuvo a partir de la interpolación de los valores de cada muestra a la curva patrón.

5.10. Determinación de la leptina en el tejido adiposo

Para la valoración de la leptina tisular se utilizó un RIA comercial (Linco Research, St Louis, MO) y por tanto se siguió el mismo protocolo que para las valoraciones plasmáticas de esta hormona.

Tras su extracción, las muestras de tejido adiposo se congelaron inmediatamente en nitrógeno líquido y se conservaron en un congelador de –80ºC hasta su utilización. Para la determinación

ISBN: 978-84-691-1902-0/DL: T-355-2008

de la leptina se preparó un homogenado de tejido en un medio tamponado a pH fisiológico, en presencia de inhibidores de proteasas (leupeptina, aprotinina, fenilmetilsulfonida) necesarios para mantener intactas las estructuras proteicas y evitar su degradación debida a los enzimas específicos, y en presencia de detergentes (Tritón X100) que permiten la eliminación de los lípidos del tejido, los cuales podrían interferir en las valoraciones posteriores (Kern 1995).

Material

- Tubos de polipropileno de 10 mL
- Tubos de polipropileno de 3 mL
- Homogeneizador manual de cristal
- Centrífuga refrigerada a 4000 rpm (J.P. Selecta, Barcelona, España)
- Pipetas Pasteur de cristal
- Criotubos con tapón de rosca de 2,5 mL
- Cajas de congelador
- Micropipeta de 1 mL
- Puntas para micropipetas de 1mL
- Balanza analítica (A&D Instruments, Abingdon, Oxon, Reino Unido)
- Congelador de -80ºC (Sanyo, Sakata Oizumi-Machi, Japón)

Reactivos

- Tampón fosfato PBS 10x (10 mM NaPi; 0,150 M NaCl; pH 7,4):
  - cloruro sódico (ClNa) 80 gr
  - cloruro potásico (KCl) 2 gr
  - bifosfato sódico (Na_2HPO_4) 14,4 gr
  - fosfato postásico dibásico (KH_2PO_4) 2,4 gr
  - llevar hasta 1 L con agua bidestilada y ajustar el pH a 7,2

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

- Aprotinina (50 μg/mL)
- Leupeptina (50 μg/mL)
- Fenilmetilsulfonida (1mg/mL)
- Tritón X100

Protocolo

Se pesaron aproximadamente 150-250 mg de cada muestra y se homogeneizaron manualmente en 700 μL de tampón de homogeneización (10μL Aprotinina, 20μL Leupeptina, 45μL PMSF, 10μL Tritón X100, 915μL PBS 1x) en un tubo de polipropileno de 10 mL. Posteriormente se centrifugaban las muestras durante 5 minutos a 4ºC y a 4000 rpm y se eliminaba la capa de lípidos aspirando el sobrenadante con una pipeta Pasteur y recuperándolo en un tubo de 3 mL. Se repetía la centrifugación para limpiar mejor la muestra. Cada homogenado se repartía en dos criotubos y se congelaba a -80ºC hasta su valoración.

La valoración de la leptina se realizó siguiendo el mismo protocolo que en las muestras de plasma y los resultados se expresaron en ng de leptina/mg de tejido adiposo.

5.11. Determinación del TNFα plasmático

El TNFα plasmático se determinó mediante un ELISA tipo sandwich no competitivo (PharMingen, San Diego, USA). Esta técnica se basa en la utilización de un anticuerpo anti-TNFα que se fija, por un lado a la placa de ELISA y por otro lado al TNFα de la muestra. Tras eliminar la muestra no unida mediante lavados sucesivos con un tampón que contiene detergente, se añade el anticuerpo específico biotinilado que se unirá al antígeno en cantidad proporcional a su concentración y permitirá la posterior detección colorimétrica con estreptavidina peroxidasa.

ISBN: 978-84-691-1902-0/DL: T-355-2008

Material

- Placas de ELISA de poliestireno de 96 pocillos
- Micropipetas de 50μL, 200μL y 1000 μL
- Puntas para micropipetas
- Pipeta multicanal para 8 puntas
- Tubos eppendorfs de 2 mL
- Tubos polipropileno de 10 mL
- Lector de placas de ELISA con filtro para 450nm y 550nm

Reactivos

- carbonato sódico ($NaC0_3$)
- carbonato sódico hidratado ($NaHC0_3$)
- tampón fosfato PBS:
    - NaCl 80 gr
    - Na_2HPO_4 11,6 gr
    - K_2HPO_4 2 gr
    - KCl 2 g
    - Llevar a 10 L y ajustar el pH a 7,0
- Suero bovino fetal (FBS)
- Tween 20
- PharMingen's TMB Substrate Reagent Set (BD Biosciences, Life Science Research, Heidelberg, Alemania)
- Ácido sulfúrico (H_2SO_4)

Protocolo

En cada pocillo de la placa de ELISA se dispensaban 50μL de anticuerpo anti-TNFα sin

ISBN: 978-84-691-1902-0/DL: T-355-2008

biotinilar y se dejaba incubar toda la noche para favorecer las uniones del anticuerpo a la placa. Posteriormente se eliminaba el exceso de anticuerpo mediante sucesivos lavados y se procedía al bloqueo de la placa con PBS-10% FBS, dejándose incubar durante 1 hora a temperatura ambiente. Seguidamente se procedía al lavado del exceso de anticuerpo y se dispensaban 50 μL de cada uno de los puntos de la patrón del mismo kit comercial y 50μL de cada una de las muestras de plasma en los diferentes pocillos, dejando incubar a temperatura ambiente durante 2 horas. Transcurrido este tiempo y tras un nuevo lavado de la placa se añadían 50 μL de la solución de anticuerpo biotiniliado y del conjugado estreptavidina-peroxidasa y se dejaba incubar a temperatura ambiente durante 1 hora. Se eliminaba el exceso de anticuerpo y se añadían 50 μL del sustrato de la reacción, el cual se dejaba actuar durante 30 minutos a temperatura ambiente. Finalmente se detenía la reacción colorimétrica añadiendo a cada pocillo 50μL de una solución 2N de ácido sulfúrico y se realizaban las lecturas al espectofotómetro con un filtro de 450 nm y una corrección de filtro de 550nm.

La concentración de TNFα de cada muestra expresada en pg/mL se obtenía tras la extrapolación de cada lectura a la curva patrón normalizada.

5.12. Determinación del TNFα en tejido adiposo

Para la valoración del TNFα en el tejido adiposo se utilizaron los mismos homogenados que para la determinación de la leptina tisular y el mismo protocolo que la valoración del TNFα plasmático. Los resultados se expresaron en ng de TNFα /mg de tejido adiposo.

5.13. Determinación de la actividad lipoproteína lipasa adipocitaria

La actividad lipoproteína lipasa se determinó en un homogenado de tejido adiposo mediante el método descrito por Ramírez (Ramírez 1985). El método de valoración utilizado se basa en la utilización de trioleïna tritiada (un triacilglicerol marcado con ^{3}H en sus ácidos grasos) como

ISBN: 978-84-691-1902-0/DL: T-355-2008

sustrato de la reacción, de manera que como consecuencia de la actividad enzimática se obtienen ácidos grasos libres. Mediante un sistema de partición líquido-líquido se consiguen separar los ácidos grasos liberados de los triglicéridos no hidrolizados. La reacción se detiene con disolventes inorgánicos y mediante un tampón de pH básico se extraen los ácidos grasos.

Estas determinaciones fueron realizadas por la Dra. Julia Peinado-Onsurbe en el laboratorio de Bioquímica y Biología Molecular de la Facultad de Biología, Universidad de Barcelona.

Material

- Contador de radiación beta (TRI-CARB 150 Packard)

Reactivos

- Tampón de valoración de la actividad LPL:
  - 27,8 mM PIPES
  - 55,5 mM MgCl2.6H2O
  - 0,55 mg BSA-FFA/mL
  - 5 U/mL Heparina sódica
  - 3,33 % suero pre-calentado
  - llevar a pH 7,0
- Estoc LPL:
  - 49,5 mg trioleína (TOG) = 6,67 mM
  - 120 μL glicerol tri [9,10-(n)-3H] oleato (5mCi/mL)
  - llevar a 8 mL con tolueno
- Solución de detención de la reacción:
  - metanol:cloroformo:heptano (1,45:1,21:1)
- Tampón borato-carbonato: H_3BO_3-KCO_3 0,1 M pH 10,5

ISBN: 978-84-691-1902-0/DL: T-355-2008

Protocolo

En cada valoración se utilizaron 7 μL de homogenado que se añadían al tubo de valoración el cual contenía 180μL de sustrato, se agitaba vigorosamente y se dejaba incubar durante 30 minutos en un baño a 25ºC. La reacción se detenía añadiendo a cada tubo 3,5 mL de una mezcla v/v de metanol/cloroformo/heptano.

Para separar los ácidos grasos libres se añadía 1 mL de tampón borato-carbonato y tras una centrifugación de la muestra a 2000 rpm durante 10 minutos y a 4ºC se conseguía la separación en dos fases. La fase inferior, que contenía la mezcla cloroformo-heptano, mantenía disueltos los triglicéridos no hidrolizados, mientras que la fase superior de metanol y agua contenía el oleato liberado en la reacción. Un total de 0,3 mL de la fase superior eran utilizados para el contage de radiación beta.

5.14. CUANTIFICACIÓN DE DNA EN HOMOGENADOS DE TEJIDO ADIPOSO

Para la cuantificación del DNA se utilizó el colorante Hoechst 33342, una bisbenzimida soluble que presenta una elevada permeabilidad de membrana de modo que no requiere una extracción previa de DNA. Este fluorescente tiene una energía de excitación a 350 nm y emite fluorescencia en el espectro del UV, a 460 nm. La sensibilidad del Hoechst es aproximadamente de 5 ng/mL.

Material

- Fluorímetro (Fluoroskan Ascent)
- Placas de ELISA de 96 pocillos
- Hoechst 33342 (1 mg/mL)
- Tris-EDTA 1x ph 7,5
- DNA stock (1 mg/mL)

ISBN: 978-84-691-1902-0/DL: T-355-2008

Protocolo

Se construyó un patrón de DNA de 20, 40, 60, 80, 100 ng/μL a partir de una solución de DNA comercial. Se prepararon diluciones de los homogenados de tejido en relación 2,5 μL de homogenado en 197,5 μL de TE. En cada pocillo se dispensaron, por duplicado, 200 μL del patrón y de las muestras y se añadieron 50 μL de Hoechst 33342 diluido 1/200 en Tris-EDTA. Se dejaron incubar las muestras durante 1 hora en una estufa a 37ºC. Transcurrido este tiempo se determinaron las concentraciones de DNA en el fluorímetro.

5.15. Determinación de la concentración de los receptores solubles de TNFα

Para determinar la concentración de las formas solubles de los receptores de TNFα se utilizaron placas comerciales de EASIAs (Enzyme Amplified Sensitivity Immunoassay) (Biosource Europa SA, Nivelles, Bélgica). Este método utiliza la capacidad de un pool de anticuerpos monoclonales dirigidos contra epítopos específicos de los sTNFR y la posterior formación de complejos anticuerpo-antígeno-anticuerpo tras la unión con un segundo anticuerpo monoclonal específico conjugado con peroxidasa. Estos complejos marcados se detectan a través de una reacción cromogénica con tetrametilbenzidina a una longitud de onda de 450 nm, dando una absorbancia proporcional a la concentración de receptores de la muestra. El límite de sensibilidad de la técnica era de 50 pg/mL para el sTNFR y de 0,1 ng/mL para el sTNFR. Los coeficientes de variación interensayo e intraensayo eran inferiores a 8,9 y a 6,5 respectivamente para ambos tipos de receptores. Todas las medidas fueron realizadas por duplicado.

