UNIVERSITAT DE BARCELONA
FACULTAT DE BIOLOGIA
DEPARTAMENT DE FISIOLOGIA

REGULACIÓ NUTRICIONAL I ENDOCRINA DE LA LIPÒLISI I LA LIPOPROTEÏNA LIPASA EN LA TRUITA IRISADA (*Oncorhynchus mykiss*) I L'ORADA (*Sparus aurata*)

Tesi Doctoral

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Memòria presentada per
Amaya Albalat Ribé
Per obtar al grau de
Doctor en Biologia

Tesi realitzada sota la direcció de la Dra. Isabel Navarro Álvarez del Departament de Fisiologia, Facultat de Biologia. Adscrita al Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, programa de Fisiologia (bienni 2001-2003).

Dra. Isabel Navarro Amaya Albalat

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ÍNDEX

INTRODUCCIÓ

En peixos, els principals òrgans d'acumulació de lípids són el múscul, el fetge i el teixit adipós (Sheridan i Kao, 1998). La importància d'un o altre teixit com a òrgan de reserva d'energia depèn principalment de l'espècie. Així, trobem espècies, com l'arengada (*Clupea harengus*) on el principal òrgan d'emmagatzement de lípids és el múscul, i altres com el bacallà (*Gadus morhua*) que preferentment acumulen els lípids en el fetge (Jobling i col., 1991). En un primer moment, es va postular que les espècies que habiten en el fons marí i estan caracteritzades per una baixa activitat acumularien els lípids preferentment en el fetge mentre que les espècies més actives acumularien els lípids majoritàriament en el múscul esquelètic (Braekken, 1959). Tot i això, Sheridan (1988) apuntà que aquestes consideracions ecològiques no expliquen la gran varietat de patrons de distribució lipídics que mostren les espècies de peixos que viuen en ambients similars. En el cas de l'orada (*Sparus aurata*) (Santhina i col., 1999) i la majoria dels salmònids, sembla que el compartiment majoritari de reserva seria el múscul esquelètic i el teixit adipós perivisceral (sobretot en peixos cultivats i alimentats de forma artificial), i no el fetge (Takama i col., 1994; Jobling i col., 1998). Tot i aquestes diferències, en les espècies que hi és present, el teixit adipós juga un paper fonamental en el balanç energètic de l'animal mitjançant la seva capacitat d'emmagatzemar i mobilitzar lípids com a font d'energia. Els lípids s'acumulen majoritàriament en forma de triacilglicerols (TAG) i en menor grau en forma de fosfolípids i esterols (Sheridan, 1988).

La quantitat de teixit adipós s'ha vist que varia de forma molt considerable segons l'estat fisiològic, reproductiu, migratori i d'hivernació del peix. Així, d'una banda s'ha observat un augment del teixit adipós visceral en peixos alimentats amb dietes d'alt contingut lipídic (Company i col., 1999) mentre que el dejuni provoca una disminució del lípid visceral i el contingut lipídic en diversos teixits (Navarro i Gutiérrez, 1995). A més, s'ha descrit en moltes espècies, com en la truita alpina (*Salvelinus alpinus*), una forta mobilització dels lípids que afecta la quantitat de teixit adipós present en els animals durant els períodes de migració i la posta d'ous (Jobling i col., 1998).

En l'aqüicultura mediterrània, les espècies de més interès són majoritàriament carnívores. Això vol dir que la seva dieta en el medi natural és alta en proteïnes. Aquestes espècies, utilitzen proteïnes per créixer i un cop cobertes les necessitats estructurals les proteïnes restants s'utilitzen com a font d'energia. Donat que les

proteïnes són el component més car de les dietes, dins l'aqüicultura s'ha intentat incrementar els nivells de lípids i de carbohidrats en les dietes per tal que serveixin com a proveïdors d'energia enfront de les proteïnes que anirien destinades principalment a creixement muscular ('sparing effect') (Cho i Kaushik, 1990). Aquestes alteracions en les proporcions de proteïnes/lípids que s'ofereixen en la dieta poden provocar canvis en la composició dels animals. En aquest sentit, dietes d'elevat contingut lipídic produeixen un augment del teixit adipós en els peixos (Company i col., 1999; Gelineau i col., 2001).

Aquests augments en la quantitat de teixit adipós o del contingut lipídic dels teixits en els peixos pot ser un factor força important dins de la indústria de l'aqüicultura. De fet, i de forma cada cop més important, un factor totalment necessari per a l'expansió de nous mercats és una alta qualitat dels productes que es venen. En aquest sentit, un augment en la quantitat de teixit adipós o un elevat contingut lipídic en els teixits (per exemple en el múscul esquelètic) pot produïr canvis en les propietats físiques i organolèptiques del peix, a més d'una dificultat afegida a l'hora de la conservació del producte. Tot això, en definitiva, pot comportar una disminució en la percepció de la qualitat del peix, que pot restar valor al producte final, tot i que val a dir que la definició de 'qualitat del peix' depèn molt de les preferències dels consumidors dels diferents països (Gjedrem, 1997; Einen i Skreede, 1998).

D'altra banda, un dels factors limitants en l'expansió de l'aqüicultura és la dependència d'aquest sector en la farina de peix per a la fabricació dels pinsos (Hardy, 1999). Aquesta dependència és un problema important a resoldre per diversos motius. En primer lloc, la quantitat de farina de peix ha minvat en els últims anys, cosa que ha suposat l'encariment d'aquest producte i en segon lloc, la qualitat de la farina de peix és variable. Donat que no és sostenible mantenir el sector de l'aqüicultura en base a l'obtenció de farina de peix s'ha estat estudiant la possibilitat de substituir la proteïna animal dels pinsos per altres fonts alternatives, com per exemple per proteïnes d'origen vegetal (Smith i col., 1988). Fins al moment, sembla que una substitució parcial de la proteïna animal per proteïna d'origen vegetal és possible tot i que es produeixen efectes en el metabolisme dels peixos (Gomes i Kaushik, 1990; Médale i col., 1998). En aquest sentit, és important que a part d'un creixement acceptable, es tingui en compte les variacions de la qualitat i composició química del filet, les propietats organolèptiques així com les variacions en els depòsits de grassa, ja que alguns estudis han mostrat que hi poden haver canvis substancials en aquests paràmetres (Francesco i col., 2004).

De totes formes, tot i la importància i repercusió econòmica que pot tenir el teixit adipós en peixos d'interès per a l'aqüicultura, relativament pocs estudis han evaluat la regulació metabòlica d'aquest teixit.

El teixit adipós està format principalment per adipòcits units entre ells per fibres de col.lagen, i per la fracció estroma-vascular, que està composada per aquelles cèl.lules que no floten en un medi aquós, és a dir, pre-adipòcits, fibroblasts, eritròcits i cèl.lules endotel.lials entre d'altres (Cannon i Nedergaard, 2001). En peixos, els adipòcits poden ser bi- o multi-nucleats (Sheridan i Harmon, 1994) i a diferència dels adipòcits de mamífers presenten una gran variabilitat de tamanys (Zhou i col., 1996). El metabolisme d'aquest teixit està basat per una banda en la seva capacitat d'hidrolitzar lípids, mecanisme que es coneix amb el nom de lipòlisi i, per una altra banda, en la seva capacitat lipogènica i d'acumular lípids. En aquest sentit, se sap que el teixit adipós dels peixos conté lipases responsables de la mobilització dels lípids com la triacilglicerol lipasa (TAG lipasa) (Sheridan i Allen, 1984) així com lipases responsables de la captació de lípids com la lipoproteïna lipasa (LPL) (Back i col., 1983). Així, Sheridan (1994) presenta el model de funcionament d'un organ de deposició lipidica que hem representat en la Figura 1.

En un primer moment, l'existència d'una TAG lipasa sensible a hormones o HSL, va ser enigmàtica (Sheridan, 1988). Murat i col. (1985) no van veure cap efecte en la lipòlisi d'adipòcits de peix després d'estimular-los amb diferents agents típicament lipolítics en mamífers, com les catecolamines i el glucagó. Però, més recentment, alguns estudis han mostrat com tant trossos de teixit adipós aïllats (Harmon i Sheridan, 1992b) com adipòcits (Vianen i col., 2002) si que són capaços de respondre a diferents hormones. Tanmateix, a nivell genètic s'ha mostrat la localització del gen de la HSL en el cromosoma 5 de l'anguila dels camps d'arròs (*Monopterus albus*) encara que no es va demostrar la seva funcionalitat (Ji i col., 2003).

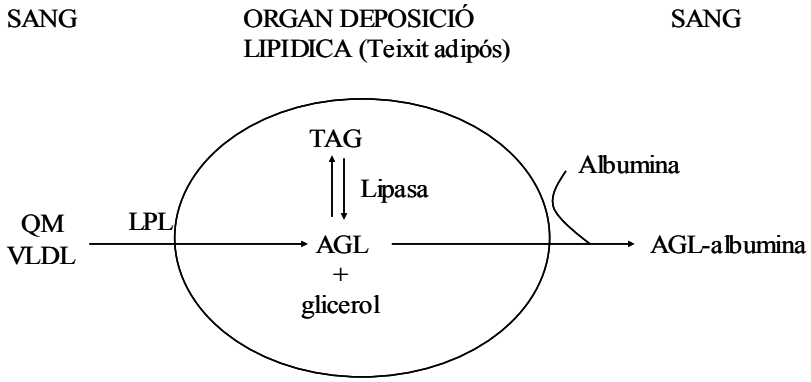

Figura 1. Esquema del model de funcionament d'un organ de deposició lipídica com el teixit adipós extret de Sheridan (1994). QM: quilomicrons; VLDL: lipoproteïnes de molt baixa densitat; LPL: enzim lipoproteïna lipasa; TAG: triacilglicerols; AGL: àcids grassos lliures.

I. MOBILITZACIÓ DELS LÍPIDS O LIPÒLISI EN EL TEIXIT ADIPÓS

La mobilització dels lípids o lipòlisi consisteix en la hidròlisi dels TAG en àcids grassos lliures (AGL) i glicerol. Els AGL poden ser parcialment re-esterificats dins el mateix adipòcit o alliberats i transportats a través de la sang units a albúmina a altres òrgans o teixits perifèrics on poden ser utilitzats (Allen, 1976). Així podem estudiar experimentalment la lipòlisi com: (1) una disminució del lípid total o els TAG en els teixits, (2) un augment de l'activitat de la TAG lipasa dels teixits, (3) un increment *in vitro* de l'alliberació dels AGL en el medi o com (4) un increment dels AGL en plasma (Sheridan, 1988). En el cas que l'estudi de la lipòlisi es faci *in vitro*, amb trossos de teixit adipós o adipòcits, es considera recomanable, independentment de l'espècie estudiada, la mesura del glicerol enfront dels AGL com a índex de la lipòlisi (Vaughan i Steinberg, 1963; Murat i col., 1985). Els adipòcits manquen de l'enzim gliceroquinasa, cosa que suposa que el glicerol no pot tornar a ser utilitzat per la cèl.lula mentre que els AGL poden ser alliberats o parcialment reciclats, és a dir, re-esterificats, dins la mateixa cèl.lula.

En mamífers, els mecanismes intracel.lulars involucrats en la lipòlisi (Figura 2) inclouen l'activació de l'adenilat ciclasa (AC) que produeix un increment del AMP cíclic (AMPc) intracel.lular donant lloc a l'activació de la proteina quinasa A (PKA) (Egan i col., 1990), la qual fosforila a les perilipines (Greenberg i col., 1991) i l'HSL (Stralfors i Belfrage, 1983). Les perilipines, en condicions basals, es situen a la superfície de les gotes lipídiques creant una barrera protectora per a la interacció de l'HSL amb la gota lipídica, mentre que quan són fosforilades permeten l'accés de l'HSL sobre els lípids (Holm i col., 2000). Així, s'ha demostrat que la PKA fosforila certs residus de la zona reguladora de l'enzim HSL a l'extrem C-terminal (Shen i col., 1998; Anthonsen i col., 1998), donant lloc a la translocació de l'enzim a la superfície de les gotes lipídiques. A més, a part de la PKA sembla que l'augment d'AMPc pot activar també la via de les MAP quinases (MAPKs) que podrien estar implicades en la regulació de la lipòlisi en el teixit adipós (Greenberg i col., 2001).

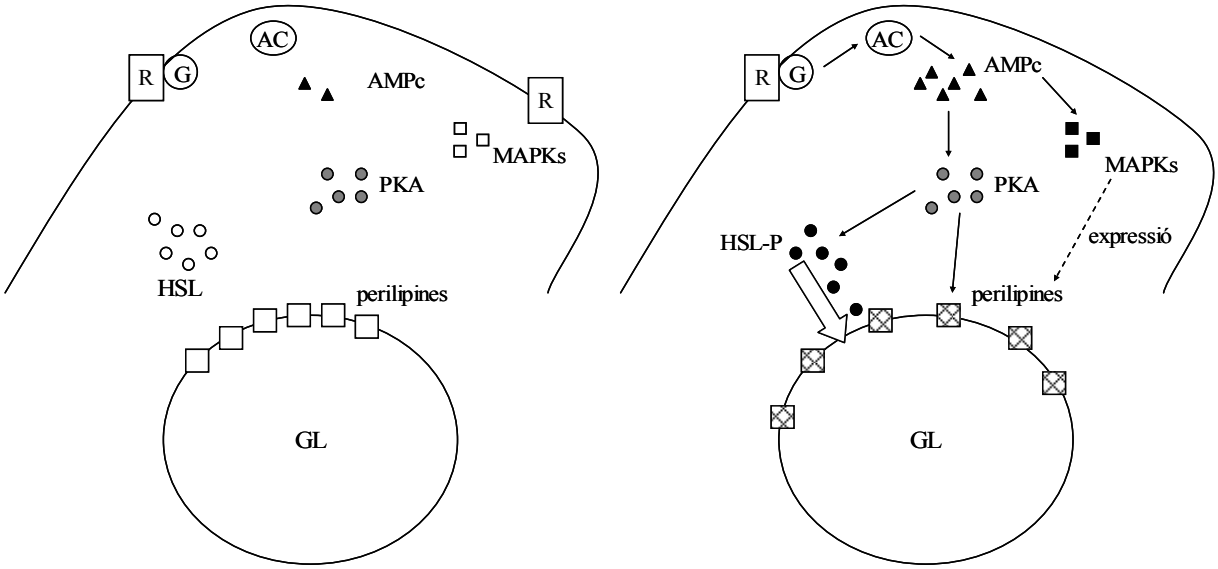

Figura 2. Mecanismes intracel.lulars involucrats en la activació de la lipòlisi basat parcialment en l'esquema presentat per Holm i col. (2000). GL: gota lipídica; HSL: lipasa sensible a hormones; PKA: proteïna quinasa A; R: receptor; G: proteïna G; AC: adenilat ciclasa; cAMP: AMP cíclic; MAPKs: quinases activadores de la mitosi.

En mamífers, la lipòlisi presenta una regulació nerviosa, metabòlica i endocrina. Pel que fa a la regulació endocrina, en aquests vertebrats superiors, les catecolamines, hormones tiroïdals, glucocorticoids, hormona de creixement i prolactina actuen com a hormones clarament lipolítiques (Jensen, 1997; Vernon, 2000). En peixos, la informació sobre la regulació de la lipòlisi és escassa is sovint contradictòria. Tot i això, sembla que els mecanismes de regulació del teixit adipós dels peixos podrien ser similars als descrits en mamífers.

Regulació nutricional de la lipòlisi

En la Figura 3, hem fet un sumari del metabolisme dels AGL en els mamífers. Els AGL plasmàtics augmenten en el dejuni degut principalment a un augment de la lipòlisi en el teixit adipós, conjuntament amb una disminució de la re-esterificació dels AGL (Frayn i col., 1995).

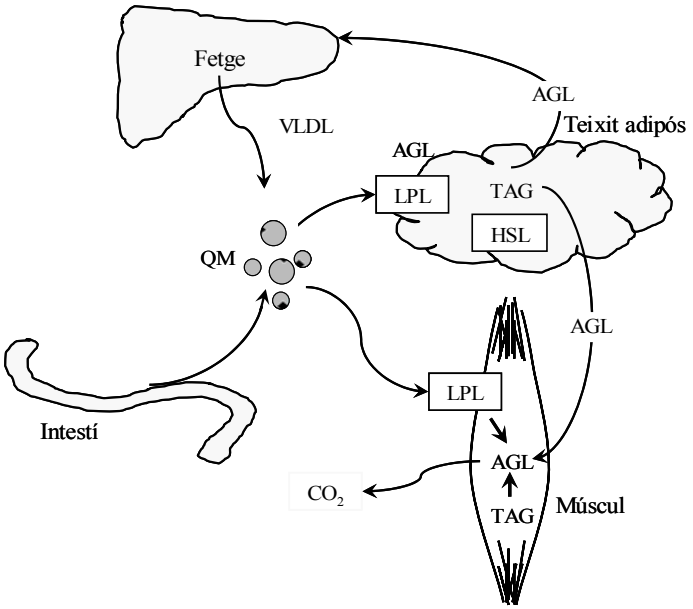

Figura 3. Esquema del metabolisme dels AGL en mamífers modificat del treball de Frayn (1998). En mamífers, en situacions de dejuni, l'aport més important d'AGL en plasma és a través de l'augment de la lipòlisi en el teixit adipós mentre que en l'estat post-prandial l'augment momentani d'AGL en plasma ve donat principalment per l'acció de la LPL. VLDL: lipoproteïna de molt baixa densitat; QM: quilomicrons; AGL: àcids grassos lliure; LPL: enzim lipoproteïna lipasa; TAG: triacilglicerols; HSL: lipasa sensible a hormones.

D'altra banda, després de la ingesta, els AGL alliberats per la LPL de l'endotel.li, a partir principalment dels QM i les VLDL que viatgen pel plasma, són una font important dels AGL plasmàtics que seran captats pels diferents teixits (Frayn, 1998). Els efectes observats *in vivo* s'han corroborat amb estudis *in vitro*, on utilitzant adipòcits aïllats, s'ha vist que la lipòlisi basal d'adipòcits de rates dejunades és més elevada que la lipòlisi d'adipòcits de rates alimentades (Morimoto i col., 1998; Szkudelski i col., 2004). Resultats similars es van obtenir en marmotes (*Marmota marmota*), on adipòcits aïllats del teixit adipós subcutani presentaven una lipòlisi major durant el període hivernant (animals dejunats) comparat amb el període no hivernant (animals alimentats) (Cochet i col., 1999).

En peixos s'ha descrit en animals dejunats un augment dels AGL en el plasma (Bilinski i Gardner, 1968; Leatherland i Nuti, 1981), que tornen a disminuïr ràpidament quan els animals són re-alimentats (la disminució és aparent a les 24 hores) (Pottinger i col., 2003). En trossos de fetge, Harmon i Sheridan (1992a) van observar un augment de l'activitat TAG lipasa en truites irisades (*Oncorhynchus mykiss*) dejunades respecte a

l'obtinguda en truites alimentades mostrant que l'estat nutricional afecta clarament la lipòlisi en el fetge. Pel que fa al teixit adipós, en el treball dels mateixos autors es va veure un augment de la lipòlisi en trossos de teixit adipós provinent d'animals dejunats enfront a teixit adipós provinent d'animals alimentats, tot i que l'augment no era significatiu.

D'altra banda, s'ha observat que canvis en la composició de la dieta poden produir alteracions en el metabolisme lipídic. Així, s'ha vist que dietes amb alt contingut lipídic provoquen un augment en la quantitat de teixit adipós visceral en l'orada (*Sparus aurata*) (Company i col., 1999). Altres estudis utilitzant dietes amb proteïna 100% d'origen vegetal van observar una disminució en la quantitat de lípid corporal total (Gómez-Requeni i col., 2004). Mentre que altres autors, treballant amb juvenils d'orada (*Sparus aurata*) van observar un efecte contrari en dietes amb alt contingut de farina de blat de moro (Pereira i Oliva-Teles, 2003).

Regulació de la lipòlisi en situacions de sepsis

En situacions d'infecció, es produeixen en mamífers canvis profunds que donen lloc a alteracions importants en el metabolisme lipídic i de les lipoproteïnes. Així, de forma consistent s'ha descrit una augment dels TAG en plasma caracteritzat per un augment de les VLDL. Aquest increment de les VLDL pot ser degut a un augment de la seva síntesi en el fetge o a una disminució en la seva taxa d'eliminació (revisat per Khovidhunkit i col., 2004). D'aquesta manera a dosis baixes de lipopolisacàrid o LPS (un component de la paret de les bacteries gram-negatives) es produeix un augment de la síntesi de les VLDL en el fetge, com a resultat d'una estimulació en la producció hepàtica *de novo* d'AGL i una activació de la lipòlisi del teixit adipós. Mentre que a dosis altes de LPS es produeix una disminució en la taxa d'eliminació de les VLDL, degut principalment a una inhibició de l'activitat LPL en plasma (Feingold i col., 1992). Pel que fa a la resposta específica del teixit adipós, en humans s'ha descrit un augment de la lipòlisi en situacions de sepsis crítica, afavorint doncs un augment dels AGL en plasma (Miles, 1993), i a més, l'administració de LPS és capaç d'estimular la lipòlisi del teixit adipós.

Les citoquines clau responsables de la coordinació de la resposta immune inclouen els factors de necrosi tumorals (TNFα i TNFβ), les interleuquines (Ils) i els interferons (IFNα, IFNβ, IFNγ). Sembla, que els efectes de les citoquines sobre el metabolisme lipídic podrien ser directes, així s'ha descrit una acció directa per exemple del TNFα sobre la lipòlisi del teixit adipós (Zhang i col., 2002) o sobre la síntesi de les VLDL en el fetge (Feingold i Grunfeld, 1987).

En peixos, s'ha descrit que una adminstració de LPS *in vivo* produeix una activació dels sistema immune amb un augment de la proliferació policlonal de limfòcits i un augment de la capacitat fagocítica dels macròfags en diferents espècies (Warr i Simon, 1983; Salati i col., 1987). A nivell de canvis en el metabolisme lipídic, la informació és escassa, però sí que s'ha observat un augment dels AGL en el plasma de la tilapia (*Oreochromis mossambicus*) degut a una adminstració de LPS (Balm i col., 1995). Tot i aquestes observacions, no hi han estudis sobre l'efecte directe que podria tenir el LPS o certes citoquines, com el TNFα sobre el metabolisme lipídic del teixit adipós o del fetge en peixos.

Regulació hormonal de la lipòlisi

Hormones pancreàtiques: Insulina i glucagó

En els mamífers, la insulina és una hormona anabòlica que estimula la captació de glucosa després de la ingesta en fetge i múscul així com la captació de AGL i glucosa en el teixit adipós. En peixos, la insulina circulant, a l'igual que en mamífers, augmenta després de la ingesta (Navarro i col., 1993), dismimueix en períodes de dejuni (revisat per Navarro i Gutiérrez, 1995) i en general sembla que juga un paper anabòlic similar al descrit en mamífers (Mommsen i Plisetskaya, 1991). Tot i això, cal mencionar que també s'han descrit efectes atípics d'aquesta hormona especialment pel que fa a la regulació de la gluconeogènesi en algunes espècies de peixos (revisat per Mommsen i Plisetskaya, 1991).

Pel que fa al paper de la insulina en el metabolisme lipídic, en uns primers estudis es va veure que una administració d'insulina provocava una disminució dels AGL

plasmàtics en vàries espècies de peixos (revisat per Ince, 1983). A més, els efectes de la insulina sobre la lipòlisi en fetge mostraren un paper anti-lipolític d'aquesta hormona. Plisetkaya i col. (1989) van veure com una insuficiència pancreàtica d'insulina produïa un increment de l'activitat TAG lipasa del fetge en dues espècies de salmó (*Oncorhynchus kisutch* i *O. tshawytscha*) mentre que en estudis realitzats *in vitro* amb trossos de fetge, la insulina va ser capaç d'inhibir l'estimulació de la lipòlisi produïda pel glucagó en truita irisada (*Oncorhynchus mykiss*) (Harmon i Sheridan, 1992a).

En el teixit adipós de la truita comuna (*Salmo trutta*), Planas et al. (2000a) van detectar receptors específics d'insulina que eren regulats segons els nivells circulants de l'hormona. Pel que fa als efectes de la insulina en la lipòlisi del teixit adipós la informació és escassa, tot i així Harmon i Sheridan (1992b) van veure que la lipòlisi en trossos de teixit adipós de truita irisada (*Oncorhynchus mykiss*) incubats amb insulina era menor que la lipòlisi en mostres control, tot i que de forma no significativa, però si que la insulina era capaç de revertir els efectes lipolítics del glucagó utilitzant el mateix model.

Per una altra banda, el glucagó en els mamífers és una hormona típicament lipolítica que actua a través de l'activació de l'adenilat ciclasa (Perea i col., 1995). En peixos, el glucagó en plasma augmenta de forma bifàsica després de la ingesta (Navarro i col., 1993; Pérez-Sánchez i col., 1989) així com en períodes de dejuni curts (revisat per Navarro i Guitérrez, 1995), mentre que en períodes de dejuni llargs els nivells de glucagó en plasma disminueixen. Aquest fet, que en un primer moment sembla contradictori, no atura la mobilització de reserves que es dóna durant el dejuni, possiblement degut als nivells molt més baixos d'insulina (revisat per Navarro i col., 2002). Els efectes del glucagó sobre el metabolisme lipídic en peixos, a diferència dels mamífers, han donat resultats contradictoris. A nivell plasmàtic el glucagó augmenta els AGL en el plasma del lluç de riu (*Esox lucius*) (Ince i Thorpe, 1975) mentre que no es veu efecte en el plasma de l'anguila (*Anguilla japonica*) (Chan i Woo, 1978). En el cas dels salmònids, sembla que el glucagó, pels resultats obtinguts en experiments *in vivo*, és lipolític tot i que els efectes varien estacionalment (Plisetskaya i col., 1989). En el fetge, els resultats concorden en un paper lipolític del glucagó en la truita irisada (*Oncorhynchus mykiss*) (Harmon i Sheridan, 1992a). D'altra banda, en la mateixa espècie però en trossos de teixit adipós Harmon i Sheridan (1992b) van observar que el glucagó augmentava l'activitat TAG lipasa i l'alliberació d'AGL mentre Migliorini i col. (1992) treballant amb trossos de teixit adipós del peix tigre (*Hoplias malabaricus*) i

Murat i col. (1985) treballant amb adipòcits de vàries espècies de peixos, no van veure cap efecte d'aquesta hormona sobre la lipòlisi.

Hormona de Creixement i Somatolactina

Les hormones hipofisàries, com l'hormona de creixement (GH), la somatolactina (SL) i la prolactina sembla que poden jugar un paper important en la regulació del metabolisme lipídic en peixos però els efectes estan molt supeditats a la durada de l'exposició, les variacions estacionals, la ritmicitat diurna i la interacció amb altres hormones (revisat per Sheridan, 1994).

La GH està relacionada, conjuntament amb el factor de creixement tipus insulina-I (IGF-I) amb efectes promotors del creixement (Le Bail i col., 1993), però a la vegada també té efectes lipolítics i diabetogènics. Tant en mamífers com en peixos, els nivells plasmàtics de GH augmenten durant el dejuni i aquest augment de la GH conjuntament amb una disminució de la insulina afavoreixen un augment dels AGL en plasma que podran ser utilitzats per teixits perifèrics (revisat per Pérez-Sánchez i Le Bail, 1999). Així, en exposicions llarges de GH, els AGL augmenten en el plasma del carpí (*Carassius auratus*) (Minick i Chavin, 1970) mentre que en la truita irisada (*Oncorhynchus mykiss*) la GH estimula la lipòlisi del fetge *in vitro* (O'Connor i col., 1993). Tot i això, el paper de la GH en el metabolisme lipídic ha donat de vegades resultats contradictoris (Sheridan, 1988) i fins el moment, no es disposa d'informació sobre el paper directe d'aquesta hormona sobre el teixit adipós.

La somatolactina (SL) és una hormona hipofisària de la familia de la GH. Els nivells plasmàtics d'aquesta hormona varien segons l'estat reproductiu (Taniyama i col., 1999), d'exercici (Kakizawa i col., 1995) i d'estrés (Rand-Weaber i col., 1993) de l'animal, fet que la posiciona com un possible candidat en la regulació de la mobilització de l'energia en peixos. De fet, en l'orada els nivells plasmàtics de SL i GH presenten una relació inversa i sembla que podrien funcionar conjuntament en la regulació de l'acumulació dels lípids (Mingarro i col., 2002), tot i això, el paper directe de la SL en la lipòlisi i en concret sobre la lipòlisi en el teixit adipós queda per estudiar.

Factor de Necrosis Tumoral (TNFα)

En mamífers, el TNFα és una citoquina important en la resposta immune i els processos inflamatoris. Però a més, s'ha vist que juga un paper clau en la homeostasi de la glucosa i el metabolisme lipídic del teixit adipós (Sethi i Hotamisligil, 1999). Així, com s'ha mencionat anteriorment, dins dels efectes descrits en mamífers destaca com agent lipolític en adipòcits (Zhang i col., 2002). El mecanisme pel qual el TNFα estimula la lipòlisi no és del tot conegut tot i que Zhang i col. (2002) van mostrar que el TNFα estimulava la lipòlisi a través de l'activació de les MAPKs conjuntament amb un augment de l'AMPc i una disminució de l'expressió de la fosfodiesterasa-3B (PDE3B). Més recentment, s'ha descrit que l'augment de la lipòlisi per TNFα és degut a una down-regulació de l'expressió de les perilipines a través de l'activació de les MAPKs p44/42 i JNK en adipòcits d'humans (Rydén i col, 2004).

En peixos, el TNFα ha estat identificat en diverses espècies com la truita irisada (*Oncorhynchus mykiss*) (Laing i col., 2001), la carpa (*Cyprinus carpio*) (Saeji i col., 2003) i l'orada (*Sparus aurata*) (Garcia-Castillo i col., 2002). A més, concretament en el cas de la truita irisada, s'han descrit dos gens codificants de TNFα (Zou i col., 2002; Bobe i Goetz, 2001; Laing i col., 2001).

D'altra banda, també en peixos, com en el cas dels mamífers, s'ha trobat expressió d'ARN missatger (ARNm) de TNFα en monòcits de truita i en macròfags prèviament estimulats amb LPS (MacKenzie i col., 2003). A més, s'ha detectat activitat TNF en sobrenedants de macròfags de truita irisada (*Oncorhynchus mykiss*) estimulats amb LPS (Qin i col., 2001). Tot i això, estudis sobre els possibles efectes del TNFα fora del sistema immune són molt limitats i concretament, el possible paper d'aquesta citoquina dins el metabolisme lipídic del teixit adipós no ha estat examinat.

Glucocorticoids i Hormona adrenocorticoide (ACTH)

Els resultats que es tenen sobre el paper del cortisol en el metabolisme lipídic dels peixos indiquen que en general funciona com a hormona lipolítica tot i que la informació és escassa. *In vivo* s'ha trobat que el cortisol augmenta els AGL en el plasma de l'anguila americana (*Anguilla rostrata*) mentre que no es veuen efectes en l'anguila

europea (*Anguilla anguilla*). Tanmateix, exposicions llargues de cortisol promouen la mobilització dels lípids en el salmó (*Oncorhynchus kisutch*) juvenil mentre que no es veuen efectes en els adults. Per tant, sembla que és important tenir en compte diversos factors si es volen comparar els efectes d'aquesta hormona entre diferents estudis (revisat per Sheridan, 1994).

Tot i la limitada informació que hi ha sobre el possible paper de l'ACTH en el metabolisme lipídic dels peixos certament els resultats no són clarificadors. Per una banda, Minick i Chavin (1970) van veure un augment dels AGL en plasma entre 6 i 24 hores després d'una administració d'ACTH en el carpí (*Carassius auratus*), mentre que altres estudis realitzats en carpa (*Cyprinus carpio*) no van veure cap efecte d'aquesta hormona (Farkas, 1967). Els resultats *in vitro* tampoc són definitoris, ja que ni Farkas (1969) ni Murat i col. (1985) van observar cap efecte estimulador d'aquesta hormona en els respectius estudis amb trossos de teixit i adipòcits respectivament.