5.16. Determinación de los niveles de insulina en plasma

Los niveles de insulina plasmáticos se determinaron mediante un método comercial basado en la competencia establecida entre la insulina de la muestra y una insulina humana marcada con I^{125}, por los epítopos de unión de un anticuerpo específico. La separación de la fracción de

ISBN: 978-84-691-1902-0/DL: T-355-2008

anticuerpo unido a insulina y anticuerpo libre se consigue por una simple centrifugación.

Material

- Lector de radiación gamma (COBRA II)
- Centrífuga refrigerada (J.P. Selecta, Barcelona, España)

Reactivos

- Rat insulin assay system with Amerlex ™-m magnetic separation (BIOTRAK, Amersham, Little Chalfont, Buckingamshire, Inglaterra)

Los valores de insulina obtenidos en ng/mL tras la extrapolación de las medidas de cpm a la curva patrón del kit de insulina, fueron transformados a unidades de sistema internacional (UI) mediante los siguientes factores de corrección (Burtis 1994):

$$(ng/mL)*172,2 = (pmol/L)$$

$$\mu UI/L*6,945 = (pmol/L)$$

Este método, aún siendo específico para la insulina murina, tiene un 189% de reacción cruzada con la insulina humana, de modo que, mediante una sencilla corrección permite calcular la concentración circulante de esta hormona en humanos.

$$(X\ \mu UI/L)*(100/189)$$

5.17. Cuantificación de la expresión de leptina y TNFα en tejido adiposo

La cuantificación de la concentración de mRNA de leptina y TNFα en el tejido adiposo se realizó mediante la combinación de la transcripción reversa y la reacción en cadena de la polimerasa (RT-PCR). Esta técnica combina la síntesis de DNA complementario a partir del

ISBN: 978-84-691-1902-0/DL: T-355-2008

RNA total o del mRNA con la posterior amplificación de este cDNA. Para ello se realizó una extracción previa de RNA total del tejido basándonos en el método descrito por Chomczynski y Sacchi (Chomczynski 1987).

5.17.1. Extracción de RNA

A partir de muestras de tejido adiposo de entre 125 y 250 mg de peso se procedió a la extracción del RNA total mediante el reactivo TriPure (Boheringer Mannheim), el cual, mediante una separación en fase líquida, permite la extracción diferencial del RNA, DNA y proteínas de la muestra. El reactivo TriPure es una solución monofásica que permite en un único paso la lisis celular mediante la acción del isotiocianato de guanidina y la posterior separación de proteínas y DNA por extracción con fenol ácido. Esto aporta una mayor rapidez metodológica así como una menor pérdida de material genético debido a la eliminación de pasos intermedios innecesarios.

Puesto que el tejido adiposo tiene un elevado contenido lipídico capaz de interferir en la RT-PCR, se añadió al protocolo convencional una primera centrifugación de los homogenados tisulares a 12000 g durante 5 minutos y a 4ºC. Este primer paso permite la eliminación de las grasas liberadas durante el proceso de homogeneización del tejido.

Material

- Politrón
- Centrífuga refrigerada (J.P. Selecta, Barcelona, España)
- Espectofotómetro Gene-Quant

Reactivos

- TriPure (Boheringer Mannheim GmbH, Alemania)
- Etanol absoluto
- Isopropanol
- Cloroformo

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

Protocolo

Debido a la dificultad para homogeneizar completamente el tejido adiposo humano manualmente por su elevado contenido fibroso, se optó por un método mecánico que, aún siendo más agresivo, permite obtener una mejor disrupción del tejido sin alterar la calidad del material genético obtenido. Para ello se utilizó un homogeneizador automático. Toda la manipulación del tejido adiposo y todos los procesos necesarios para la obtención del RNA se realizaron en estrictas condiciones de esterilidad y a 4ºC para evitar al máximo la contaminación y degradación de la muestra.

La homogeneización del tejido se realizó como paso previo a la extracción de RNA, en no más de 8 muestras a la vez para no demorar en exceso el tiempo de exposición de la muestra a agentes contaminantes y a razón de 1 mL de reactivo TriPure por cada 125-250 mg de tejido adiposo. Tras la centrifugación de las muestras a 12000g durante 5 minutos y a 4ºC para eliminar la primera capa de lípidos se dejaba actuar el reactivo durante 10 minutos manteniendo siempre la muestra en frío. Posteriormente se añadían 200 microlitros de cloroformo y tras una agitación vigorosa para mezclar bien las fases se dejaba incubar durante 15 minutos. Una ulterior centrifugación a 12000 g durante 15 minutos separaba el RNA en una fase superior acuosa de la cual, tras añadir 500 microlitros de isopropanol y guardar a –20ºC durante toda la noche, se podía precipitar el RNA. Este precipitado de RNA se lavaba con etanol 75% para eliminar todos los restos de isopropanol que podrían interferir en la RT-PCR y se dejaba secar al aire entre 15 y 25 minutos. Se consideraba que la muestra estaba suficientemente seca cuando no se observaban gotitas de etanol ni en las paredes ni en el fondo del tubo. Este RNA se resuspendía en 50 microlitros de agua estéril y se guardaba inmediatamente a –80ºC para la posterior amplificación.

La concentración del RNA se calculó midiendo la absorbancia a 260 nm en un espectofotómetro (Gene Quant, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, Inglaterra) de una dilución 1/100 del precipitado previamente disuelto. El

ISBN: 978-84-691-1902-0/DL: T-355-2008

grado de pureza respecto a proteínas y DNA se estimó mediante el cociente entre la absorbancia a 260 nm y la absorbancia a 280 nm medidas en el mismo aparato. Los ratios obtenidos oscilaron entre 1,4 y 2,0, siendo las concentraciones de RNA obtenidas muy variables.

5.17.2. RT-PCR para la leptina y el TNFα

5.17.2.1. Transcripción Inversa

La transcripción inversa aprovecha la capacidad de algunos enzimas víricos para sintetizar DNA monocatenario a partir de RNA en presencia de *primers* o cebadores y en unas condiciones de pH y concentración de sales adecuadas. Para éste método utilizamos una transcriptasa reversa comercial derivada del virus de la leucemia murina (Roche Diagnostics GmbH, Alemania) y random hexamers como cebadores.

Material

- Termociclador Progene (Techne, Cambridge)

Reactivos

- Expand Reverse Transcriptase 50U/μL (Roche Diagnostics GmbH, Alemania)
- Ditiotreitol, DTT (Roche Diagnostics GmbH, Alemania)
- Mezcla equimolecular de desoxirribonucleótidos trifosfato (dNTPs) 10mM cada uno (Boheringer Manheim, Alemania)
- Random hexamers (Perkin Elmer Branchburg, New Jersey, USA)
- Tampón 5x (50 mM Tris-HCl, 100mM NaCl, 1 mM EDTA, 10 mM ditiotreitol, 0,05% polydocanol (v/v), 50% glycerol (v/v), pH 8,4) (Roche Diagnostics GmbH, Alemania)
- RNAse Inhibitors (Perkin Elmer Branchburg, New Jersey, USA)

Esta reacción se realizó a partir de 0,5 microgramos de RNA total en un volumen final de 6 microlitros para la leptina y de 5,5 μL para el TNFα, en presencia de DTT en un termociclador

ISBN: 978-84-691-1902-0/DL: T-355-2008

Progene. Los tránscritos resultantes fueron directamente amplificados mediante PCR o bien congelados a –80ºC para la posterior amplificación. Las condiciones de temperatura utilizadas para la transcripción reversa fueron las descritas en la Tabla 1.

Tabla 1. Condiciones de tiempo y temperatura utilizadas para la transcripción reversa.

Proceso	Temperatura	Tiempo
Desnaturalización	90 ºC	1 min
Retrotranscripción	30 ºC	10 min
Retrotranscripción	42 ºC	45 min
Elongación	99 ºC	5 min

5.17.2.1.1. Transcripción reversa del gen de la leptina

En un tubo de PCR se dispensaron 0,5 μg de RNA total, se llevaron a un volumen final de 4 μL con agua estéril. Se desnaturalizó la muestra durante 1 minuto a 90ºC. A cada muestra se añadieron 6 μL de la mezcla para la transcripción reversa: 2μL de 5xbuffer, 1μl de una solución 25 mM de dNTPs, 1μL de ditiotreitol, 1μL de random hexamers, 0,5 μL de inhibidores de RNAsas y 0,5 μL de transcripatsa reversa. Las muestras se colocaron en el termociclador y se procedió a su retrotrasncripción según las condiciones descritas en la Tabla 1.

5.17.2.1.2. Transcripción reversa del gen del TNFα

En un tubo de PCR se dispensaron 0,5 μg de RNA total, se llevaron a un volumen final de 4,5 μL con agua estéril. Se desnaturalizó la muestra durante a 1 minuto a 90ºC. A cada muestra se añadieron 5,5 μL de la mezcla para la transcripción reversa: 2μL de 5xbuffer, 1μl de una solución 25 mM de dNTPs, 1μL de ditiotreitol, 1μL de random hexamers, 0,5 μL de inhibidores de RNAsas y 0,5 μL de transcriptasa reversa. Las muestras se colocaron en el termociclador y se procedió a su retrotranscripción según las condiciones descritas en la Tabla 1.

5.17.2.2. PCR semicuantitativa

La PCR semicuantitativa permite amplificar específicamente segmentos de DNA de doble

ISBN: 978-84-691-1902-0/DL: T-355-2008

cadena mediante dos cebadores oligonucleotídicos situados en las regiones extremas del fragmento que se desea amplificar. La repetición cíclica de los procesos de desnaturalización, unión y extensión de los cebadores permite realizar copias sucesivas y exactas de manera exponencial. Puesto que se co-amplifican en la misma reacción dos genes distintos, la relación entre la expresión del uno y el otro permite establecer un ratio de cuantificación.

Material

- Termociclador Progene (Techne, Cambridge)
- Tubos para PCR de 0,2 mL (Perkin Elmer Branchburg, New Jersey, USA)
- Autoclave (J.P. Selecta, Barcelona, España)
- Agitador vórtex Heidolph (Heidolph Instruments GmbH&Co KG, Walspersdorfer, Alemania)

Reactivos

- Expand High Fidelity PCR System 3,5 U/μL (Roche Diagnostics GmbH, Alemania)
- Expand HF Buffer 10x (20 mM Tris-HCl pH 7,5, 100mM KCl, 0,1 mM EDTA, 1 mM ditiotreitol, 0,5% Tween 20 (v/v), 0,5% Nonidet P40 (v/v), 50% glycerol (Roche Diagnostics GmbH, Alemania)
- Cloruro de magnesio 25 mM (Roche Diagnostics GmbH, Alemania)
- Agua bidestilada (Milli-Q) autoclavada

5.17.2.2.1. Síntesis de los oligonucleótidos

Las secuencias de los oligonucleótidos para la leptina (OBH12, OBH2) y para la actina (BACC20, BACC21) fueron cedidas por el laboratorio del Prof A. Palou (Departamento de Biología Fundamental y Ciencias de la Salud, (UIB). La secuencia de los oligonuceótidos para el TNFα (TNF1, TNF2) fue cedida por el Prof. Marià Alemany (Departamento de Bioquímica y Fisiología, Facultad de Biología, UB). Las secuencias de los *primers* utilizados estan basados en las secuencias para el gen de la obesidad (Isse N, 1995; GenBank D63710) para el gen del TNFα (Nedwin GE, 1985; GenBank X02910) y para el gen de la beta-actina (Nakajima-Ijima S

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

1985: GenBank M10277). Estos cebadores se sintetizaron en los laboratorios TIB MOLBIOL (Konto, Berlín). Las secuencias de estos oligonucleótidos se detallan en la Tabla 2.

Tabla 2. Secuencia de los oligonucleótidos sintéticos utilizados.

Cebador	Orientación	Secuencia	longitud	TM (°C)
TNF1	Sentido	5'-GAGTGACAAGCCTGTAGCCCATGTTGTAGC-3'	30	69,5
TNF2	Antisentido	5'-GCAATGATCCCAAAGTAGACCTGCCCAGAC-3'	30	69,5
		Longitud del fragmento amplificado 454 pb		
OBH12	Sentido	5'- TTCACACACGCAGTCAGTCTC-3'	22	68,4
OBH2	Antisentido	5'-ACAGAGTCCTGGATAAGGGGT-3'	21	64,7
		Longitud del fragmento amplificado 482 pb		
BACC20	Sentido	5'-GTGGTGGTGAAGCTGTAGCC-3'	20	66,6
BACC21	Antisentido	5'-GAGAAGATGACCCAGATCATGT-3'	22	61,6
		Longitud del fragmento amplificado 258 pb		

Protocolo

Para la reacción de amplificación se utilizaron 10 μL de la solución del tránscrito inverso en un volumen final de 25 μL según las condiciones de temperatura establecidas en las Tablas 3 y 4.

Tabla 3. Condiciones de amplificación para la reacción de PCR del gen de la leptina.

Proceso	Temperatura (°C)	Tiempo (min)	Nº ciclos
Desnaturalización	94	3	1
Desnaturalización	94	1	
Annealing	63,5	1	30
Elongación	72	2	
Elongación final	72	10	1

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

Tabla 4. Condiciones de amplificación para la reacción de PCR del gen del TNFα.

Proceso	Temperatura (ºC)	Tiempo (min)	Nº ciclos
Desnaturalización	94	3	1
Desnaturalización	94	1	
Annealing	65	1	33
Elongación	72	2	
Elongación final	72	10	1

5.17.2.2.2.Amplificación del gen de la leptina

Se utilizaron 10 μL del producto de la transcripción reversa y se añadieron 15μl de la mezcla de reaccción: 2,5μl de 10xbuffer, 1,5μl de Cl_2Mg 25mM, 0,3μl de cada uno de los *primers* de leptina, 0,3μl de cada uno de los *primers* para la beta-actina, 0,3μl de Transcriptasa Reversa, 9,5 μl de agua estéril. Se procedió a la amplificación del gen según las condiciones descritas en la Tabla 3.

5.17.2.2.3. Amplificación del gen del TNFα

Se utilizaron 10 μL del producto de la transcripción reversa y se añadieron 15μl de la mezcla de reacción: 2,5μl de 10xbuffer, 2μl de Cl_2Mg 25mM, 0,5μl de cada uno de los *primers* del TNFα, 0,1μl de cada uno de los *primers* para la beta-actina, 0,3μl de Transcriptasa Reversa, 9,5 μl de agua estéril. Se procedió a la amplificación del gen según las condiciones descritas en la Tabla 4.

5.17.3. Electroforesis de DNA

La electroforesis horizontal en gel de agarosa permite separar, identificar y si es preciso purificar fragmentos de DNA de diferentes tamaños en función de la concentración de agarosa del gel. Estos fragmentos se detectan mediante la tinción con bromuro de etidio, una sustancia fluorescente que se intercala entre las bases de la doble hélice de DNA dando fluorescencia cuando se irradia con luz ultravioleta, siendo la intensidad de esta fluorescencia proporcional a la concentración de ácido nucleico. La fluorescencia del bromuro de etidio no

ISBN: 978-84-691-1902-0/DL: T-355-2008

permite detectar bandas que contengan menos de 1 ng de DNA (Sharp 1973), pero este límite de sensibilidad es suficiente para la cuantificación de productos de PCR.

Material

- Sistema de electroforesis horizontal, mini-sub cell GT (Bio-Rad, California)
- Fuente de alimentación para electroforesis, Power Pac 300 (Bio-Rad, California)
- Horno microondas
- Transiluminador de luz ultravioleta
- Cámara fotográfica Polaroid

Reactivos

- Tampón TAE 50X (solución de trabajo 1x):
  - Tris base 242 gr
  - Ácido acético glacial 57,1 gr
  - 0,5M EDTA pH 8,0 100 mL
  - Llevar a 1 L con agua destilada
- Agarosa de baja electroendosmosis
- Bromuro de etidio 10 mg/mL (Bio-Rad, California)
- Marcador de peso molecular 100 pb DNA Ladder
- Tampón de carga (50% Glicerol, 50% agua bidestilada, 0,012 mg de azul de bromofenol)

Protocolo

Se preparó el gel de agarosa a una concentración final de 1,5% en TAE 1X. Se añadían 5 μL de bromuro de etidio y tras una vigorosa agitación se montaba el gel. Tras 40 minutos de solidificación se procedía a cargar una mezcla de 15 μL de producto de PCR y 5 μL de tampón de carga en cada pocillo. Como referencia del tamaño de DNA de los fragmentos analizados se cargaban 2 μL de marcador de peso molecular en el primer pocillo de cada gel. La

ISBN: 978-84-691-1902-0/DL: T-355-2008

electroforesis se realizó durante 40 minutos a 75V en tampón TAE 1X.

Las bandas de DNA se visualizaban en un transiluminador de luz ultravioleta de 312 nm de longitud de onda y mediante una cámara fotográfica Polaroid acoplada a un sistema informático, se captaban y almacenaban las imágenes en soporte informático con formato TIFF.

5.17.4. Semicuantificación del producto de PCR

La cuantificación de la expresión génica de leptina y TNFα se determinó por densitometría midiendo la intensidad de las distintas bandas detectadas en el gel mediante el software KODAC DIGITAL SCIENCE (KODAC, Science Park, New Haven CT). Los valores de expresión de leptina y TNFα se normalizaron con los niveles de mRNA de beta-actina, expresándose en los resultados como el cociente entre las densidades obtenidas para cada uno de estos genes y la densidad del mRNA de la beta-actina.

5.18. Estudio morfométrico adipocitario

5.18.1. Preparación del tejido adiposo para microscopía óptica

5.18.1.1. Proceso de fijación de la muestra

El proceso de fijación de un tejido permite evitar los cambios estructurales que tienen lugar cuando un fragmento de tejido es extraido del organismo vivo y por tanto detener los procesos de descomposición y autolisis. La fijación se desarrolla a nivel de proteínas, lípidos y carbohidratos pero los mecanismos de acción difieren en función del sustrato y del tipo de fijador utilizado. Así pues, debido al elevado contenido lipídico de las células adipocitarias fue necesaria la utilización de un doble sistema de fijación.

El glutaraldehido se utilizó para fijar las estructuras proteicas aprovechando las propiedades de reticularización de estas estructuras y el contenido lipídico se fijó mediante el tetraóxido de osmio, el cual permite la formación de puentes de unión entre moléculas de naturaleza lipídica

ISBN: 978-84-691-1902-0/DL: T-355-2008

(Burck 1969).

Material

- Bisturí
- Frascos de 50 mL con tapón de rosca
- Frascos de cristal de 10 mL con tapón hermético
- Pipetas Pasteur de cristal

Reactivos

- Glutaraldehido 25%
- Tampón fosfato pH 7,4
  - 8 mL fosfato potásico 0,1 M — 1,36 g $PO4H_2K$; 100 mL agua destilada
  - 42 mL fosfato sódico 0,1 M — 1,78 g PO_4HNa_2; 100 mL agua destilada
- Tetraóxido de osmio ($Os0_4$)
- Solución de fijación: 50 mL tampón fosfato pH 7,4; 5 mL glutaraldehido 25%

Protocolo

En el mismo momento de la extracción de la muestra de grasa se separaba un fragmento de aproximadamente 100 mg para el estudio morfológico de la muestra. Este fragmento se seccionaba en fragmentos más pequeños (de aproximadamente $2mm^3$) y se dejaban durante 24 horas y a temperatura ambiente inmersos en 50 mL de solución de fijación. Antes de iniciar la fijación con tetraóxido de osmio se eliminaba el exceso de glutaraldehido con tampón fosfato y se dejaba durante 2 horas con una solución de OsO_4 a 4ºC y protegido de la luz. Una vez fijadas todas las estructuras se eliminaba el exceso de osmio mediante dos lavados de media hora cada uno con tampón fosfato.

ISBN: 978-84-691-1902-0/DL: T-355-2008

Debido al escaso poder de penetración del tetraóxido de osmio (0,5 mm por hora) era importante obtener fragmentos de tejido adiposo muy pequeños. Consideramos que la muestra estaba bien fijada cuando adoptaba una coloración negra brillante y una mayor dureza.

5.18.1.2. Inclusión del tejido adiposo en parafina

Para obtener cortes histológicos suficientemente delgados y uniformes que permitan posteriormente la visualización de las estructuras biológicas es necesario que la muestra adquiera una consistencia adecuada. Esto se consigue utilizando la inclusión en parafina, una sustancia con un punto de fusión entre 45ºC y 60ºC que saponifica a temperatura ambiente. Esta sustancia, puesto que es químicamente inactiva, no modifica la estructura química de la muestra y permite su conservación indefinida. El principal inconveniente es que para la inclusión del tejido en parafina, éste debe calentarse, y puesto que la parafina no es soluble en agua y prácticamente tampoco en alcohol, se requiere una deshidratación de la muestra mediante pasos sucesivos en alcoholes de grado creciente y una posterior eliminación del alcohol generalmente con xilol, cloroformo o benzol.

Material

- Inclusor de parafina con brazo automático programado en secuencias de cambio de 1 hora (Technicon, Especialidades Médicas MYR, Barcelona)
- Moldes de parafina
- Microtomo tipo Minot (Reichert-Jung)

Reactivos

- Etanol absoluto
- Xilol
- Parafina Poliwax con punto de fusión a 57ºC

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

Protocolo

Para la deshidratación de las muestras los fragmentos se depositaban en los cestos del inclusor y se programaba el brazo mecánico de manera que la muestra estuviera 1 hora en cada una de las soluciones de etanol necesarias para la fijación (etanol de 30º, 50º, 70º) 2 horas en etanol absoluto, 2 horas en xilol, 2 horas en parafina a 57ºC.

Cada fragmento de tejido se montaba en un bloque de parafina y se dejaba solidificar a temperatura ambiente. Una vez liberados los bloques de los moldes, con la ayuda de un bisturí se piramidizaban las caras para hacer más accesible la muestra a la hoja del microtomo y de este modo facilitar los cortes histológicos.

5.18.1.3. Obtención de cortes histológicos

Existen diferentes tipos de microtomos que difieren pricipalmente en el grosor mínimo que permiten cortar. Para realizar cortes de 6 μm de grosor se utilizó un microtomo manual de deslizamiento tipo Minot (de cuchilla fija y bloque móvil).

Material

- Pincel fino
- Portaobjectos
- Placa calefactora a 37ºC
- Estufa a 37ºC

Protocolo

Para cada una de las muestras se montaba el bloque en el microtomo y se encaraba con la hoja para cortar. Se cortaban secuencialmente cortes de 6 micras de grosor y se depositaban encima del portaobjetos cubierto con una fina película de agua previamente temperada a unos 37ºC para facilitar la extensión del corte y evitar los desagradables doblamientos de la muestra. A su vez, el calor contribuye a una mayor adhesión de la muestra al portaobjetos.

ISBN: 978-84-691-1902-0/DL: T-355-2008

Tras eliminar el exceso de agua del portaobjetos se dejaban las preparaciones al menos durante 3 horas en una estufa a 37ºC para conseguir la máxima adhesión. De este modo las muestras quedaban listas para la tinción.

5.18.1.4. Tinción y montaje de las preparaciones

Las estructuras biológicas no tienen suficiente contraste como para poder ser diferenciadas al visualizarse a través del microscopio óptico, por eso se requieren métodos de coloración artificial que tiñen específicamente algunas estructuras celulares.

Para estas muestras se utilizó la hematoxilina de Harris, un colorante vegetal obtenido a partir del palo de campeche. Esta sustancia es incolora y debe ser oxidada a hemateína la cual, en contacto con los iones férricos y a pH ácido produce una coloración violeta. La fijación del colorante a la muestra se obtiene incrementando el pH con lavados en agua corriente.