Catecolamines i Hormones Tiroïdals

En mamífers, les catecolamines estimulen la producció d'AGL tant en plasma com en teixit adipós (Butcher i col., 1965). En peixos, els resultats són contradictoris. D'aquesta manera els estudis realitzats *in vivo* mostren que una administració de catecolamines produeix un increment (Larsson, 1973), una baixada (Farkas, 1969) o cap efecte (Perrier i col., 1972) sobre els nivells d'AGL en plasma. Treballs més recents, en carpes (*Cyprinus carpio*) canulades recolzen els resultats que mostren a l'adrenalina, i especialment la noradrenalina com hormones anti-lipolítiques en peixos (Van Raaij i col., 1995). Per una altra banda, en fetge la noradrenalina va ser capaç d'estimular l'alliberació d'AGL en el medi ràpidament (en qüestió de minuts) mentre que l'adrenalina no va tenir cap efecte en trossos de fetge de salmó (*Oncorhynchus kisutch*) (Sheridan, 1987). En el teixit adipós, els efectes *in vitro* de les catecolamines han estat investigats en trossos de teixit i en adipòcits. En trossos de teixit adipós Migliorini i col. (1992) no va veure cap efecte de les catecolamines sobre l'alliberació d'AGL en el medi i en adipòcits els resultats mostren un efecte nul (Murat i col., 1985) o una disminució de la lipòlisi degut a l'acció de les catecolamines a través dels receptors β-adrenèrgics (Vianen i col., 2002). Sembla que aquest efecte anti-lipolític de les catecolamines

s'explicaria com un mecanisme de protecció en els peixos per evitar un augment d'AGL i d'altres intermediaris de la β-oxidació que podrien causar danys cel.lulars en casos on les catecolamines augmentessin, com per exemple, en condicions d'hipòxia, una situació que es dóna de forma més comú en peixos que en mamífers (revisat per Van den Thillart i col., 2002).

Els efectes de les hormones tiroïdals mostren en general una tendència a la mobilització de lípids ja sigui *in vivo*, on 24 hores després d'una injecció de T3 es veu un increment dels AGL en el plasma de la carpa (*Cyprinus carpio*) o *in vitro* en diversos teixits de salmó (*Oncorhynchus kisutch*) incloent el teixit adipós (Sheridan, 1986).

II. MECANISMES DE DEPOSICIÓ LIPÍDICA EN EL TEIXIT ADIPÓS: IMPORTÀNCIA DE LA LIPOPROTEïNA LIPASA

Com s'ha mencionat anteriorment, els peixos, a diferència dels mamífers, acumulen els lípids en diferents òrgans o teixits. Els principals òrgans de deposició lipídica en els peixos són el teixit adipós, el múscul i el fetge. En aquests teixits, els lípids s'acumulen majoritàriament en forma de TAG. Per a l'estudi de la deposició lipídica el paper de l'enzim lipoproteïna lipasa (LPL) és realment important.

La LPL és un enzim glicoproteic amb activitat triacilglicèrid hidrolasa i fosfolipasa. La principal funció de la LPL és la hidròlisi dels TAG continguts en els QM i les VLDL que circulen per la sang. En mamífers, la seva acció dóna lloc a l'alliberació d'AGL que poden ser re-esterificats i guardats com a reserva d'energia en teixits, com el teixit adipós, o utilitzats com a font d'energia per altres òrgans perifèrics, com el múscul i el cor (Auwerx i col., 1992). Per tal de poder desenvolupar la seva funció, la LPL després de ser sintetitzada per diversos teixits és secretada fora de la cèl.lula, on a través de l'espai intersticial viatja fins a la superfície luminal de l'endoteli, que és on realitza la seva funció (Figura 4). Un cop en l'endoteli, la LPL s'uneix a proteoglicans de tipus heparan i dermatan sulfat. S'ha descrit que l'activitat LPL és estimulada per l'apoproteïna C-II que actua com a cofactor i també per l'heparina (activador no fisiològic). L'heparina té la capacitat de solubilitzar i estabilitzar l'enzim i facilita la interacció amb el substracte (Bengtsson-Olivecrona i Olivecrona, 1985).

En peixos, la primera informació que es va tenir sobre l'existència d'aquest enzim va ser obtinguda per Skinner i col. (1980) que van veure com després d'una injecció d'heparina el sèrum de la truita irisada (*Oncorhynchus mykiss*) contenia activitat LPL, una propietat típica descrita en mamífers. Posteriorment, l'enzim va ser caracteritzat a nivell bioquímic per Skinner i Youssef (1982). Fins a l'actualitat, s'ha detectat activitat LPL en teixit adipós, múscul vermell i blanc, cor, cervell, fetge i en ovaris vitel.logènics de la truita (Black i col., 1983; Black i Skinner, 1986; Black i Skinner, 1987; Lindberg i Olivecrona, 1995).

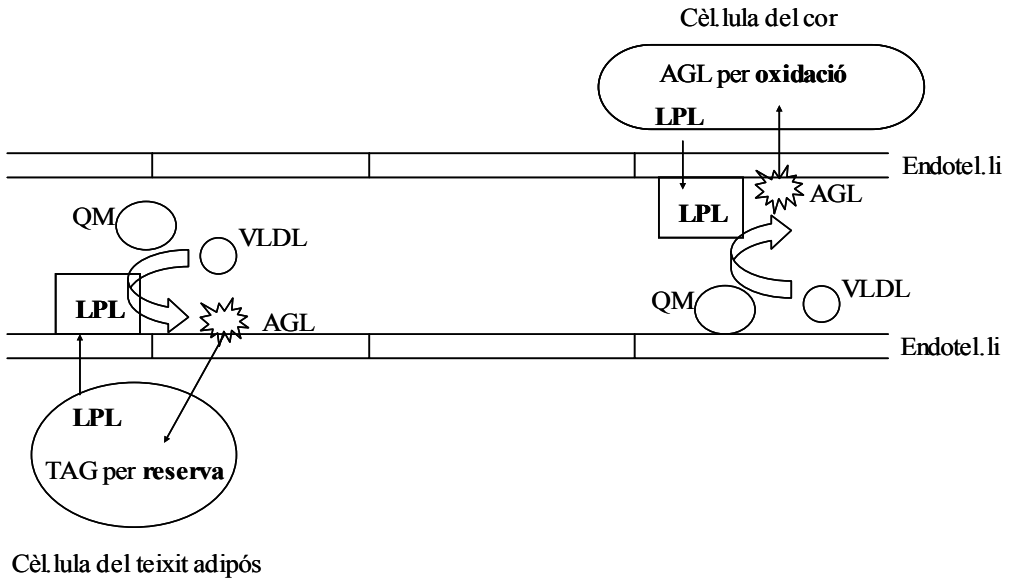

Figura 4. Esquema de l'efecte de la LPL en dos tipus cel.lulars diferents com són les cèl.lules del teixit adipós i les cèl.lules del cor en mamífers. QM: quilomicrons, VLDL: lipoproteïna de molt baixa densitat, AGL: àcids grassos lliures i LPL: lipoproteïna lipasa. Modificat del treball De Soley i Llobera, Esquemes bàsics de regulació del metabolisme. Textos docents. Publicacions U.B. (1996).

A nivell molecular, Arnault i col. (1996) van obtenir una seqüència parcial de la LPL en el peix zebra (*Bachydanio rerio*) i en la truita irisada (*Oncorhynchus mykiss*); tot i això, va ser a l'any 2002 que Lindberg i Olivecrona van determinar la seqüència de nucleòtids sencera de la LPL en la truita irisada (*Oncorhynchus mykiss*). El mateix any, Oku i col. (2002) van caracteritzar el gen de la LPL en l'orada vermella (*Pagrus major*). Pel que fa a les característiques de l'enzim, és interessant mencionar que la LPL de la truita irisada (*Oncorhynchus mykiss*) és poc estable a 37 ºC mentre que a 25 ºC l'enzim és molt més estable i presenta una gran afinitat per l'heparina, fins i tot més que la LPL bovina (Lindberg i Olivecrona, 2002).

La regulació de l'activitat LPL ha estat àmpliament estudiada en mamífers. Sembla que la regulació respon principalment a estats nutricionals, d'estrés, canvis hormonals i ambientals i està en funció del teixit (Howard i col., 1993), mentre que la regulació de la LPL en peixos ha estat molt poc estudiada.

Regulació nutricional de l'activitat LPL

En mamífers, després de la ingesta, l'activitat LPL augmenta en teixit adipós conjuntament amb nivells més elevats d'insulina en plasma (Eriksson i col., 2003). En múscul, s'han observat respostes diferents. En alguns estudis, s'ha vist que l'activitat LPL disminueix en múscul després de la ingesta (Picard i col., 1999; Ladu i col., 1991) mentre que en altres estudis no s'han vist canvis significatius (Lanza-Jacoby i col., 1997; Ong i col., 1994). En canvi, en situacions de dejuni, l'activitat LPL disminueix en teixit adipós, cosa que permet que els AGL siguin preferentment destinats a altres teixits essencials com el múscul esquelètic i el cor (Lladó i col., 1999; Eckel, 1987). Així, en el dejuni l'activitat LPL no varia o augmenta en el múscul i el cor (Ruge i col., 2005; Sudgen i col., 1993).

En peixos, hi ha un únic estudi sobre la regulació de l'activitat LPL realitzat per Black i Skinner (1986) que van observar com després de sotmetre a unes truites irisades (*Oncorhynchus mykiss*) un dejuni de 8 setmanes l'activitat LPL disminuïa significativament en teixit adipós i fetge mentre que no hi havia canvis significatius en múscul vermell i cor. A nivell molecular, Liang i col. (2002) van veure canvis en els nivells d'ARNm de la LPL en fetge i teixit adipós segons la condició nutricional, mentre que els nivells de lípids a la dieta afectaven únicament els nivells d'ARNm de la LPL en el fetge. Així, segons els estudis previs realitzats en peixos, sembla que la regulació de la LPL és depenent del teixit com en el cas dels mamífers però fins al moment no hi ha informació sobre la regulació hormonal que hi hauria governant aquests efectes observats.

Regulació de l'activitat LPL en situacions de sepsis

En mamífers, com s'ha mencionat anteriorment, les situacions d'infecció provoquen com a primer efecte un increment dels TAG en plasma (Grunfeld i col., 1992). Així, l'administració de LPS produeix una hipertrigliceridèmia que ja es detecta a les 2 hores després de l'admininstració i és manté almenys durant 24 hores (revisat per Khovidhunkit i col., 2004). Aquest efecte és degut bàsicament a un augment de la

lipòlisi en el teixit adipós que estimula la síntesi de VLDL en el fetge (Lanza-Jacoby i col., 1998) i també és degut a canvis en la taxa d'eliminició dels triglicèrids en plasma (Lanza-Jacoby i col., 1997).

Pel que fa als efectes sobre l'activitat LPL, com ja s'ha comentat anteriorment dosis altes de LPS disminueixen l'activitat LPL (Feingold i col., 1992). Sembla que aquest efecte podria estar mediat pel TNFα donat que s'ha descrit un efecte directe inhibidor del TNFα sobre l'activitat LPL en teixit adipós en rosegadors (Feingold i col., 1994). Tot i això, sembla que aquesta disminució de l'activitat LPL en teixit adipós no es pot relacionar directament amb l'augment de TAG que es dóna en plasma en situacions d'infecció, donat que el TNFα disminueix l'activitat LPL però en qüestió d'hores, mentre que l'augment de TAG en plasma és molt ràpid (revisat per Khovidhunkit i col., 2004).

Pel que fa als efectes d'una infecció o una administració de LPS en el metabolisme lipídic dels peixos hi ha molt poca informació. Tot i això, s'ha descrit un increment dels TAG en plasma en truites irisades infectades amb *Serratia liquefaciens* (una bacteria gram negativa) (Aydin i col., 2001). Tanmateix, pel que fa a possibles efectes sobre l'activitat LPL no hi ha informació fins al moment.

Regulació hormonal de l'activitat LPL

Hormones pancreàtiques: Insulina

En mamífers la insulina és una hormona clau en la regulació de l'activitat LPL. Així, en adipòcits de rata, la insulina funciona com agent estimulador de l'activitat LPL (Ong i col., 1988). D'altra banda, en el múscul, sembla que la regulació també es dona a través de la insulina (Picard i col., 1999) ja sigui directa o indirectament, a través de la seva acció en el metabolisme de la glucosa en múscul (Pollare i col., 1991) o dels AGL (Ferraro i col., 1993).

En peixos, els nivells d'insulina augmenten de forma significativa després de la ingesta (Navarro i col., 1993) i disminueixen durant períodes de dejuni (Navarro i col., 1992; Sundby i col., 1991). Així en períodes de dejuni la baixada de la insulina en

plasma inhibeix la reserva d'energia i afavoreix la mobilització de reserves. Fins al moment, s'ha descrit una funció anti-lipolítica d'aquesta hormona en trossos de teixit adipós de la truita irisada (*Oncorhynchus mykiss*) (Harmon i Sheridan, 1992b). Tot i això, no s'ha estudiat si la insulina podria tenir algun efecte sobre l'activitat LPL en peixos tot i que s'ha vist que en situacions de dejuni l'activitat LPL en el teixit adipós de la truita irisada (*Oncorhynchus mykiss*) també disminueix a l'igual que en mamífers (Black i Skinner, 1986).

Factor de Necrosi Tumoral (TNFα)

En mamífers, s'ha descrit que el mateix teixit adipós pot sintetitzar i alliberar TNFα, que podria funcionar com un autèntic 'adipostat' (Bulló-Bonet i col., 1999). Els resultats mostren que el TNFα disminueix l'activitat LPL en teixit adipós tant *in vivo* (Semb i col., 1987) com *in vitro* (Fried i Zechner, 1989; MacKay i col., 1990). Pel que fa a l'efecte en altres teixits, s'ha descrit que l'administració *in vivo* de TNFα no té cap efecte sobre l'activitat LPL del múscul del diafragma o del cor, mentre que l'acció sobre el teixit adipós es dóna vàries hores després de l'administració del TNFα (Grunfeld i col., 1989).

Tot i això, el possible paper del TNFα sobre l'activitat LPL del teixit adipós no ha estat examinat fins al moment en peixos a pesar de la seva importància en mamífers.

III. CAPTACIÓ DE GLUCOSA EN PEIXOS

La conversió de glucosa a lípids és un procés que té lloc fàcilment quan les condicions alimentàries són òptimes. Tant si la glucosa es oxidada per a l'obtenció d'energia, com si és utilitzada com a substrat lipogènic, els teixits han de ser capaços de captar aquesta glucosa.

Podem dir que els peixos tenen, sobretot si ho comparem amb els mamífers, una capacitat limitada d'utilitzar glucosa, fet que s'accentua en el cas de les espècies carnívores (Moon, 2001). Aquest és un problema en la indústria de l'aqüicultura ja que els carbohidrats són la font d'energia més barata que es pot oferir en la dieta. Així diversos estudis van mostrar com peixos alimentats amb dietes amb alt contingut en carbohidrats provocaven un hiperglicèmia permanent en plasma i un menor creixement (Cowey i Walton, 1989).

En mamífers, els nivells de glucosa en plasma estan regulats de tal manera que les concentracions de glucosa varien relativament poc. En aquesta regulació la insulina hi juga un paper clau (Khan, 1996). Es per això, que es podria pensar que la baixa capacitat del peixos per digerir la glucosa seria deguda a uns nivells baixos d'insulina (Palmer i Ryman, 1972). Però contràriament al inicialment pensat, els peixos presenten nivells d'insulina en plasma inclús superiors als que presenten els mamífers (Gutiérrez i col., 1984; Mommsen i Plisetskaya, 1991). Tanmateix, la secreció d'insulina en peixos respon a estímuls similars als descrits en mamífers com la glucosa, tot i que cal dir que en peixos els aminoàcids són més potents que la glucosa estimulant la secreció d'insulina (Carneiro i col., 1993).

Per tant, sembla que la captació de glucosa per part dels teixits podria ser un factor limitant (Abblet i col., 1983). Tot i això, s'han descrit receptors d'insulina en teixits clau per al metabolisme de la glucosa com són el múscul esquelètic, el fetge i el teixit adipós (Navarro i col., 1999; Planas i col., 2000a). A més, i de forma interessant s'ha vist que aquests receptors es regulen en funció de la quantitat d'insulina circulant. En múscul blanc, i de form característica en peixos, nivells alts d'insulina comporten un augment del nombre de receptors o 'up-regulation' (Parrizas i col., 1994; Baños i col., 1998) mentre que en múscul vermell l'efecte és totalment el contrari ('down-regulation'), possiblement per protegir aquest teixit d'una excessiva resposta a l'hormona (Baños i col., 1997). Tot i aquesta regulació, sí que s'ha vist que tant en

múscul esquelètic com en teixit adipós, el nombre de receptors és menor si es compara amb els mamífers.

Un altre factor clau en la utilització de la glucosa per part dels teixits és l'entrada de glucosa en les cèl.lules dels teixits diana. Per entrar dins la cèl.lula, la glucosa necessita de la presència de transportadors de glucosa en membrana. En mamífers s'han identificat i caracteritzat dos tipus de transportadors de glucosa. Un són els cotransportadors de sodi-glucosa i els altres són els transportadors de difusió facilitada o també anomenats GLUTs. Els transportadors de sodi i glucosa són els responsables de la captació de glucosa procedent de la dieta en el lumen intestinal i la re-absorció renal en el tub proximal de la nefrona. El transport de glucosa és actiu i es dóna en contra del seu gradient de concentració. D'altrabanda, els GLUTs medien el transport de glucosa de forma facilitada, és a dir, a favor del gradient de concentració. En aquest cas el transport s'ha vist que és saturable, estereospecífic, bidireccional i inhibible per certs compostos com la citocalasina B.

En peixos, diversos estudis han mesurat la captació de glucosa en diferents sistemes cel.lulars com eritròcits (Soengas i Moon, 1995), enteròcits (Soengas i Moon, 1998) i miòcits (Castillo i col., 2004). En aquests sistemes s'ha vist que el transport és saturable, estereospecífic i inhibible per citocalasina B, suggerint doncs que en peixos el transport també es dóna a través de GLUTs.

A nivell molecular, els resultats ho confirmen. D'aquesta manera, en peixos s'han clonat diversos membres de la família dels GLUTs (Teerijoki i col., 2000; Teerijoki i col., 2001; Krasnov i col., 2001) i de forma molt interessant s'ha clonat i caracteritzat un GLUT homòleg al GLUT4 de mamífers capaç de respondre a la insulina en el teixit adipós del salmó (*Oncorhynchus kisutch*) (Capilla i col., 2004).

Regulació hormonal de la captació de glucosa

Insulina

Com ja s'ha mencionat anteriorment, l'homeostasi de la glucosa en mamífers està finament regulada. En aquest grup de vertebrats, la insulina és l'hormona reguladora més important en l'homeostasi de la glucosa. La insulina, en resposta a nivells alts de

glucosa estimula la captació d'aquest compost en el múscul i el teixit adipós. En aquesta estimulació hi juga un paper clau el GLUT4 que respon a la insulina. En condicions basals, aquest transportador es troba emmagatzemat en vesícules intracel.lulars i quan es estimulat per la insulina el transportador es transloca a la membrana plasmàtica (Cushman i Wardzala, 1980). La via de senyalització ha estat molt estudiada i sembla que s'han identificat fins al moment dos mecanismes. Un es dóna a través de la fosfatidilinositol-3-quinasa (PI3K), que activa posteriorment molècules com la quinasa depenent de fosfoinositol (PDK1) (Figura 5), mentre que l'altre mecanisme inclou l'activació de la proteïna G petita de la família Rho (GTP-binding protein TC10) a les caveoles a través de la fosforilació de la Cb1 (revisat per Khan i Pessin, 2002).

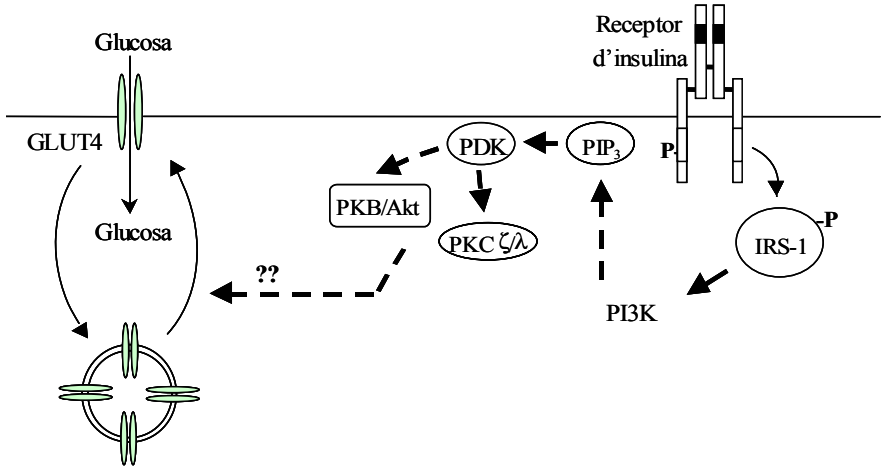

Figura 5. Esquema de la via de senyalització de la insulina a través del mecanisme de la PI3K que dóna lloc a un augment de la translocació del GLUT4 a la membrana plasmàtica produïnt una estimulació de la captació de la glucosa (segons Baumann i Saltiel, 2001).

En peixos, s'han idenficat receptors d'insulina en múscul (Parrizas i col., 1994) i teixit adipós (Planas i col., 2000a). A més, s'ha identificat la presència d'un GLUT homòleg al GLUT4 de mamífers en múscul (Planas i col., 2000b) i teixit adipós (Capilla i col., 2004) de salmònids. A nivell funcional, estudis realitzats amb miòcits de truita irisada (*Oncorhynchus mykiss*) van mostrar com la insulina era capaç d'estimular la captació de glucosa (Castillo i col., 2004) suggerint doncs la presència funcional d'un transportador sensible a insulina en el múscul de la truita. De totes formes, pel que fa al teixit adipós, els estudis han estat molt limitats i només trobem el treball de Christiansen i col. (1985) on no van veure cap efecte de la insulina sobre la captació de glucosa en adipòcits aïllats de la truita irisada (*Oncorhynchus mykiss*).

Factor de creixement tipus insulina (IGF-I)

La funció principal de l'IGF-I és la de mediar els efectes reguladors i promotors del creixement induïts per la GH. Així, l'IGF-I té una gran acció mitogènica però a més exerceix efectes metabòlics similars als que produeix la insulina. A concentracions fisiològiques, s'ha vist que l'IGF-I estimula la proliferació cel.lular i en certs teixits la diferenciació, mentre que a concentracions altes l'IGF-I estimula el transport de glucosa.

En peixos, s'han identificat receptors d'IGF-I en múscul (Parrizas i col., 1994) i teixit adipós (Planas i col., 2000a) en major nombre que els receptors d'insulina. En aquest grup de vertebrats, els efectes biològics de la insulina i l'IGF-I estan més entrecreuats que en mamífers per la qual cosa sembla que l'IGF-I podria jugar un paper important en la regulació del metabolisme de la glucosa. Fins al moment s'ha observat que l'IGF-I és capaç de disminuir els nivells de glucosa en plasma en la truita de fontana (*Salvelinus fontinalis*) (Skyrud i col., 1989) i d'aminoàcids en l'anguila (*Anguila japonica*) (Duan, 1998), mentre que a nivell *in vitro* aquest pèptid és capaç d'estimular la captació de glucosa en el múscul del barramundi (*Lates calcarifer*) (Degger i col., 2000) i en miòcits de la truita irisada (*Oncorhynchus mykiss*) inclús en major grau que la insulina (Castillo i col., 2004). Pel que fa al paper directe de l'IGF-I sobre el teixit adipós manca informació (Sheridan, 1994) tot i que donada la presència de receptors en aquest teixit, aquest podria ser un teixit diana tant per l'acció mitogènica com l'acció metabòlica de l'IGF-I.

OBJECTIUS

L'objectiu general d'aquesta tesi ha estat l'obtenció de preparacions d'adipòcits aïllats de peix per tal d'obrir les portes a l'estudi del metabolisme lipídic i glucídic del teixit adipós en peixos amb un sistema *in vitro*. Per altra banda, el nostre interès també s'ha centrat en l'estudi de la regulació de l'activitat lipoproteïna lipasa, enzim clau en el metabolisme lipídic. Les espècies de peixos escollides per tal fi han estat la truita irisada (*Oncorhynchus mykiss*) i l'orada (*Sparus aurata*) degut principalment a la seva importància en el sector de l'aqüicultura.

Seguint aquest objectiu general, els objectius concrets d'aquesta tesi són:

I. Posar a punt l'aïllament i l'obtenció d'adipòcits a partir de teixit adipós mesentèric de la truita irisada i l'orada i establir les condicions per a la mesura de la lipòlisi *in vitro*.

II. Estudiar la regulació nutricional i evaluar el paper de les hormones pancreàtiques (insulina i glucagó) en la lipòlisi d'adipòcits aïllats de la truita irisada.

III. Conèixer la regulació nutricional, l'efecte de diferents dietes formulades amb proteïna animal i vegetal, i la regulació hormonal (insulina, glucagó i hormona de creixement) en la lipòlisi d'adipòcits aïllats de l'orada.

IV. Analitzar els efectes d'una situació d'infecció induïda i en concret mesurar el paper directe del factor de necrosi tumoral alfa (TNFα) sobre el metabolisme lipídic del teixit adipós de la truita irisada.

V. Estudiar la regulació nutricional i el possible paper de la insulina sobre l'activitat lipoproteïna lipasa en diferents teixits de la truita irisada i l'orada.

VI. Posar a punt la mesura del transport de glucosa en adipòcits aïllats de la truita irisada i analitzar els possibles efectes de la insulina i el factor de creixement tipus insulina (IGF-I).

RESULTATS

I. REGULACIÓ NUTRICIONAL I HORMONAL DE LA LIPÒLISI EN PEIXOS

Regulation of lipolysis in isolated adipocytes of rainbow trout (*Oncorhynchus mykiss*): the role of insulin and glucagon

A. Albalat, J. Gutiérrez, I. Navarro

Comparative Biochemistry and Physiology Part A (*In press*) (2005)

Nutritional and hormonal control of lipolysis in isolated gilthead seabream (*Sparus aurata*) adipocytes

A. Albalat, P. Gómez-Requeni, P. Rojas, F. Médale, S. Kaushik, G.J. Vianen, G. Van den Thillart, J. Gutiérrez, J. Pérez-Sánchez, I. Navarro

American Journal of Physiology – Regulatory Integrative and Comparative Physiology 289: 259-267 (2005)

Control of adipose tissue lipid metabolism by tumor necrosis factor-α in rainbow trout (*Oncorhynchus mykiss*)

A. Albalat, C. Liarte, S. MacKenzie, L. Tort, J.V.Planas, I. Navarro

Journal of Endocrinology 184: 527-534 (2005)

Regulació de la lipòlisi en adipòcits aïllats de la truita irisada (*Oncorhynchus mykiss*): el paper de la insulina i el glucagó

Regulation of lipolysis in isolated adipocytes of rainbow trout (*Oncorhynchus mykiss*): the role of insulin and glucagon

Regulació de la lipòlisi en adipòcits aïllats de la truita irisada (*Oncorhynchus mykiss*): el paper de la insulina i el glucagó

Resum

En el present estudi, hem examinat els efectes de la insulina i el glucagó sobre la lipòlisi de la truita irisada (*Oncorhynchus mykiss*). Amb aquesta finalitat, es van aïllar adipòcits a partir del teixit adipós mesentèric i van ser incubats en absència (lipòlisi basal) o en presència de diferents concentracions d'insulina i glucagó. A més, per tal d'elucidar els efectes d'aquestes hormones *in vivo* sobre la lipòlisi dels adipòcits, es va realitzar un experiment de dejuni i una injecció intraperitoneal de glucagó.

La lipòlisi basal, mesurada com l'alliberació de glicerol en el medi d'incubació, va augmentar de forma proporcional amb el nombre de cèl.lules i amb el temps d'incubació. La viabilitat cel.lular va ser verificada mesurant l'activitat lactat dehidrogenasa (LDH) en el medi.

La insulina (a les concentracions de 35 i 350 nM) va disminuïr la lipòlisi d'adipòcits aïllats de la truita irisada *in vitro*, mentre que el glucagó va ser clarament lipolític a les concentracions de 10 i 100 nM.

A més, l'hipoinsulinèmia produïda gràcies a l'experiment de dejuni, així com la injecció de glucagó, van incrementar la lipòlisi mesurada en adipòcits aïllats aproxidament 1.5- i 1.4-vegades, respectivament, respecte a la lipòlisi d'adipòcits de peixos control.

En conclusió, les dades presentades en aquest estudi demostren que la lipòlisi, mesurada en adipòcits aïllats de truita irisada, pot ser regulada per la insulina i el glucagó. Els resultats no només indiquen que la insulina és una hormona important en la deposició lipídica a través del seus efectes anti-lipolítics en adipòcits de la truita irisada, sinó que a més presenten al glucagó com una hormona lipolítica, com indiquen els experiments tant *in vitro* com *in vivo*.

Regulation of lipolysis in isolated adipocytes of rainbow trout (*Oncorhynchus mykiss*): the role of insulin and glucagon

Amaya Albalat, Joaquim Gutiérrez, Isabel Navarro

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Running title: Hormonal control of lipolysis in isolated rainbow trout adipocytes

Key words: adipocyte, fasting, glucagon, glycerol, insulin, LDH, lipolysis, rainbow trout

Please address all correspondence to I. Navarro

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Fax: 34+ 93+ 4110358

Email: mnavarro@ub.edu

Abstract

In the present study, we have examined the effects of insulin and glucagon on the lipolysis of rainbow trout (*Oncorhynchus mykiss*). To this end, adipocytes were isolated from mesenteric fat and incubated in the absence (basal lipolysis) or presence of different concentrations of insulin and glucagon. In addition, to further elucidate the effects of these hormones *in vivo* on adipocyte lipolysis, both fasting and intraperitoneal glucagon injection experiments were performed.

Basal lipolysis, measured as the glycerol released in the adipocyte medium, increased proportionally with cell concentration and incubation time. Cell viability was verified by measuring the release of lactate dehydrogenase (LDH) activity in the medium.

Insulin (at doses of 35 and 350 nM) decreased lipolysis in isolated adipocytes of rainbow trout *in vitro*, while glucagon was clearly lipolytic at concentrations of 10 and 100 nM.

Furthermore, hypoinsulinemia induced by fasting, as well as glucagon injection, significantly increased lipolysis in isolated adipocytes approximately 1.5- and 1.4-fold, respectively, when compared with adipocytes from control fish.

Our data demonstrate that lipolysis, as measured in isolated adipocytes of rainbow trout, can be regulated by both insulin and glucagon. These results not only indicate that insulin is an important hormone in lipid deposition via its anti-lipolytic effects on rainbow trout adipocytes, but also reveal glucagon as a lipolytic hormone, as shown by both *in vitro* and *in vivo* experiments.

1. Introduction

Adipose tissue is among the most important lipid storage organs found in numerous teleost species (Sheridan, 1988). In salmonids, adipose tissue is distributed abundantly in the abdominal cavity, surrounding the digestive tract and pyloric caeca (Sheridan and Harmon, 1994). In addition, adipocytes are packed in myosepta, especially in the belly flaps. The visceral fat and lipid content of various tissues is reduced during fasting (Navarro and Gutiérrez, 1995) or during parr-smolt transformation, indicating a mobilization of lipid storage during periods of high-energy demand and catabolism of body reserves. On the other hand, an excess of visceral fat tissue storage can result from high lipid levels in the diet (Company et al., 1999). In fish, the enzymatic machinery responsible for lipid mobilization and deposition in adipose tissue is similar to that found in mammals (Sheridan and Harmon, 1994). However, the endocrine factors directly involved in their regulation are not well understood. Among the hormones known to enhance lipid mobilization, glucagon can stimulate liver triacylglycerol lipase activity in coho salmon *(Oncorhynchus kisutch)* (Plisetskaya et al., 1989a). By contrast, insulin enhances lipid storage in fish tissues, as shown by various *in vivo* studies revealing increased lipogenesis rates in liver and muscle, although visceral fat has not been analyzed (reviewed by Navarro et al., 2005). In salmonids, insulin and glucagon regulate lipid metabolism *in vitro* in isolated hepatocytes (Mommsen and Plisetskaya, 1991; Harmon and Sheridan, 1992a). However, information regarding the actions of hormones on fish adipose tissue metabolism remains scarce, and *in vitro* studies have thus far been limited to examining hormonal effects in adipose tissue slices of relatively few fish species. Harmon and Sheridan (1992b), for instance, reported that insulin and glucagon modulated lipolysis via triacylglycerol lipase in the adipose tissue of rainbow trout (*Oncorhynchus mykiss*), while no effects were found from glucagon on adipose tissue lipolysis in wolf fish (*Hoplias malabaricus*) (Migliorini et al., 1992). Although the mammalian adipocyte has been well characterized at both the metabolic and hormonal control levels, a suitable *in vitro* model of fish adipocytes has not been fully developed. Few reports have analyzed the morphological characteristics, size, and distribution of adipocytes in fish (Zhou et al., 1996; Fauconneau et al., 1997). Catecholamines regulate lipolysis in isolated adipocytes from tilapia (*Oreochromis mossambicus*) (Vianen et al., 2002). More

recently, we have reported the effects of diet and several hormones in isolated adipocytes from gilthead sea bream (*Sparus aurata*), demonstrating the lipolytic effects of glucagon and growth hormone (Albalat et al., 2005).