Material

- Cubetas de tinción
- Cubre-objetos

Reactivos

- Etanol absoluto
- Hematoxilina de Harris
- Xilol
- Hidróxido amonio
- DePeX

Protocolo

Para el desparafinado de la muestra esta se sumergía durante 1 hora en xilol. El exceso de xilol se eliminaba mediante dos lavados de 10 minutos cada uno en etanol absoluto, un lavado

ISBN: 978-84-691-1902-0/DL: T-355-2008

de 10 minutos con etanol de 70º y un lavado de 10 minutos en agua destilada para favorecer la total hidratación de la muestra. Posteriormente se filtraba la hematoxilina y se dejaba teñir la muestra durante 10 minutos. Se eliminaba el exceso de colorante con un lavado de 5 minutos en agua corriente y se diferenciaba la tinción durante 3 segundos con una solución de etanol:clorhídrico 99:1. Se procedía a lavar la muestra durante 7 minutos con agua destilada y se sumergía 15 segundos con agua amoniacal.

El montaje de la preparación con DePeX requiere una previa deshidratación que se consigue con pasos sucesivos con etanol de grado creciente (5 minutos en etanol absoluto, 5 minutos con etanol de 70º) y la posterior sustitución del etanol por xilol (30 segundos en xilol:etanol 1:1, 30 segundos en xilol:fenol 4:1, 5 minutos en xilol). Antes de cubrir las muestras con el cubreobjetos, se secaba el exceso de xilol en una estufa o al aire libre.

5.18.2. Análisis morfométrico de los adipocitos

El estudio morfológico de las muestras procesadas para microscopia óptica se realizó mediante un microscopio electrónico Reichert-Jung, modelo POLIWAR 2. Este microscopio incorpora una cámara de vídeo que permite enviar la imagen a un ordenador y almacenarla sobre un soporte informático (Visilog) para ser posteriormente analizada.

La calidad de las muestras no permitió automatizar el proceso de análisis de imagen en el programa VISILOG, con lo cual se optó por el sistema semi-automático de planimetría (MOP VIDEOPLAN, Kontron Embedded Computers AG, Oskar-von-Miller-Straße, Eching) (Gore 1979). De este modo, las imágenes captadas en el microscopio eran grabadas en formato TIFF y posteriormente tratadas con el paquete Adobe Photoshop. Este programa nos permitía aumentar el tamaño de la imagen y modificar el contraste de la muestra de modo que se pudieran diferenciar correctamente los perfiles celulares. Este análisis de la imagen obtenida permitía eliminar el ruido de fondo debido a la tinción con hematoxilina que nos distorsionaba las imágenes en el estudio automatizado. Posteriormente las imágenes obtenidas se imprimían

ISBN: 978-84-691-1902-0/DL: T-355-2008

a 300 píxels (Hewlett-Packard 4L) y se analizaban por planimetría. El planímetro fue programado para calcular las áreas, los perímetros y los diámetros de cada uno de los perfiles, a la vez que calculaba el sumatorio de estas variables y el número de perfiles medidos.

Las áreas de medida para cada captación eran escogidas en función de la calidad de la imagen y se medían un mínimo de 8 áreas para cada paciente. Se consideraban de buena calidad todos aquellos campos de visión que presentaran el máximo número de perfiles celulares intactos, el máximo número de células y que no presentaran superposiciones o dobleces de la muestra (Ver LÁMINA 1).

Las condiciones de medida se estandarizaron en un ocular WPK 10x, un objetivo 10x con una corrección de ampliación de 1. La captación de la imagen se realizó siempre con las mismas condiciones de luz, monitorizadas a través de una consola fotográfica Reichert-Jung acoplada al microscopio óptico (sensibilidad de 400 ASA y un tiempo de exposición de 2,6 segundos).

Las mismas condiciones de captación de imágenes y procesamiento informático fueron utilizadas sobre un portaobjetos milimetrado de 1mm (Olympus Ref OB-M-1/100) que fue utilizado para la conversión de unidades máquina a micras. El coeficiente de variación intraensayo fue de 1,16%.

5.18.2.1. Corrección de los diámetros medios celulares

En la estimación de los diámetros medios celulares a partir de medidas directas debemos asumir algunos errores inherentes al método y al propio observador. Por un lado, los cortes histológicos de cada una de las células no se realizan en su plano ecuatorial, y por otro lado es difícil diferenciar entre los diámetros más pequeños correspondientes a los casquetes celulares y los espacios intersticiales. De modo que, asumiendo la esfericidad de los adipocitos (Reverter 1993) y la distribución normal de los diámetros celulares, podemos aplicar los métodos de corrección descritos por Giger y Riedwil (Giger 1970).

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

Así se obtiene una primera estimación del diámetro medio corrigiendo el histograma generado a partir de los valores observados, por aquellos diámetros de tamaño pequeño que no hemos considerado en la muestra estudiada, según la fórmula derivada de la integración de la función de diámetro:

$$D_1 = (4*d)/\pi$$

Donde,

D_1= es la corrección del diámetro medido, d es la media de los diámetros observados

con una desviación estándar estimada de:

$$s_1 = (d-D_1)/3$$

Donde,

D_1= es la corrección del diámetro medido, d es la media de los diámetros observados

Una segunda estimación del diámetro medio se consigue corrigiendo la primera estimación del diámetro D_1 por los diámetros mayores no computados. Para ello nos referimos al normograma descrito por Giger y Riedwyl en 1970. Si se considera $P<0,1$ para la cola de la derecha de la curva normal, podemos asumir que D_2 es prácticamente igual a D_1. En este estudio no se realizó la segunda corrección para el valor del diámetro medio.

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
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RESULTADOS

UNIVERSITAT ROVIRA I VIRGILI
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SECCIÓN 1 .

Leptin concentrations do not correlate with fat mass nor with metabolic risk factors in morbidly obese females

Diabetes, Nutrition & Metabolism (sometido)

UNIVERSITAT ROVIRA I VIRGILI
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Leptin concentrations do not correlate with fat mass nor with metabolic risk factors in morbidly obese females

Running Head: Leptin and morbid obesity.

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Keywords: Leptin, morbid obesity, fat mass, lipid profile, insulin.

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UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
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Abstract

Aims: To investigate the determinants of leptinemia in a cohort of morbid obese females compared to those of normal weight and mild-to-moderate obesity, and the relationships between leptin and metabolic derangements associated with obesity.

Methods: Recruited females were: moderately obese (n = 44; BMI 25-40 kg/m^2), morbidly obese (n = 34; BMI ≥ 40 kg/m^2) and normal-weight volunteers (n = 12; BMI 19-25 kg/m^2). Fat mass assessed by bioelectrical impedance and waist-to-hip ratio (WHR) were determined in all subjects. Biochemical determinations included plasma leptin, lipoprotein profile, fasting insulin and cortisol.

Results: Plasma leptin values were significantly increased in morbid obese patients (54.95± 1.8 ng/mL) compared to those moderately obese (30.2±1.7 ng/mL; *P<0.001*) and to controls (9.77±1.4 ng/mL; *P<0.001*). Percentage body fat and age explained 65% of the variance in leptin values. When adjusted for these variables no differences in leptin concentrations were found between groups. No relationships between leptin concentrations and anthropometric or biochemical variables were observed when the morbidly obese group was analyzed separately but when removed from the overall analysis (BMI < 40 kg/m^2) plasma leptin was significantly and positively related with anthropometric variables (BMI, percentage body fat and WHR), total cholesterol, LDL-cholesterol, plasma triglycerides, γGT, GOT and uric acid; and negatively with HDL-cholesterol. For obese patients no significant differences were observed in the adjusted leptin values with respect to the presence of diabetes, dyslipidemia or hypertension.

Conclusions: Circulating leptin concentration is not a marker of adiposity in morbid obesity and, further, other known metabolic consequences of obesity are not related to leptin in the morbid obesity state.

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INTRODUCTION

Leptin, the obesity (ob) gene product, has been implicated in body-weight regulation in mice and its deficiency associated with the development of obesity (1). In obese humans, however, increases in adipose tissue expression of leptin mRNA and, concomitantly, of circulating leptin concentrations have been demonstrated, as well as strong correlations between leptin values and various measures of obesity such as body fat or body mass index (2-4). Rather than being seen as merely a marker of adiposity, a more active role in body-weight control has been proposed for leptin and which is inferred from the observation that different factors, physiological and/or pathological, can affect its concentrations (5-8). Most studies, even when conducted on relatively large cohorts, have been performed using lean or mildly-obese subjects and, often, the higher ranges of adiposity have not been addressed. Morbid obesity represents an extreme state of the disease that only some patients attain and is estimated to occur in no more than 0.5% of obese persons; making it a relatively uncommon disorder (9). These patients, or a sub-population of them, could have some specific characteristics that predispose to a progression toward the morbidly obese state. Although decreased leptin production has been reported recently in two severely obese children (10) and in three massively obese members of a turkish family (11), the role of leptin in the development, or maintenance, of morbid obesity has not been fully investigated.

Leptin concentrations have been related to some of the metabolic risk factors associated with obesity (12-14). These relationships, however, could be artefactual with adiposity acting as a confounding factor. Hence we investigated the determinants of leptinemia in a group of morbidly obese females and compared the findings with normal-weight and mild-to-moderately obese females all of whom were weight-stable and characterized on the basis of body composition and metabolic phenotype. A secondary aim was to evaluate the relationships between leptin concentrations and other biochemical markers of metabolic derangement associated with obesity, particularly in relation to the presence of morbid obesity.

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
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MATERIALS AND METHODS

Thirty-four morbidly obese (body mass index (BMI) ≥40 kg/m^2) and 44 moderately obese (BMI 25-40 kg/m^2) females were recruited from among those attending the Clinics of Obesity and Nutrition at the Hospital de St. Joan de Reus. Twelve healthy, normal-weight females (BMI 19-25 kg/m^2) recruited from among the medical staff served as a control group. All subjects were free of inflammatory or infectious disease at the time of admission, had thyroid hormones and basal cortisol levels within our reference range and none were in receipt of anti-inflammatory medication, insulin, steroid preparations or hormone replacement. Patients were requested to maintain their normal eating habits in the week prior to admission and all of them reported being weight-stable over the previous three months. Obese patients were subsequently classified on the basis of metabolic complications of obesity.

After admission to the metabolic ward, fasting blood samples were taken at 08:00h for biochemical and hormone analyses. Body composition measurements were then performed. Height and weight were measured and BMI (in kg/m^2) was calculated. Waist girth was measured at the minimum circumference between the iliac crest and the rib cage, hip girth at the maximum width over the greater trochanters and the waist-to-hip ratio (WHR) was then calculated. According to the consensus document of the Spanish Society for the Study of Obesity (SEEDO), abdominal fat distribution for a female population was defined as a WHR > 0.9 (15). Whole body impedance at 50KHz was measured using a tetrapolar bioelectrical impedanciometer (Human-Im Scan^R, Dietosystem, Spain) early in the morning under fasting conditions and after voiding as we have described elsewhere (16). From these measurements, fat-free-mass (FFM) was calculated using the gender-specific equations validated by Segal et al (17). Fat mass (FM) was calculated as the difference between body weight and FFM. The mean coefficient of variation for within-patient impedance measurements in our laboratory was 0.71%. On all occasions, the observed impedance deviated from the expected value by < 3.5 Ohms.

ISBN: 978-84-691-1902-0/DL: T-355-2008

Radioimmunoassay, using commercial kits, was used to determine serum leptin concentrations (Linco Research, St Louis, MO), fasting insulin (Amersham, Little Chalfont, UK), and cortisol (DPC, LA, USA). Within and between assay variation were 4.98% and 4.5% for leptin, 5.05% and 13.1% for insulin, 4.31% and 5.2% for cortisol, respectively. Plasma lipids (triglycerides, cholesterol) and high- low- and very low-density lipoprotein cholesterol (HDLc, LDLc and VLDLc, respectively), fasting glucose, uric acid concentrations and liver function tests were assayed by the hospital's routine chemistry laboratory.

Statistical analysis was performed using the SPSS/PC package. Leptin concentrations being skewed, the values were log-transformed so as to approach a normal distribution and geometric means are presented here. Differences in mean values between groups were assessed by one-way analysis of variance (ANOVA) and Scheffe post-hoc tests, and the contingency table chi-square test was used to analyze qualitative traits. Regression analyses were conducted for quantitative variables and regression coefficients (r) were derived. A multiple stepwise regression analysis was conducted to identify the factors affecting leptin concentrations and in which age, weight, BMI, fat mass, percentage body fat, waist and hip circumferences, WHR, insulin and cortisol were entered as independent variables. The unexplained residual of leptin values in each subject was calculated by the general linear model procedure introducing age and percentage body fat as covariates since these were the only variables that significantly correlated with leptin concentrations. The adjusted leptin values for each individual were calculated by adding the individual's residual leptin value to the mean leptin value of the whole group or to the mean leptin value of the obese patients when this group was analyzed separately. Statistical significance was accepted at $P<0.05$.

The study protocol was approved by the Ethics Committee of the Hospital St. Joan and each subject gave written consent to participation.

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

RESULTS

Table 1 shows the characteristics of the study group. Significant differences in all anthropometric measurements were observed between groups. Control subjects were younger than the patient groups but no differences in age were observed between the patient groups. Fasting glucose, lipid concentrations, urate and hepatic enzymes were significantly increased in patients compared to controls and slightly higher in morbid obese patients than in their moderately obese counterparts although the differences between the groups of patients were not statistically significant. Fasting insulin and plasma leptin concentrations were significantly increased in morbid obese patients compared, either, to control subjects or to moderately obese patients. As shown in Table 2, plasma leptin concentrations were strongly correlated with BMI, fat mass, percentage body fat (Figure 1) and waist circumference but not with WHR in the overall study group. In the multiple stepwise regression analyses, percentage of body fat and age were the only variables that accounted for the variability in leptin values. Taken together, these two variables explained 65% of the variance in leptin. When adjusted for the degree of adiposity and age, no significant differences in leptin values between any of the groups were found.

As shown in Table 2, when subjects with BMI <40 kg/m^2 were considered separately, plasma leptin was strongly related with all anthropometric variables (BMI, percentage body fat and WHR). When the morbid obese group was analyzed separately, leptin values were inversely related only with WHR with no significant relationships with BMI or percentage body fat. A positive relationship was observed between leptin and fasting insulin for the overall study group but this association was not significant when the analysis was performed separately for subjects below and above the BMI cut-off of 40 kg/m^2. Similarly, plasma leptin correlated significantly with lipid profiles, hepatic enzymes and uric acid concentrations in the group of subjects with BMI <40 kg/m^2 but, except for a negative correlation with fasting glucose (r=-0.39, P<0.05), no relationship between leptin and any of these biochemical variables was

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
ISBN: 978-84-691-1902-0/DL: T-355-2008

observed for the morbidly obese group. When adjusted for adiposity and age in the overall study group, leptin was not related to any of the biochemical nor hormone parameters assessed and only a negative relationship with fasting glucose (r=- 0.29, $P<0.05$) was observed.

In the patient groups, 23 % were classified as diabetic, 32.5% as dyslipidemia, 38% as hypertensive, 9.5% as hyperuricemic, 18% had a previous history of cholelithiasis and 43% with abdominal fat distribution. Between groups, NIDDM and hypertension were more frequent in the morbid obese subjects than in the moderately obese (12 from 34 vs 6 from 44 and 17 from 33 vs 12 from 44 respectively; $P<0.05$). The frequencies of dyslipidemia and cholelithiasis were lower in the morbid obese group than in the moderately obese group (8 from 33 vs 17 from 44 and 4 from 31 vs 9 from 42, respectively) although the differences were not statistically significant. On adjustment for age and percentage body fat, leptin values were lower in the diabetic group of patients compared to the non-diabetic (33.1 ± 1.5 ng/mL vs 40.7 ± 1.6 ng/mL) and slightly lower in those with abdominal adipose distribution (36.3 ± 1.5 ng/mL vs 40.7 ± 1.6 ng/mL); the differences not being statistically significant. When compared with respect to the presence or absence of hypertension, hyperuricemia, dyslipidemia or cholelithiasis, the adjusted leptin values were not significantly different between the patient groups (data not shown).

DISCUSSION

The results of the present study suggest that, contrary to the findings in general populations, total adiposity is not a significant determinant of leptinemia in morbidly obese females.

ISBN: 978-84-691-1902-0/DL: T-355-2008

Several studies describe the positive relationship between leptinemia and adiposity in general populations and which suggest that, in humans, serum leptin concentrations reflect the amount of body adipose tissue (3,4,18). A strong relationship between leptin levels and adiposity indices (BMI, percentage body fat) was observed in the present study when the overall study group was assessed. However, these relationships disappear when the morbidly obese patients are assessed separately (q.v. the dispersion observed in *Figure 1* of those with BMI $\geq$40 kg/m^2). This same lack of relationship for subjects in the high range of adiposity can be seen in the graphical data presented in published reports but with respect to which no comments appear in the text (4,19). To-date, most of the studies have been performed in lean or mildly obese subjects and the higher ranges of adiposity have not been addressed. A recent study, conducted in a large sample of black subjects (BMI ranged from 14-62 kg/m^2), reported an exponential relationship between plasma leptin and fat mass i.e. a loss of linearity in this relationship at the upper extreme of the BMI distribution curve (20). In two studies conducted on extremely obese females (12,21) and in which, incidentally, fat mass had not been assessed, the reported relationship between leptin concentrations and BMI appeared weaker than expected. Further, no relationship between leptin gene expression and BMI had been observed in omental or subcutaneous adipose tissue from very obese patients (22). This suggests that metabolic responses in the morbidly obese differ considerably from those of the general population. In the present study (in which body composition was measured) leptin concentrations appeared to be independent of the degree of adiposity for the morbidly obese group of patients. It needs be noted that the estimation of fat mass with Bio-electrical Impedance Analysis is liable to increased inaccuracy when applied to the massively-obese patient but, nevertheless, this finding is validated by the same lack of correlation observed with BMI in this group of patients. It may be suggested that this lack of correlation could be due to the narrower range of adiposity represented in the morbid obesity group. However, with respect to the moderately obese group, leptin is still strongly related to overall adiposity ($r=0.59$, $P<0.01$) and BMI ($r=0,67$, $P<0.01$) even though the range of BMI is narrower than that of the morbid obesity group (25.9 to 39.5 *vs* 40.3 to 55.8 kg/m^2, respectively).

ISBN: 978-84-691-1902-0/DL: T-355-2008

Since short-term negative energy balance has been shown to induce acute changes in leptin levels (23,24) the patients were requested to maintain their normal eating patterns and not to diet in the week prior to the study. In addition, possible energy restriction was indirectly evaluated by determining hydroxybutyrate concentrations in all subjects. Two morbidly obese patients admitted to some energy restriction, as did two from the overweight group. Two further patients in the moderately obese group, one control and one morbidly obese subject had hydroxybutyrate values beyond the ketosis range (25). No significant differences in absolute or adjusted leptin values were observed between these individuals and their non-ketotic counterparts and when these were excluded from the statistical analysis, the leptin values were still strongly related with adiposity in the moderately obese group but not in the morbid obesity group. Moreover, no clinical evidence of recent changes in body composition had been noted in our study subjects since this was a selection criterion for inclusion into the study.

Insulin has been suggested as a factor in the regulation of leptin synthesis and secretion (26-29). Since the number of diabetics was significantly higher in the morbid obesity group (and with a greater degree of hyperinsulinemia) it could that this factor induced the high variability of leptinemia despite a similar degree of adiposity in these subjects. However, the same magnitude of relationship between leptin and insulin was observed for the overweight as for the morbidly obese patients. Moreover, when diabetic and non-diabetic patients were compared, no differences in the adjusted leptin concentrations were observed. This finding is in agreement with previous studies such as that conducted by Haffner *et al.* (30) on a large sample of diabetic and non-diabetic Mexican-Americans. Similarly, Clement *et al* (12) in a study of 241 morbidly obese patients, failed to find differences in leptin concentrations between normoglycemic, glucose intolerant and well-controlled diabetic patients of similar BMI; only patients with poorly-controlled diabetes showed significantly lower leptinemia. In our study, only four diabetic patients were not under good metabolic control (HbA1c $\geq$ 8.8%) and, as with the study of Clement *et al,* these patients presented with lower adjusted leptin

ISBN: 978-84-691-1902-0/DL: T-355-2008

levels than their well-controlled diabetic counterparts (23.44 ± 1.19 ng/mL vs 41.69 ± 1.1 ng/mL). Even after their exclusion from the statistical analysis, no differences in leptin levels were observed between diabetic and non-diabetic patients. Although an effect of insulin resistance on the high variability observed at the high end of the range of adiposity cannot be excluded from these data, its exact role would require further, longitudinal studies.

In our morbid obese patients, leptin concentrations were not related to indices of general adiposity but with a negative correlation only with waist-to-hip ratio. This negative association between leptin and abdominal fat deposit in morbid obese subjects is consistent with that reported by Lönnqvist in a sub-population of females but not in males (21). Since some metabolic diseases are related to abdominal fat content, it could be suggested that, in markedly obese females, leptin could play a protective role against some metabolic derangement associated with obesity. Lönnqvist *et al.* suggested that this hypothesis could be supported by the lack of relationship between leptin and markers of cardiovascular risk in morbidly obese patients. In our morbid obesity group, no relationship was observed between circulating leptin concentrations and lipid profile or with insulin values. This cannot be interpreted as a direct protective effect of leptin *per se* since leptin does not correlate with these markers in the overall study group when the effect of fat mass is controlled for. Moreover, when our subjects were classified on the basis of other secondary complications of obesity, no differences in leptin levels were observed irrespective of the presence or absence of any such complications.

In conclusion, in morbidly obese patients the plasma leptin concentrations although increased do not reflect the amount of adipose stores, and, as such, factors other than simple adiposity need to be invoked to explain the variation in leptin values. Whether this is the result of a distorted leptin production in massive obesity or an adaptive mechanism that prevents further weight gain when the morbid state is reached remains to be elucidated. Longitudinal studies

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
Mònica Bulló Bonet
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are needed to explore the possible protective effect in the extreme range of adiposity as well as associated factors affecting leptin production in morbidly obese patients.

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ISBN: 978-84-691-1902-0/DL: T-355-2008

Table 1. Characteristics of the study subjects

	Controls (n=12)	Moderate obese (n=44)	Morbid obese (n=34)
Age (y)	30.67 (9.5)	45.36 (11.8) *	42.0 (9.9) *
Body Mass Index (kg/m^2)	21.09 (1.6)	34.10 (4.0)$^{\#\,\S}$	46.38 (4.3)$^{\#}$
Body fat (%)	26.61 (2.9)	43.60 (3.6)$^{\#\,\S}$	50.71 (2.2)$^{\#}$
Waist/hip	0.79 (0.04)	0.91 (0.1)$^{\#}$	0.88 (0.1)*
Fasting glucose (mmol/L)	4.83 (0.3)	6.12 (1.2)	6.86 (2.7)*
Plasma cholesterol (mmol/L)	4.86 (0.7)	5.77 (1.1)*	5.35 (0.8)
LDL-cholesterol (mmol/L)	2.61 (0.6)	3.62 (1.03)*	3.24 (0.6)
VLDL-cholesterol (mmol/L)	0.35 (0.2)	0.66 (0.3)*	0.63 (0.3)*
HDL-cholesterol (mmol/L)	1.91 (0.3)	1.36 (0.4)$^{\#}$	1.38 (0.3)$^{\#}$
Triglycerides (mmol/L)	0.76 (0.4)	1.53 (0.8)*	1.45 (0.7)*
Urates (μmol/L)	153.4 (32.9)	248.6 (79.0)$^{\#}$	239.5 (63.0)$^{\#}$
GOT (μkat/L)	0.22 (0.05)	0.49 (0.2) *	0.48 (0.3)*
γGT (μkat/L)	0.17 (0.06)	0.32 (0.2)	0.36 (0.3)*
Fasting insulin (nmol/L)	0.19 (0.3)	0.31 (0.2)‡	0.66 (0.7)*
Cortisol (nmol/L)	570.7 (294.2)	403.6 (209.2)*	349.1 (152.5)*
Leptin (ng/mL)[a]	9.77 (1.4)	30.20 (1.7)$^{\#\S}$	54.95 (1.8)$^{\#}$

Results are expressed as mean (standard deviation). [a]Leptin values are presented as the geometric mean. *P<0.05 and $^{\#}P$<0.001 vs control subjects $^{\ddagger}P$<0.05 and $^{\S}P$<0.001 vs morbid obese patients (Scheffé test after significant analysis of variance).