In the present study, we assessed the role of insulin and glucagon on adipose tissue lipolytic activity, as measured in isolated mesenteric adipocytes from rainbow trout.

2. Materials and Methods

2.1. Animals and experimental conditions

Mesenteric adipose tissue for the obtention of isolated adipocytes was obtained from rainbow trout (*Oncorhynchus mykiss*), provided by the fish farm 'Truites del Segre' (Lleida, Spain) and maintained in our facilities at the University of Barcelona, Spain. The adipocytes were isolated from mesenteric fat tissue because is the tissue were these cells are more abundant and easy to isolate. Animals were fed *ad libitum* by hand once a day with a standard commercial diet and were acclimated for 10 days before any experiments were conducted. Fish were kept under natural light conditions, latitude (40º 5' N; 0º 10' E), and at a temperature of 15 ± 1ºC.

2.2. In vitro studies:

In the experiments for standardizing the conditions for the study of adipocyte lipolysis and in experiments using *in vitro* incubation of hormones, mesenteric adipose tissue was obtained from fish with an average weight of 260 g. The procedure of isolation and incubation of adipocytes is explained in section *2.4.* Unless otherwise indicated cells incubations were conducted in triplicate and results represent the average of triplicate values from three independent experiments. For each experiment a pool of adipose tissue from 4 animals on average were used.

For experiments to study *in vitro* hormone effects cells were incubated with either porcine insulin (3.5, 35 and 350 nM) or bovine glucagon (1, 10 and 100 nM), both obtained from Sigma (Madrid, Spain). The reason for use mammalian hormones is that fish glucagon and insulin are not available.

2.3. In vivo studies:

2.3.1. Effects of fasting: In an initial experiment, fish with an average weight of 360 g were either fed *ad libitum* with a standard commercial diet (n=13), or fasted for 21 days (n=13). Animals were maintained under the same conditions described above, and sampled after the experimental period. Control fish were fasted 24 h before sampling.

Adipocyte isolation experiments were performed in triplicate (on three consecutive days) and each day 4 or 5 fish from fasted group and 2 fish from control group (due to the high adipose tissue content) were used.

2.3.2. Glucagon administration: In a second experiment, fish were injected intraperitoneally (1 µl/g body weight) with either glucagon (75 ng/g body weight) (n=4) or saline solution (n=4), and sampled 3 h after injection. Bovine glucagon (Sigma, Madrid, Spain), dissolved in saline, was used in this study. Adipose tissue from each group of fish was pooled in order to obtain adipocyte preparations. All fish were fasted 24 h before the experiment.

In both experiments 2.3.1. and 2.3.2., blood samples were obtained from the caudal vein by means of heparinized syringes after the respective experimental period. Plasma was collected, and following blood centrifugation (700 g, 10 min), was split into three fractions, for glucose, FFA, and insulin analysis, respectively. All plasma aliquots were frozen until the analyses were conducted. Fish were killed with a blow to the head, weighed, lengthed and mesenteric adipose tissue was obtained to isolate adipocytes. In addition, whole livers were extracted and weighed.

Experiments were conducted in accordance with the Catalan government's 'Departament de Medi Ambient i Habitatge; Generalitat de Catalunya' regulations regarding the treatment of experimental animals (No. 2215).

2.4. Adipocyte isolation

Adipocytes were isolated as described by Vianen et al., (2002) with minor modifications. Fat tissue was cut into thin pieces and incubated for 60 min in Krebs-Hepes buffer pre-gassed with 5% CO_2 in O_2 (pH 7.4) containing collagenase type II

(130 U/ml) and 1% bovine albumin serum (BSA) in a shaking water bath at 15°C. The cell suspension was filtered through a double layer of nylon cloth, and then washed three times by flotation. Finally, the cells were carefully resuspended at the desired concentration in Krebs-Hepes buffer containing BSA 2% using a Fuchs-Rosenthal counting chamber. 400 µl aliquots of this final adipocyte suspension were incubated for 6 hours in polypropylene tubes in a shaking bath (15°C), in the absence or presence of different hormones

2.5 Analysis of lipolysis

At the end of the incubation period, the tubes were quickly placed on ice, and following a short centrifugation (1800 g; 2 min; 4ºC), cell-free aliquots were immediately placed in perchloric acid, yielding a final concentration of 2%. Neutralized supernatant was extracted for measurement of glycerol concentration (as an index of lipolysis), using a spectophotometric method with glycerokinase and glycerol phosphate dehydrogenase (Wieland, 1984; Tebar et al., 1996). All products were obtained from Sigma Aldrich (Madrid, Spain). For free fatty acid (FFA) analysis, cell-free aliquots of the medium were used to directly measure FFA with the WAKO test commercial kit.

2.6. Viability of adipocyte preparations

Cell viability was verified by spectrophotometrically analyzing the lactate dehydrogenase (LDH) activity released in the medium (Stralfors, 1990; Bottcher and Fürst, 1996). LDH activity was measured both at the beginning (0 hours) and end (6 hours) of the incubation time. Results are expressed as the percentage of LDH activity versus total LDH activity. To measure total LDH activity, the medium used during the cell incubation was removed and replaced by the same volume of lysis medium (incubation medium with 2% triton). In addition, to ensure complete lysis of adipocytes, cell suspensions were passed through a syringe, and following centrifugation (1800 g; 2 min; 4ºC), supernatant LDH was measured. Positive controls were obtained by incubating adipocytes for 6 hours (total incubation time) in the presence of triton 0.2%.

2.7. Biochemical analysis of plasma parameters

Plasma glucose concentration was determined using the glucose oxidase colorimetric method (GLUCOFIX; Menarini Diagnostics, Firenze, Italy) (Huggett and Nixon, 1957; Sala-Rabanal et al.., 2003), while plasma free fatty acids were analyzed using a commercial enzymatic method (NEFA-C, Wako Test). Plasma insulin levels were measured by radioimmunoassay using bonito insulin as a standard and rabbit anti-bonito insulin as an antiserum (Gutiérrez et al., 1984).

2.8. Statistical Analysis

Data are presented as the mean ± standard error of mean (S.E.M.). Results from the *in vitro* studies were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. *In vivo* experiments: fasting and glucagon administration experiments were analyzed using the paired t-test (t-Student). Differences were considered significant when $P<0.05$. All statistical analyses were performed using SPSS 11.5 for Windows.

3. Results

3.1. Standardizing protocol for the study of adipocyte lipolysis

Figure 1A shows a photomicrograph of isolated trout adipocytes at a magnification of 100×. To visualize the nuclei, adipocytes were stained with Hoechst (4 μg/ml) (Figure 1B). The average diameter of adipocytes was 44.64 ± 1.74 μm (diameters ranged from 7.12 to 112.96 μm), with adipocytes measuring from 10 to 20 μm proving to be the most abundant, as shown in Figure 1C.

The amount of glycerol released in the medium increased with cell concentration, from $3.3x10^5$ cells/ml to $13.2x10^5$ cells/ml (Figure 2A). A concentration of $6.6x10^5$ cells/ml was selected to ensure that subsequent studies would have suitable measurements.

Time course experiments running from 1 to 7 hours incubation exhibited a linear increase in lipolysis levels (Figure 2B). A 6-hour incubation period was chosen for posterior hormonal control experiments.

Glycerol and FFA levels, measured simultaneously in the same cell preparations, are presented in Figure 3. The ratio FFA: glycerol varied from 3.03 ± 0.06 (2 h incubation) to 2.48 ± 0.07 (6 h incubation).

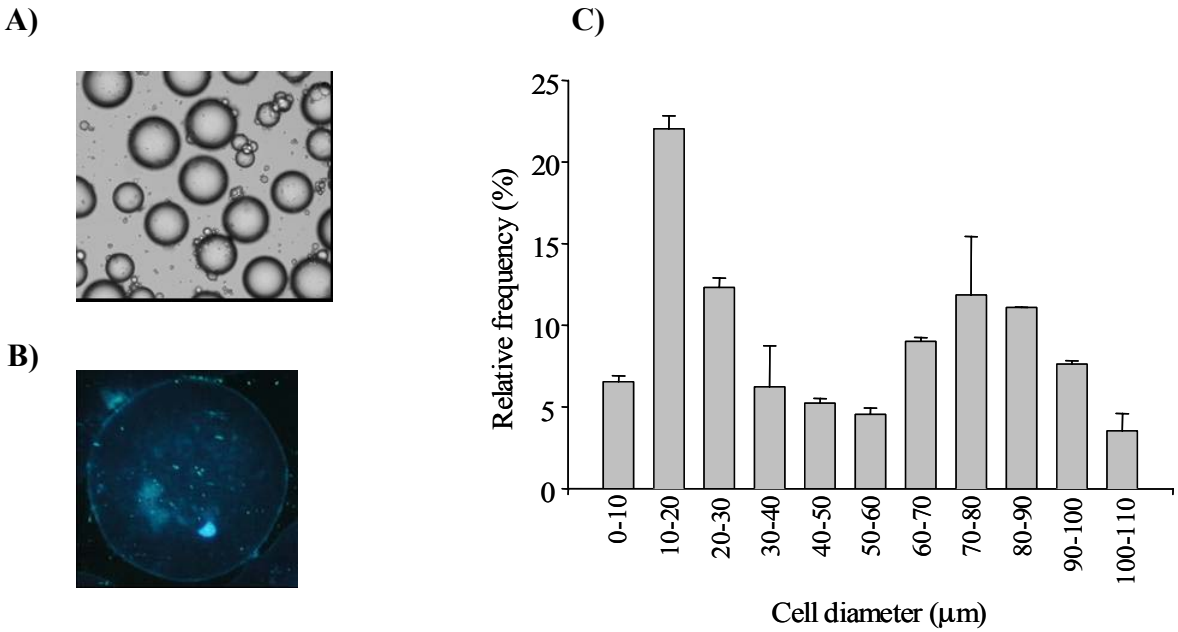

Figure 1. Photomicrographs of rainbow trout adipocytes: (A) suspension of unfixed and unstained adipocytes (×100); (B) a single adipocyte stained with Hoescht (4 μg/ml); and (C) distribution of diameters (μm) of rainbow trout adipocytes. Values are the mean ± S.E.M. of three individual distributions.

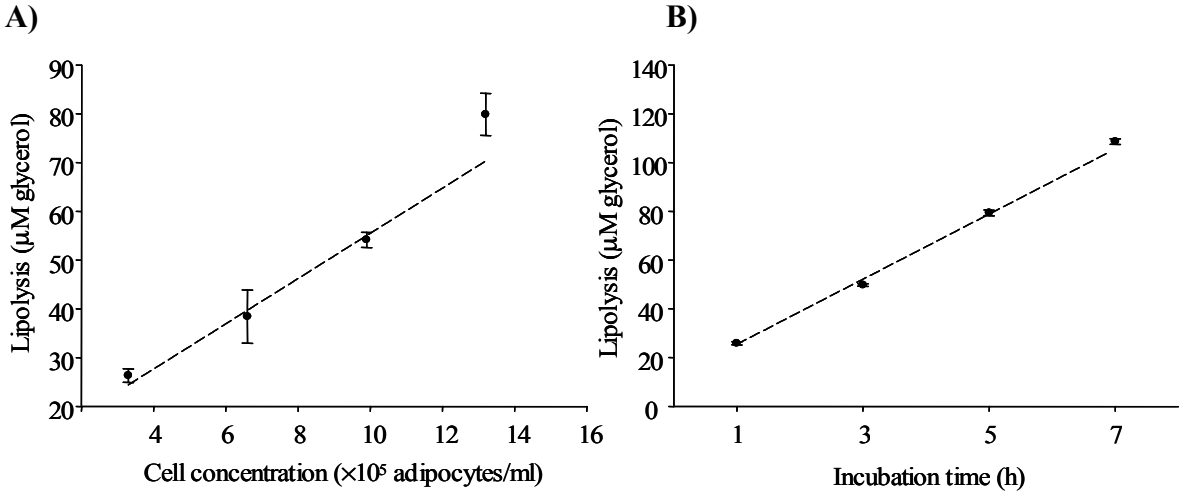

Figure 2. Effects of increasing adipocyte concentrations on basal lipolysis (A) and time course of basal lipolysis in rainbow trout adipocytes (B). Values are the mean ± S.E.M. of triplicates from a representative experiment. Results were fitted to a linear regression, (A) r^2 = 0.85 and (B) r^2 = 0.99.

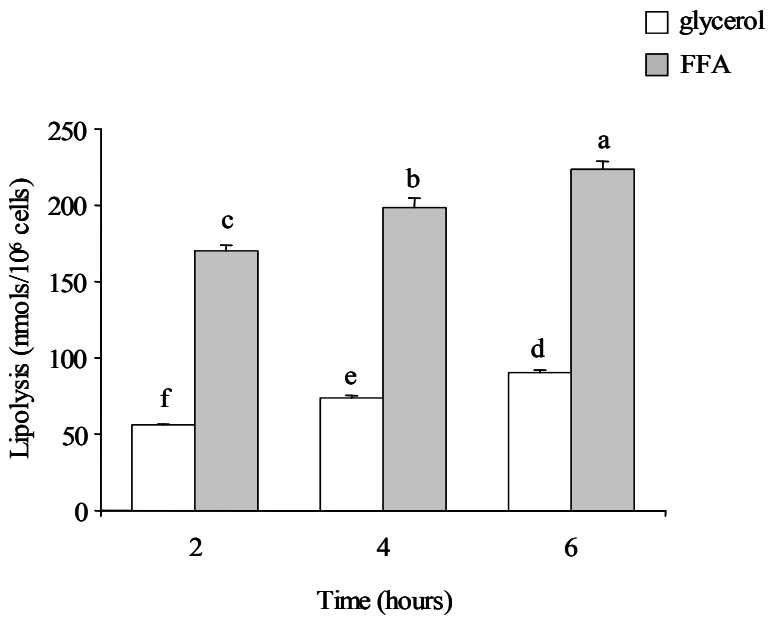

Figure 3. Time course of basal lipolysis as simultaneously measured by glycerol and FFA release in rainbow trout adipocytes. Values are the mean ± S.E.M. of tetraplicates from a representative experiment.

In Figure 4, LDH activity in the cell medium is expressed as the percentage of LDH activity versus total LDH activity (in the presence of 2% triton). No significant differences were found between the LDH released in the medium at 0 or 6 h of incubation, indicating that the cell suspensions retained a high viability during the incubation periods. Furthermore, adipocytes incubated for 6 h in the presence of triton 0.2% (positive control) exhibited significantly higher LDH values, thereby demonstrating the suitability of this method for measuring the viability of fish adipocytes.

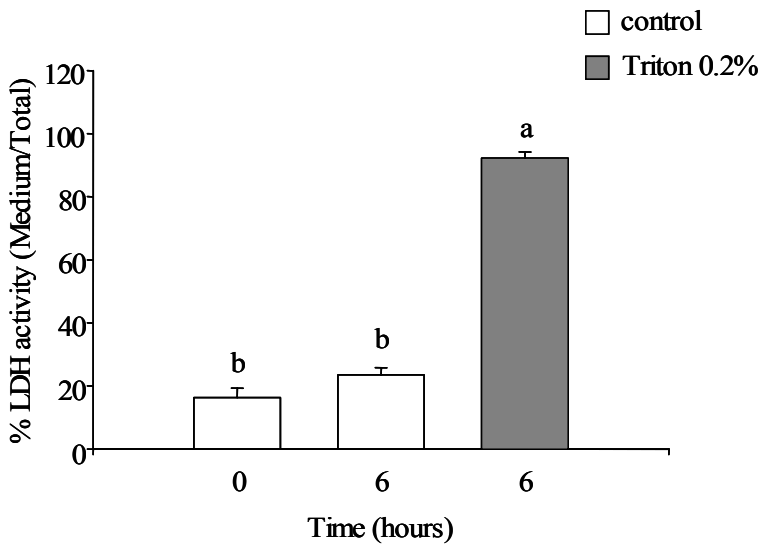

Figure 4. LDH activity in adipocyte medium expressed as % LDH in the medium versus total LDH (measured after lysis of adipocytes with triton 2%). Samples were obtained both at the beginning of the incubation (0 h) and following 6 hours of incubation (6 h). Positive controls were obtained by incubating adipocytes for 6 hours in the presence of 0.2% triton. Values are the mean ± S.E.M. of triplicates from 3 independent experiments. Values were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values not sharing a common letter are significantly different ($P<0.05$).

3.2. In vitro incubations with insulin and glucagon

The effects of insulin and glucagon incubations on adipocyte lipolysis are shown in Figure 5. Glucagon stimulated lipolysis at 10 and 100 nM (135.45 ± 6.19 and 143.75 ± 11.67% above controls, respectively). On the other hand, insulin had an antilipolytic effect at concentrations of 35 and 350 nM (81.00 ± 9.45 and 84.06 ± 2.4% above basal levels, respectively).

3.3. In vivo studies:

3.3.1. Effects of fasting:

Final weight and hepatosomatic index (HSI) were significantly lower in fasted fish than in control fish (Table 1). FFA plasma levels increased with fasting (approximately

2-fold), while glucose levels were maintained. Insulin plasma decreased to undetectable levels in fasted fish (Table 1).

Isolated adipocytes from fasted fish showed higher lipolysis levels compared with adipocytes from normally fed fish. Adipocytes isolated from fasted fish exhibited a lipolytic rate of 15.95 ± 0.80 nmols glycerol/h/10^6cells, while adipocytes from control fish exhibited a lipolytic rate of 11.09 ± 0.47 nmols glycerol/h/10^6cells (Figure 6).

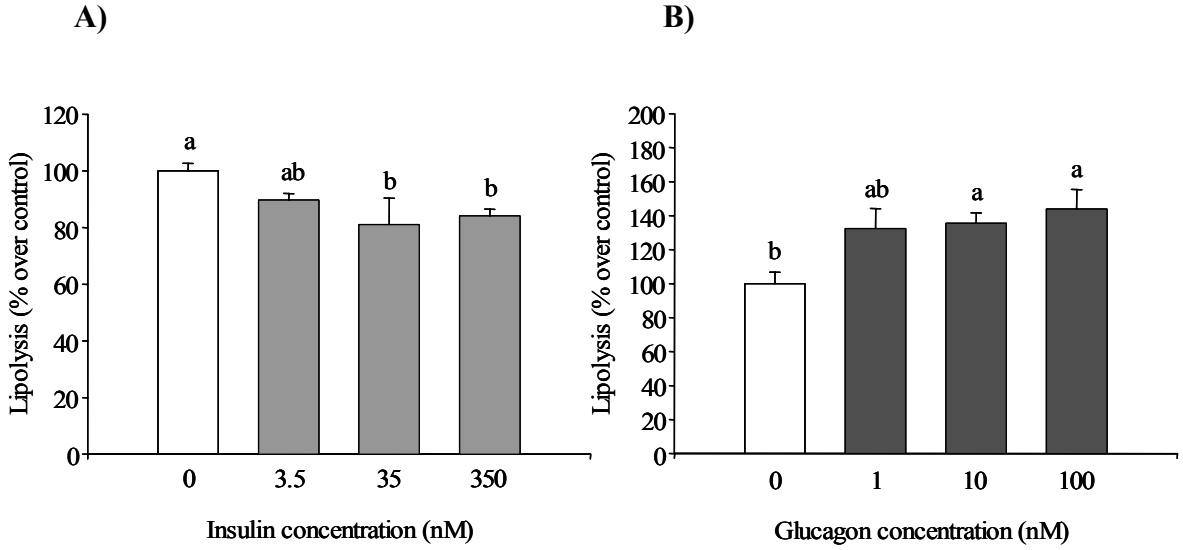

Figure 5. Effects of 6 hours of incubation with insulin (A) and glucagon (B) on adipocyte lipolysis in rainbow trout. Values are the mean ± S.E.M. of triplicates from 3 independent experiments. Values were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values not sharing a common letter are significantly different ($P<0.05$).

3.3.2. Effects of i.p glucagon administration:

Morphological parameters of rainbow trout used in the glucagon injection experiment are shown in the Table 2. Administration of a 75 ng/Kg i.p. dose of glucagon induced a significant increase in glucose plasma levels, from 145.04 ± 13.65 mg/dl in control fish to 208.14 ± 11.11 mg/dl in glucagon-injected fish. Plasma FFA levels increased in glucagon-injected fish approximatelly 2-fold compared with saline-treated controls. Furthermore, a significant decrease in circulating insulin levels was observed in glucagon-treated fish versus saline-treated controls, as shown in Table 2.

Adipocytes isolated from glucagon-treated fish exhibited higher levels of glycerol release (23.07 ± 0.10 nmols glycerol/h/10^6cells) compared with controls (16.13 ± 0.29 nmols glycerol/h/10^6cells), with an increase of 143.08% over controls (Figure 7).

Table 1. Morphological and plasmatic parameters of rainbow trout in the fasting experiment. Values are the mean (n=6 in fed and n=13 in fasted fish) ± S.E.M. Asterisk (*) indicates significant differences between fed and fasted groups (P<0.05). Values were analyzed using the paired t-test (t-Student).

	Fed	Fasted
Morphological parameters		
Final body mass (g)	448.98 ± 15.52	285.69 ± 8.31*
Length (cm)	31.08 ± 0.55	28.12 ± 0.38
Liver (g)	5.99 ± 0.34	2.40 ± 0.13*
Mesenteric fat (g)	7.48 ± 1.43	3.53 ± 0.40*
HSI (%)[1]	1.34 ± 0.07	0.84 ± 0.03*
MFI (%)[2]	1.71 ± 0.31	1.25 ± 0.14
CF (%)[3]	1.52 ± 0.11	1.29 ± 0.05*
Plasmatic parameters		
Glucose (mg/dl)	94.43 ± 3.24	86.78 ± 4.39
FFA (meq/l)	0.318 ± 0.02	0.621 ± 0.04*
Insulin (ng/ml)	18.34 ± 1.41	n.d.

[1] Hepatosomatic index = (liver wt./fish wt.) × 100
[2] Mesenteric fat index = (mesenteric fat wt./fish wt.) × 100
[3] Condition factor index = (fish wt./(fish length)^3) x 100

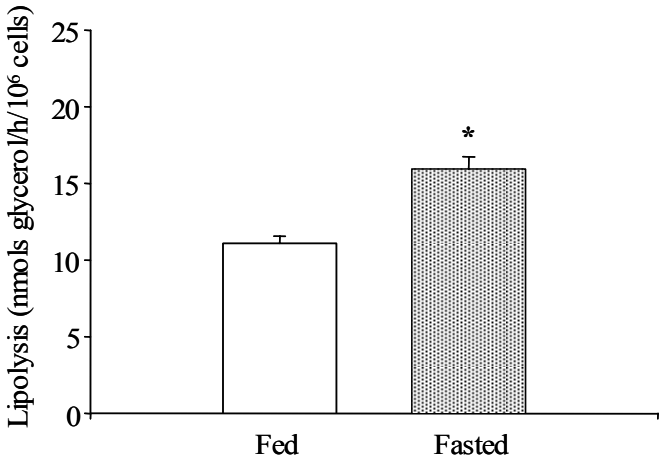

Figure 6. Effects of fasting on the basal lipolysis of rainbow trout adipocytes incubated for 6 hours. Values are the mean ± S.E.M. of tetraplicates from 3 independent experiments using fish from the *in vivo* experiment. Asterisk (*) indicates significant differences between fed and fasted groups (P<0.05). Values were analyzed using the paired t-test (t-Student).

Table 3. Morphological and plasmatic parameters of rainbow trout in the glucagon-injection experiment. Values are the mean (n=4 in control and n=4 in glucagon-injected fish) ± S.E.M. Asterisk (*) indicates significant differences between control and glucagon-injected groups (P<0.05). Values were analyzed using the paired t-test (t-Student).

	Control	Glucagon
Morphological parameters		
Final body mass (g)	295.30 ± 32.42	273.40 ± 23.25
Length (cm)	26.17 ± 0.67	25.83 ± 0.60
Liver (g)	3.58 ± 0.29	3.23 ± 0.70
Mesenteric fat (g)	7.75 ± 1.57	5.95 ± 0.44
HSI (%)[1]	1.26 ± 0.21	1.16 ± 0.16
MFI (%)[2]	2.59 ± 0.39	2.18 ± 0.03
CF (%)[3]	1.63 ± 0.06	1.58 ± 0.04
Plasmatic parameters		
Glucose (mg/dl)	145.04 ± 13.65	208.14 ± 11.11*
FFA (meq/l)	0.265 ± 0.03	0.678 ± 0.04*
Insulin (ng/ml)	10.33 ± 0.56	5.65 ± 0.62*

[1] Hepatosomatic index = (liver wt./fish wt.) × 100
[2] Mesenteric fat index = (mesenteric fat wt./fish wt.) × 100
[3] Condition factor index = (fish wt./(fish length)^3) x 100

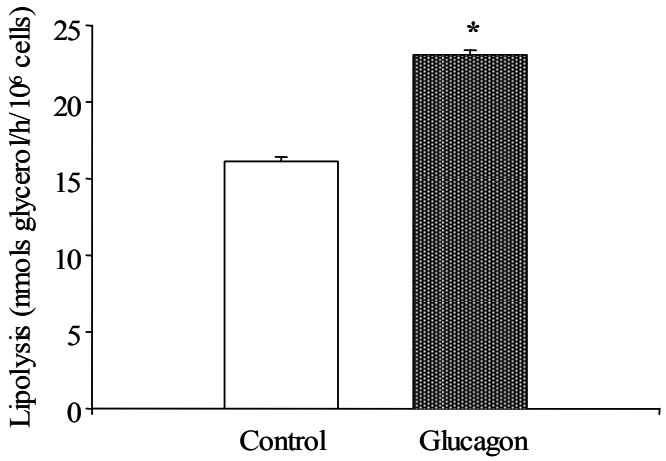

Figure 7. Effects of glucagon *in vivo* administration (3 hours after the injection) on basal lipolysis of rainbow trout adipocytes incubated for 6 hours. Values are the mean ± S.E.M. of heptaplicates. Asterisk (*) indicates significant differences between control and glucagon-injected groups (P<0.05). Values were analyzed using the paired t-test (t-Student).

4. Discussion

In the present study we have isolated mesenteric adipocytes from rainbow trout and studied the effects of insulin and glucagon on lipolysis measured in this *in vitro* system. Variability in the size of adipocyte cells found in trout appears to be a general characteristic of fish. Populations of small adipocytes were previously found in both rainbow trout (*Oncorhynchus mykiss*) (Fauconneau et al., 1997) and in Atlantic salmon (*Salmo salar*) (Zhou et al., 1996). This trait differs from the typically uniform adipocyte size found in rat and other mammalian isolated adipocytes (Robdell, 1964). In mammals, it has been described that changes in adipose cell size could contribute to explain differences in lipid mobilization (Leibel et al., 1989; Coppack et al., 1994; Tsujita et al., 1995). However, in fish there are no studies concerning the relation between adipose cell size and lipolysis. Therefore, the reason for such high levels of small cells in fish adipose tissue remains unknown.

The rate of lipolysis in trout adipocytes increased with cell concentration and incubation time, as has been reported in mammalian isolated adipocytes (Tebar et al., 1993). These correlations with time and cell concentration are similar to those found for sea bream adipocytes (Albalat et al., 2005), although levels of glycerol release in this particular sparid were, in fact, lower than that those found in trout, despite the higher incubation temperature used for seabream adipocytes (22ºC).

An approximate release ratio for glycerol:FFA of 3 was observed in adipocyte incubations at time 2 hours, which is the expected ratio when a complete degradation of triacylglycerols occurs, with both non-esterified fatty acids and glycerol as the two end products of lipolysis (Steinberg, 1963). However, this ratio decreased as incubation time increased (up to 6 hours), suggesting that a certain level of FFA-re-esterification is produced during the incubation process (van Harmelen et al., 1999). Such re-esterification might result from the fact that not all of the fatty acids were retained by the albumin from the adipocyte incubation buffer, and that glycerophosphates were formed from glucose. This finding suggests that, under our conditions, the measurement of glycerol released in medium would offer a better estimation of the lipolytic rate than FFA release.

The relatively low levels of LDH released in the incubation medium are regarded as a good indication of cell viability in isolated adipocytes and other cell models

(Kinutani et al., 1985; Ueda et al., 2004). In addition, LDH release in adipocytes incubated in the presence of triton for 6 hours (positive control), exhibited significantly greater LDH compared with control adipocytes, confirming that incubation time did not significantly alter cell viability. Furthermore, similar incubation times (between 5-6 hours) have also been used in adipocyte preparations of both sea bream (Albalat et al., 2005) and tilapia (Vianen et al., 2002).

Insulin decreased basal lipolysis at 35 and 350 nM in trout adipocytes. This effect is consistent with the antilipolytic effects observed by Harmon and Sheridan (1992b) in slices of adipose tissue of rainbow trout, although higher doses of mammalian insulin were employed in that study. Nevertheless, the dose used in trout adipocyte incubations exceed that of circulating levels of insulin usually found in trout (Navarro et al., 2002), thus extrapolation of results obtained *in vitro* to a physiological situation has to be made always with caution. Moreover, it is possible that the adipocytes would be more sensitive to the homologous hormone. Insulin action in trout adipocytes is consonant with the reported presence of specific insulin receptors in trout adipose tissue (Planas et al., 2000). This observation contrasts with the nearly null action of insulin on basal lipolysis in sea bream adipocytes under similar conditions (Albalat et al., 2005). One possible explanation is that the tissue is exhibiting a species-specific response to insulin. In support of this hypothesis it has to be added that also insulin plasma levels in both species have reported to be different, with lower values in sea bream compared to trout; furthermore, a lower stimulation of insulin secretion by amino acids has been found in sea bream (Navarro et al., 2002). Besides, the possibility of seasonal differences in insulin effects cannot be discarded, since considerable variations in insulin receptor numbers have been observed over the course of a year (Planas et al., 2000). In addition, insulin metabolic regulation clearly varies in trout, depending on the season (Navarro et al., 1992). Other studies that indicate anti-lipolytic and anabolic effects of insulin on lipid metabolism in fish are mostly indirect. When insulin insufficiency was induced in coho salmon, it resulted in markedly enhanced liver lipolytic activity (Plisetskaya et al., 1989b), while insulin injection induced the incorporation of C^{14} glucose into the liver and muscle of northern pike (*Esox lucius*) (Ince and Thorpe, 1976). Interestingly, obesity and mesenteric fat accumulation are the principal characteristics of the so-called "cobalt" variant of rainbow trout. The lack of pars intermedia, as well as the increased plasma levels of insulin in these fish, has been postulated to depress lipid mobilization and obesity (Yada et al., 2002). Unfortunately, and contrary to mammals, no models of

fish obesity exist that can help us understand the hormonal regulation of fat storage and catabolism.

While glucagon has been shown to elicit a lipolytic response by elevating plasma FFA in various fish species, in others glucagon failed to affect lipid metabolism (reviewed by Plisetskaya and Mommsen, 1996). The direct action of glucagon on fish adipose tissue has only been demonstrated in salmon adipose tissue slices (Harmon and Sheridan, 1992b), and more recently in seabream adipocytes (Albalat et al., 2005). The present study demonstrates a lipolytic action in mesenteric isolated adipocytes in trout. Nevertheless, only the lowest dose used in the present study could be considered physiological (Navarro et al, 2002) although it can not be discarded a higher response using teleost hormone.

Interestingly, adipocyte cells from glucagon-treated fish also exhibited higher lipolysis levels than those from control fish, indicating glucagon's lipolytic effects in adipose tissue *in vivo* are maintained in isolated adipocytes as well. A significant increase in plasma glucose levels was observed in glucagon-injected fish. This increase coincides with the well-known hyperglycemic effect of glucagon in fish, suggesting that glucagon treatment was effective in the present experiment. Similar hyperglycemic effect was found by Magnoni et al. (2001), whereby bovine glucagon was tested in trout at the same dose, which can be considered physiological (Plisetskaya et al., 1989a), accompanied by several effects in brain metabolism. In the present study, the *in vivo* action of glucagon may rely partially on direct effect, and partially on effects mediated by insulin, since plasma insulin levels were significantly lower in glucagon-injected fish. Consistent with these findings, glucagon at doses of 1-100 ng/g decreased circulating insulin levels in coho and chinook salmon (*Oncorhynchus kisutch* and *O. tshawytscha*) (Plisetskaya et al., 1989a) in the majority of experiments. Nevertheless, glucagon exerted no effects in other fish species, such as eel (*Anguilla anguilla*) (Ince and Thorpe, 1977) and results from other studies in fish suggest that insulin and glucagon secretion might act relatively independent of each other (Plisetskaya et al., 1991; Novoa et al., 2004). What remains clear, however, is that the typical mammalian interrelation of these two pancreatic hormones does not exist in piscine systems. Moreover, the insulinotropic action of glucagon and glucagon-like peptide, characteristic of mammals, is not found in fish, when tested *in vitro* in isolated pancreatic islets (Mommsen and Plisetskaya, 1993).