Table 2. Correlation of fasting plasma leptin with body composition parameters and with hormonal or biochemical markers of metabolic derangements associated with obesity in subjects with body mass index below or above 40 kg/m^2

	Overall study group (n=90)		BMI < 40 kg/m^2 (n=56)		BMI ≥ 40 kg/m^2 (n=34)	
	Leptin	**Log Leptin**	**Leptin**	**Log Leptin**	**Leptin**	**Log Leptin**
BMI	0.56**	0.75**	0.70**	0.82**	-0.06	-0.01
Fat mass	0.56**	0.74**	0.69**	0.80**	0.016	-0.01
%Body fat	0.53**	0.77**	0.64**	0.77**	-0.06	0.03
Waist circumference	0.47**	0.72**	0.65**	0.77**	-0.28	-0.20
Waist-to-hip ratio	-0.03	0.17	0.36**	0.46**	-0.39*	-0.39*
Fasting insulin	0.38**	0.34**	0.19	0.20	0.27	0.24
Plasma cholesterol	0.11	0.24*	0.37**	0.43**	-0.003	0.08
HDL cholesterol	-0.25*	-0.41**	-0.40**	-0.51**	-0.01	-0.01
LDL cholesterol	0.17	0.32**	0.44**	0.50**	-0.009	0.09
Triaglycerides	0.06	0.18	0.26	0.32*	-0.18	-0.22
γGT	0.27*	0.35**	0.31*	0.37**	0.21	0.28
GOT	0.08	0.19	0.16	0.28*	0.01	0.06
Urates	0.27*	0.43**	0.57**	0.54**	-0.07	0.06

*P<0.05 ** P<0.01

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
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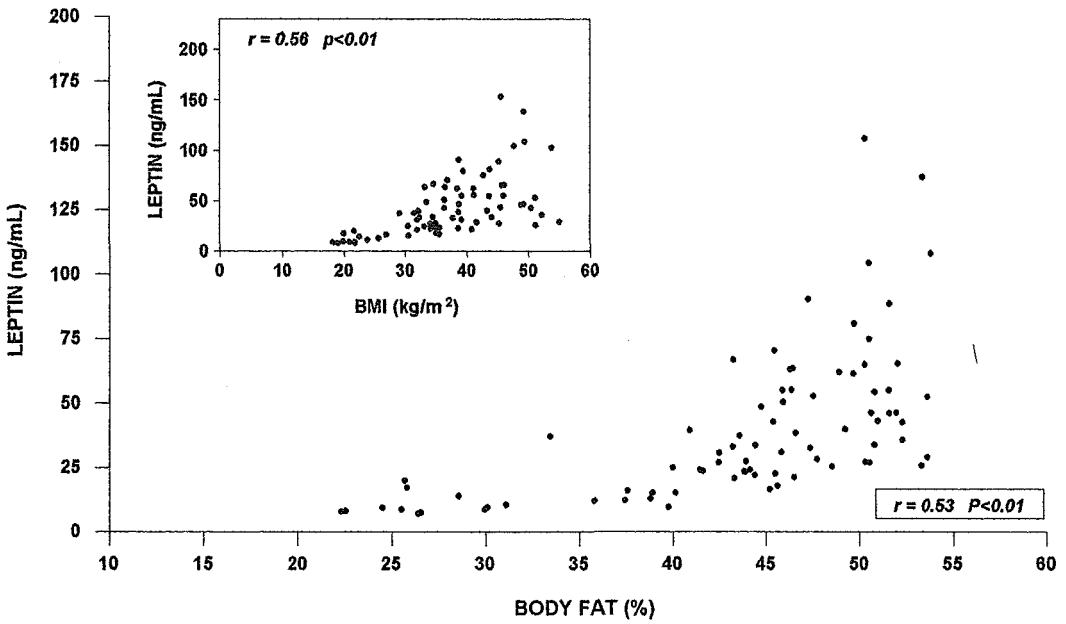

Figure 1.

Relationship between plasma leptin concentrations and body mass index or percentage of body fat in the overall study group (n=90)

UNIVERSITAT ROVIRA I VIRGILI
EFECTO DE LA ADIPOSIDAD SOBRE EL SISTEMA TNFα-LEPTINA
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SECCIÓN 2 .

Plasma acyl-estrone levels are altered in obese women

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PLASMA ACYL-ESTRONE LEVELS ARE ALTERED IN OBESE WOMEN

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ABSTRACT

A group of obese women (BMI>27 kg/m^2; N=73) was studied together with lean controls (BMI <27 kg/m^2; N=25). Three groups were defined by the compliance with: BMI lower than 27 kg/m^2 , glycaemia lower than 5.5 mM and insulinaemia lower than 0.2 nM (controls, group 1, N=19). The subjects with BMI>27 kg/m^2, glucose >5.5 mM and insulin >0.2 nM constituted group 3 (N=41), and those with BMI>27 with glycaemia and/or insulinaemia lower than the limits set constituted group 2 (N=32). The women in group 3 had higher fat content, BMI and fat-free mass than those in group 2 and the controls. There were no changes in most plasma parameters, such as free estrone and β-estradiol. Leptin levels were higher in groups 2 and 3 than in controls. In controls, leptin and acyl-estrone levels were well correlated with BMI and fat content; this correlation was not found in groups 2 and 3 for acyl-estrone, although it was found for leptin. Acyl-estrone levels were lower than expected in most obese women when compared to those of controls, suggesting an altered availability or function of this hormone. In obese women, acyl-estrone levels –and probably function– are lower than expected, contrasting with maintained leptin-BMI correlations. The role of insulin in the control of body weight, perhaps through acyl estrone-mediated effects, should be re-evaluated.

INTRODUCTION

The regulation of fat deposition by insulin is a key factor in the control of body weight (1). Obesity is characterized by an altered insulin function, mainly through increases in insulin resistance (2), typified by higher secretion, levels and extrahepatic removal (3). The obese also tend to show increased glucose levels, a consequence of insulin resistance, which often develops into diabetes. The altered insulin function is either a consequence of obesity or a causal factor in its development (1); but in any case the intensity of insulin alterations is a distinctive trait of the metabolic consequences of obesity (4,5) and its associated pathology: the metabolic syndrome (6).

Leptin, the product of Ob gene (7) is produced mainly in adipose tissue (7,8), in such a way that the circulating levels can be correlated with the fat mass (9,10). This relationship is somewhat altered in severe obesity in humans (11). In some animal models, such as the ob/ob mice, obesity is the consequence of lack of leptin secretion (7,12) or defects in its receptors –Zucker fa/fa rat, db/db mouse– accompanied by very high circulating leptin levels (13). There is no doubt that leptin is a main factor in the body weight control system (14), as well as an index of fatness (15). Our previous research has shown that oleoyl-estrone (OE), a hormone synthesized in the adipose tissue (16), induces the loss of body fat both in normal (17), dietary-obese (18) and genetic-obese (19) rats, sparing protein and maintaining thermogenesis (20). OE decreases the expression of leptin (21), but leptin enhances the synthesis and storage of OE in adipose tissue (16). In addition, OE administration may alter the ponderostat setting (22), which suggests that OE is a signal from adipose tissue participating downstream of leptin in the system controlling the body weight (22). The circulating levels of OE in normal-weight and mildly obese humans are strongly correlated with the BMI, fat mass and circulating leptin (23), but obese rats show lower OE than expected and altered leptin levels, in line with its purported role as a ponderostat signal (22).

Here we used the degree of alteration of insulin functionality as a parameter for the classification of severe obesity. We used this factor as a correlate of the intensity of energy homeostasis impairment and the alteration of the body weight control system. We attempt to determine whether in severe human obesity the BMI-OE correlation is maintained, or altered as found in obese rats, as a sign of the anomalies affecting the body weight control system.

MATERIALS AND METHODS

Subjects and Study Design

Obese and overweight women (body mass index [BMI]>27 kg/m^2) were recruited among those attending the Clinics of Obesity and Nutrition at the Hospital de St. Joan de Reus (N=73). The control group consisted of normal- and low-weight women (BMI <27 kg/m^2) (N=25), and was recruited from the same patients' pool (women suffering anorexia nervosa but otherwise healthy, N=8) and the medical staff. All subjects were free of inflammatory or infectious disease at the time of the study, had normal thyroid hormone levels and were not receiving anti-inflammatory medication, insulin, or other hormonal treatment. Patients and controls were requested to maintain their normal eating habits in the week prior to the study and all reported that their body weight remained stable over the previous three months.

Fasting blood samples were taken at 8.00 am for biochemical and hormone analyses. Body composition measurements were then performed.

TABLE 1
Essential Measures of the Subjects Included in the Study

parameter	units	group 1 BMI<27; [glc]<5.5 & [ins]<0.2	group 2 BMI>27; [glc]<5.5 or [ins]<0.2	group 3 BMI>27; [glc]>5.5 & [ins]>0.2
N		19	32	41
age	y	29 ± 3	43 ± 2 ‡	44 ± 2 ‡
weight	kg	49.9 ± 2.2	94.0 ± 3.2 ‡	104.7 ± 2.9 ‡*
fat-free mass	kg	38.6 ± 1.3	50.3 ± 1.3 ‡	53.8 ± 1.2 ‡*
fat mass	kg	11.3 ± 1.1	43.8 ± 2.0 ‡	50.0 ± 1.8 ‡*
body fat	% BW	21.8 ± 1.4	46.0 ± 0.7 ‡	47.8 ± 0.5 ‡*
BMI	kg/m^2	19.3 ± 0.8	38.0 ± 1.3 ‡	41.3 ± 1.0 ‡*
waist/hip ratio	-	0.81 ± 0.01	0.89 ± 0.01 ‡	0.90 ± 0.02 ‡

Statistical significance ($p<0.05$) of the differences between groups: ‡ differences between group 1 and groups 2 or 3; * differences between groups 2 and 3

The patients were classified using as discriminating factor both a standard measure of obesity: BMI higher or lower than 27 (24), as well as the glycaemia-insulinaemia data. Three groups were defined. Group 1: BMI lower/equal than 27, glycaemia lower/equal than 5.5 mM and insulinaemia lower/equal than 0.2 mM; i.e. non-obese with normal glycaemia and insulinaemia (N=19); the controls that had a BMI<27 but did not meet both the glycaemia and insulinaemia criteria (N=6) were discarded. Group 2: BMI higher than 27, and either glycaemia lower/equal than 5.5 mM or insulinaemia lower/equal than 0.2 mM; i.e. obese with normal or mildly altered glycaemia and insulinaemia (N=32). Group 3: BMI higher than 27, glycaemia higher than 5.5 mM and insulinaemia higher than 0.2 mM; i.e. obese with altered glycaemia and insulinaemia (N=41) (Table 1).

Anorexic patients were included in the study in order to obtain a wider range of BMI values. No significant differences with the other women in the control group were found for the hormonal parameters studied other than those related to their varying body weight.

The study protocol was authorized by the Ethics Committee of the Hospital St. Joan and each subject included in the study gave a voluntary, fully-informed, written consent.

Body Composition Analyses

Height was measured to the nearest mm with a wall-mounted stadiometer, weight was determined on a standard clinical balance with an accuracy of ± 100 g, and BMI (in kg/m^2) was calculated. Waist girth was measured at the minimum circumference between the iliac crest and the

rib cage, hip girth at the maximum width over the greater trochanters and the waist-to-hip ratio was then calculated.