Fasting induced a decrease in insulin levels as expected, as well as in weight and HSI. Increased plasma FFA is an indication of tissue lipolysis. During fasting in trout, mesenteric fat is mobilized first, then lipids from liver or muscle fat depots (Jezierska et al., 1982). In the present study, visceral adipose tissue contributed to lipid mobilization since a clear increase in the lipolytic rate occurred in adipocytes from fasted fish compared with control fish. Plasma insulin levels in fasted trout may fall below 0.5-2.5 ng/ml, according to the sensitivity of the radioimmunoassay. These extremely low insulin levels may contribute to lipid mobilization from visceral adipose tissue.

In summary, the data reported in this study validate the use of isolated rainbow trout adipocytes as a tool for studying the nutritional and hormonal regulation of lipolysis.

The results of our *in vitro* experiments indicate that insulin is an anti-lipolytic hormone in adipose cells, and that glucagon increases lipolysis levels in rainbow trout adipocytes. Furthermore, adipocytes from fasted and glucagon-injected rainbow trout exhibited higher lipolysis levels when compared with control fish adipocytes, which indicates that metabolism of isolated adipose cells depends on hormonal changes *in vivo*.

This *in vitro* system opens new possibilities for further studies on lipid metabolism in rainbow trout, as well as in other fish species.

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Control nutricional i hormonal de la lipòlisi en adipòcits aïllats d'orada (*Sparus aurata*)

Nutritional and hormonal control of lipolysis in isolated gilthead seabream (*Sparus aurata*) adipocytes

Control nutricional i hormonal de la lipòlisi en adipòcits aïllats d'orada (*Sparus aurata*)

Resum

En aquest treball, hem examinat els efectes de la composició de la dieta i el dejuni sobre la lipòlisi d'adipòcits aïllats d'orada (*Sparus aurata*). Tanmateix, hem analitzat els efectes de la insulina, el glucagó i l'hormona de creixement en la lipòlisi d'adipòcits aïllats de peixos prèviament alimentats amb dietes de diferent composició.

La lipòlisi basal, mesurada com l'alliberació de glicerol en el medi d'incubació, va augmentar proporcionalment amb el nombre de cèl.lules i amb el temps d'incubació, validant la capacitat d'aquest model *in vitro* per a l'estudi de la regulació hormonal. Les orades van ser alimentades amb dues dietes diferents, la dieta FM (amb proteïna principalment provinent de farina de peix) i la dieta PP (amb 100% proteïna d'origen vegetal) durant 6 setmanes. Passat aquest període, cada grup va ser dividit en dos grups més: alimentat i dejunat (durant 11 dies). La lipòlisi basal va ser més alta en adipòcits provinents de peixos alimentats amb la dieta PP respecte la dieta FM. Els adipòcits d'animals dejunats presentaren uns nivells de lipòlisi més elevats (casi 3 vegades) que la dels adipòcits d'animals alimentats independentment de la dieta prèviament subministrada.

Els efectes hormonals van ser similars en els diferents grups: el glucagó va augmentar la lipòlisi mentre que la insulina no va tenir cap efecte. L'hormona de creixement va ser clarament lipolítica tot i que l'increment relatiu de glicerol respecte el control va ser menor en adipòcits aïllats d'animals dejunats respecte als animal alimentats.

Els resultats demostren per primera vegada que la lipòlisi mesurada en adipòcits aïllats d'orada, està afectada segons l'estat nutricional del peix. A més, els resultats suggereixen que el glucagó i especialment l'hormona de creixement juguen un paper important en el control de la lipòlisi dels adipòcits.

Nutritional and hormonal control of lipolysis in isolated gilthead seabream (*Sparus aurata*) adipocytes

A. Albalat[1], P. Gómez-Requeni[2], P. Rojas[1], F. Médale[3], S. Kaushik[3], G.J. Vianen[4], G. Van den Thillart[4], J. Gutiérrez[1], J. Pérez-Sánchez[2] and I. Navarro[1]

[1]Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

[2]Instituto Acuicultura Torre de la Sal (CSIC), Castellón, Spain

[3]Laboratoire de Nutrition des Poissons, Unitée Mixte INRA-IFREMER, 64310 Saint-Pée-sur-Nivelle, France

[4]Department of Integrative Zoology, Institute Biology, Leiden University, The Netherlands

Running title: Control of lipolysis in isolated gilthead seabream adipocytes

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Please address all correspondence to I. Navarro

[1]Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Fax: 34+ 93+ 4110358

Email: mnavarro@ub.edu

Abstract

We examined the effects of diet composition and fasting on lipolysis of freshly isolated adipocytes from gilthead seabream (*Sparus aurata*). We also analyzed the effects of insulin, glucagon and growth hormone (GH) in adipocytes isolated from fish fed with different diets.

Basal lipolysis, measured as glycerol release, increased proportionally with cell concentration and time of incubation which validates the suitability of these cell preparations for the study of hormonal regulation of this metabolic process. Gilthead seabream were fed two different diets, FM (100% of fish meal) and PP (100% of plant protein supplied by plant sources) for 6 weeks. After this period, each diet group was divided into two groups: fed and fasted (for 11 days). Lipolysis was significantly higher in adipocytes from PP fed fish than in adipocytes from FM fed fish. Fasting provoked a significant increase in the lipolytic rate of about 3-fold in isolated adipocytes regardless of nutritional history.

Hormone effects were similar in the different groups: glucagon increased the lipolytic rate while insulin had almost no effect. GH was clearly lipolytic, although the relative increase in glycerol over control was lower in isolated adipocytes from fasted fish compared to fed fish. Taking together all this data, we demonstrate for the first time that lipolysis, measured in isolated sea bream adipocytes, is affected by the nutritional state of the fish. Furthermore, our data suggest that glucagon and especially GH play a major role in the control of adipocyte lipolysis.

1. Introduction

Adipose tissue plays a central role in energy homeostasis in storing lipids in the form of triacylglycerols, and in mobilizing them via breakdown into free fatty acids (FFAs) and glycerol (34). Adipose tissue is one of the most important lipid stores in several teleosts, although in some species liver or muscle also constitute lipid storage organs (33). In salmonids, adipose tissue is distributed primarily in the abdominal cavity, associated with the mesenteric and pyloric caeca (31). In gilthead seabream, adipose tissue is also located periviscerally. It is known that fish adiposity changes seasonally and is affected by trophic status. High fat feeds can lead to increases in visceral fat (4) resulting in reduced product yield and quality of cultured fish (7). In gilthead seabream, replacing fish meal with plant protein seems to alter lipid metabolism and results in smaller fat depots (10).

Endocrine control of adipose tissue mobilization and storage remains almost unexplored in fish, although insulin and glucagon are clearly involved (12, 24, 28). Key hepatic enzymes in lipid metabolism such as hepatic lipase and acetyl-co A carboxylase are also regulated by pancreatic hormones *in vitro* in isolated hepatocytes, or *in vivo* in injected fish (23, 13). Several *in vivo* studies suggest that growth hormone and somatolactin act together, in a complementary way, to regulate fat stores in gilthead seabream (20, 4). However, the knowledge on the direct actions of hormones on fish adipose tissue is limited, and the results are contradictory. Harmon and Sheridan (12) reported that insulin and glucagon are able to regulate the level of lipolysis through triacylglycerol lipase in adipose tissue pieces of rainbow trout. Migliorini et al. (19) found that neither catecholamines nor glucagon affected the levels of lipolysis in slices of adipose tissue in the wolf fish, *Hoplias malabaricus*. More recently, it was reported that noradrenaline and isoproterenol decreased lipolysis in adipocytes from tilapia (41) and rainbow trout (1). However, the role of possible lipolytic/antilipolytic hormones in the endocrine control of adiposity in teleosts remains to be examined in detail.

The objective of this study was to assess how nutritional status and diet composition affect the level of lipolysis in isolated adipocytes of gilthead seabream (*Sparus aurata*). Secondly, the effects of insulin, glucagon and growth hormone on lipolysis were analyzed in these cells. This work is part of a more extensive study in

which the effects of dietary plant protein supply in growth performance, nitrogen-metabolism and GH liver axis activity have been monitored.

2. Material and Methods

2.1. Animals and experimental conditions

Isoproteic and isolipidic diets were formulated with fish meal (diet FM) and different plant protein (PP) sources (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) to replace 100% of fish meal protein (diet PP). Indispensable amino acids (IAA) were added to plant protein-based diets, meeting the theoretical IAA requirement (Table 1).

Table 1. Ingredient and chemical composition (g/kg) of the two diets (FM and PP)

Ingrédients (g/kg)	FM	PP
Fish Meal, (low-temperature)	704	
Corn gluten meal	-	250
Wheat gluten	-	250
Extruded Peas (Aquatex)	-	120
Rapeseed meal (Primor 00)	-	27
Lupin, sweet white	-	7
Extruded whole wheat	142	16
Fish oil	124	158
Binder (Na Alginate)	10	10
Mineral premix [a]	10	10
Vitamin premix [a]	10	10
CaHPO4.2H20 (18%P)	-	51
Amino acid mix [b]	-	91
Chemical composition		
Dry matter (DM), g/kg	940	901
Crude protein (CP), g/kg DM	461	428
Crude fat, g/kg DM	165	168
Gross energy, MJ/kg DM	204	217
Sum of essential amino acids (EAA)	246	248
Sum of dispensable amino acids (DAA)	232	232
EAA/DAA ratio	1.1	1.1

[a] According to National Research Council. *Nutrient requirements of Fish*. Washingtong DC: National Academy Press, 1993.

[b] Amino acid mix contained (L –Arg HCl, 15.9; L-His, 4.8; L-Lys, 32.5; DL-Met, 5.4; L-Trp, 2.9; L-Thr, 10.4; L-Ile, 8.4 and L-Val, 11)

Experiments were performed to standardize conditions for adipocyte incubations in adult seabream. Fish were acclimated to laboratory conditions at the Instituto de Acuicultura de Torre de la Sal, Castellón, Spain. Fish were fed daily with a commercial diet. Experiments were performed in June under natural conditions of light (16h light: 8h dark) and temperature (22ºC), latitude (40º 5' N; 0º 10' E). Water flow was 20 L/min, and oxygen content of outlet water remained above 85% air saturation.

To study the effects of dietary protein sources, fish of 96-98 g initial body mass were distributed in four 500-L tanks with groups of 40 fish per tank. Water temperature ranged from 23 to 25ºC over the course of a 6-week trial (August–September 2002). Fish from two tanks were fed the FM diet and fish from the other two tanks were fed the diet. Each diet was offered to apparent visual satiety in one meal per day (10:00 h), and feed consumption was recorded daily. After this period, each dietary group was divided into two subgroups: one group was continued to be fed to apparent visual satiety (FM and PP fed groups) and the other group was fasted for 11 days (FM and PP fasted groups).

Adipocyte isolation experiments were performed in triplicate (on three consecutive days) and each day 5 or 6 fish from each experimental group were used. Fish were killed with a sharp blow on the head and blood samples were taken by caudal puncture using heparinized syringes. Control fish were sampled 24h after the last meal. The fish were weighed immediately, and the adipose tissue was dissected. The adipose tissue was weighed and 10 g of adipose tissue from 5-6 animals was pooled and used for each experiment and condition. Blood samples were centrifuged and different aliquots of plasma were kept at –20ºC until the day of analysis.

2.2. Adipocyte isolation

Adipocytes were isolated as described by Vianen et al. (41), with some minor modifications. Fat tissue was cut into thin pieces and incubated for 60 min in polypropylene tubes with Krebs-HEPES buffer pre-aerated with 5% CO_2 in O_2 (pH 7.4) containing collagenase type II (130 U/ml) and 1% bovine albumin serum (BSA), in a shaking water bath at 18°C. The cell suspension was filtered through a double layer of nylon cloth and then washed three times. Finally, cells were carefully resuspended at the

desired concentration in Krebs-HEPES buffer containing 2% BSA using a Fuchs-Rosenthal counting chamber. Aliquots of 400 µl of this final adipocyte suspension were incubated for 6 hours in polypropylene tubes (4 tubes for each basal condition and 3 tubes for each concentration of hormone tested) in a shaking bath (22ºC) in the absence or presence of different hormones. At the end of the incubation, tubes were rapidly placed on ice and after a short centrifugation (1800 g; 2 min; 4°C) cell-free aliquots of the medium were immediately placed into perchloric acid to give a final concentration of 2%. Neutralized supernatants were taken for the measurement of glycerol concentration (as an index of lipolysis) using an enzymatic method with glycerokinase and glycerol phosphate dehydrogenase as described by Tebar et al. (36). Results are the average of tetraplicates for basal conditions and triplicates for each concentration of hormone tested in three independent experiments.

Suspensions were stained with Trypan blue and examined under light microscope at different times to check the integrity and viability of the cells (fig 1A). To visualize the nuclei, adipocytes were stained with Hoechst (4µg/ml) and with May-Grünwald/Giemsa.

2.3. Biochemical analyses of plasma parameters

Plasma glucose concentration was determined by the glucose oxidase colorimetric method (GLUCOFIX; Menarini Diagnostics, Firenze, Italy) (15; 29) and plasma free fatty acids were analyzed using a commercial enzymatic method (NEFA-C, Wako Test). Plasma insulin levels were measured by radioimmunoassay using bonito insulin as standard and a rabbit anti-bonito insulin as antiserum (11).

2.4. Materials

Hormones used were: porcine insulin (10 and 100 nM) from Sigma (Madrid, Spain), porcine glucagon (10 and 100 nM) from Sigma and recombinant gilthead seabream growth hormone (0.1-10 nM) was produced as described elsewhere (18).

2.5. Statistical analysis

All data are presented as means ± standard error of mean (S.E.M.). Results were

analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test when variances were homogeneous, and otherwise by the Games-Howell test (following Levene's test for the study of the homogeneity of variance). The effects of the hormones were analyzed by paired t-test. .

3. Results

3.1. Standardizing protocol for the study of adipocyte lipolysis

Figure 1A shows a photomicrograph of isolated gilthead seabream adipocytes with a magnification of 100×. Figure 1B and 1C show single adipocytes with the double nuclei characteristic of fish adipocytes (31) stained with Hoechst and May-Grünwald/Giemsa. Glycerol released to the medium increased proportionally with cell concentration, between $3x10^5$ cells/ml and $12x10^5$ cells/ml (Figure 2A). A concentration of $6x10^5$ cells/ml was selected to ensure that the subsequent studies would have suitable measurements.

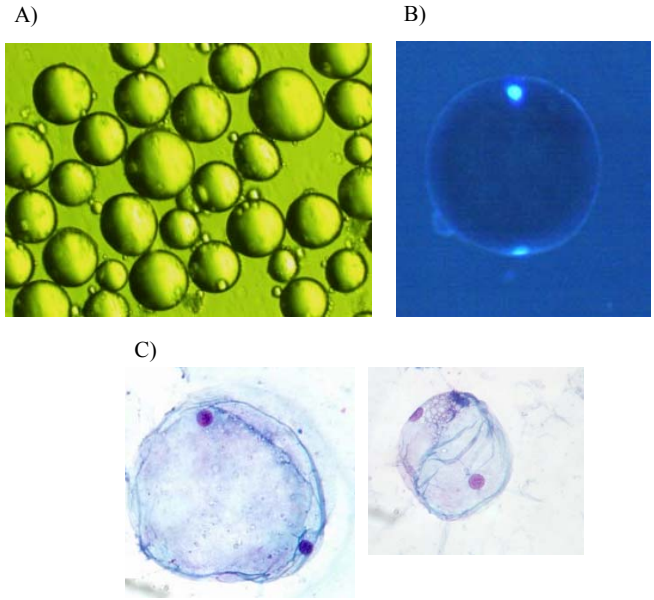

Figure 1. Photomicrographs of seabream adipocytes, suspension of unfixed and unstained adipocytes (×100) (A); a detail of a single fat cell stained with Hoechst (B) and two adipocytes stained with May-Grünwald/Giemsa (C).

Time course experiments from 1 to 7 hours of incubation showed a linear increase in the level of lipolysis (Figure 2B). A period of incubation of 6 hours was selected since previous results showed that this time is optimal for assessing either the inhibitory or stimulatory action of hormones (data not shown).

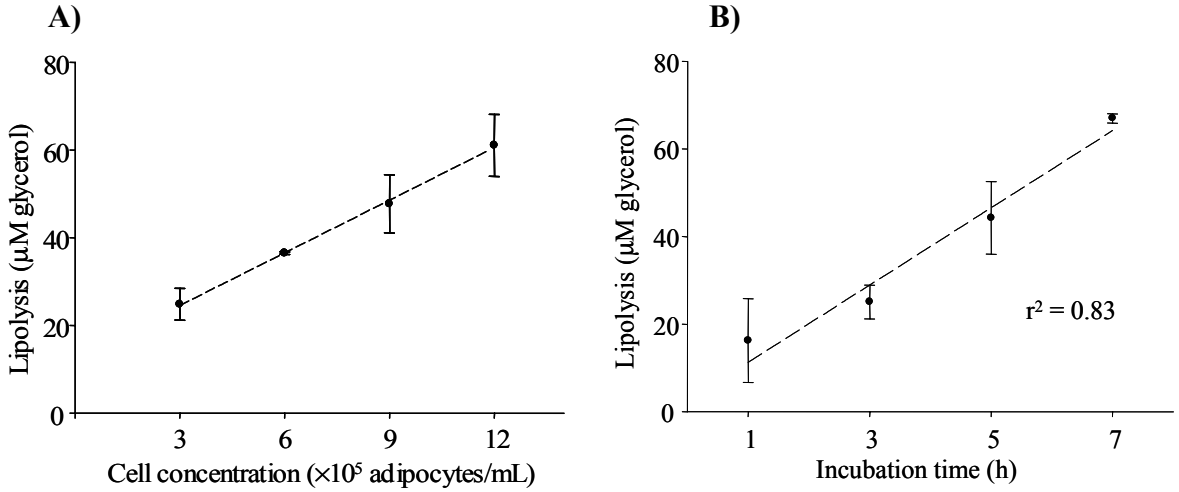

Figure 2. Effect of increasing concentrations of adipocytes on basal lipolysis (A) and time course of basal lipolysis of gilthead seabream adipocytes up to 7 hours of incubation time (B), values are the mean (n=3) ± S.E.M. of a representative experiment. Results were fitted to linear regression, (A) r^2= 0.99 and (B) r^2= 0.83.

3.2. Effects of diet and fasting on morphological parameters, plasma metabolites and insulin levels, and isolated adipocyte lipolysis

Fish fed the PP diet had significantly lower liver mass than fish fed FM diet. No significant differences were found in body mass, adipose tissue content, and hepatosomatic index between fish fed PP and those fed diet FM (Table 2). Fasting for 11 days induced a decrease in body mass and a clear decrease in liver weight of about 50%, provoking a marked fall in the hepatosomatic index in both groups (Table 2).

Plasma insulin levels in fed seabream were similar between dietary groups and decreased with fasting only in the fasted PP group (Figure 3A). Glycemia levels were similar in all conditions, ranging from 5.03 to 6.53 mmol/l. Plasma FFA levels increased significantly with fasting in both groups (Figure 3B).

Table 2. Morphological parameters of gilthead seabream used in the experiments. Values are average (n=17 in fed and n=17 in fasted FM and n =15 in fed and n=15 in fasted PP) ± S.E.M. Results were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. Data for HSI and MFI were arc-sine transformed before statistical analysis was done.Values not sharing a common letter are significantly different (P<0.05).

	FM fed	FM fasted	PP fed	PP fasted
Final body mass (g)	227.12 ± 5.02^{a}	203.65 ± 8.24^{a}	208.93 ± 7.15^{a}	175.18 ± 6.52^{b}
Length (cm)	19.22 ± 0.13	18.62 ± 0.17	18.86 ± 0.17	18.19 ± 0.23
Liver (g)	3.75 ± 0.22^{a}	1.62 ± 0.09^{c}	3.07 ± 0.18^{b}	1.34 ± 0.07^{c}
Mesenteric fat (g)	2.65 ± 0.23^{a}	2.14 ± 0.21^{ab}	2.29 ± 0.23^{ab}	1.69 ± 0.18^{b}
HSI (%)[1]	1.64 ± 0.08^{a}	0.82 ± 0.03^{b}	1.47 ± 0.07^{a}	0.76 ± 0.03^{b}
MFI (%)[2]	1.18 ± 0.12	1.08 ± 0.09	1.11 ± 0.11	0.94 ± 0.09

[1] Hepatosomatic index = (liver wt./fish wt.) × 100

[2] Mesenteric fat index = (mesenteric fat wt./fish wt.) × 100

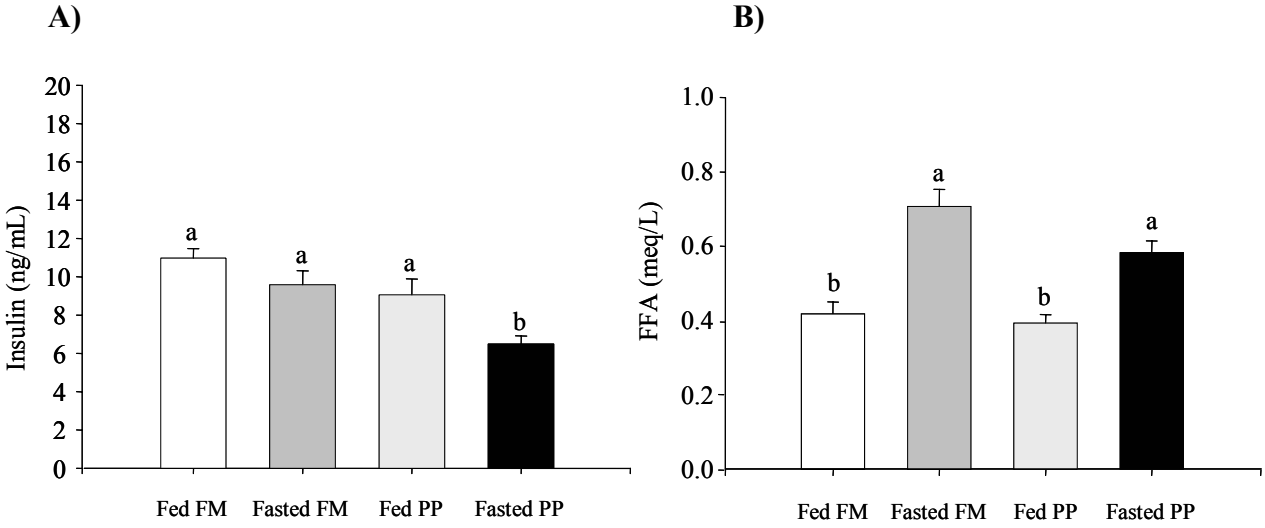

Figure 3. Plasma levels of insulin (A) and free fatty acids (B) in gilthead seabream fed (after overnight fasting) and fasted (for 11 days) from FM and PP groups. Each value is the mean (n=17 in fed and n=17 in fasted FM and n =15 in fed and n=15 in fasted) ± S.E.M. Statistical analysis was done as in Table 2. Values not sharing a common letter are significantly different (P<0.05).

The lipolytic rate, measured as glycerol released to the medium, was significantly higher in adipocytes from fish fed the diet PP in fed state (Figure 4). Fasting provoked an almost 3-fold increase in the lipolytic rate in isolated adipocytes: from 5.71 to 16.25 nmols glycerol/h/10^6 cells in FM group and from 8.58 to 21.47 nmols glycerol/h/10^6 cells in PP group (Figure 4).

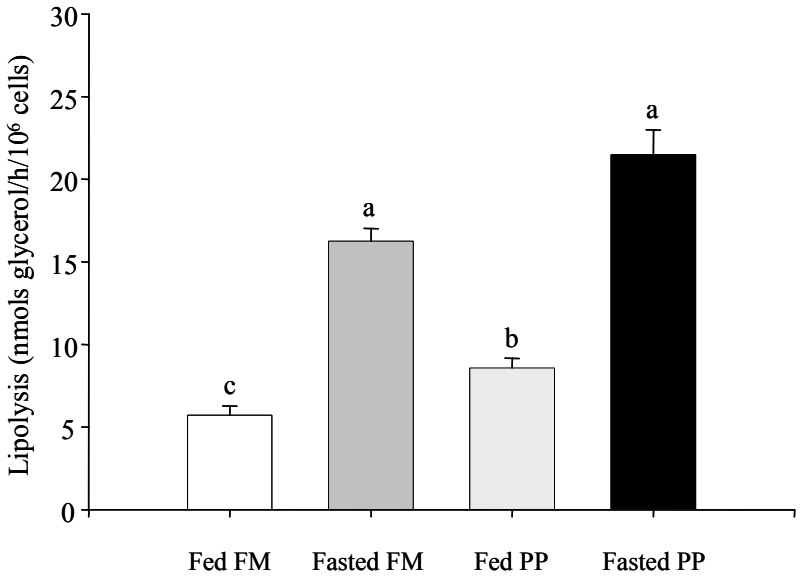

Figure 4. Basal lipolysis after 6 h of incubation of adipocytes from fed and fasted gilthead seabream FM and groups. Values are the average of tretraplicates from 3 independent experiments ± S.E.M. Results were analyzed by one-way analysis of variance (ANOVA), followed by the Games-Howell test. Values not sharing a common letter are significantly different (P<0.05).

3.3. Effects of incubations with hormones in isolated adipocytes

The effects of hormones on lipolysis of adipocytes from the FM fed group are shown in Figure 5A and after the fasting period in Figure 5B. In the FM fed group, insulin did not induce any significant change in lipolysis. Glucagon led to a significant increase in lipolysis in fed fish at the higher dose (100 nM). Growth hormone was clearly lipolytic (increases of between 192-249% over control) in FM fed animals, although no clear dose response was observed. The relative response to GH was lower in cells from fasted fish, in which increases varied from 135 to 176% over control.

The effects of hormones on the lipolysis of adipocytes from PP fed and fasted fish are shown in Figures 6A and 6B respectively. Insulin (10 nM) decreased lipolysis in PP fed fish while this hormone had no effect in fasted fish. The lipolytic effect of glucagon was significant after incubations with 10 nM glucagon in both fed and fasted fish. GH stimulated lipolysis in the fed PP group at two of the three concentrations tested, while in the fasted PP group only a significant higher lipolysis was observed after incubation with a hormone concentration of 1 nM.

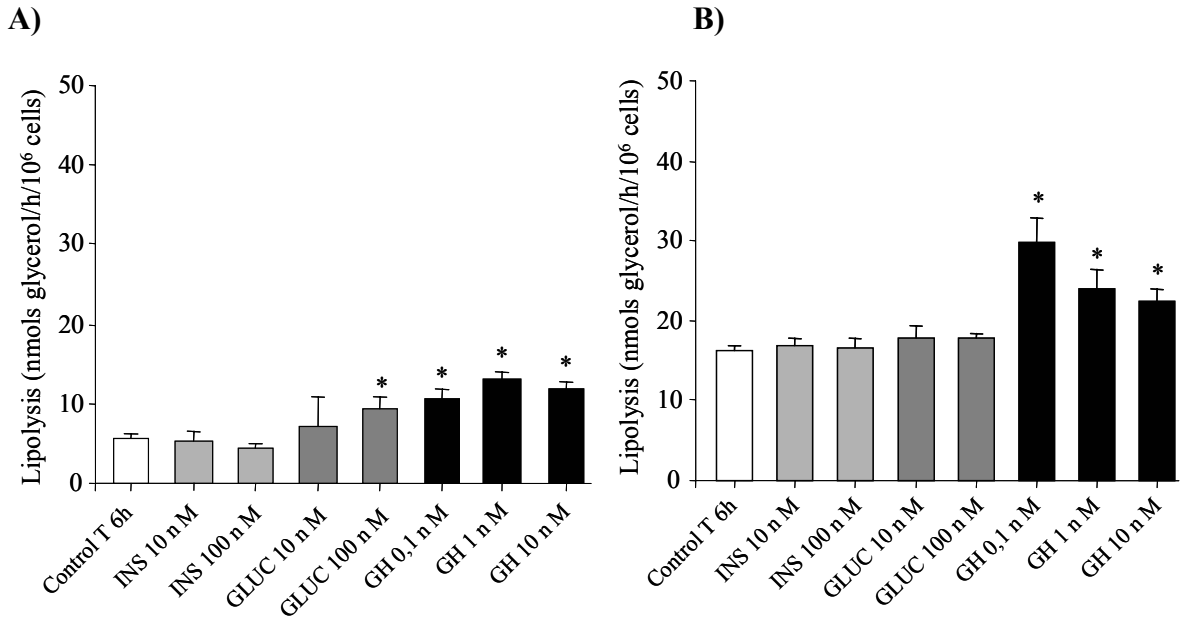

Figure 5. Hormone effect on adipocyte lipolysis of fish from the group fed FM diet (A) and of fasted fish from the same group (B). Each value is the mean ± S.E.M. of triplicates from 3 independent experiments. Results were statistically analyzed by paired t-test, (*) indicates significant differences between control and each of the hormones tested.

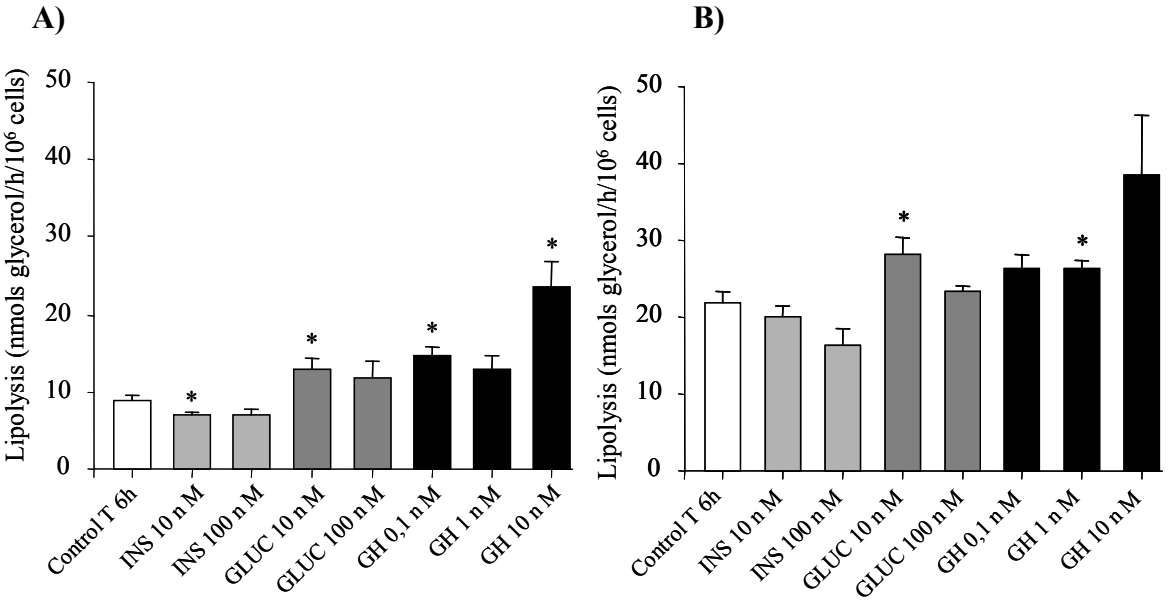

Figure 6. Hormone effect on adipocyte lipolysis of fish from the group fed PP diet (A) and fasted fish from the same group (B). Each value is the mean ± S.E.M. of triplicates from 3 independent experiments (n=9). Results were statistically analyzed as in figure 5, (*) indicates significant differences between control and each of the hormones tested.

4. Discussion

Ectotherms, which have relatively low basal metabolic rates, store substantial amounts of lipid in their livers, but also in the mesenteric adipose tissue, which has gained metabolic importance through vertebrate evolution. Adipose tissue is distributed in the abdominal cavity located periviscerally in many fish species, including gilthead seabream. Few studies have analyzed the morphology and distribution of adipose cells in visceral fat and muscle of salmonids (42, 9). Though there is abundant literature on the endocrine control of adipocyte function in either isolated or cultured mammalian adipose cells (16, 40), this *in vitro* model has not been fully studied in fish (22, 38, 41).

Our finding that the rate of lipolysis was proportional to cell density and linear with incubation time, as observed in mammals (37), together with microscopic characteristics and the specific stain, confirm that gilthead seabream isolated adipocytes are suitable for the study of the regulation of lipolysis by different effectors. Considering the time of incubation, the lipolytic rates calculated in gilthead seabream isolated adipocytes (5.71 nmols/h/10^6 cells) was lower than those previously reported in rainbow trout adipocytes in similar experimental conditions (8.9-12.8 nmols/h/10^6 cells) working at different temperatures of incubation (rainbow trout adipocytes at 15°C and seabream adipocytes at 22°C) (1). The lipolytic activity in the fish species studied until now is lower than in mammals (reviewed by Van den Thillart et al., (38)). However, the differences in temperature of incubation in mammalian and piscine systems (37ºC versus 15-22ºC) have to be taken into account.

A growth trial of 12 weeks previously performed during April-July in gilthead seabream adapted to the same diets under similar experimental conditions resulted in a reduction in growth rate from 1.86% (FM) to 1.56% (PP) (10). Although the factors responsible for altered feeding behavior in fish fed substituted diets are not clear (anti-nutritional factors, low palatability and AA composition) it appears that the effect is more pronounced over time. Thus the shorter period of adaptation to diet (6 weeks) in the present study may explain the lower impact of diet on growth rate. The levels of lipolysis in adipocytes from PP fed fish were higher than in fish fed FM. These results are in agreement with earlier observations regarding lipid metabolism in seabream (10) and other teleostean species fed with diets with high plant protein content (17, 6): a decrease in lipid deposition and mesenteric fat together with a clear hypo-cholesterolemia, and a decrease in postprandial plasma triglyceride levels. Since

changes in the rate of adipocyte lipolysis were weak, these changes were not reflected in the levels of plasma FFA, which remained similar in both groups in the fed condition.

The decrease in weight and relative liver size after fasting corroborates the finding that food deprivation significantly affects the metabolic status of seabream (24). Our data also show that previous nutritional history has a significant effect on catabolism induced by fasting since fish from the PP group responded differently from the FM fed group. Seabream in the PP group were the most affected by fasting, presenting the lowest body weight, liver weight, and plasma insulin levels. Plasma FFA increased in both fasted groups, while glucose was maintained. An increase in plasma FFA together with normoglycemia has been also reported in fasted salmon, *Oncorhynchus kisutch*. This increase was found to be related to lipid depots mobilization and increase in gluconeogenesis (32). Furthermore, and in agreement with the increase in plasma FFA irrespective of the previous diet, there was a clear increase in lipolytic activity in seabream adipocytes from fasted fish, indicating a significant contribution of adipose tissue to lipid mobilization. Nevertheless, other tissues such as liver and muscle may play an important role in the observed increase of plasma FFA. To our knowledge, this is the first demonstration that lipolysis measured in isolated adipocytes is affected by the nutritional status of fish. This effect has been reported in mammalian species, such as rats, in which a short fasting induced an increase of plasma FFA concommitant with increases in basal lipolysis levels in isolated adipocytes from adipose tissue (21). This breakdown of triglycerides and the decrease in abdominal fat tissue weight indicate a relatively rapid mobilization (after 11-13 days fasting) of lipid depots to meet energy demands in seabream in comparison with other species (24). The previous adaptation does not seem to affect the lipid mobilization capacity during fasting, since the increase in glycerol release in fasted fish in relation to fed fish was similar in both groups. Nevertheless, the maximum levels of lipolysis were observed in fasted PP with the minimum values of plasma insulin, which suggested a possible role of this hormone in the regulation of lipolysis in adipocytes.

Under our conditions insulin did not affect notably the rate of lipolysis *in vitro*. Insulin is involved in the stimulation of glucose carbon conversion into lipid in hepatocytes (30) of rainbow trout. Antilipolytic actions of insulin have been reported in incubations of salmon liver and adipose tissue pieces (12). Besides, specific insulin receptors have also been characterized in trout adipose tissue (27). In fact, mammalian insulin has been shown to stimulate glucose uptake in isolated trout adipocytes (3). A

possible explanation of the observed low or absence of response of adipocyte lipolysis to insulin could be related to the effects described in mammals by Morimoto et al. (21), who reported that insulin preferentially decreased lipolytic rate of adipocytes previously increased by other hormones such as catecholamines, rather than affecting the basal lipolytic activity in isolated rat adipocytes. In the same way, in hepatocytes from fed rainbow trout, insulin depressed glucagon-stimulated lipolysis, while basal lipolysis was not affected (13).

Both glucagon and GH are lipolytic in seabream cell incubations, GH being more potent in activating glycerol release to the medium. Nevertheless, the fact that the GH used was homologous to the species studied has to be taken into account. The relative increase in lipolysis after GH incubations in fasted fish was lower than in fed fish, which could be a consequence of the already higher rate of lipolysis in fasted fish. From our data we cannot deduce a change in hormone sensitivity, although in other fish models, such as hepatocytes, fasting affected the responsiveness to hormone stimulation (13, 26).

Early studies performed in fish adipose tissue slices or adipocytes showed an absence or a very low response of this tissue to hormones such as catecholamines, glucagon and ACTH (8, 22). Only recently, an inhibition of lipolytic rate after catecholamine incubation has been observed in isolated adipocytes from tilapia, which has been associated with resistance to hypoxia (41). The observed effects of glucagon in our study agree with the lipolytic action through stimulation of liver triacylglycerol lipase in fish (33). Besides, glucagon stimulated lipid breakdown in liver and adipose tissue pieces from rainbow trout (12). It appears then that glucagon is able to mobilize triglyceride depots not only from liver, but also from adipose tissue, as in mammals, although glucagon has a stronger lipolytic potency in mammalian systems (25, 36).

Lipolytic actions of GH are in agreement with the anti-lipogenic effects previously found for this hormone, such as inhibition of acetyl-coenzyme A carboxylase in the liver of gilthead seabream (39). Its lipolytic function correlates well with the increases in GH plasma levels in experimentally fasted seabream (5) or trout (2).

In summary, lipolysis of adipocytes isolated from adipose tissue of seabream is clearly modulated by the nutritional condition of the fish. These cells increase the lipolytic activity in presence of glucagon and GH. However, further studies are needed to understand fully the endocrine regulation of adipose tissue metabolism in fish.

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Control del metabolisme lipídic del teixit adipós pel factor de necrosis tumoral-α (TNFα) en la truita irisada (*Oncorhynchus mykiss*)

Control of adipose tissue lipid metabolism by tumor necrosis factor-α in rainbow trout (*Oncorhynchus mykiss*)

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Resum

El factor de necrosi tumoral-α (TNFα) és una citoquina amb múltiples funcions biològiques, que en mamífers inclouen la modulació del metabolisme del múscul i del teixit adipós. En peixos, el TNFα ha estat identificat en diverses espècies. Tot i això, pocs estudis han examinat el paper del TNFα fora del sistema immunològic en peixos.

En aquest treball, hem analitzat l'efecte del TNFα recombinant humà i de medis condicionats de macròfags de truita irisada estimulats amb lipopolisacàrid (LPS)-(MCM-LPS) sobre la lipòlisi d'adipòcits aïllats de la truita irisada (*Oncorhynchus mykiss*). A més, s'ha estudiat els efectes d'una injecció de LPS *in vivo* sobre el metabolisme lipídic del teixit adipós.

El TNFα recombinant humà va estimular la lipòlisi dels adipòcits de truita irisada d'una manera dosi i temps depenent. De forma similar, els LPS-MCM van estimular la lipòlisi dels adipòcits de truita irisada respecte a medis condicionats control. Els experiments amb inhibidors específics de la via de les MAP quinases van mostrar que la p44/42 i la p38 estan parcialment involucrades en els efectes lipolítics del TNFα. D'altra banda, la lipòlisi d'adipòcits provinents d'animals injectats amb LPS va ser superior a la lipòlisi d'adipòcits d'animals control 24 hores després de la injecció mentre que no es va veure cap efecte a les 72 hores. A més, l'activitat lipoproteïna lipasa (LPL) del teixit adipós de truites irisades injectades amb LPS va ser inferior a la dels controls 24 hores després de la injecció.

En resum, aquests resultats suggereixen que el TNFα juga un paper important en el control del metabolisme lipídic en la truita irisada estimulant la lipòlisi *in vitro* i *in vivo* i inhibint l'activitat LPL del teixit adipós *in vivo*.

Control of adipose tissue lipid metabolism by tumor necrosis factor-α in rainbow trout (*Oncorhynchus mykiss*)

A. Albalat[1], C. Liarte[2], S. MacKenzie[2], L. Tort[2], J.V.Planas[1], I. Navarro[1]

[1]Dpt. de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Spain

[2]Dpt. de Biologia Cel·lular, de Fisiologia i d'Immunologia de la Universitat Autònoma de Barcelona, Spain

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Please address all the correspondence to I. Navarro

[1]Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, 08028, Spain

Fax: 34+ 93+ 4110358

Email: mnavarro@ub.edu

Abstract

Tumor necrosis factor-α (TNFα) is a cytokine with multiple biological functions which in mammals has been shown to modulate muscle and adipose tissue metabolism. In fish, TNFα has been identified in several species. However, few studies have examined the role of TNFα in fish outside the immune system. In this study, we assessed the effects of human recombinant TNFα and conditioned media from rainbow trout LPS-stimulated macrophages (LPS-MCM) on lipolysis in isolated rainbow trout adipocytes. Furthermore, we studied the effects of an LPS injection *in vivo* on lipid metabolism. In our study, hrTNFα stimulated lipolysis in trout adipocytes in a time- and dose-dependent manner. Similarly, LPS-MCM stimulated lipolysis in trout adipocytes when compared with control conditioned medium. Experiments using specific inhibitors of the MAPK pathway showed that p44/42 and p38 are partially involved in the lipolytic effects of TNFα. On the other hand, adipocytes from LPS-injected rainbow trout showed higher basal lipolysis than adipocytes from control fish after 24 hours, while this effect was not seen at 72 hours. Furthermore, lipoprotein lipase (LPL) activity in adipose tissue of LPS-injected fish was lower than in the controls at 24 hours. These data suggest that TNFα plays an important role in the control of lipid metabolism in rainbow trout by stimulating lipolysis *in vitro* and *in vivo* and by down-regulating LPL activity of adipose tissue *in vivo*.

1. Introduction

Tumor necrosis factor belongs to a large family of structurally related proteins called 'TNF Ligand Superfamily'. In mammals, two forms of TNF have been identified and characterized (called α and β) which share 30% of protein sequence homology and which are known to act through the same receptors (Vilcek & Lee 1991).

In fish, the first gene identified that codified for TNFα was in the Japanese flounder (*Paralychthys olivaceus*) (Hirono *et al.* 2000). Further studies have identified and characterized TNFα mRNA in rainbow trout (*Oncorhynchus mykiss*) (Laing *et al.* 2001), brook trout (*Salvelinus fontinalis*) (Bobe & Goetz, 2001), carp (*Cyprinus carpio*) (Saeji *et al.* 2003) and sea bream (*Sparus aurata*) (Garcia-Castillo *et al.* 2002). More recently, Zou *et al.* (2002) have provided evidence for the presence of two different TNF genes in rainbow trout, the novel TNF1 and the previously characterized TNF2 (Laing *et al.* 2001, Bobe & Goetz 2001). Interestingly, TNFα mRNA expression has been reported in primary trout monocytes (Zou *et al.* 2002, MacKenzie *et al.* 2003) and *in vitro* differentiated macrophages after stimulation with lipopolysacharide (LPS) (MacKenzie *et al.* 2003). Furthermore, TNF-like activity has been found in supernatants of rainbow trout macrophages stimulated with LPS. These supernatants were able to enhance neutrophil migration and macrophage respiratory burst activity (Qin *et al.* 2001). These tools have been useful since due to the lack of homologous TNFα peptides in fish, studies covering the biological functions of TNFα in these species have been mainly performed using mammalian recombinant TNFα or macrophage culture supernatants after being stimulated with LPS (Goetz *et al.* 2004). Recently, recombinant trout TNF1 and TNF2 proteins have been produced and they have shown to induce gene expression of a number of proinflammatory factors in freshly isolated head kidney leucocytes and the macrophage cell line RST11. Furthermore these proteins enhanced leucocyte migration and phagocytic activity *in vitro* in a dose dependent manner (Zou *et al.* 2003).

In mammals, TNFα is secreted mainly by macrophages and monocytes but it can also be synthesized by other cell types. This cytokine plays an important role in the immune response and in inflammatory processes. However, TNFα has been increasingly recognized as a key modulator of glucose homeostasis and lipid metabolism in adipose tissue (Sethi & Hotamisligil 1999). In fact, some authors have

suggested that TNFα produced by the adipocyte itself acts as a true adipostat (Bulló-Bonet *et al.* 1999). Described effects of TNFα on lipid metabolism include stimulation of lipolysis in human adipocytes (Zhang *et al.* 2002), inhibition of the expression of enzymes involved in lipogenesis such as acetyl-CoA carboxylase and fatty acid synthase and inhibition of lipoprotein lipase (LPL) activity (Semb *et al.* 1987, Grundfeld *et al.* 1989).

The mechanisms by which TNFα stimulates lipolysis are still largely unknown. In fact, TNFα is a potent activator of mitogen-activated protein kinases, including ERK-1 and ERK-2 (p42/44), c-Jun NH_2-terminal kinase and p38 kinase (Wallach *et al.* 1999). In human adipocytes, Zhang *et al.* (2002) demonstrated that TNFα activated ERK and increased lipolysis, a mechanism which was blocked using two specific MEK inhibitors such as PD98059 and U0126. However, more research is needed in order to clarify the importance of the different MAPK pathways on the activation of lipolysis by TNFα.

Outwith the immune system, studies concerning the biological actions of TNFα in fish are scarce. However, some studies have already shown that TNFα could be a potentially important factor for immune-endocrine interactions in fish (Lister & van de Kraak 2002). In the present paper, we have investigated the role of TNFα in adipose tissue lipid metabolism in rainbow trout. To this end, we have examined the effects of TNFα in lipolysis on freshly isolated trout adipocytes using human recombinant TNFα (hrTNFα) and conditioned media from rainbow trout LPS-stimulated macrophages. Furthermore, in order to elucidate the mechanisms involved in TNFα stimulated lipolysis different MAPK inhibitors have been used. Finally, we studied the possible effects of LPS, a stimulus known to induce the expression of TNFα in trout macrophages (MacKenzie *et al.* 2003), administered *in vivo* on lipolysis of rainbow trout adipocytes and LPL activity in trout adipose tissue. The data presented in this study suggest that TNFα plays an important role in lipid metabolism in trout by stimulating lipolysis *in vitro* and *in vivo* and by down-regulating LPL activity in adipose tissue *in vivo*.

2. Material and methods

2.1. Animals and experimental conditions

Mesenteric adipose tissue for *in vitro* studies was obtained from rainbow trout (*Oncorhynchus mykiss*) of an average weight of 266.45 ± 14.55 g, acclimated to laboratory conditions at the facilities of the University of Barcelona, Spain. Animals were fed *ad libitum* by hand once a day and acclimated for 10 days before the experiments were conducted. Fish were kept under natural conditions of light, latitude (40º 5' N; 0º 10' E) and at 15 ± 1ºC of temperature.

For the LPS injection *in vivo* experiment, rainbow trout of an average weight of 154.89 ± 6.74 g were separated in two groups (12 fish in each group). The day of the experiment, one group was injected intraperitoneally (i.p.) with LPS (6 mg/Kg) a dose previously shown to induce the activation of the immune system in fish (MacKenzie *et al.* 2004; Ribas *et al*. 2004*)*, and the other group was injected with saline (control group). Fish were anesthesized with 2-phenoxy-ethanol and immediately sacrificed by a cranial blow at 24 and 72 hours after the injection and blood was removed by caudal puncture using heparinized syringes. Immediately, fish were weighed and the adipose tissue was extracted to perform the adipocyte isolation. Furthermore, a portion of adipose tissue from each fish was separated and kept in liquid nitrogen for LPL activity analysis. At the end of the sampling, blood samples were centrifuged and different aliquots of plasma were kept at –20ºC until the day of analysis.

Experiments were conducted according to the Catalan government 'Departament de Medi Ambient i Habitatge; Generalitat de Catalunya' regulations concerning treatment of experimental animals (No. 2215).

2.2. Adipocyte isolation

Adipocytes were isolated by the method of Robdell (1964) with some minor modifications. Fat tissue was cut into thin pieces and incubated for 60 min in polypropylene tubes with Krebs-Hepes buffer pre-gassed with 5% CO_2 in O_2 (pH 7.4) containing collagenase type II (130 U/ml) and 1% bovine albumin serum (BSA), in a

shaking water bath at 15°C. The cell suspension was filtered through a double layer of nylon cloth and then washed three times by flotation. Finally, cells were carefully resuspended in Krebs-Hepes buffer containing 2% BSA at a density of $6x10^5$ cells/ml using a Fuchs-Rosenthal counting chamber. Aliquots of 400 µl of this final adipocyte suspension were incubated in polypropylene tubes in a shaking bath in the absence or presence of hrTNFα up to 6 hours at 15°C. In a similar manner, adipocytes were incubated in the presence of hrTNFα and specific inhibitors of the MAPK pathway (PD98059 and SB203580). Adipocytes were pre-incubated for 20 min with the inhibitors before the addition of the cytokine.

At the end of the incubation time, tubes were rapidly placed on ice and cell-free aliquots of the medium were placed into enough perchloric acid to give a final concentration of 2%. Neutralized supernatants were taken for the measurement of glycerol concentration as index of lipolysis using a spectophotometric method (Wieland 1984, Tebar *et al.* 1996). All products were obtained from Sigma Aldrich (Madrid, Spain). Control and experimental conditions were conducted in triplicates; results are the average of triplicates from three independent experiments conducted with different adipocyte preparations. Previous experiments showed that basal lipolysis was proportional to cell density from $3x10^5$ to $12x10^5$ adipocytes/ml and also linear with incubation time for at least 7 hours (Albalat *et al.* 2002).

2.3. Isolation of macrophages and production of TNF-containing supernatants

Rainbow trout macrophages were isolated from the head kidney and cultured as previously described (MacKenzie *et al.* 2003).

To obtain supernatants from LPS-activated macrophages, macrophages were incubated at a density of 1 x 10^7 cell/ml in DMEM high glucose medium (PAA Laboratories) and stimulated with LPS (10 µg/ml) for 12 hours at 18°C under 5% CO_2. This concentration of LPS has been previously shown to be effective in stimulating TNFα expression in trout macrophages (MacKenzie *et al.* 2003). Following the incubation, the medium was collected and centrifuged for 10 min at 2000 g at 4°C. Supernatants were pooled and 4 ml of cell-free medium was concentrated by filtration using Amicon Ultra-4 filters (Millipore) to retain molecules with a molecular weight higher than 30 KDa (such as the trimeric form of TNFα, weight 51 KDa). The

concentrated media were diluted (1/100) with Krebs buffer and used to incubate freshly-prepared trout adipocytes. Adipocytes were incubated with LPS stimulated media and non-stimulated media for 6 hours at 15°C in a shaking bath.

2.4. Lipoprotein lipase assay

A portion of adipose tissue from each fish was homogenized in 9 volumes of homogenization buffer (10 mM HEPES, 1 mM EDTA and 1 mM DTT) at pH 7.4 and containing 5 U/ml of heparin. Homogenates were centrifuged at 36 700 g at 4°C for 20 min and the clear intermediate phase (between the fat droplets and the pellet) was used for the LPL activity assay. LPL activity was measured as previously described (Lindberg & Olivecrona 1995). LPL activity was performed with 10% Intralipid labelled with tri[9, 10-^{3}H]oleoglycerol by sonication in ice. The assay mixture (total volume 200 µl) contained 10 µl of the labelled intralipid, 5 % (vol/vol) pre-heated rat serum as apo C-II source, 0.1 M NaCl, 0.15 M Tris-HCl, heparin (0,02%), bovine serum albumin (BSA, 60 mg/ml) and 25 µl of sample. After a 2h incubation at 25°C, the reaction was stopped by the addition of 2 ml isopropanol/heptane/1M H_2SO_4 (40:48.3:1) and 0.5 ml water, and the free fatty acids were extracted as described (Bengtsson-Olivecrona & Olivecrona 1991). For each sample, four replicates were measured and activities were expressed as mU/g adipose tissue considering that 1 mU is equivalent to 1 nmol of fatty acid released per min.

2.5. Insulin radioimmunoassay

Plasma insulin levels were measured by radioimmunoassay (RIA) that used bonito insulin as standard and rabbit anti-bonito insulin antibodies as antiserum according to Gutiérrez *et al.* (1984).

2.6. Statistical Analysis

All data are presented as mean ± standard error (S.E.) (n = 6 or 9 as stated). Results obtained were analyzed by one-way analysis of variance (ANOVA) followed by

Tukey's test or by Student-Newman-Keuls test as indicated in the figures. Differences were considered significantly different when P<0.05.

3. Results

3.1. Dose-response of rhTNFα at different incubation times

hrTNFα was able to stimulate lipolysis of rainbow trout adipocytes in a dose dependent manner after 6 hours of incubation beginning at 50 ng/ml, with a maximal stimulation of over 2-fold when adipocytes were incubated with hrTNFα at a concentration of 100 ng/ml (Fig. 1). Incubation of adipocytes with the highest concentration of TNF tested had no effects on the viability of the cells judged by cytological examination. Furthermore, lactate dehydrogenase (LDH) activity in the medium, commonly used as a marker of cell viability, was measured and LDH in the medium of control and TNF-exposed adipocytes was not significantly different showing that TNFα did not affect viability of the cells after 6 hours of incubation (data not shown).

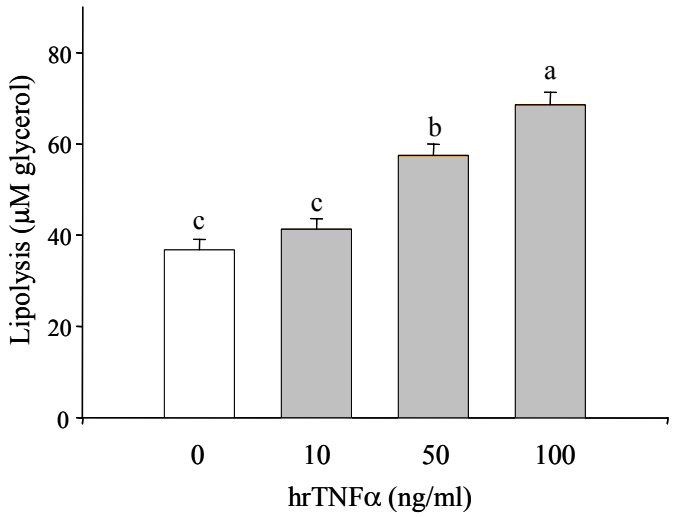

Figure 1. Effects of hrTNFα on lipolysis (measured as glycerol released in the medium) in rainbow trout isolated adipocytes after 6 h of incubation. Each point represents the average ± S.E. of triplicates from 3 independent experiments. Values not sharing a common letter are significantly different (*P*<0.05; Tukey's test).

3.2. Time-course of rhTNFα on lipolysis in rainbow trout adipocytes

In order to study the time course effects of hrTNFα on lipolysis in rainbow trout adipocytes we used the concentration of 50 ng/ml of rhTNFα. As we show in Fig. 2, rhTNFα at 50 ng/ml significantly ($p<0.05$) stimulated glycerol release from adipocytes at times as short as 30 min and the stimulatory effect was maintained up to 7 hours of incubation time. However, since basal lipolysis (glycerol released in the medium in the absence of hormone) also increased with time, the maximum stimulation of rhTNFα was obtained already at 1 hour of incubation.

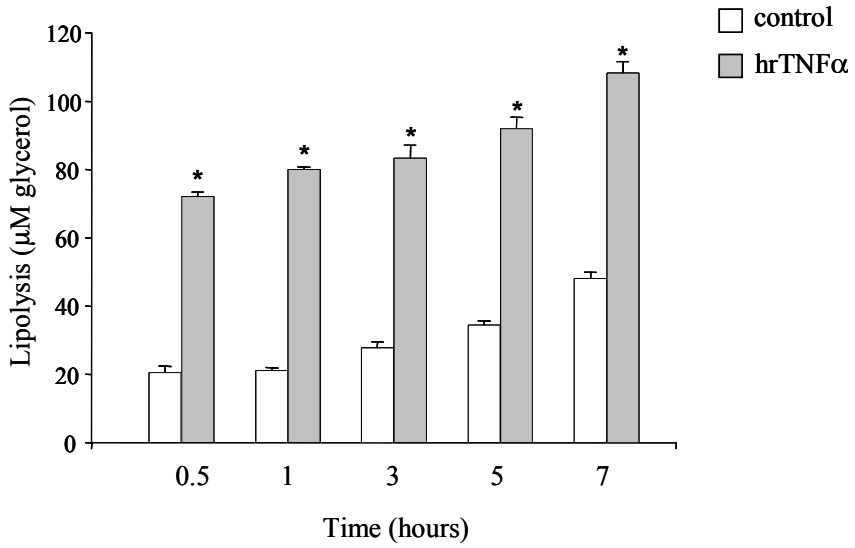

Figure 2. Time course of the effect of hrTNFα on lipolysis in rainbow trout isolated adipocytes. Values are the average ± S.E. of triplicates from a representative experiment from a total of 3 independent experiments. Values with (*) indicate significant differences between its corresponding control, ($P<0.05$; t-student).

3.3. Effect of conditioned medium from control and LPS-stimulated rainbow trout macrophages in lipolysis in rainbow trout adipocytes

Rainbow trout macrophages were incubated with control DMEM medium or with DMEM containing LPS (10 μg/ml) for 12 hours. Conditioned media were concentrated and tested on freshly prepared isolated trout adipocytes for 6 hours. As shown in Fig. 3, incubation of trout adipocytes with conditioned medium from LPS-stimulated macrophages markedly stimulated lipolysis when compared with control-conditioned medium (control-MCM). Furthermore, the stimulation observed was not due to the

presence of LPS but due to other factors secreted in the medium by the macrophages since LPS added alone (10 μg/ml) did not significantly affect lipolysis in rainbow trout adipocytes incubated for the same period of time (control, 100 ± 6.17 % ; LPS, 90.45 ± 6.01 % over control).

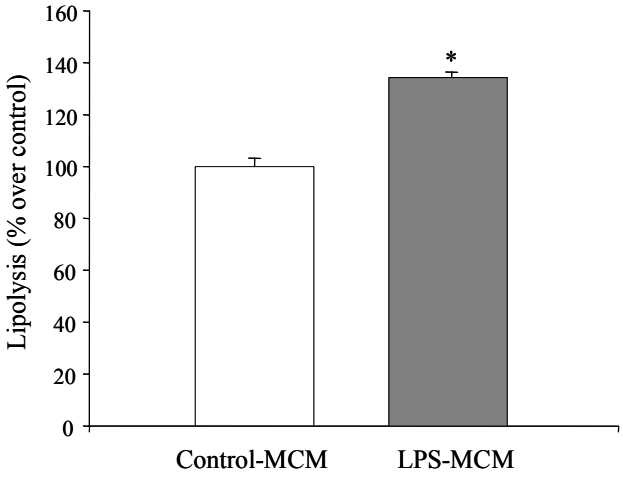

Figure 3. Effect of macrophage-conditioned media in rainbow trout adipocytes. Adipocytes were incubated with control macrophage-conditioned medium (control-MCM) and with LPS-stimulated macrophage conditioned-medium (LPS-MCM). Data are expressed as percent change with respect to the control group, which has been set at 100 % (43.92 ± 3.22 μM glycerol) and represent average ± S.E. from triplicates from 2 independent experiments (n = 6). Asterisk (*) indicate significant differences between control, (P<0.05; t-student).

3.4. TNFα stimulated lipolysis is partially inhibited by MAPK inhibitors

To elucidate the signals involved in hrTNFα-induced lipolysis we used two specific inhibitors of the MAPK pathway, SB203580 for p38 and PD98059 for MEK1, the upstream kinase that activates p42/44. In these experiments, the adipocytes were pre-incubated with the inhibitors for 20 min before the addition of rhTNFα. As shown in Fig. 4, none of the inhibitors at the highest concentration tested (50 μM) had any effect on basal lipolysis. Interestingly, SB203580 (Fig. 4A) had an inhibitory effect (at the two concentrations tested) on rhTNFα-stimulated lipolysis (50 ng/ml). However, this inhibitory effect was not observed when rhTNFα was added at a higher concentration (100 ng/ml). On the other hand, PD98059 (Fig. 4B) displayed a similar pattern since the highest concentration tested (50 μM) was able to partially inhibit the stimulatory effect of rhTNFα at 50 ng/ml but not at 100 ng/ml. Nevertheless, none of

the inhibitors tested were able to inhibit completely the stimulatory effects of rhTNFα in the conditions studied.

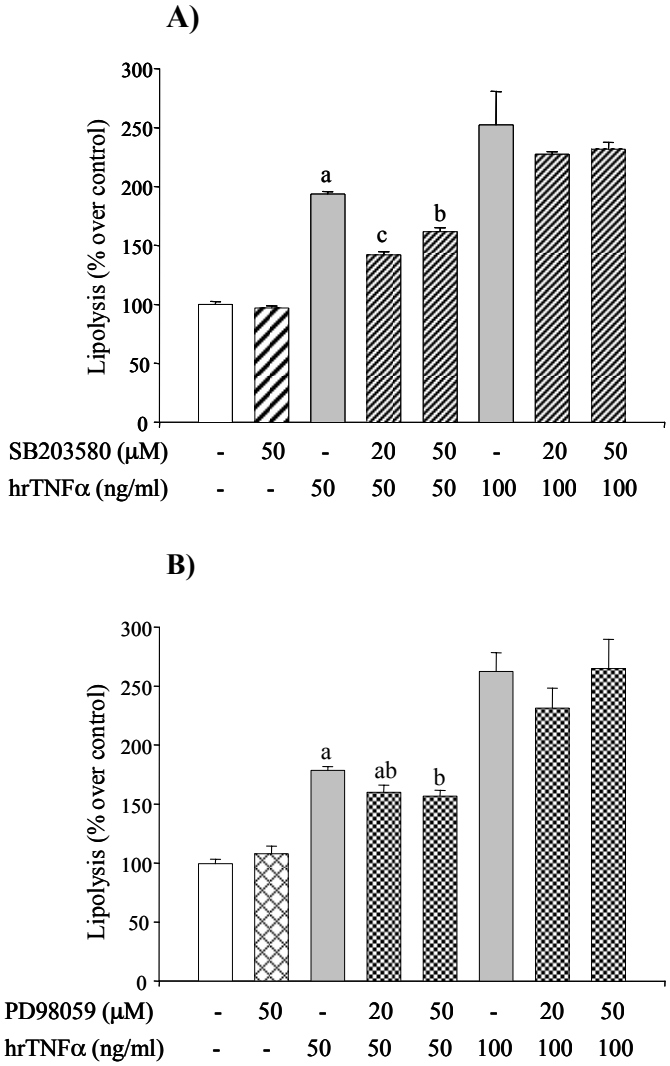

Figure 4. Effect of SB203580 (A) and PD98059 (B) on TNF-stimulated lipolysis. Inhibitors were pre-incubated for 20 min before the addition of the cytokine and adipocytes were incubated for 6 h. Data are expressed as percent change with respect to the control group, which has been set at 100 % (33.21 ± 1.63 and 30.35 ± 1.63 μM glycerol for SB203582 and PD98059, respectively) and represent average ± S.E. from triplicates from 2 independent experiments (n = 6) for each inhibitor used. Values not sharing a common letter are significantly different (P<0.05; Tukey's test).

3.5. Effect of in vivo LPS administration in rainbow trout adipocyte lipolysis

When administered *in vivo*, LPS (6 mg/Kg, i.p. injection) was able to stimulate basal *in vitro* lipolysis of rainbow trout adipocytes isolated 24 h after the injection, as

shown in Fig. 5. This stimulation, however, was not observed 72 h after the injection. Furthermore, there was no significant difference in basal lipolysis in adipocytes from control (saline-injected) fish at 24 and 72 hours after the injection.

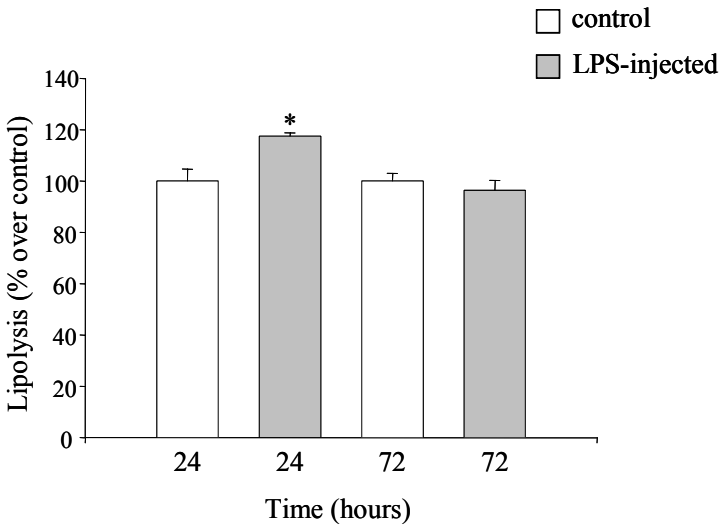

Figure 5. Effects of LPS administration *in vivo* on basal lipolysis in isolated adipocytes. Each point represents the average ± S.E. from five replicates of samples from 6 fish per group. Data are expressed as percent change with respect to the control group, which has been set at 100 % (71.79 ± 3.34 and 72.54 ± 2.20 μM glycerol at 24 and 72h, respectively). Values with (*) indicate significant differences between its corresponding control, (P<0.05; t-student).