Whole body impedance at 50KHz was measured using a tetrapolar bioelectrical impedanciometer (Human-Im ScanR, Dietosystem, Spain) as we have described elsewhere (25). All measurements were performed early in the morning, under fasting conditions and after voiding, in accordance with the National Institutes of Health Technology Assessment Conference Statement (26). From these data, fat-free-mass (FFM) was calculated using the gender-specific equations validated by Segal *et al.* (27). Fat mass was the difference between body weight and FFM.

Metabolite and Hormone Analyses

Plasma hormones were measured through radioimmunoassay: free and acyl-esterified estrone (28), leptin (HL81K kit, Linco Research, St Charles, MO USA), insulin (Biotrak RPA547 kit, Amersham, Little Chalfont, UK), and β-estradiol (TKE22 Coat-a-count kit, DPC, Los Angeles CA, USA). Plasma was also used for the measurement of total cholesterol, total protein, glucose, urea, creatinine, uric acid, triacylglycerols and aspartate transaminase activity, by standard automated procedures (ITC Diagnostics IZASA, Sant Andreu de la Barca, Spain); 3-hydroxybutyric acid (310UV kit, Sigma, St Louis, MO USA), free (nonsterified) fatty acids (NEFAC kit, Wako Chem., Neuss, Germany) and lactate (29) were also measured.

Differences in mean (± SEM) values between groups were assessed by unpaired t-test. Correlation and regression analyses were performed for quantitative variables using the SPSS 8.0 program. Statistical significance was accepted at $p<0.05$.

RESULTS

Table 1 includes the anthropometric measurements, BMI and other body composition-derived measures. As expected, there were differences between groups 1 and 2 / 3 for all parameters, but groups 2 and 3 also showed significant differences in weight, fat-free mass, fat tissue mass, percentage of body fat and BMI, but not in waist-hip ratio.

Table 2 shows the plasma levels of metabolites. There were no differences in protein, urea, creatinine, triacylglycerols, free fatty acids or 3-hydroxybutyrate. There were differences between nonobese (group 1) and obese (groups 2 and 3) for glucose, uric acid, and aspartate transaminase activity, all highest in group 3. Cholesterol levels in group 3 were also higher than in group 1. There were also differences between groups 2 and 3 in glucose, lactate and uric acid: in all cases the highest values were those of group 3.

Table 3 presents the levels of circulating hormones in normal-weight and obese women. There were no differences in the levels of free estrone or β-estradiol. Obese (groups 2 and 3) women showed higher levels of insulin and leptin than the nonobese (group 1). Esterified estrone levels in group 3

TABLE 2
Plasma Parameters of Normal-Weight and Obese Women

parameter	units	group 1 BMI<27; [glc]<5.5 & [ins]<0.2	group 2 BMI>27; [glc]<5.5 or [ins]<0.2	group 3 BMI<27; [glc]<5.5 & [ins]<0.2
N		19	32	41
protein (total)	g/L	74 ± 1	73 ± 1	72 ± 1
glucose	mM	4.7 ± 0.1	5.6 ± 0.2 ‡	7.0 ± 0.4 ‡✳
lactate	mM	1.39 ± 0.13	1.32 ± 0.10	1.69 ± 0.08 ✳
urea	mM	5.3 ± 0.4	5.3 ± 0.2	5.7 ± 0.2
uric acid	µM	173 ± 18	220 ± 11 ‡	263 ± 13 ‡✳
creatinine	µM	81 ± 4	74 ± 2	76 ± 1
triacylglycerols	mM	1.5 ± 0.8	1.3 ± 0.1	1.6 ± 0.1
free fatty acids	mM	0.22 ± 0.06	0.28 ± 0.04	0.38 ± 0.05
3-hydroxybutyrate	mM	0.51 ± 0.16	0.47 ± 0.11	0.62 ± 0.19
cholesterol (total)	mM	4.9 ± 0.3	5.4 ± 0.2	5.8 ± 0.2 ‡
aspartate transaminase	IU	0.26 ± 0.02	0.46 ± 0.05 ‡	0.51 ± 0.04 ‡

Statistical significance ($p<0.05$) of the differences between groups: ‡ differences between group 1 and groups 2 or 3; ✳ differences between groups 2 and 3.

TABLE 3
Plasma Hormones in Normal-weight and Obese Women

parameter	units	group 1 BMI<27; [glc]<5.5 & [ins]<0.2	group 2 BMI>27; [glc]<5.5 or [ins]<0.2	group 3 BMI>27; [glc]>5.5 & [ins]>0.2
N		19	32	41
insulin	nM	0.094 ± 0.007	0.26 ± 0.03 ‡	0.63 ± 0.10 ‡✳
glucose/insulin ratio	–	53.2 ± 3.2	16.8 ± 1.7 ‡	30.2 ± 3.9 ‡✳
leptin	µg/L	7.3 ± 1.0	49.6 ± 7.2 ‡	48.3 ± 5.0 ‡
estrone (free)	nM	0.84 ± 0.19	0.83 ± 0.15	0.95 ± 0.12
estrone (esterified)	nM	166 ±15	196 ± 25	215 ±18 ‡
β-estradiol	nM	0.28 ± 0.07	0.27 ± 0.06	0.23 ± 0.03

Statistical significance ($p<0.05$) of the differences between groups: ‡ differences between group 1 and groups 2 or 3; ✳ differences between groups 2 and 3

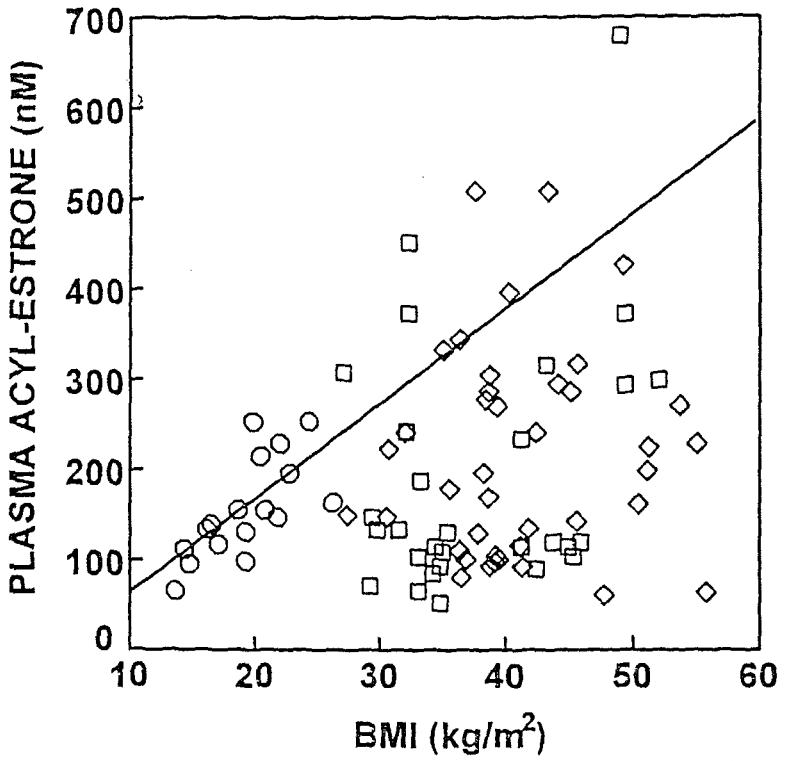

FIGURE 1
Relationship Between Plasma Acyl-estrone Levels and the BMI of Women.

Group 1: circles; Group 2: squares; Group 3: diamonds. The calculated regression line for Group 1 has been drawn. Statistical analysis of the relationships between acyl estrone levels and BMI:

group:	1	2	3
correlation coefficient, r	0.684	0.312	0.045
significance, p	0.003	0.099	0.784

were also higher than in group 1. The only differences between groups 2 and 3 were in insulin and in the ratio of glucose versus insulin, both higher in group 3 but still lower than in group 1.

Figure 1 shows the relationships between plasma acyl-esterified estrone levels and the BMI. The correlation was good for non-obese women (group 1) but this effect was not observed in the obese. Thus there is a clear correlation between acyl-estrone levels and BMI at low BMI values, i.e. this is true for nonobese women, but the linearity disappears with obesity, the values being lower, for most of the women in groups 2 and 3 than expected from the regression line drawn using the nonobese controls.

Figure 2 shows the crossed correlations between some parameters that define obesity and the levels of the main hormones controlling the energy distribution: leptin, insulin and acyl-estrone. Group 1 shows the highest degree of correlation between the data. There is a clear correlation between acyl-estrone and both BMI and the percentage of fat. Leptin levels were also correlated with BMI and

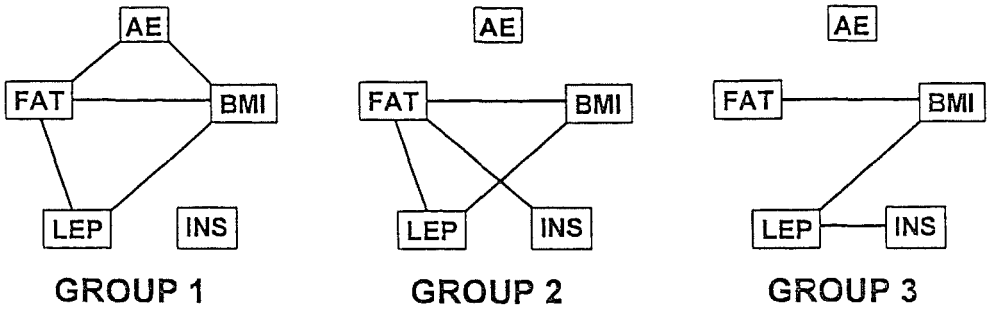

FIGURE 2
Interrelationship Between Body Weight Measures and Hormonal Factors in Lean And Obese Women.

The lines represent a significant relationship between the parameters connected ($p<0.05$); lack of connecting line indicates that no such correlation has been found.
AE = plasma acyl-estrone; FAT = % fats in body weight; BMI = body mass index; LEP = plasma leptin; INS = plasma insulin.

percentage of fat. Insulin was not correlated with either of the other parameters. Leptin maintained its correlation with BMI in groups 2 and 3, but its correlation with percentage of fat was maintained only in group 2. Plasma acyl-estrone was not correlatied with any other parameter in groups 2 and 3. In group 3 insulin was correlated with leptin. There was a significant correlation in group 1 between BMI and age, and in group 3 between acyl-estrone and age (data not shown).

DISCUSSION

The criteria used for classification of the subjects into three groups include the widely accepted criterion that normality in body weight extends up to BMI values of 27 kg/m^2 (24), this figure marking the border between overweight and (mild) obesity. The other criteria used: glycaemia higher than 5.5 mM and insulinaemia higher than 0.2 mM, were selected on the basis of values within the "normal" range, but having a high probability of altered glucose metabolism in predisposed populations (30). The aim was to discriminate not between well-defined situations but between trends, so that the group identified as having a somewhat altered insulin-glucose relationship (group 3) would be discriminated by both markers; this would leave the remaining cases –in which the tendency was not as markedly consistent– in an intermediate group (group 2). Applying the same criteria to the non-obese subjects, a group evolved in which the low glucose and low insulin criteria were not meet as in the controls (group 1) and was discarded because it was too small (N=6, 2 of which were anorexic) and wide dispersion of data. This pruning of controls helped to make the control group 1 much more consistent. The application of essentially metabolic parameters as discriminating factors thus resulted in the

establishment of three groups which, curiously, showed marked differences in weight, fat-free and fat mass. This already points towards a well-established trend: the close relationship between the control of energy handling –including fat deposition– and insulin functionality (1,5).

There was a clear trend towards higher cholesterol, glucose and uric acid levels, all indicators of a deteriorating overall metabolic control in group 3, and to a lesser extent in group 2. The higher levels of aspartate transaminase found in the obese also point in the same direction. The presence of high lactate levels in the group having higher fat mass (group 3) is consistent with the high sensitivity of adipose tissue glycolysis to adrenergic stimulation (31), which results in higher lactate production by this tissue, another factor directly related to high fat content (32).