In order to elucidate whether the LPS injection affected not only lipolysis in isolated adipocytes but other mechanisms important for lipid metabolism, we measured LPL activity in adipose tissue at 24 h, which is when the LPS effect on lipolysis was clearly observed. LPL activity was significantly lower in adipose tissue from the LPS-injected group (99.79 ± 13.25 mU/g adipose tissue) when compared to control-injected group (152.96 ± 10.10 mU/g adipose tissue). Since insulin is an important regulator of adipose LPL activity in mammals, we measured the levels of insulin in plasma of the animals used in the experiment at 24 h. However, plasma insulin levels were not significantly different between control and LPS-injected fish (4.75 ± 0.32 and 5.94 ± 0.32 ng/ml respectively). Therefore, the observed changes in adipose tissue lipid metabolism do not appear to be due to changes in plasma insulin levels.

4. Discussion

In the present study, we show that hrTNFα stimulates lipolysis in rainbow trout adipocytes in a dose dependent manner. In fact, several studies have previously demonstrated that TNFα is able to increase the rate of lipolysis in different mammalian cell types such as 3T3-L1 adipocytes (Souza *et al.* 2003, Ogawa *et al.* 1989), human adipocytes (Zhang *et al.* 2002) and rat adipocytes (Gasic *et al.* 1999). However, to our knowledge, this is the first time that TNFα is shown to stimulate lipolysis in fish adipocytes.

In our study, rhTNFα, at a concentration of 100 ng/ml, caused a 2-fold stimulation of lipolysis in rainbow trout adipocytes, which is comparable to the lipolytic response of human fat cells to rhTNFα (10-100 ng/ml) after a 48 h incubation (Rydén *et al.* 2002). In addition, other studies using mammalian adipocytes have demonstrated that maximal TNFα-stimulated lipolysis is obtained after 6-24h of incubation (Zhang *et al.* 2002, Hauner *et al.* 1995). Nevertheless, because a mammalian peptide was used in the present experiments we can not ensure that the stimulation and time course response would be identical with a homologous peptide. The apparent difference in the effective time to observe effects of TNFα between mammal and fish adipocytes (30 min) could be related to the existence of different intracellular activation mechanisms. In fact, the mechanism(s) by which TNFα stimulates lipolysis are not fully understood and they are strongly dependent on the cell type. In human preadipocytes, Rydén *et al.* (2002) found that TNFα–induced lipolysis involves the activation of the MEK1/2-ERK1/2 and JNK pathway but not the p38 pathway. Inhibitors of MEK1/2 and JNK such as PD98059 and dimetylaminopurine inhibited TNFα-induced lipolysis in human preadipocytes (Rydén *et al.* 2002). Similar results were obtained in 3T3-L1 adipocytes using inhibitors of the ERK pathway (Souza *et al.* 2003). In addition, Zhang *et al.* (2002) showed that PD98059 and U0126, another specific inhibitor of MEK 1/2, inhibited not only TNFα-induced lipolysis but also basal lipolysis in human differentiated adipocytes. In the present study, we found that PD98059 did not have any effect on basal lipolysis but that it partially blocked the lipolytic effects of rhTNFα in rainbow trout adipocytes. In a similar manner, SB203580 had no effect on basal lipolysis but was able to partially block rhTNFα-induced lipolysis in rainbow trout adipocytes. These observed effects of SB203580 differ from those observed in human preadipocytes where no effect of this

inhibitor was observed on basal or TNFα–induced lipolysis (Rydén *et al.* 2002). However, in the present study none of the inhibitors tested were able to completely block rhTNFα-mediated lipolysis. Therefore, we suggest that rhTNFα stimulates lipolysis in rainbow trout adipocytes at least in part through activation of ERK1/2 and p38 kinase.

The effects of rhTNFα on lipolysis in rainbow trout adipocytes were further confirmed by the results obtained with macrophage-conditioned media in rainbow trout adipocytes. Trout adipocytes incubated in the presence of LPS-stimulated macrophage conditioned medium had higher basal lipolysis than adipocytes incubated with control macrophage-conditioned medium. The active factor(s) in LPS-stimulated macrophage-conditioned medium responsible for the induced lipolysis in trout adipocytes were not determined in this study. However, MacKenzie *et al.* (2003) have demonstrated that LPS increases the expression of TNFα mRNA in *in vitro* differentiated macrophages. Furthermore, supernatants harvested from trout macrophages stimulated with LPS exhibit TNF-like activities measured as enhanced neutrophil migration and enhanced macrophage respiratory burst activity (Qin *et al.* 2001). Outwith the immune system, Lister *et al.* (2002) found that trout macrophage-conditioned medium significantly inhibited hCG-stimulated testosterone production by goldfish (*Carassius auratus*) testis pieces *in vitro*, a typical feature described in mammals when cytokine levels are increased. In our study, LPS alone did not have any effect on basal lipolysis of rainbow trout adipocytes. Given these results, we suggest that at least one of the possible active factors for the observed induced lipolysis could be the presence of TNFα in LPS-stimulated macrophage-conditioned medium. However, since the active factor(s) responsible for the induced lipolysis were not determined we should consider that it is possible that other cytokines, such as IL-1β, present in the LPS-stimulated supernatants could be in part responsible of the observed lipolytic effect.

Finally, we examined the effects of a single dose injection of LPS on rainbow trout adipocyte basal lipolysis. Adipocytes from LPS-injected fish had higher basal lipolysis compared to adipocytes from control fish at 24 h after the LPS injection. Very few studies have checked the possible effects of an *in vivo* injection of LPS in adipocyte lipolysis, even in mammals. Pond & Mattacks (1998) observed an increase in basal lipolysis in guinea-pig adipocytes surrounding the popliteal lymph nodes after being activated with a subcutaneous injection of LPS. On the other hand, Porter *et al.* (2002)

showed that exposure of adipose-tissue explants to TNFα for 24 h produced an increase in adipocyte glycerol release in a short term incubation.

In addition to the effects on lipolysis, we found that *in vivo* treatment with LPS inhibited LPL activity in rainbow trout adipose tissue at 24 h. LPL is a key enzyme that provides fatty acids from triglycerides to peripheral tissues such as adipose tissue (Enerbäck & Gimble 1993). In mammals, it is clearly recognised that there are mainly two situations where adipose tissue LPL is down-regulated. One is food deprivation and the other is trauma/sepsis/LPS administration, which is a response primarily mediated by TNFα (Wu *et al.* 2004). The results obtained in our study are in agreement with several studies which reported that LPL activity is inhibited by TNFα in mammalian adipose tissue (Grunfeld *et al.* 1989; Semb *et al*. 1987) and with the results presented by Kawasaki *et al.* (2004) where an LPS injection in rats provoked a decrease in LPL activity in adipose tissue and an increase in hormonal sensitive lipase (HSL) 2h after the LPS injection. Importantly, the decrease observed in LPL activity by LPS administration in the present study could not be related to changes in plasma insulin levels since similar insulin levels were found in the saline and LPS-injected groups. Nevertheless, from our data it is not possible to conclude that insulin did not affect LPL activity since insulin was only measured at one sampling time (24h). More data in plasma insulin levels between the injection and the 24h point would be necessary in order to clarify a possible role of insulin in the observed effect in LPL activity. Moreover, future studies should investigate whether hrTNFα has direct effects on LPL activity in isolated adipocytes.

In summary, hrTNFα stimulated lipolysis in isolated rainbow trout adipocytes by a signalling mechanism that involved, at least in part, activation of ERK1/2 and p38 kinase. Moreover, conditioned medium from LPS-stimulated trout macrophages was able to induce an increase in the lipolytic rate in isolated rainbow trout adipocytes. Finally, in vivo LPS administration induced an increase in lipolysis in isolated rainbow trout adipocytes and a down-regulation of LPL activity in rainbow trout adipose tissue 24 h after the injection, suggesting that several mechanisms important for lipid metabolism are altered due to the administration of LPS. We suggest that the *in vivo* effects of LPS on lipid metabolism are probably mediated by TNFα, which could be secreted by macrophages or by adipose tissue itself (MacKenzie *et al.* 2003, Sewter *et al.* 1999).

To our knowledge, this is the first time that the effects of TNFα are evaluated in fish adipocytes. Although more research is needed, we suggest that TNFα could be a key modulator of lipid metabolism in fish and that the metabolic activity of TNFα has been conserved during the evolution from fish to mammals.

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II. REGULACIÓ NUTRICIONAL I HORMONAL DE LA LIPOPROTEÏNA LIPASA EN PEIXOS

Regulation of lipoprotein lipase (LPL) activity in rainbow trout (*Oncorhynchus mykiss*) tissues

A. Albalat, J. Sánchez-Gurmaches, J. Gutiérrez, I. Navarro

General and Comparative Endocrinology (*Submitted*) (2005)

Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (*Sparus aurata*)

A. Albalat, A. Saera-Vila, J. Gutiérrez, J. Pérez-Sánchez, I. Navarro

(*In preparation*)

Regulació de l'activitat lipoproteïna lipasa (LPL) en diferents teixits de la truita irisada (*Oncorhynchus mykiss)*

Regulation of lipoprotein lipase (LPL) activity in rainbow trout (*Oncorhynchus mykiss*) tissues

Regulació de l'activitat lipoproteïna lipasa (LPL) en diferents teixits de la truita irisada (*Oncorhynchus mykiss*)

Resum

La lipoproteïna lipasa (LPL) es considera un enzim clau en la deposició lipídica i el metabolisme de molts teixits però la informació sobre l'activitat LPL i la seva regulació en peixos és molt escassa. En el present estudi, hem examinat la regulació nutricional de l'activitat LPL realitzant un experiment post-prandial i un experiment de dejuni en la truita irisada (*Oncorhynchus mykiss*). Degut a que la insulina juga un paper important en la regulació nutricional de l'activitat LPL en mamífers, els efectes d'aquesta hormona van ser analitzats *in vivo* realitzant un administració intraperitoneal d'aquesta hormona. A més, hem portat a terme estudis *in vitro* utilitzant trossos de teixit adipós per tal de clarificar el paper directe de la insulina i el factor de necrosi tumoral-α (TNFα) com a possibles reguladors de l'activitat LPL en la truita irisada.

L'activitat LPL del teixit adipós va augmentar en resposta a la ingesta a les 4 hores després de l'administració de l'aliment, i va disminuïr a nivells basals a les 6 hores. D'altrabanda, no es va veure una resposta clara en el múscul vermell ni en el múscul blanc, on els valors de l'activitat LPL van ser menors, especialment en el múscul blanc. A més, el dejuni va produïr una disminució de l'activitat LPL en teixit adipós juntament amb valors baixos d'insulina plasmàtica. L'administració intraperitoneal d'insulina va estimular l'activitat LPL en el teixit adipós 3 hores després de la injecció, mentre que no hi va haver cap resposta en els músculs vermell i blanc.

Finalment, els estudis *in vitro* utilitzant trossos de teixit adipós indicaren que la insulina és capaç d'estimular la proporció de LPL en estat actiu a nivell extracel.lular. D'altrabanda, el TNFα no va afectar l'activitat LPL utilitzant el mateix model *in vitro*.

En conclusió, aquestes dades indiquen que l'activitat LPL està regulada de manera diferencial segons el teixit després de la ingesta, i apunten a la insulina com un regulador important de l'activitat LPL en el teixit adipós de la truita irisada.

Regulation of lipoprotein lipase (LPL) activity in rainbow trout (*Oncorhynchus mykiss*) tissues

A. Albalat, J. Sánchez-Gurmaches, J. Gutiérrez and I. Navarro

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Running title: Regulation of LPL activity in rainbow trout

Key words: LPL activity, nutritional regulation, insulin, rainbow trout

Please address all correspondence to I. Navarro

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Fax: 34+ 93+ 4110358

Email: mnavarro@ub.edu

Abstract

Lipoprotein lipase (LPL) is considered a key enzyme in the lipid deposition and metabolism of many tissues. Information on LPL activity and its regulation in fish remains very scarce. In the present study, we have examined the nutritional regulation of LPL activity by conducting post-feeding and fasting experiments in rainbow trout (*Oncorhynchus mykiss*). As insulin plays an important role in the nutritional regulation of LPL activity in mammals, the effects of this hormone were tested *in vivo* by intraperitoneal administration. Moreover, we conducted *in vitro* studies using fat pads of rainbow trout to better clarify the direct role of insulin and tumor necrosis factor-α (TNFα) as possible regulators of LPL activity in rainbow trout.

LPL activity in adipose tissue increased in response to feeding, 4 hours after ingestion of food, then decreasing to basal levels at 6 hours. No clear response was found in either red or white muscles, where LPL values were lower. Moreover, fasting produced a down-regulation of LPL activity in adipose tissue, concomitant with low levels of plasma insulin. While insulin administration stimulated LPL activity of adipose tissue 3 hours after injection, no response was observed in red or white muscles. Finally, *in vitro* studies using fat pads revealed that insulin significantly stimulated the proportion of LPL in active conformation at the extracellular level. On the other hand, TNFα did not greatly affect LPL activity using this *in vitro* model.

These data indicate that LPL activity is regulated in a tissue-specific manner following food intake, and suggest that insulin is an important regulator of LPL activity in the adipose tissue of rainbow trout.

1. Introduction

Lipoprotein lipase (LPL) is a glycoprotein enzyme belonging to a large family of lipases, including pancreatic, hepatic and endothelial lipases (Rader and Jaye, 2000). LPL in its active form appears to be located at the surface of the vascular endothelium, as well as in LPL-synthesizing cell tissues (Olivecrona and Bengtsson-Olivecrona, 1987; Rodrigues et al., 1992), where hydrolyses triglycerides from circulating chylomicrons and very low density lipoproteins (VLDL). These triglycerides are hydrolysed into free fatty acids (FFA), which can then be re-esterified and stored, as occurs in adipose tissue, or used as an energy source by peripheral tissues, mainly in muscle and heart (Auwerx et al., 1992). For this reason, LPL is considered a key enzyme in the lipid deposition and metabolism for the entire organism.

In fish, partial genomic structures in rainbow trout (*Oncorhunchus mykiss*) and zebrafish (*Brachydanio rerio*) LPL genes were reported by Arnault et al. (1996). However, the full nucleotide sequence for rainbow trout LPL was presented by Lindberg and Olivecrona (2002), while the LPL gene of red sea bream (*Pagrus major*) was characterized by cDNA and genomic structure analysis by Oku et al. (2002). Nevertheless, few studies have measured LPL activity in fish. In one early study, Skinner et al. (1980) found that the serum of trout contained lipoprotein lipase activity following heparin injection. This enzyme was further characterized by Skinner and Youssef (1982). LPL activity was also found in the liver of adult trout and cod (Black et al., 1983) and in extra-hepatic tissues such as adipose tissue, red and white muscles, heart, brain, liver and vitellogenic ovaries of rainbow trout (*Oncorhynchus gairdneri*) (Black et al., 1983; Black and Skinner, 1986; Black and Skinner, 1987; Lindberg and Olivecrona, 1995) being adipose tissue, (main organ for fat storage in many species of fish) the tissue with the highest LPL activity.

Regulation of LPL appears to be very complex and responds to dietary or hormonal changes and environmental conditions being clearly tissue-specific (Howard et al., 1993; Auwerx et al., 1992). In mammals such as human and rodents, adipose tissue LPL activity is down-regulated during fasting (Lladó et al., 1999; Bergö et al., 1996), while remaining unchanged or increasing in skeletal muscle and heart (Ruge et al., 2005; Sugden et al., 1993). In these species, the observed effects have been correlated to circulating levels of glucocorticoids and insulin (reviewed by Enerbäck

and Gimble, 1993). In similar fashion, adipose tissue LPL activity increases in humans following food intake, in tandem with increased plasma insulin levels (Eriksson et al., 2003). In fact, several studies have clearly demonstrated that insulin is one of the main regulators of LPL (Farese et al., 1991), capable of stimulating LPL activity in adipose tissue (Ong et al., 1988). Apart from fasting, LPL has been shown to be down-regulated in mammals in other settings related to the activation of an immunological response, such as during trauma/sepsis/lipopolisacharide (LPS) administration. In such cases, it appears that LPL activity response is mainly controlled by tumor necrosis factor-alpha (TNFα). Moreover, it has been shown in mammals that LPL activity is inhibited directly by TNFα in adipose tissue (Grunfeld et al., 1989; Semb et al., 1987).

There are very few studies concerning the nutritional regulation of LPL activity in fish. Black and Skinner (1986), working with rainbow trout, demonstrated how during fasting LPL activity decreased in adipose tissue and liver, but not in heart or red muscle. More recently, Liang et al. (2002) not only found that feeding condition altered liver and visceral adipose LPL mRNA levels, but also that dietary lipid levels altered liver LPL mRNA levels of red sea bream (*Pagrus major*). Their findings show that the LPL gene expression of red sea bream is regulated in a tissue-specific manner by diet. Little is known, however, about the hormonal regulation underlying these observed effects, or the role of other possible regulators such as TNFα. Regarding this last issue, our group has recently discovered that a single administration of LPS, a stimulus known to induce TNFα expression of in trout macrophages (MacKenzie et al., 2003), decreased LPL activity in rainbow trout adipose tissue (Albalat et al., 2005a). However, the direct effects of TNFα on adipose tissue LPL activity were not addressed in that study.

The objectives of the present study were to determine both the nutritional regulation and the role of insulin and other potential regulators such as TNFα on LPL activity in rainbow trout. To examine the nutritional regulation of LPL activity in different tissues, post-feeding and fasting experiments were conducted in adult rainbow trout, while plasma insulin levels were determined in parallel. Moreover, insulin was administered *in vivo* and the effects on LPL activity were measured in adipose tissue and muscle of rainbow trout. Finally, *in vitro* studies were performed, incubating insulin and TNFα in fat pads of rainbow trout in order to clarify the direct role of these potential regulators on LPL activity. Our results demonstrate not only that feeding condition regulates LPL activity in rainbow trout in a tissue-specific manner, but also

show that insulin is an important regulator of adipose tissue LPL activity *in vivo* and *in vitro*.

2. Materials and methods

2.1. Animals and experimental conditions

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the fish farm 'Truites del Segre' (Lleida, Spain) and acclimated to laboratory conditions in our facilities at the University of Barcelona, Spain. During this period, animals were fed *ad libitum* by hand once a day and acclimated for 15 days before any experiments were conducted. Fish were kept under natural conditions of light, latitude (40º 5' N; 0º 10' E), and at a temperature of 15 ± 1ºC.

1. Post-feeding experiment: Rainbow trout (n = 20) with an average weight of 290 g were fed with a commercial diet to apparent visual satiety (10:00h), and adapted to this feeding schedule for 10 days. On the day of the experiment, fish were fed and killed (5 animals at each sampling time) 2, 4, 6, and 36 hours after feeding.

2. Fasting experiment: Rainbow trout with an average weight of 360 g were separated into two groups (19 fish per group): one group was fed daily to apparent visual satiety (10:00h) using a commercial diet (fed group), while the other was deprived of food for a period of 21 days (fasted group). On the day of the experiment, control fish were sampled 24 h after the last meal.

3. In vivo injection of insulin: Rainbow trout (n = 22) with an average weight of 310 g were separated into two groups (11 fish in each group). On the day of the experiment, fish received a single intraperitoneal injection (1 µl/g body wt.) of vehicle (PBS) or porcine insulin (2.16 nmol/100 g body wt.), a dose shown to reduce glucose plasma levels in rainbow trout in previous studies (Plisetskaya et al., 1985). After 3 and 6 hours, fish from control and insulin-injected groups were killed, and blood and tissue samples were taken.

In these experiments, fish were killed with a sharp blow to the head, and blood samples were taken by caudal puncture using heparinized syringes. Fish were weighed, and adipose tissue, red muscle and white muscle were rapidly extracted and frozen in

liquid nitrogen. Finally, liver was weighed, blood samples were centrifuged at 4°C and different aliquots of plasma were kept at -80°C until the day of the analysis. In the post-feeding experiment, the stomach of each fish was carefully dissected and weighed with its content, in order to measure total stomach weight.

4. In vitro experiments: Rainbow trout with an average weight of 250 g and acclimated to laboratory conditions were killed and adipose tissue was rapidly removed and washed in Krebbs buffer. Adipose tissue was then cut into small pieces and 1 g samples were collected and incubated in Krebbs buffer supplemented with BSA 4% (4 ml), with or without porcine insulin (500 nM) or human recombinant TNFα (50 ng/ml) (Sigma Aldrich) for 4 hours at 15°C in a shaking bath. In each experiment, all conditions (fat pads incubated with or without hormone) were conducted in triplicate, with results representing the mean of two independent experiments. Following incubation, medium was removed and fresh medium containing heparin (5 U/ml) was added in a ratio of 2 ml/g of adipose tissue. Fat pads with fresh heparin-containing medium were incubated in an ice-cold shaking bath for 60 min in order to release all membrane-bound LPL into the medium. LPL was measured in heparin-containing buffer (heparin-releasable fraction, HR-LPL), as well as in heparin-washed adipose tissue pieces following homogenization (non-heparin-releasable fraction, NHR-LPL) as previously described by Ballart et al., (2003). Total LPL activity was calculated by adding the LPL values of both fractions.

The viability of fad pads was determined by measuring lactate dehydrogenase (LDH) activity in those fad pads containing medium.

2.2. Lipoprotein lipase assay

From *in vivo* studies, portions of adipose tissue, as well as red and white muscles, from each fish were homogenized in 9 volumes of homogenization buffer (10 mM HEPES, 1 mM EDTA and 1 mM DTT) at pH 7.4 and containing 5 U/ml of heparin. Homogenates were centrifuged at 36,700g at 4°C for 20 min and the clear intermediate phase (between the fat droplets and pellet) was used for LPL activity assays. For *in vitro* studies, LPL activity was measured in HR-LPL and NHR-LPL fractions and LPL activity was measured as previously described (Lindberg and Olivecrona 1995; Arantzamendi et al., 2003). LPL activity measurements were conducted with 10%

Intralipid, labelled with tri[9, 10-^{3}H]oleoglycerol by sonication in ice. The assay mixture (total volume 200 µl) contained 10 µl of the labelled intralipid, 5% (vol/vol) pre-heated rat serum as apo C-II source, 0.1 M NaCl, 0.15 M Tris-HCl, heparin (0.02%), bovine serum albumin (BSA, 60 mg/ml), and 25 µl of sample. Following 2 h incubation at 25°C, the reaction was stopped by the addition of 2 ml isopropanol/heptane/1M H_2SO_4 (40:48.3:1) and 0.5 ml water, with free fatty acids extracted as previously described (Bengtsson-Olivecrona and Olivecrona 1991). For each sample, four replicates were measured and activities were expressed as mU/g adipose tissue, based on the calculation that 1 mU is equivalent to 1 nmol of fatty acid released per min.

2.3. Biochemical analyses of plasmatic parameters

Plasma glucose concentration was determined by the glucose oxidase colorimetric method (GLUCOFIX; Menarini Diagnostics, Firenze, Italy) (Huggett and Nixon, 1957; Sala-Rabanal et al., 2003). Plasma free fatty acids were analyzed using a commercial enzymatic method (NEFA-C, Wako Test). Plasma insulin levels were measured by radioimmunoassay (RIA) using bonito insulin as a standard and rabbit anti-bonito insulin as an antiserum (Gutiérrez et al., 1984).

2.4. Statistical analysis

Data are reported as mean values ± standard error of mean (SEM). In the post-feeding and insulin injection experiments, differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. Results from fasting experiment, as well as from the *in vitro* experiments were analyzed by paired t-test (Student's t-test). A p-value lower than 0.05 was considered statistically significant.

3. Results

3.1. Experiment 1: Post-feeding levels of plasma metabolites, insulin levels and LPL activity in different tissues

Body weight, length, and the weight of mesenteric fat for rainbow trout used in the post-feeding experiment were as follows: 287.33 ± 12.64 g; 27.38 ± 0.66 cm; and 3.97 ± 0.35 g, respectively. The total stomach weight of rainbow trout was similar at 2, 4, and 6 hours after feeding. However, the greatest stomach weight occurred 2 hours after feeding (27.17 ± 2.41 g), decreasing almost 3-fold (10.34 ± 2.29 g) 36 hours after feeding, as shown in Figure 1. Plasma insulin levels were high and similar between 2 and 6 hours after feeding, decreasing to basal levels (2.56 ± 0.74 ng/ml) 36 hours after feeding. While glycemia exhibited similar levels throughout the post-feeding period, glucose concentration was highest 4 hours after feeding. These levels were not, however, significantly different, with the lowest levels occurring 36 hours after feeding (basal levels). Finally, plasma FFA levels were similar at the sampling times studied (Table 1).

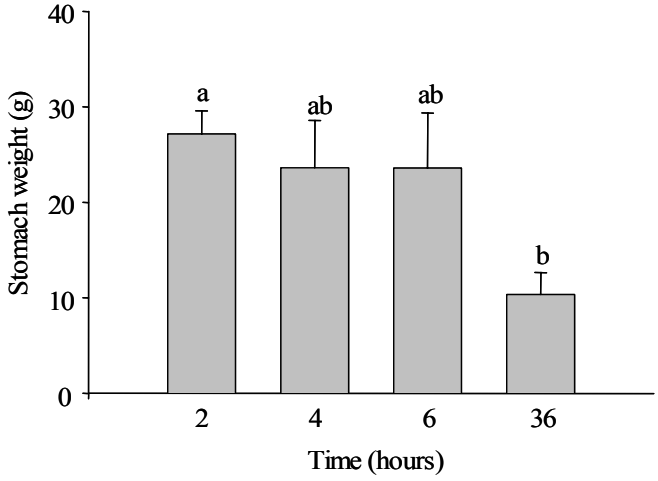

Figure 1. Total stomach weight of rainbow trout from the post-feeding experiment sampled at different time points. Stomach weight was measured by carefully dissecting the stomach of each fish with its content. The number of fish sampled at each time point was n = 5. Values are the mean ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$).

Table 1. Plasmatic parameters of rainbow trout used in the post-feeding experiment sampled at different time points (2, 4, 6 and 36 hours after feeding). Values are the mean (n = 5 for each time sampled) ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$).

	2h	4h	6h	36h
Glucose (mg/dl)	93.09 ± 10.86	120.36 ± 12.87	116.34 ± 6.72	108.17 ± 5.87
FFA (mEq/L)	0.39 ± 0.01	0.48 ± 0.02	0.44 ± 0,05	0.49 ± 0.02
Insulin (ng/ml)	9.74 ± 0.46 [a]	10.80 ± 0,77 [a]	11.97 ± 0.44 [a]	2.56 ± 0.74 [b]

LPL activity in adipose tissue began to increase 2 hours after feeding, with maximum values (978.87 ± 24.35 mU/g) occurring at 4 hours. Afterwards, LPL activity decreased at 6 hours after feeding to values close to basal levels (Figure 2). Although a similar pattern was observed in red muscle, with the highest LPL activity values occurring 2 and 4 hours after feeding, in this case the differences between sampling times were not significant. In white muscle, LPL activity was very low (compared to adipose tissue or red muscle), high variability was noted between animals, and the observed changes in LPL activity were not significantly different.

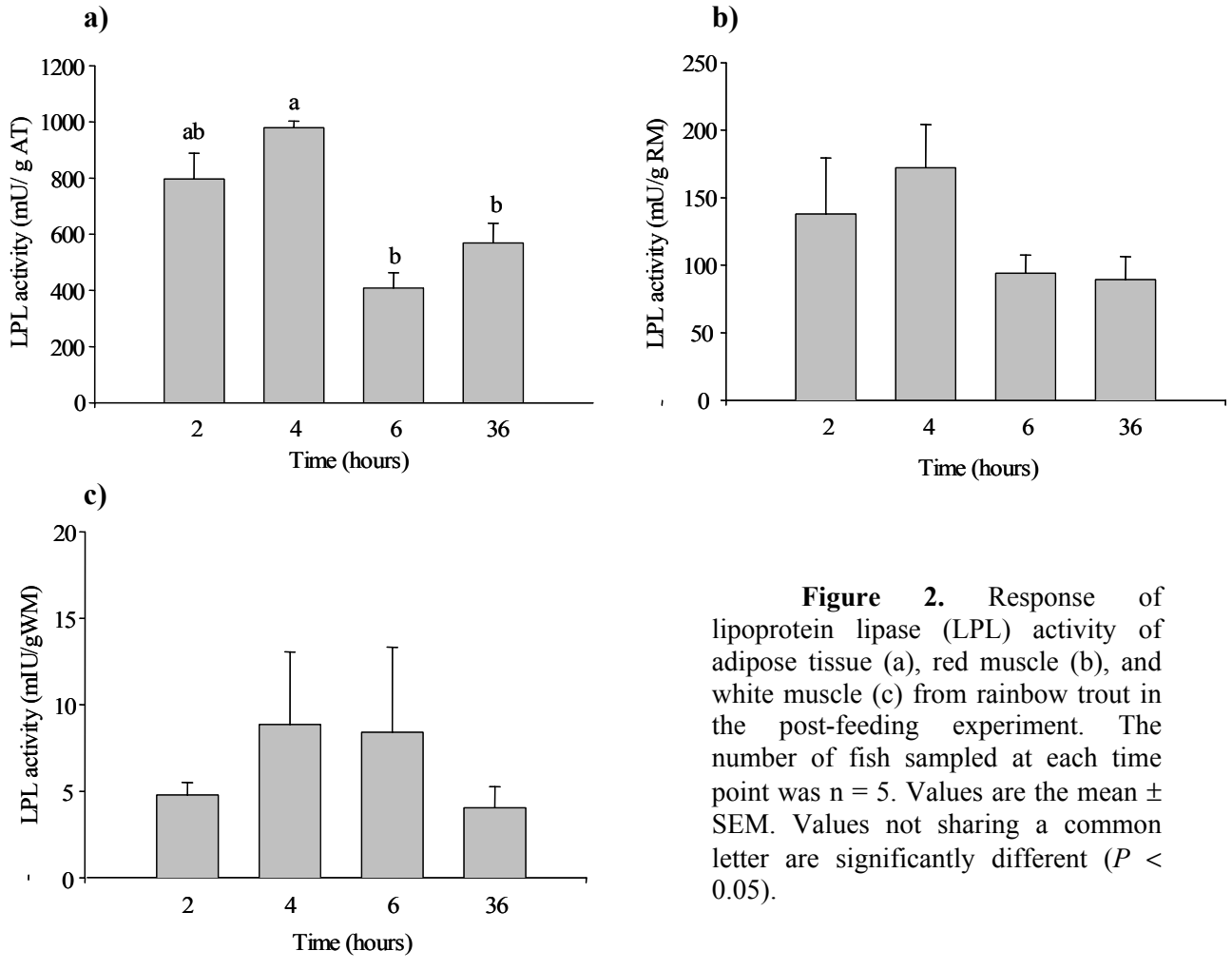

Figure 2. Response of lipoprotein lipase (LPL) activity of adipose tissue (a), red muscle (b), and white muscle (c) from rainbow trout in the post-feeding experiment. The number of fish sampled at each time point was n = 5. Values are the mean ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$).

3.2. Experiment 2: Effects of fasting on morphological parameters, plasma metabolites, insulin levels, and LPL activity in adipose tissue

Fasting for 3 weeks provoked a 50% decrease in liver weight and mesenteric adipose tissue content. In addition, body weight, HSI and condition factor index were significantly lower in fasted fish compared to fed fish, as shown in Table 2. Glycemia levels were slightly lower in fasted fish, while plasma FFA levels were nearly 2-fold higher in fasted animals (Table 2). Furthermore, fasting produced important changes in plasma insulin levels, with the concentration decreasing from 18.34 ng/ml in fed fish, to non-detectable levels in fasted fish.

Table 2. Effects of fasting on morphologic and plasma parameters of rainbow trout fed or fasted for 21 days. Values are the mean (n = 6 in fed and n = 13 in fasted groups) ± SEM. Asterisk (*) indicates significant differences between fed and fasted groups ($P < 0.05$; t-student).

	Fed	Fasted
Final body mass (g)	448.98 ± 15.52	285.69 ± 8.31*
Length (cm)	31.08 ± 0.55	28.12 ± 0.38
Liver (g)	5.99 ± 0.34	2.40 ± 0.13*
Mesenteric fat (g)	7.48 ± 1.43	3.53 0.40*
HSI (%)[1]	1.34 ± 0.07	0.84 ± 0.03*
MFI (%)[2]	1.71 ± 0.31	1.25 ± 0.14
CF (%)[3]	1.52 ± 0.11	1.29 ± 0.05*
Plasma parameters		
Glucose (mg/dL)	94.43 ± 3.24	86.78 ± 4.39
FFA (meq/L)	0.318 ± 0.02	0.621 ± 0.04*
Insulin (ng/mL)	18.34 ± 1.41	n.d.

[1] Hepatosomatic index = (liver wt./fish wt.) x 100
[2] Mesenteric fat index = (mesenteric fat wt./fish wt.) x 100
[3] Condition factor index = (fish wt./(fish length)^3) x 100

The effects of fasting on LPL activity in adipose tissue are shown in Figure 3. LPL activity measured in adipose tissue was significantly lower in fasted rainbow trout compared to fed fish. Fasting caused an almost 3-fold decrease in adipose tissue LPL activity; specifically, from 400.04 ± 35.03 in fed fish to 144.34 ± 14.55 mU/g adipose tissue in fasted fish.

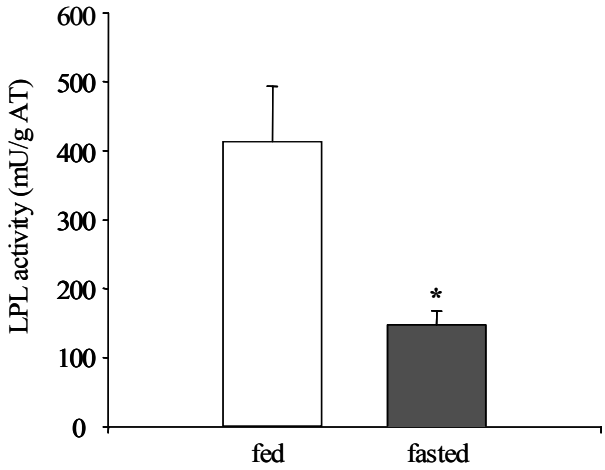

Figure 3. Effects of fasting on lipoprotein lipase (LPL) activity of adipose tissue from rainbow trout. Values are the mean ± SEM. The number of fish in each group was n = 6 in fed and n = 13 in fasted groups. Asterisk (*) indicates significant differences between fed and fasted groups ($P < 0.05$).

3.3. Experiment 3: Effects of insulin injection on plasma metabolites, insulin levels, and LPL activity

Body weight, length, and the weight of the mesenteric fat for rainbow trout used in the insulin injection experiment were as follows: 309.04 ± 16.38 g; 26.79 ± 0.41 cm; and 3.84 ± 0.34 g, respectively. Fish injected with insulin presented significantly lower plasma glucose levels 3 and 6 hours after the injection, compared to the control group (Figure 4).

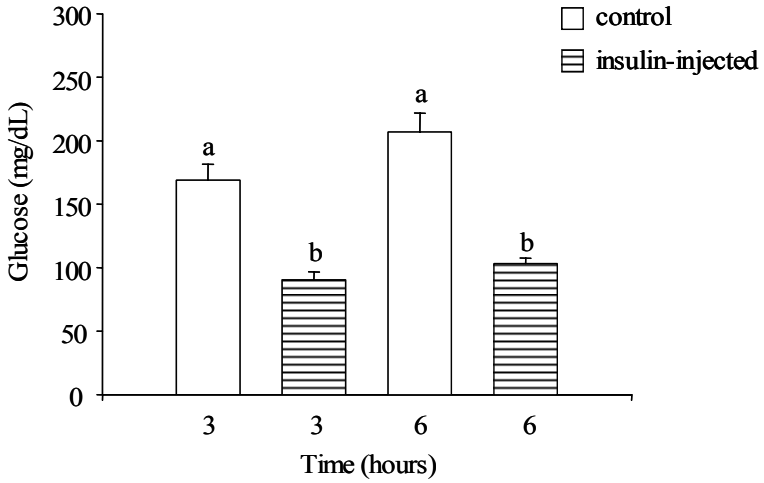

Figure 4. Effects of insulin administration *in vivo* in plasma glucose levels of rainbow trout sampled at 3 and 6 hours after injection. The number of fish sampled at each condition was n = 5-6. Values are the mean ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$).

Insulin generated significantly increased LPL activity in adipose tissue 3 hours after injection (Figure 5a). LPL activity in control-injected fish was 185.70 ± 37.12 mU/g, while in insulin-injected fish activity was 455.93 ± 121.52 mU/g. Although six hours after injection, LPL activity was still higher in insulin-injected fish compared to the control group, the difference was not significant. In red muscle, there was no response in LPL activity to insulin administration (Figure 5b). As in the post-feeding experiment, LPL activity was lower in red muscle compared to adipose tissue.

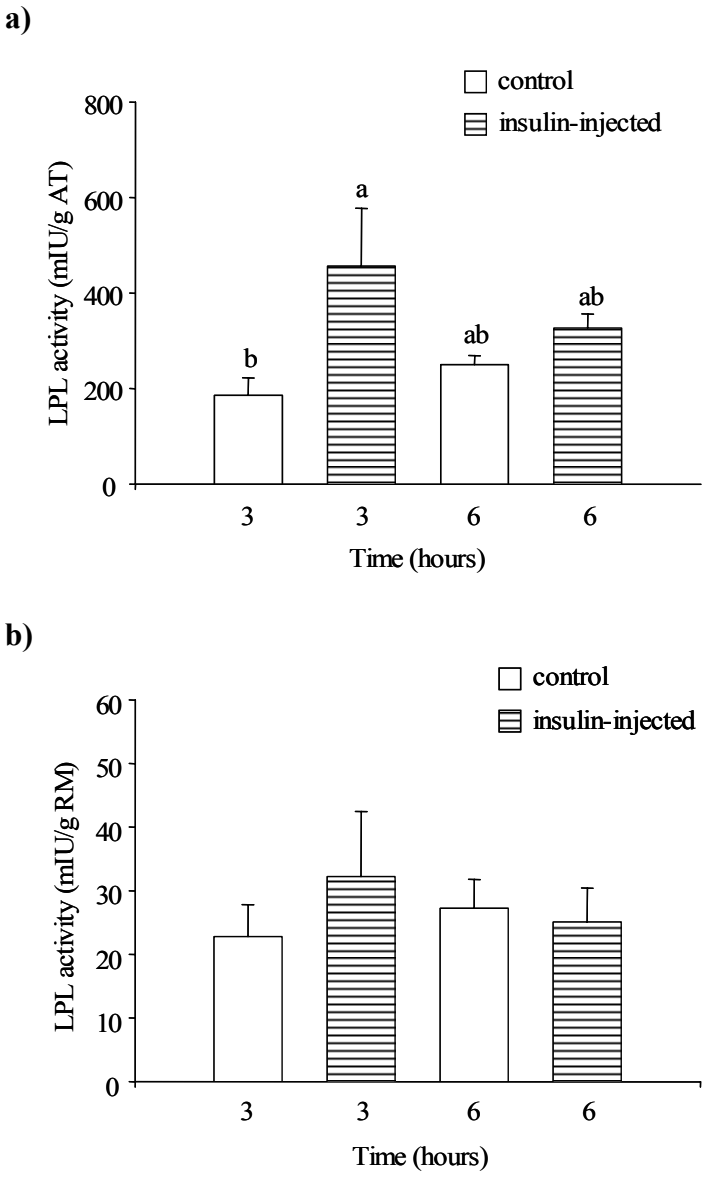

Figure 5. Response of rainbow trout lipoprotein lipase (LPL) activity of adipose tissue (a) and red muscle (b) to insulin 3 and 6 hours after injection. The number of fish sampled at each condition was n = 5-6. Values are the mean ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$).

Finally, LPL activity was measured in white muscle with no differences observed between control and insulin-injected groups (data not shown), as the activity was very low (with values ranging from 11.54 ± 0.91 to 6.44 ± 3.32 mU/g).

3.4. In vitro experiments: Effects of insulin and TNFα on different fractions of LPL activity in isolated fat pads

To evaluate the direct effects of insulin and TNFα on LPL activity, small portions of adipose tissue from rainbow trout were incubated in the absence or presence of insulin and TNFα for 4 hours. This incubation time did not affect the integrity of the fat pads, since LDH activity in the medium was similar at both the start and end of the incubation period (data not shown). As presented in Figure 6, LPL activity was higher in the NHR-LPL fraction, compared to the HR-LPL fraction. Total LPL activity was calculated by adding the values from both fractions, which were consistent with those LPL values obtained by directly measuring LPL activity in the adipose tissue homogenates from the same pool of adipose tissue (data not shown). As shown in Figure 6a, insulin increased significantly LPL activity when expressed as % of HR/Total LPL activity. While not reaching significant levels, insulin did stimulated HR-LPL activity, which is thought to represent the active form of the enzyme found at the extracellular level. Moreover, insulin tended to lower NHR-LPL activity, which is believed to represent intracellular LPL in active conformation (Ballart et al., 2003). In contrast, TNFα decreased the LPL activity measured in both fractions. Furthermore, the % of HR/Total LPL activity was also lower in TNFα–incubated fat pads, although not significantly.

4. Discussion

Absolute values for LPL activity recorded in this study correspond well with those found in rainbow trout by both Lindberg and Olivecrona (1995) and Arantzamendi et al. (2003). Adipose tissue exhibited the highest LPL activity values when compared to red and white muscles, consistent with several previous studies (Aranzamendi et al., 2003;

Lindberg and Olivecrona, 1995; Black and Skinner, 1986), and confirming the different ability of rainbow trout tissues to take up triacylglycerol from circulating lipoproteins (Black et al., 1983).

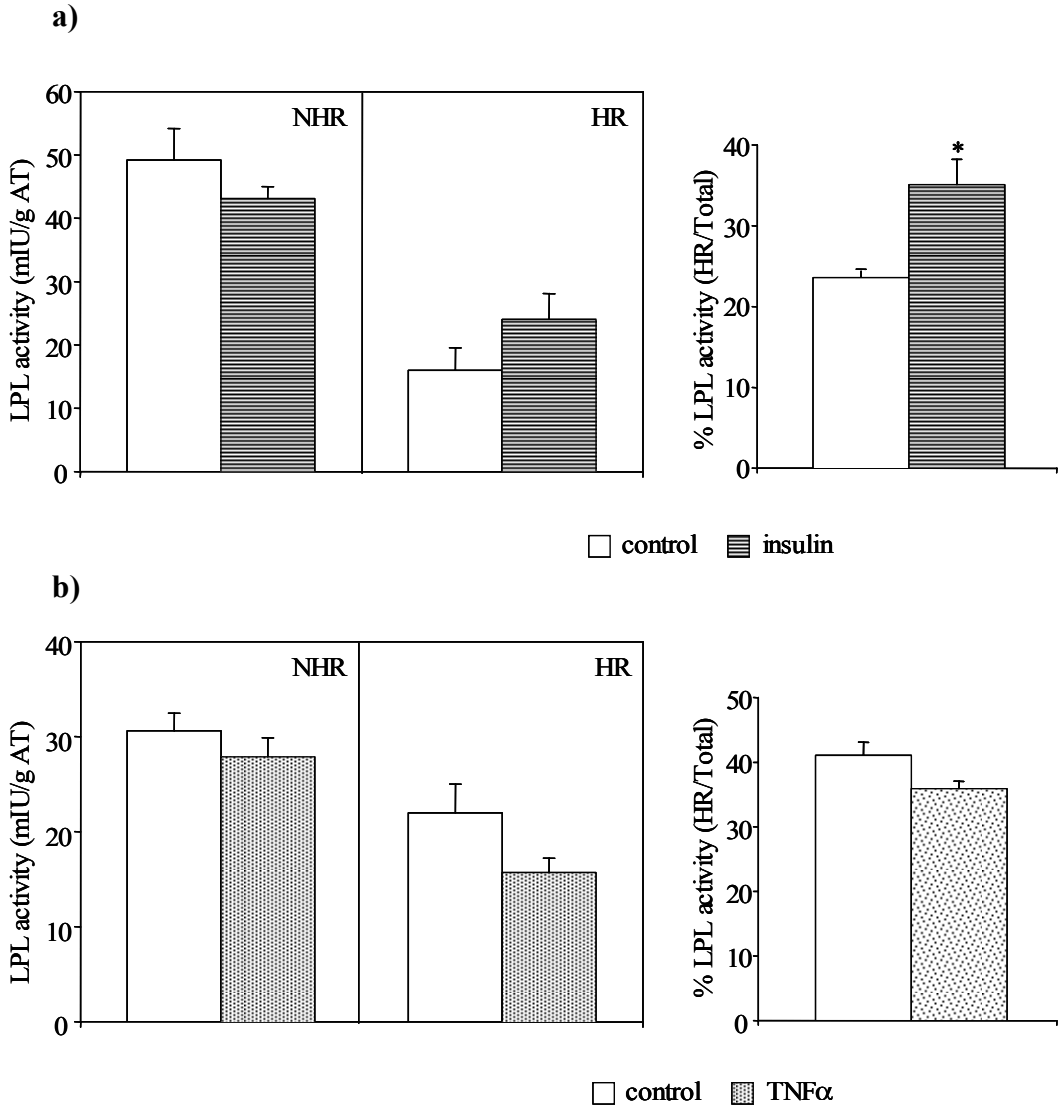

Figure 6. Effects of insulin (a) and TNFα (b) *in vitro* on HR-LPL, NHR-LPL, and % (HR/Total) of rainbow trout adipose tissue. Total LPL activity was calculated by the addition of both fractions. Values are the mean ± SEM of triplicates from 2 independent experiments. Asterisk (*) indicates significant differences between control and insulin or TNFα–treated groups ($P < 0.05$).

It is well known that following food intake, LPL activity increases in mammalian adipose tissue (Eckel, 1987). This postprandial regulation of LPL activity is mediated by insulin, which increases after meal intake and has been shown to be a necessary and sufficient condition for the tissue-specific response of LPL activity (Picard et al., 1999).

In the present study, the LPL activity of rainbow trout exhibited a demonstrable response to feeding. In adipose tissue, LPL activity increased 2-4 hours after feeding, in tandem with high insulin levels, which may trigger activation of the enzyme (Picard et al., 1999). Nevertheless, insulin levels were still high at 6 hours, while LPL activity was down-regulated, suggesting that other factors could contribute to the modulation of LPL activity in rainbow trout adipose tissue during the postprandial period. Although insulin appears to be one of the principal regulators of LPL activity in mammals, other studies have pointed out that other agents (i.e. endogenous factors from adipose tissue or postprandial metabolites) could be involved in the postprandial regulation of LPL. Within this context, Wu et al. (2004) observed increased endogenous adipose tissue TNFα levels in rats 6 hours after their last meal, in tandem with a rapid down-regulation of LPL activity in adipose tissue at this same time point. Their finding suggests that TNFα is involved in the rapid down-regulation of adipose tissue LPL activity during short-term food deprivation. Moreover, other studies have proposed that regulation of LPL activity by fatty acids could be an important event during fasting/feeding periods (Amri et al., 1996).

In regard to the other tissues studied, food intake provoked an increase in LPL activity in red muscle, synchronized with that observed in adipose tissue, although in this case, the activity level was not significant. Moreover, LPL activity in white muscle was not affected by feeding.

This lower or nil response of red and white muscles to food intake is consistent with the fact that LPL activity in mammalian muscle generally does not increase after feeding, while it is activated in catabolic conditions related to lipid use as an energy source (Borensztajn, 1987; Nikkila, 1987). Thus far, this remains the first time that LPL activity has been simultaneously measured in fish adipose tissue, and in red and white muscles in response to feeding and short-term fasting (36 hours).

Based on this tissue-specific regulation, mammalian skeletal muscle response to feeding has yielded different results. While several studies have found that feeding decreases LPL activity in muscle (Picard et al., 1999; Ladu et al., 1991; Kotlar and Borensztajn, 1977), others found no alteration in muscle LPL activity (Lanza-Jacoby et al., 1997; Ong et al., 1994). The regulation of LPL in this tissue is mediated by insulin (Picard et al., 1999), either directly or indirectly through its action on muscle glucose (Pollare et al., 1991) or fatty acid metabolism (Ferraro et al., 1993).

In the present study, the slight increase observed in trout red muscle LPL activity could stem from the fact that in rainbow trout this tissue not only contains substantial reserves of stored lipids (review by Sheridan, 1988), but is also a metabolically more active tissue in terms of lipid deposition (Robinson and Mead, 1973) compared to white muscle. Similar results have been described in mammals where, although there is no physical separation of red and white muscles, LPL activity has been shown to differ depending on the muscle fiber type. In this sense, LPL activity in mammalian muscle is greater in highly oxidative or 'red' muscle fibers than in less oxidative or 'white' muscle fibers (reviewed by Cortright et al., 1997).

During fasting, fish adipose tissue metabolism is directed to increase lipolytic activity (Albalat et al., 2005b), thereby inhibiting lipid uptake and LPL activity. While earlier studies described a clear down-regulation of adipose tissue LPL activity in rainbow trout due to long term fasting (8 weeks), no effect was observed in red muscle (Black and Skinner, 1986). Those results were confirmed in the present study, since LPL activity decreased in the trout adipose tissue of fish fasted for 3 weeks. This decrease in adipose tissue LPL activity during fasting would allow such vital organs or tissues as red muscle and heart to continue using lipids as an energy source to maintain their vital functions.

The observed down-regulation of LPL activity in adipose tissue under fasting conditions was accompanied by a clear reduction of plasma insulin levels. This decline during food deprivation has been well documented (Navarro et al., 1992; Sundby et al., 1991) in fish, and reflects the need for maintaining homeostasis, reducing nutrient storage, and mobilizing reserves. This decrease in plasma insulin levels in fasted rainbow trout suggests that this hormone could also play an important role in the down-regulation of adipose tissue LPL activity in fasted fish. In mammals, however, LPL activity is regulated by other hormones, like glucagon (Borensztajn et al., 1973). Therefore, it is important not to discard the possibility that other endocrine factors may also be involved in these observed effects during the fasting process.

This potentially positive role of insulin on adipose tissue LPL activity in fish would be consistent with the anabolic function this hormone plays in mammals and fish. In this context, the antilipolytic actions of insulin have been observed during incubations of salmon liver and adipose tissue pieces (Harmon and Sheridan, 1992), as well as in rainbow trout isolated adipocytes (Albalat et al., 2002). To better evaluate whether insulin acts as a regulator of LPL activity in fish, it was administrated

intraperitoneally. Insulin administration induced hypoglycemia, as previously described (Mommsen and Plisetskaya, 1991), thus demonstrating the effectiveness of hormone administration in our experiment. Interestingly, while insulin increased LPL activity in adipose tissue 3 hours after the injection, no effects were observed in red and white muscle. These results are consistent with those obtained in the postprandial setting, wherein adipose tissue LPL response was much more evident than in muscle.

In humans, acute insulin administration produced increased LPL activity in adipose tissue and decreased LPL activity in skeletal muscle (Farese et al., 1991); similar results were reported in rats (Gorski and Stankiewick-Choroszucha, 1982). Based on the results of our study, it appears not only that the increased LPL activity observed in trout adipose tissue permits the preferential direction of lipoproteins to this tissue for storage, but also that the regulation of LPL in muscle is not directly mediated by insulin levels.

The next step was to further explore whether insulin had a direct effect on LPL activity in adipose tissue, as suggested by the results from our *in vivo* experiment. To this end, we used adipose tissue slices as an *in vitro* model.

Incubation of fat pads has already been utilized in mammals to study the regulation of LPL activity (Ballart et al., 2003; Ueki et al., 1993), and in fish to study the hormonal regulation of lipolysis (Harmon and Sheridan, 1992). The *in vitro* results presented in this study demonstrated the direct effects of insulin on LPL activity, expressed as the percentage of heparin-releasable LPL activity vs. total activity. Other studies have distinguished between heparin and non-heparin releasable LPL activity (Ballart el al., 2003; Rosato et al., 1997) being the latter fraction the one with highest values in agreement with the results obtained from the present work. The difference between the two fractions is the location of the LPL enzyme. Heparin-releasable LPL is thought to represent the active form of the enzyme found at pericellular and extracellular levels (on adipose tissue cell surfaces, or on the adipose tissue extracellular matrix), while the non-heparin releasable fraction is regarded as the active form of LPL located at the intracellular level (Ballart et al., 2003). In our experiments, insulin increased the proportion of active LPL at the extracellular level, where functionality actually develops, vs. total LPL activity. Similar results were obtained by Apple and Fried (1992) in cultured human tissue fragments, whereby insulin increased both HR-LPL activity and total LPL activity. Additional studies (Rosato et al., 1997) found that insulin stimulated HR-LPL activity of adipocytes from control and septic rats. In this

particular case, however, adipocytes were employed instead of fad pads, and the incubation time with insulin was longer (20 hours).

Finally, the possible role of TNFα on LPL activity was analyzed using this same *in vitro* model. While in the case of TNFα no significant effects were observed, a tendency towards lower heparin-releasable LPL activity was noted in fat pads incubated with TNFα compared with control samples. In fact, the results from several studies suggested that this cytokine would effect a higher down-regulation: (a) In mammals, TNFα was found to decrease LPL activity in the adipose tissue of several species (Fried and Zechner, 1989; Semb et al., 1987); (b) LPS was shown to decrease LPL activity in the adipose tissue of both rainbow trout (Albalat et al, 2005a) and mammals (Bagby and Pekala, 1987), an effect thought to be mediated by TNFα (MacKenzie et al., 2003). Based on these findings, it was reasonable to expect TNFα to exert a negative effect on adipose tissue LPL activity. However, since a tendency towards lower HR-LPL activity was observed, additional studies should be conducted (i.e. longer incubation periods) to ensure that TNFα has no effect on adipose tissue LPL activity.

In summary, LPL activity in rainbow trout is regulated in a tissue-specific manner by feeding condition. LPL activity in adipose tissue increased in response to feeding, while only a very low response was observed in red muscle. Furthermore, LPL activity in adipose tissue was down-regulated in fasted animals. In these settings, changes in plasma insulin levels suggested that this hormone could act as a positive regulator of LPL activity in adipose tissue, although other factors may also be involved.

The *in vivo* administration of insulin, as well as the *in vitro* results obtained in this study, confirmed the positive role of insulin on LPL activity of adipose tissue. Moreover, LPL activity in red muscle was not affected by insulin administration, suggesting that in this tissue such activity is not regulated directly by insulin. Finally, under our experimental conditions, TNFα did not significantly alter LPL activity *in vitro*. However, more research is needed to determine whether this particular cytokine, or indeed other possible negative regulators of LPL activity, is at work in the adipose tissue of fish.

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Regulació per la insulina de l'activitat i l'expressió de la lipoproteïna lipasa (LPL) en l'orada (*Sparus aurata*)

Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (*Sparus aurata*)

Regulació per la insulina de l'activitat i l'expressió de la lipoproteïna lipasa (LPL) en l'orada (*Sparus aurata*)

Resum

La lipoproteïna lipasa (LPL) és un enzim important en el metabolisme lipídic mitjançant la seva capacitat d'hidrolitzar els triacilglicerols que circulen en forma de lipoproteïnes. En peixos, la informació sobre la regulació nutricional de la LPL i els mecanismes hormonals implicats en aquesta regulació és molt reduïda. En aquest estudi, hem analitzat la regulació nutricional de la LPL en l'orada (*Sparus aurata*) i hem estudiat el paper de la insulina com a possible modulador de l'activitat i l'expressió de la LPL valorats simultàniament per primer cop en peixos.

El dejuni de 2 setmanes va provocar una disminució de l'activitat LPL en teixit adipós juntament amb una baixada de la insulina plasmàtica, mentre que no es van observar canvis en l'activitat LPL en el múscul vermell. Per tal d'elucidar el possible paper de la insulina, es va provocar experimentalment una hiperinsulinèmia mitjançant una injecció d'arginina i d'insulina. Tot i això, l'arginina va estimular de forma predominant la secreció de glucagó enfront la secreció d'insulina en aquesta espècie i l'activitat LPL en el teixit adipós no va canviar significativament. De forma alternativa, l'administració *in vivo* d'insulina va donar lloc a un augment en l'activitat LPL 3 hores després de la injecció, mentre que l'activitat no va variar en el múscul vermell. Els canvis observats en l'activitat LPL en el teixit adipós van anar acompanyats per un augment dels nivells d'ARNm, tot i que els canvis en l'expressió de l'enzim foren més lents. En el múscul vermell, no es van observar canvis en els nivells d'ARNm de la LPL suggerint que en aquest teixit la insulina no juga un paper directe en la regulació de la LPL.

Aquest estudi mostra que l'activitat LPL és regulada nutricionalment i indica la importància de la insulina com a modulador de l'activitat i l'expressió de la LPL en el teixit adipós de l'orada.

Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (*Sparus aurata*)

A. Albalat[1], A. Saera-Vila[2], J. Gutiérrez[1], J. Pérez-Sánchez[2] and I. Navarro[1]

[1]Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

[2]Instituto Acuicultura Torre de la Sal (CSIC), Castellón, Spain

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Please address all correspondence to I. Navarro

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Fax: 34+ 93+ 4110358

Email: mnavarro@ub.edu

Abstract

Lipoprotein lipase (LPL) is a key enzyme in lipoprotein metabolism by its capacity to hydrolyze triglycerides circulating in the form of lipoprotein particles. In fish, very little information is available about the nutritional regulation of LPL and the hormonal mechanisms underlying LPL regulation. In the present study, we have analyzed the nutritional regulation of LPL in gilthead sea bream (*Sparus aurata*) and we have studied the possible role of insulin as a potential modulator of LPL activity and expression for the first time in fish.

Fasting for 2 weeks provoked a clear decrease in adipose tissue LPL activity concomitant with lower levels of plasma insulin while no effects were observed in red muscle. In order to elucidate the specific role of insulin, hyperinsulinemia was experimentally induced by arginine and insulin injections. However, arginine stimulated predominantly glucagon over insulin secretion in this fish species and LPL activity did not change significantly in adipose tissue. Instead, insulin administration induced an increase in adipose tissue LPL activity 3 hours after the injection, whereas LPL activity in red muscle was not affected. Changes in LPL activity were accompanied by an increase in LPL mRNA levels in adipose tissue of insulin-injected gilthead sea bream, although changes in LPL expression were slower than variations in LPL activity. Finally, LPL mRNA levels in red muscle were similar between control and insulin-injected gilthead sea bream suggesting that insulin does not play a direct role on the regulation of LPL in this tissue.

The current study shows that LPL activity is regulated by nutritional condition and points out the importance of insulin as a modulator of LPL activity and expression in adipose tissue of gilthead sea bream.

1. Introduction

Lipoproprotein lipase (LPL) is a glycoprotein enzyme that in mammals is produced in several tissues, such as adipose tissue, skeletal muscle, heart, macrophages, lactating mammary gland, but not in the adult liver (Garfinkel and Schotz, 1987). Once LPL is synthesized, the enzyme is secreted and transferred to the luminal surface of the capillary endothelial cells where it actually develops its function (Camps et al., 1990; Auwerx et al., 1992). LPL hydrolyzes triglycerides circulating in the form of chylomicrons and very low density lipoproteins into free fatty acids (FFA) and 2-monoacylglycerols. The resulting FFA can be utilized by different tissues, like adipose tissue, where FFA are re-esterified and stored or by other peripheral tissues, such as muscle and heart where FFA can be used as energy source (Auwerx et al., 1992). For these reasons LPL plays a central role in lipid metabolism.

In fish, LPL has been identified at molecular level in different species, including zebrafish (*Brachydanio rerio*) (Arnault et al., 1996), rainbow trout (*Oncorhynchus mykiss*) (Arnault et al., 1996; Lindberg and Olivecrona, 2002) and red sea bream (*Pagrus major*) (Oku et al., 2002). In these species, partial or complete gene sequences have been identified. Furthermore, LPL expression has been measured in a variety of tissues of rainbow trout (Lindberg and Olivecrona, 2002), red sea bream (Liang et al., 2002) and recently, in gilthead sea bream (*Sparus aurata*) (Saera-Vila et al., 2005). Very few studies have measured LPL activity in fish. So far, LPL activity has been measured in adipose tissue, red and white muscles, heart, brain and vitellogenic ovaries of rainbow trout (Black et al., 1983; Black and Skinner, 1986; Black and Skinner, 1987; Lindberg and Olivecrona, 1995). Furthermore, and contrary to the situation in mammals, LPL activity and expression has also been detected in liver of adult fish (Black et al., 1983; Liang et al., 2002; Saera-Vila et al., 2005).

In mammals, LPL is regulated in a high degree by nutritional condition and in a tissue-specific manner. LPL activity is down-regulated during fasting in adipose tissue (Lladó et al., 1999; Bergö et al., 1996) while it does not change or increase in skeletal muscle and heart (Sudgen et al., 1993; Ruge et al., 2005). In mammals, the main modulator of LPL is insulin. The action of insulin appears to be different depending on the tissue. In this sense, insulin stimulates directly the LPL activity in adipose tissue, shown by the *in vivo* and the *in vitro* studies (Picard et al., 1999; Ong et al., 1988) while

the insulin action decreases the activity in skeletal muscle of humans and rats (Farese et al., 1991; Gorski and Stankiewick-Choroszucha, 1982).

The enzyme activity can be regulated at different levels through pre- and post-translational mechanisms (Doolittle et al., 1990; Ladu et al., 1991). During fasting LPL mRNA levels decrease in adipose tissue, whereas it increases in skeletal muscle, showing a reciprocal regulation of the mRNA levels between both tissues, in parallel to the changes observed in LPL activity (Enerbäck et al., 1989; Lee et al., 1998). However, in mammals, regulation of LPL appears to be very complex and LPL activity, mRNA levels and the enzyme mass have not always been found to change in the same direction (Doolittle et al., 1990; Bergö et al., 1996). The mechanism by which insulin stimulates LPL activity in adipose tissue has been mostly attributed to positive effects of insulin on mRNA stability, thus increasing mRNA levels (Raynolds et al., 1990) and changes at post-translational level (Semenkovich et al., 1989) rather than increases in LPL gene expression (Vydelingum et al., 1983).

In fish, studies related to the regulation of LPL are scarce and none of them have looked at LPL activity and LPL expression at the same time. Nevertheless, it seems that LPL in fish is also regulated by nutritional condition. Black and Skinner (1986) and Albalat et al. (2005a) found a down-regulation of adipose tissue LPL activity due to fasting in trout while no changes were observed in red muscle. Similarly, Liang et al. (2002) found lower LPL mRNA levels in adipose tissue of red sea bream fasted for 48 hours. More recently, one study in gilthead sea bream (*Sparus aurata*) has shown variations in adipose tissue LPL expression on the basis of changes in the dietary protein sources. In that study, LPL expression was lower in adipose tissue of gilthead sea bream fed with a plant protein diet compared with fish fed with a fish meal protein diet indicating that diet composition can affect LPL expression in gilthead sea bream (Saera-Vila et al., 2005). However, there is very little information about the possible hormonal regulation underlying this nutritional regulation of LPL activity or expression in fish. In this sense, recently, we have found a stimulatory effect of insulin on LPL activity in adipose tissue of rainbow trout (Albalat et al., 2005a), suggesting that in fish, insulin could play a similar role in the regulation of LPL activity as the one described in mammals.

The objective of the present study was to study the nutritional regulation of LPL activity in gilthead sea bream and assess the possible role of insulin as a modulator of LPL activity and expression. To this end, LPL activity was measured in adipose tissue

and red muscle of fed and fasted gilthead sea bream. Furthermore, in order to evaluate the importance of insulin in this regulation, hyperinsulinemia was experimentally induced by *in vivo* administrations of arginine (a potent insulin secretagoge in fish) and insulin. The role of insulin was studied in terms of changes in LPL activity and expression simultaneously for the first time in fish.

Results indicate that LPL activity in gilthead sea bream is nutritionally regulated in a tissue-specific manner and presents insulin as an important activator of LPL in adipose tissue, by increasing LPL activity and LPL mRNA levels.

2. Materials and Methods

2.1. Animals and experimental conditions

Gilthead sea bream (*Sparus aurata*) for fasting and *in vivo* insulin administration experiments were obtained from the 'Centre d'Aqüicultura-IRTA de Sant Carles de la Ràpita', Tarragona, Spain and acclimated for 15 days to laboratory conditions in our facilities at the University of Barcelona, Spain. Fish were kept under a light regime of 12-h light, 12-h dark photoperiod in a closed-water flow system with water temperature of 22 ± 1ºC (experiments were performed in July). For the *in vivo* arginine administration experiment, gilthead sea bream were acclimated to laboratory conditions at the 'Instituto de Acuicultura de Torre de la Sal, CSIC', Castellón, Spain. Fish were kept under natural conditions of light (experiment was performed in September), latitude (40º 5' N; 0º 10' E) and water temperature (23-25ºC).

2.1.1. Fasting experiment: Gilthead sea bream of 125 g initial body mass were separated into two groups (n=6 in fed group and n=7 in fasted group): one group was fed daily in one meal per day (10:00h) to apparent visual satiety using a commercial diet (fed group) and the other was deprived of food for 2 weeks (fasted group). On the day of the experiment, control fish (sampled 24 h after the last meal) and fasted fish were killed and blood and tissue samples were collected.