There is a direct relationship between body fat and circulating levels of acyl-esterified estrone in lean and mildly obese humans of both sexes (23). This relationship, however, is limited to the non-obese as found here, since in most of the obese women studied, the circulating levels of acyl-estrone were lower than the values expected from the relationship observed in the non-obese, and the correlation of acyl-estrone with BMI was lost as a consequence of obesity.

Zucker fa/fa rats show plasma acyl-estrone levels similar to those of lean rats (22), but the administration of oleoyl-estrone results in a significant mobilization of fat with no changes in plasma parameters (19,21). Since the synthesis of oleoyl-estrone by adipose tissue is controlled by leptin (16) and oleoyl-estrone affects the expression of the Ob gene (21) it may be assumed that in these rats the alteration of leptin function lowers oleoyl-estrone levels. Oleoyl-estrone has also been found to modify the ponderostat setting in rats, which strengthens its postulated role as a ponderostat signal (16). The low-for-weight circulating acyl-estrone levels in obese women is similar to that found in genetically obese rats. These data are consistent with an altered function of acyl-estrone in obesity.

The case of acyl-estrone contrasts with that of leptin. Leptin levels were much higher in the obese than in the nonobese women, as previously observed (9,10), again in parallel to the case of Zucker fa/fa rats (33). We found a clear correlation between leptin and the parameters that determine fatness: BMI and percentage of fat. The correlations between leptin and fatness indicators were mostly maintained in the obese groups, suggesting that in women, leptin synthesis –and possibly function– was not significantly altered except in morbid obesity; this is consistent with the lack of changes in leptin levels found in obese women after these levels are adjusted for age and fat mass (unpublished). This is further supported by the unaltered leptin handling (34) and the relative scarcity of mutations in the leptin gene or its receptor (35-39) in humans. Insulin enhances the synthesis and release of leptin in humans (40,41), but the association between hyperinsulinemia and leptin is lost in obesity (42). In this study we observed, however, that although leptin levels were high in obese women, they were uncorrelated with those of insulin except for group 3, in which the alterations in insulin were more marked.

Insulin changes, practically intrinsic to obesity, have often been considered to be a consequence of the metabolic alterations of obesity rather than a key factor in the development of this condition (43).

The data presented here show that early / mild insulin alteration is consistent with obesity, and suggest that the intensity of alteration in insulin function –i.e. insulin resistance– is a key factor defining severe / complicated obesity. The correlation found in the severe obesity group (group 3) between insulin levels and those of leptin, and the low circulating acyl-estrone levels suggest that the influence of insulin on the signals that control the adjustment of body weight may be much more significant than has usually been assumed. Leptin (44,45) and oleoyl-estrone (46) are known to affect insulin levels and resistance, but their interrelationship during obesity is far from being fully understood.

In conclusion, we have found that in obese women, acyl-estrone levels –and probably function– are diminished, contrasting with maintained leptin-BMI correlations. Insulin role in the control of body weight, perhaps through acyl estrone-mediated effects, should be re-evaluated.

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SECCIÓN 3 .

Serum sTNFα receptors and leptin levels in normal-weight and obese women: effect of adiposity and diabetes

Obesity Research (sometido)

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Serum sTNFα receptors and leptin levels in normal-weight and obese women: effect of adiposity and diabetes.

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Abbreviated title: TNF-leptin system, adiposity and diabetes.

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Abstract

Objective: To evaluate the effect of adiposity and the presence of diabetes on leptin, TNFα and plasma soluble TNF receptors (sTNFR1, sTNFR2) levels.

Research Methods and Procedures: We studied a cohort of 157 diabetic and non-diabetic females with a wide range of adiposity distributed into five groups. Control: body mass index (BMI) between 19-27 kg/m^2 (n=24), obese: BMI between 27-40 kg/m^2 (n=63), obese type2-diabetes mellitus (DM): BMI between 27-40 kg/m^2 with DM (n=19), morbid obese: BMI≥40 kg/m^2 (n=29) and morbid obese type2-DM: BMI≥40 kg/m^2 with diabetes (n=22). Fasting glucose levels, plasma total triglycerides and cholesterol, high- low- and very low-density lipoprotein cholesterol were assayed by enzymatic and colorimetric methods. Plasma TNFα levels were measured by enzyme-linked immunoabsorbent assay. Insulin and leptin levels were assayed by radioimmunoenzimatic assays. And both sTNFR were measured by immunoenzimometric assays.

Results. All groups of patients showed significant increases in both sTNFR relative to control sTNFR1 was higher in morbid obese diabetic individuals compared to non-diabetic counterparts (P=0.003) while sTNFR2 was significantly different between obese and morbid obese subjects (P=0.036). Bivariate correlation analysis showed a significant relationship between both plasma soluble TNF receptors and body mass index, percentage of body fat, fasting glucose, insulin and leptin. In multivariate analysis, both soluble TNFR plasma levels were significantly associated with BMI and the presence of diabetes (R^2=0.20 for sTNFR1 and sTNFR2). When plasma leptin levels were added into the model, this protein and the presence of diabetes explained 27% of the variance of the plasma sTNFR1 levels.

Discussion: These results suggest that TNFα-leptin system could be involved in the pathophysiology of obesity and the associated insulin resistance.

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INTRODUCTION

Tumor necrosis factor alpha (TNFα) is a potent cytokine produced, mainly, by the immune system. It has been implicated in energy balance regulation in different pathological conditions such as cancer (1), AIDS (2,3), other catabolic situations (4) and in some eating disorders (5,6). This cytokine is also produced in adipose (7) and muscle tissues (8) and released into the circulation. Recently, the TNFα system has been described in the pathophysiology of obesity (9) and other associated metabolic complications such as insulin resistance and type-2 diabetes mellitus (7). TNFα expression is increased in most of the rodent models of obesity examined to-date and is related to the degree of insulin resistance (10). Moreover, an increased TNFα expression in adipocyte and muscle tissue has been reported recently in obese people (8,11) together with significant relationships between TNFα expression (or plasma protein product concentrations) and body mass index (12,13), percentage of body fat (11) and the degree of hyperinsulinemia (12). Conversely, a considerable number of investigators have failed to observe higher TNFα levels in obese compared to lean individuals (12,14).

Intracellular signal responses to TNFα are mediated by at least two known cell-surface receptors present on adipocytes and other cell types (15,16). Both receptors also exist in soluble forms that are derived, apparently, by proteolytic cleavage from the cell surface forms (17). Recent data have suggested that these soluble receptor forms can modulate TNFα function, could reflect the degree of activation of the TNFα system and be implicated in some aspects of energy metabolism (18).

Currently, there is growing interest as to which of the two TNF receptors is involved in the induction of insulin resistance in the obesity state. Preliminary studies with knockout-gene rodent models suggest that insulin resistance induced by obesity is mediated by soluble TNFR1

(10) but Hotamisligil et al (19), in a limited series of obese females (n=19), observed an increase of the sTNFR2 mRNA expression and sTNFR2 plasma levels compared to lean-body control subjects. The soluble forms of TNFR2 expression were significantly related to BMI and plasma insulin levels, suggesting that this receptor is an important factor contributing to the obesity-associated insulin resistance. Recently, an increase in the RNA expression of both types of receptors (20) or in the two plasma soluble TNFα protein receptors (14) was observed in a limited number of obese subjects compared to controls, independent of the presence of diabetes.

We report, here, a study on a large group of diabetic and non-diabetic females with a wide range of adiposity exploring: a) the effect of the adiposity and the presence of diabetes on TNF-α and soluble receptors plasma levels, and b) the determinants of the levels of these molecules in plasma.

RESEARCH METHODS AND PROCEDURES

Subjects

We studied 157 females aged between 19 and 65 years with a wide range of adiposity and who were consecutively recruited from among those attending the outpatient clinics of the University Hospital of Sant Joan de Reus, Spain. Four patient-group assignments, with respect to body mass index (BMI) and presence/absence of diabetes, were compared to a control group of healthy subjects with a BMI between 19 and 27 kg/m^2. The study subjects were defined as obese when the BMI was between 27 and 40 kg/m^2 and as morbid obese when the BMI was >40 kg/m^2. The presence of diabetes was confirmed when a previous diagnosis of NIDDM had been performed or when fasting plasma glucose of $\geq$7 mmol/L was observed on two consecutive occasions. The characteristics of all the study subjects are summarized in Tables 1 and 2. Exclusion criteria were the presence of infectious, inflammatory, neoplastic or

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systemic diseases, hypothyroidism or endocrine diseases other than diabetes, or the active use of antiobesity drugs. The study protocol was approved by the hospital ethics committee and all subjects gave written informed consent to participation in the study.

Data collection and Laboratory Procedures

Height was measured without shoes to the nearest 0.5 cm. Body weight was measured to the nearest 0.1 kg (with the subject lightly clothed) and BMI was calculated as weight/height^2. Waist circumference was measured midway between the lower rib margin and the iliac crest. Hip circumference was determined as the widest circumference measured over the greater trocanter. The waist-to-hip ratio (WHR) was then calculated. Fat free mass (FFM) was assessed as previously described by tetrapolar bioelectrical impedance at 50 KHz (Human-I Scan^R, Dietosystem, Spain) using the gender and fat-specific equations validated by Segal et al (21). Fat mass (FM) was then calculated as the difference between total body weight and FFM.

Fasting blood samples were collected in the morning between 0800h and 1000 h. Plasma and serum were separated immediately by centrifugation and aliquots were frozen at -80C for subsequent batched analysis. Plasma total triglycerides and cholesterol, high- low- and very low-density lipoprotein cholesterol (HDLc, LDLc, VLDLc) and glucose levels were assayed by the hospital's routine chemistry laboratory. A commercial radioimmunoassay kit was used to determine fasting plasma insulin (Amersham, Little Chalfont, UK). The lowest limit of detection was 48 pg/mL. The intra- and inter-assay coefficients of variation for insulin were 5.05% and 13.4%, respectively. Homeostasis model assessment of insulin resistance (HOMA IR) was then calculated as previously described (13):

HOMA IR = [fasting insulin (μU/mL) * fasting glucose (mmol/L)] / 22.5

Immunoenzymometric assays were used to determine the levels of sTNFR1 and sTNFR2 concentrations in plasma (BioSource, Fleunes, Belgium). The minimum detectable concentration was estimated to be 50 pg/mL and 0.1 ng/mL for each of the two receptors,

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respectively. The intra- and inter-assay coefficients of variation were <6.5% and <8.9% respectively for sTNFR1 and <3.3% and <6.9% respectively for sTNFR2. TNFα levels were measured by enzyme-linked immunosorbent assay (Pharmingen, San Diego, USA). The intra- and inter-assay coefficients of variation were <5.8 % and <13.8%, respectively. Plasma leptin levels were measured by a radioimmunoenzymatic assay using commercial kits (Linco Research, St Louis, MO). Within- and between-assay variations were 4.98 % and 4.5 %, respectively.

STATISTICAL METHODS

Descriptive results of continuous variables are expressed as means ± SEM. The variables with non-gaussian distributions, such as leptin and insulin levels, were logarithmic transformed to approach normal distribution. The means are expressed as geometric means. One way analysis of variance (ANOVA) with multiple comparisons was used to explore differences between groups. Relationships between two quantitative variables were assessed by Pearson's correlation coefficient. A step-wise multiple regression analyses was performed to identify the factors affecting plasma levels of soluble TNF receptors. Age, BMI, fat mass, WHR, fasting plasma glucose and insulin concentrations, and presence/absence of diabetes mellitus were entered as independent variables. The model R^2 indicates the percentage variance in the dependent variable that is explained by the independent variables included in the model. All statistical calculations were performed using the SPSS/PC software. Values were considered statistically significant with $P<0.05$.

RESULTS

Table 1 contains the anthropometric characteristics of the study groups. BMI, percentage of body-fat and waist circumference increased in relation to the degree of obesity. No significant