2.1.2. In vivo injection of arginine: Gilthead sea bream of 179 g body mass were separated into two groups: control and arginine-injected (20 fish for each group, separated in two tanks). Following an overnight fasting, fish were anaesthetised with

MS-222 (0.1 g/l) and received a single intraperitoneal injection (volume injected 500 μl) of vehicle (saline solution) or arginine (6.6 μmol/g body mass). After 3 and 6 hours, fish from control and arginine-injected groups were killed, and blood and tissue samples were taken.

2.1.3. In vivo injection of insulin: Gilthead sea bream (n = 20) of 126 g body mass were separated into two groups: control and insulin-injected (10 fish in each group). On the day of the experiment, fish were anaesthetised with MS-222 (0.1 g/l) and received a single intraperitoneal injection (1 μl/g body mass) of vehicle (saline solution) or porcine insulin (2.16 nmol/100 g body mass). Fish from control and insulin-injected groups were killed after 3 and 18 hours and blood and tissue samples were taken.

In all experiments, fish were killed with a sharp blow to the head, and blood samples were taken by caudal puncture using heparinized syringes. Fish were weighed, and adipose tissue and red muscle were rapidly extracted and frozen in liquid nitrogen. Finally, liver was weighed, blood samples were centrifuged at 3,000g for 20 min at 4°C and different aliquots of plasma were kept at -80°C until the day of the analysis.

2.2. Lipoprotein lipase assay

Portions of adipose tissue, as well as red muscle, from each fish were homogenized in 9 volumes of homogenization buffer (10 mM HEPES, 1 mM EDTA and 1 mM DTT) at pH 7.4 and containing 5 U/ml of heparin. Homogenates were centrifuged at 36,700g at 4°C for 20 min and the clear intermediate phase (between the fat droplets and pellet) was used for LPL activity assays. LPL activity measurements were conducted with 10% Intralipid, labelled with tri[9, 10-^{3}H]oleoglycerol by sonication in ice. The assay mixture (total volume 200 μl) contained 10 μl of the labelled intralipid, 5% (vol/vol) pre-heated rat serum as apo C-II source, 0.1 M NaCl, 0.15 M Tris-HCl, heparin (0.02%), bovine serum albumin (BSA, 60 mg/ml), and 25 μl of sample. Following 2 h incubation at 25°C, the reaction was stopped by the addition of 2 ml isopropanol/heptane/1M H_2SO_4 (40:48.3:1) and 0.5 ml water, with free fatty acids extracted as previously described (Bengtsson-Olivecrona and Olivecrona, 1991). For each sample, four replicates were measured and activities were expressed as mU/g adipose tissue, based on the calculation that 1 mU is equivalent to 1 nmol of fatty acid released per min.

2.3. LPL expression

Measurements of LPL transcripts of gilthead sea bream tissues (adipose and red muscle) from the insulin administration experiment were made by means of a real-time PCR (SYBR Green I), using ß-actin as housekeeping gene. Total RNA was isolated using the ABI Prism 6100 Nucleid Acid prepStation reagents, and cDNA was synthesized using ABI High Capacity Archive kit reagents (Applied Biosystems, Foster City, CA). PCR amplification and analysis were carried out with the iCycler iQTM Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Specific primers for gilthead sea bream LPL and ß-actin were made as described elsewhere (Calduch-Giner et al., 2003; Saera-Vila et al., 2005). The final volume of PCR reactions was 25 µl, using IQ SYBR Green Supermix (Bio-Rad) and specific primers at a final concentration of 0.9 µM. The real-time PCR protocol consisted of 10 min at 95ºC followed by 40 cycles of 15 sec at 95ºC and 1 min at 60ºC. Standard curves were generated by amplification of serial dilutions of known quantities of recombinant plasmids for each gene of interest. The dynamic range of standard curves spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value. Reactions were performed in triplicate, and Ct numbers were averaged. For the target and reference gene, the efficiency of the PCR reaction (90-99%) was the same for serial dilutions of standards and RT reactions. For both analysed genes, the specificity of amplified products was verified by the analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Fluorescent data, acquired during the extension phase, were analyzed with the iCyclerTM iQ Optical System Software Version 3.0a (Bio-Rad) and converted into Ct values. Each transcript level was normalized to b-actin gene using the delta-delta method (Livak and Schmittgen, 2001).

2.4. Biochemical analyses of plasmatic parameters

Plasma glucose concentration was determined by the glucose oxidase colorimetric method (GLUCOFIX; Menarini Diagnostics, Firenze, Italy) (Huggett and Nixon, 1957; Sala-Rabanal et al., 2003). Plasma free fatty acids were analyzed using a commercial

enzymatic method (NEFA-C, Wako Test). Plasma insulin levels were measured by radioimmunoassay (RIA) using bonito insulin as a standard and rabbit anti-bonito insulin as an antiserum (Gutiérrez et al., 1984). Glucagon levels were analyzed by mammalian RIA, previously validated for several species including gilthead sea bream (Gutiérrez et al., 1986; Navarro et al., 1995).

2.5. Statistical analysis

Data are reported as mean values ± standard error of mean (S.E.M.). Differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test in the cases that variances were homogeneous (Levene's test was used for the study of the homogeneity of variances), and otherwise by Games-Howell test. Results from fasting experiment were analyzed by paired t-test (Student's t-test). P-values lower than 0.05 were considered significantly different.

3. Results

3.1. Effects of fasting on LPL activity in gilthead sea bream

Fasting for 2 weeks produced a significant decrease in liver weight and hepatosomatic index (HSI) as shown in Table 1. Glycemia levels were similar between fed and fasted groups while FFA increased significantly in fasted group. Finally, plasma insulin levels were about 4-fold lower in fasted group.

In this experiment LPL activity was measured in adipose tissue and red muscle. Absolute values of LPL activity were slightly higher in adipose tissue (LPL activity in fed group 17.58 ± 3.85 mU/g) compared to red muscle (LPL activity in fed group 13.63 ± 1.32 mU/g). As shown in Figure 1A, LPL activity decreased approximately 70% in adipose tissue of fasted fish compared to fed fish. On the other hand, no differences were found in LPL activity in red muscle from fed and fasted groups (Figure 1B), that was very similar in both groups.

Table 1. Morphological and plasmatic parameters of the gilthead sea bream used in the fasting experiment. Values are the mean ± SEM (n=6 in fed and n=7 in fasted groups). Asterisk (*) indicates significant differences between fed and fasted groups ($P < 0.05$; t-student).

	Fed	Fasted
Final body mass (g)	122.78 ± 6.63	116.27 ± 7.53
Length (cm)	17.58 ± 0.35	17.14 ± 0.34
Liver (g)	1.48 ± 0.11	0.84 ± 0.12*
Mesenteric fat (g)	0.85 ± 0.13	0.57 ± 0.11
HSI (%)[1]	1.22 ± 0.09	0.72 ± 0.09*
MFI (%)[2]	0.71 ± 0.12	0.56 ± 0.08
CF (%)[3]	2.25 ± 0.06	2.29 ± 0.05
Plasma parameters		
Glucose (mg/dl)	128.09 ± 9.26	113.22 ± 3.10
FFA (meq/l)	0.52 ± 0.07	0.87 ± 0.12*
Insulin (ng/ml)	5.02 ± 1.61	1.23 ± 0.57*

[1] Hepatosomatic index = (liver wt./fish wt.) x 100
[2] Mesenteric fat index = (mesenteric fat wt./fish wt.) x 100
[3] Condition factor index = (fish wt./(fish length)^3) x 100

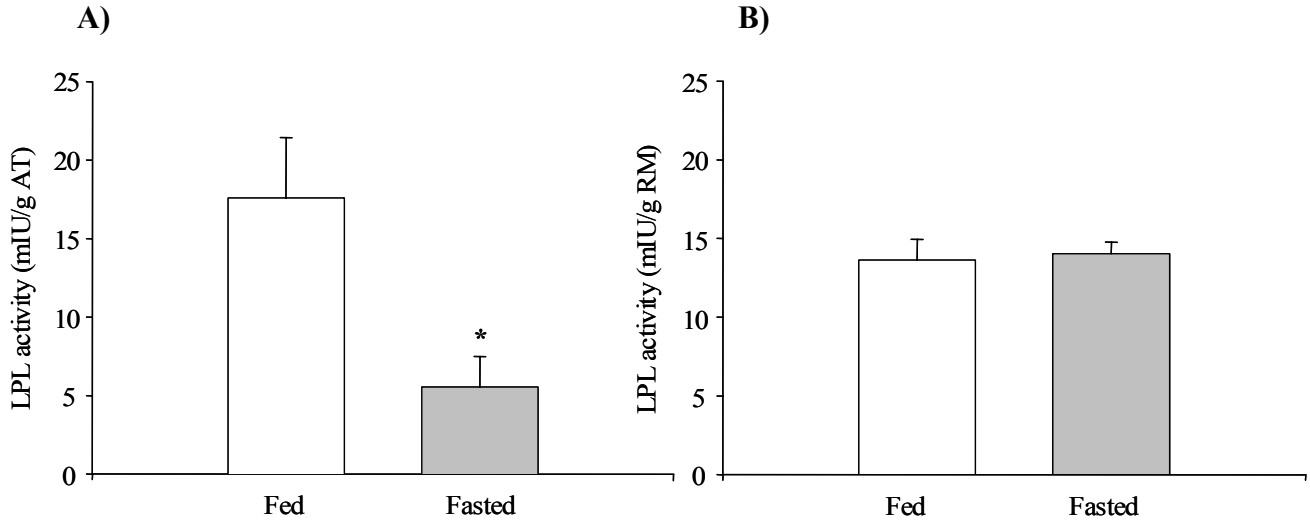

Figure 1. Effects of fasting on LPL activity in adipose tissue (A) and red muscle (B) of gilthead sea bream. Each sample was analysed in tetraplicates. Values are the mean ± SEM (n=6 in fed and n=7 in fasted groups). Asterisk (*) indicates significant differences between fed and fasted groups ($P < 0.05$; t-student).

3.2. Effects of an intraperitoneal injection of arginine on LPL activity in gilthead sea bream

Gilthead sea bream used in the *in vivo* administration of arginine had a body mass and mesenteric fat mass of: 179.23 ± 4.79 g and 1.90 ± 0.12 g, respectively. Plasma insulin levels increased significantly in arginine-treated fish 6 h after the injection concomitant with a decrease in FFA (Table 2). Besides, glycemia levels increased more than 3-fold in arginine-injected fish 3 h after the injection and levels were still higher after 6 h. A similar pattern to that of glucose was observed in plasma glucagon (Table 2).

Table 2. Plasma levels of insulin, glucagon, glucose and FFA of gilthead sea bream sampled 3 and 6 hours after a saline (control group) or arginine injection (arginine group). The number of fish sampled at each condition was n=10. Values are the mean ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$).

	Control 3h	Arginine 3h	Control 6h	Arginine 6h
Insulin (ng/ml)	1.67 ± 0.17^{ab}	2.91 ± 0.54^{a}	1.55 ± 0.13^{b}	2.91 ± 0.38^{a}
Glucagon (ng/ml)	2.58 ± 0.41^{b}	5.62 ± 1.01^{a}	1.96 ± 0.25^{b}	3.88 ± 0.8^{a}
Glucose (mg/dl)	93 ± 10.26^{c}	309.4 ± 15.16^{a}	72.6 ± 3.07^{c}	160.8 ± 30.77^{b}
FFA (mEq/l)	0.51 ± 0.04^{ab}	0.48 ± 0.06^{b}	0.68 ± 0.02^{a}	0.36 ± 0.02^{b}
Ratio glucagon/insulin	2.94 ± 0.64	3.80 ± 0.73	2.14 ± 0.22	2.53 ± 0.49

LPL activity in adipose tissue of arginine-injected fish was similar to control fish 3 h after the injection. At 6 h, a slight increase of LPL activity in arginine-treated group was found although this increase was not significant as presented in Figure 2.

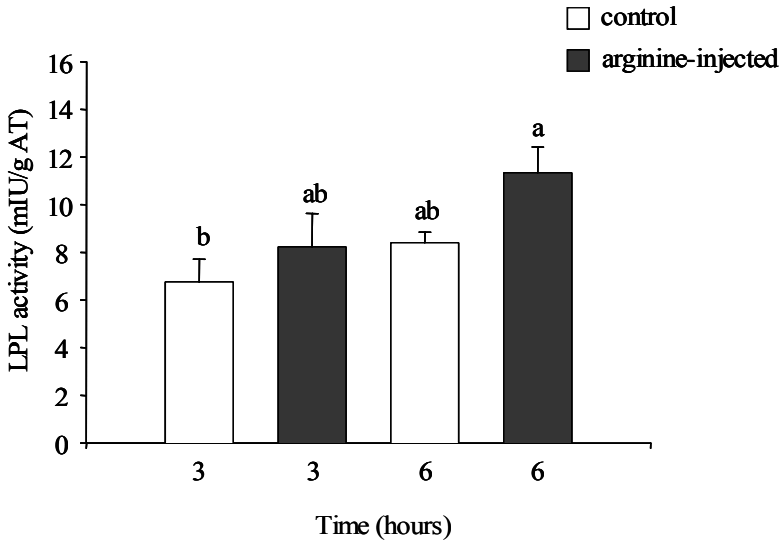

Figure 2. LPL activity in adipose tissue of control and arginine-injected gilthead sea bream. Animals were sampled 3 and 6 hours after the injection. Each sample was analysed in tetraplicates. Values are the mean ± SEM. (n=10 for each condition). Values not sharing a common letter are significantly different ($P < 0.05$).

3.3. Effects of an intraperitoneal injection of insulin on LPL activity in gilthead sea bream

Gilthead sea bream used in the *in vivo* administration of insulin had a body mass and mesenteric fat mass of: 126.30 ± 3.85 g and 0.92 ± 0.10 g, respectively. Plasma glucose levels were similar between control and insulin-injected groups while FFA decreased in insulin-injected group 3 h after the injection being significantly different compared to insulin-injected gilthead sea bream 18 h after the injection (Table 3).

Table 3. Effects of an *in vivo* administration of insulin in plasma glucose and FFA levels in gilthead sea bream sampled 3 and 18 hours after a saline (control group) or insulin injection (insulin group). The number of fish sampled at each condition was n=5. Values are the mean ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$).

	Control 3h	Insulin 3h	Control 18h	Insulin 18h
Glucose (mg/dl)	79.77 ± 2.44	78.40 ± 3.01	78.84 ± 4.56	80.51 ± 2.31
FFA (mEq/l)	0.294 ± 0.04^{ab}	0.183 ± 0.02^{b}	0.308 ± 0.04^{ab}	0.344 ± 0.05^{a}

As shown in Figure 3A, LPL activity in adipose tissue of insulin-injected fish was over 3-fold higher than LPL activity of control group. LPL activity of insulin-injected group decreased to basal levels 18 h after the injection and no differences were found between control and insulin-injected groups 18 h after the injection.

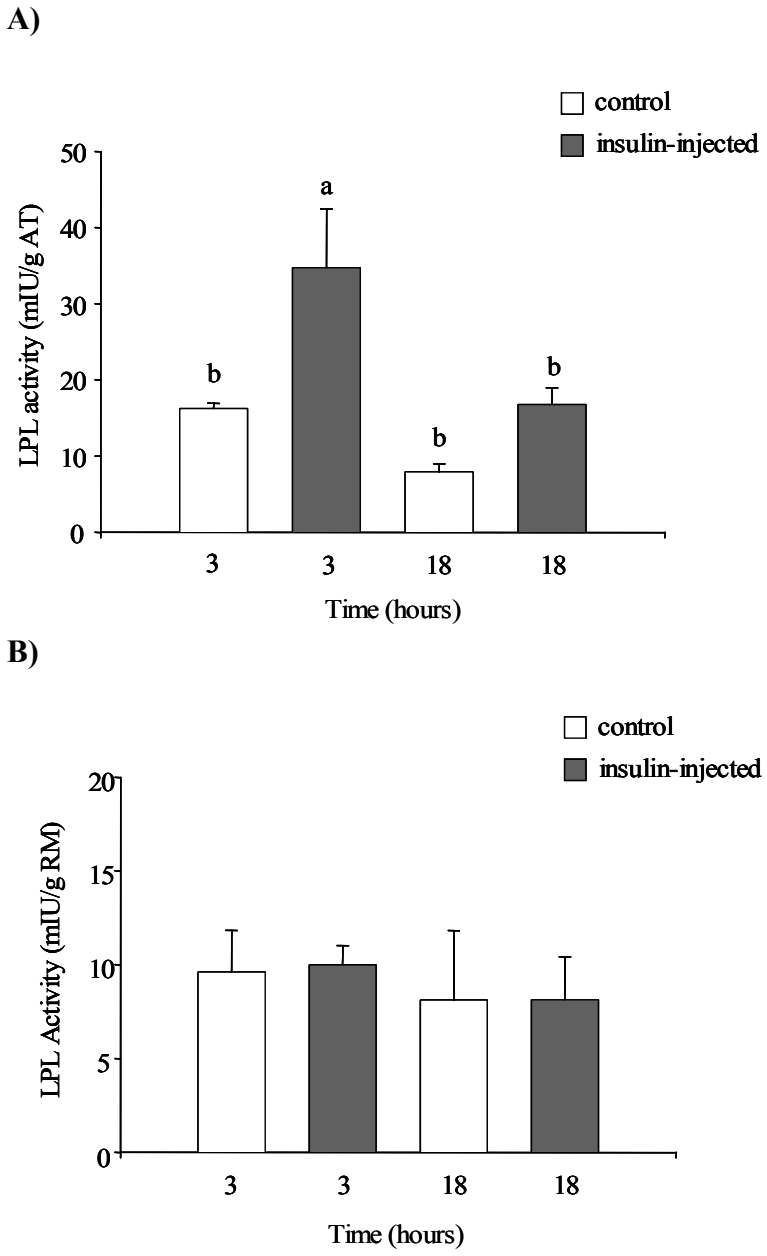

Figure 3. LPL activity in adipose tissue: AT (A) and red muscle: RM (B) of control and insulin-injected rainbow trout. Animals were sampled 3 and 18 hours after the injection. Each sample was analysed in tetraplicates. Values are the mean ± SEM. (n=5 for each condition). Values not sharing a common letter are significantly different ($P < 0.05$).

In red muscle, no differences were obtained between control and insulin-injected groups at the sampling times studied (Figure 3B). In general, and similarly to the results

obtained in the fasting experiment, LPL activity was higher in adipose tissue compared to red muscle in most of the groups, taking into account the absence of stimulation on LPL activity in red muscle.

Insulin administration provoked an increase in LPL expression in adipose tissue at both sampling times being significant 18 h after the injection. On the other hand, in red muscle, LPL expression was not affected by insulin administration neither at 3 h nor at 18 h.

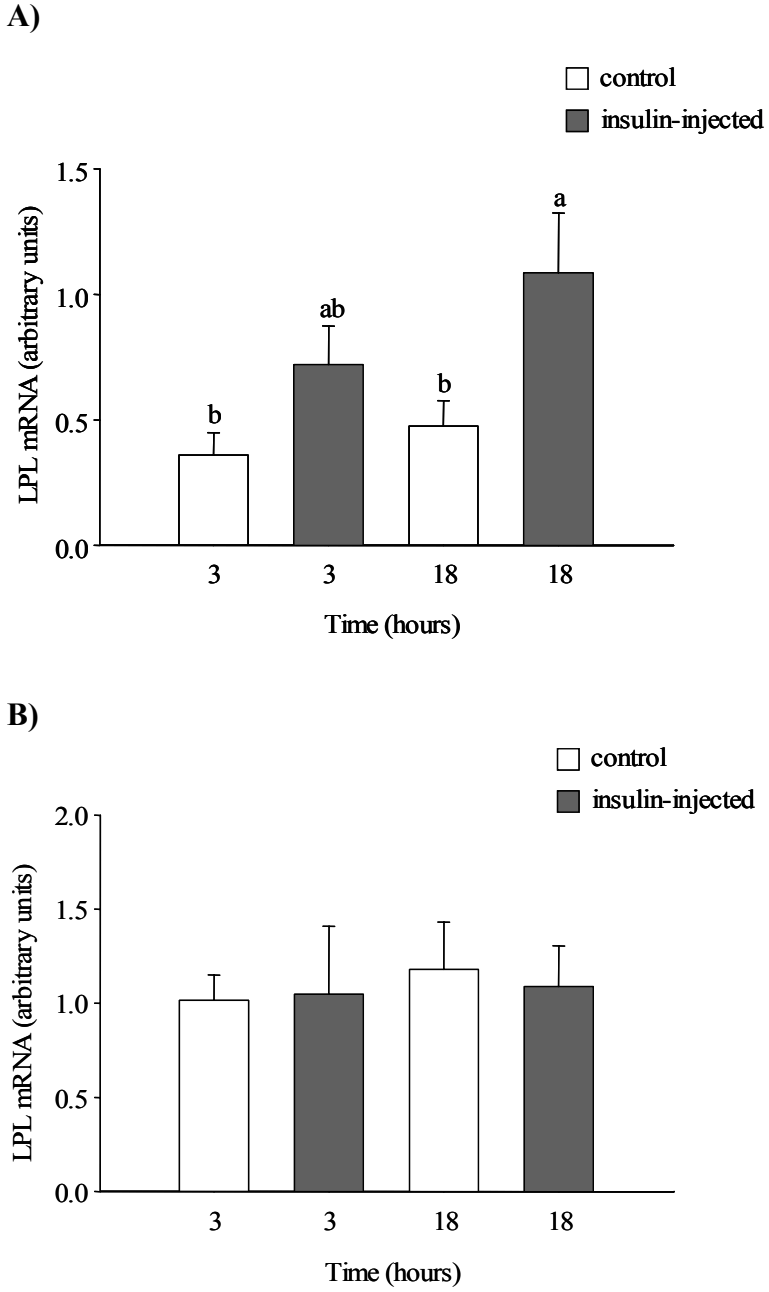

Figure 4. Expression analysis (arbitrary units) of LPL in adipose tissue (A) and red muscle (B) of control and insulin-injected rainbow trout. Animals were sampled 3 and 18 hours after the injection. Reactions were performed in triplicates. Values are the mean ± SEM. (n=5 for each condition). Values not sharing a common letter are significantly different ($P < 0.05$).

4. Discussion

This paper describes for the first time, in a fish species, that LPL activity and expression in adipose tissue are regulated by insulin. Absolute values of LPL activity in adipose tissue obtained in the present study are lower than those previously reported in rainbow trout using the same procedure (Albalat et al., 2005a) and similar to those described in an initial study in gilthead sea bream by Arantzamendi et al (2003). Furthermore, LPL activity in adipose tissue was higher compared to red muscle indicating the different ability of gilthead sea bream tissues to take up triacylglycerol from circulating lipoproteins, a characteristic already shown in trout (Black et al., 1983; Black and Skinner, 1986; Albalat et al., 2005a). In white muscle, LPL activity was not measured because activity values in rainbow trout (Albalat et al., 2005a) and preliminary results in gilthead sea bream (data not shown) showed that this tissue has very low LPL activity together with a high variability between individuals.

Fasting provoked clear alterations in gilthead sea bream metabolism as shown by significant changes in liver weight and hepatosomatic index as expected (Navarro and Gutiérrez, 1995). Glycemia levels were similar between fed and fasted groups, while FFA in plasma increased significantly in fasted group. Similar results have been reported for this and other piscine species indicating that during fasting there is an increase in the mobilization of lipids together with an increase in gluconeogenesis (Albalat et al., 2005b; Sheridan and Mommsen, 1991). Insulin levels decreased in fasted group, a feature described in fasted fish of different species (Navarro et al., 1992; Sundby and col., 1991). This decline in plasma insulin levels was accompanied by a reduction in LPL activity in adipose tissue, whereas LPL activity in red muscle was not affected by feeding condition. This decline in adipose tissue LPL activity correlates well with the reported increase in lipolysis of isolated adipose tissue cells from fasted gilthead sea bream (Albalat et al., 2005b), indicating a shift of metabolism during fasting towards lipid mobilization together with decrease in lipid accumulation. Comparable results have been reported in trout by Black and Skinner (1986) where 8 weeks of fasting induced a decrease of LPL activity in adipose tissue while no differences were found in red muscle. Results obtained in mammals, showed a similar response of LPL activity due to food deprivation. In humans, fasting of 30 hours decreases LPL activity in adipose tissue (a decrease of 50%), whereas the activity

increases in skeletal muscle (an increase of 100%) (Ruge et al., 2005), showing that LPL is regulated in a tissue-specific manner.

In order to elucidate if the lower levels of plasma insulin detected in fasted gilthead sea bream could be related to the decrease observed in adipose tissue LPL activity we experimentally tried to induce hyperinsulinemia through an intraperitoneal injection of arginine and insulin in gilthead sea bream. This arginine injection experiment was part of a more extensive work studying the effects of this amino acid in gilthead sea bream plasma hormone profiles (Vega-Rubín de Celis et al., 2004).

Arginine has been shown to be a potent insulin secretagoge in fish, a feature also described in mammals (Mommsen and Plisetskaya, 1991). In fact, and contrary to the situation in mammals, arginine and other amino acids have a stronger insulinotropic action than glucose in fish. The same dose of arginine used in this study has been successfully used in salmonids as a potent tool to increase plasma insulin levels between 3-9 fold (Baños et al., 1999; Mommsen et al., 2001). However, in the present study, arginine stimulated predominantly glucagon over insulin secretion and the effects of glucagon dominated over plasma glucose. In these conditions, LPL activity did not change significantly in adipose tissue. In fact, the effect of insulin could be masked by the action of glucagon. Interestingly, at 6 hours, LPL activity tended to be higher together with a lower glucagon/insulin molar ratio when compared to the ratio shown at 3 hours in arginine-injected fish. Nevertheless, even in mammals, information of LPL regulation by glucagon is scarce. This hormone has shown to stimulate LPL activity when administered *in vivo* (Borensztajn et al., 1973), while results obtained *in vitro* have found an inhibitory effect (Murase et al., 1981).

As arginine was not an ideal hyperinsulinemic model in this fish species, we directly administrated insulin in gilthead sea bream. Although insulin did not produce a decrease in plasma glucose levels it decreased plasma FFA which correlates well with the anti-lipolitic effects of this hormone (Plisetskaya et al., 1989). Besides, insulin does not always provoke clear changes in plasma glucose levels and glycogen tissue reserves in fish (reviewed by Navarro et al., 2005).

Insulin administration provoked an increase in LPL activity in adipose tissue after 3 hours while no effect was seen at 18 hours. In this case, the insulin effect was tissue-specific, because LPL activity in red muscle did not change due to insulin administration. These results are similar to what was observed in the fasting experiment where only changes in adipose tissue LPL activity were obtained. These data suggest

that in gilthead sea bream insulin stimulates LPL activity in adipose tissue, whereas in red muscle LPL activity is not directly mediated by plasma insulin levels. In humans, insulin regulates LPL activity in a tissue-specific manner as described by Farese et al. (1991). Direct positive effects of insulin on the activity of the enzyme have been reported *in vitro* in isolated adipocytes of rats (Ong et al., 1988). Insulin administration, increases LPL activity in human adipose tissue 6 hours after the injection, whereas muscle LPL activity decreases. In fact, in mammals, the effect of insulin on muscle LPL activity remains unclear and some authors have reported that insulin produces no changes or decreases of LPL activity in muscle (Cryer et al., 1976; Tan et al., 1977). Most likely, insulin increases LPL activity in adipose tissue to promote lipid storage and keeps lipids away from the muscle by not altering LPL activity in this tissue.

Finally, in adipose tissue LPL mRNA levels increased 3 hours after insulin administration, and significantly after 18 hours, while no effects on LPL expression were found in red muscle, confirming the idea that LPL activity in this tissue does not respond directly to plasma insulin levels in gilthead sea bream.

In mammals, regulation of LPL is complicated and regulation can exist at different levels (transcriptional, translational and post-transcriptional). Starvation decreases LPL mRNA levels together with activity in adipose tissue while both increase in other tissues, such as heart and skeletal muscle (Enerbäck and Gimble 1993; Enerbäck et al., 1988; Lee et al., 1998). However, during short-term fasting, LPL activity decreases and this down-regulation it is not accompanied by changes in LPL mRNA or protein levels. In this case some studies have found that LPL protein shifts from an active to an inactive form (Bergö et al., 1996) whereas other studies have shown that during short-term starvation there is an increase in the degradation of newly synthesized LPL (Lee et al., 1998).

The specific role of insulin has been studied in rat adipocytes, where an insulin administration increased LPL activity, LPL synthetic rates and LPL mRNA levels (Ong et al., 1988). The mechanism by which insulin increases LPL mRNA levels is not clear but it has been proposed that one possibility would be through the stabilization of LPL mRNA (Raynolds et al., 1990). Besides, results obtained in human adipose tissue fragments showed that insulin increased heparin-releasable and total LPL activity by specifically increasing the rate of LPL protein synthesis (Apple and Fried, 1992).

In fish, few studies have looked at the regulation of LPL expression and there is no information regarding the possible role of insulin. Liang et al. (2002) reported a

disminution in LPL mRNA levels in adipose tissue of starved red gilthead sea bream compared to fed fish. While other studies in trout and the present study show lower LPL activity in adipose tissue of fasted fish (Black and Skinner, 1986; Albalat et al., 2005a). Taken all these data together, it appears that down-regulation of enzyme activity could be explained at least in part by a decrease in LPL mRNA levels.

However, in the present study, LPL activity increased significantly 3 hours after the insulin injection but positive effects at transcriptional level were found at 18 hours. These results suggest that insulin could regulate LPL at different levels. At short-term, insulin would stimulate LPL activity through different mechanisms such as stimulating the secretion of the enzyme or the proportion of enzyme in active conformation (Semenkovich et al., 1989) and the long-term effect of insulin would be an increase in the expression of the enzyme by stabilization of mRNA or other unknown mechanisms. In fact, *in vitro* results with adipose tissue fragments of rainbow trout showed that insulin incubated for 4 hours stimulates the heparine-releasable LPL activity, which is considered to be the LPL in active conformation found at extracellular level (Albalat et al., 2005a).

In summary, these results show that LPL activity is altered by feeding condition and indicate that insulin can stimulate LPL activity and expression in adipose tissue of gilthead sea bream.

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III. TRANSPORT DE GLUCOSA EN ADIPÒCITS DE TRUITA

Glucose uptake in isolated adipocytes of rainbow trout (*Oncorhynchus mykiss*): Effects of insulin and insulin like growth factor-I (IGF-I)

A. Albalat, J. Sánchez-Gurmaches, E. Capilla, J.V. Planas and I. Navarro

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Captació de la glucosa en adipòcis aïllats de la truita irisada (*Oncorhynchus mykiss*): Efecte de la insulina i el factor de creixement tipus insulina-I (IGF-I)

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Captació de la glucosa en adipòcis aïllats de la truita irisada (*Oncorhynchus mykiss*): Efecte de la insulina i el factor de creixement tipus insulina-I (IGF-I)

Resum

En peixos, la informació sobre el transport de glucosa en el teixit adipós i la seva regulació hormonal és molt limitada.

En el present treball, hem analitzat el transport de glucosa mesurant la captació de 2-deoxiglucosa (2-DG) en adipòcits aïllats del teixit adipós mesentèric de la truita irisada (*Oncorhynchus mykiss*). La captació de 2-DG va augmentar amb la concentració de cèl.lules i el temps d'incubació. La citocalasina B va ser capaç d'inhibir el transport de glucosa de forma dosi-depenent suggerint doncs que en adipòcits, la captació de glucosa es dóna a través de transportadors de glucosa específics de difusió facilitada (GLUTs).

Pel que fa a la regulació hormonal, la insulina (1 i 100 nM) va estimular el transport de glucosa mesurat durant 2 i 3 hores. Cal destacar, que el IGF-I va ser més eficient que la insulina estimulant el transport de glucosa a les mateixes concentracions (1-100 nM).

Finalment, i per tal d'elucidar els mecanismes intracel.lulars involucrats en l'estimulació del transport de glucosa produït per la insulina, els adipòcits van ser pre-incubats amb wortmanina. Aquest inhibidor de la fosfatidilinositol 3' kinasa (PI3K) va bloquejar totalment l'efecte estimulador de la insulina sobre el transport de glucosa suggerint que aquesta és la principal via de senyalització en els efectes observats.

En resum, sembla que el transport de glucosa en adipòcits de la truita irisada podria estar regulat per la insulina i per l'IGF-I i a més els resultats suggereixen que la via de la PI3K és el principal mecanisme intracel.lular involucrat en els efectes estimuladors de la insulina sobre el transport de glucosa.

Glucose uptake in isolated adipocytes of rainbow trout (*Oncorhynchus mykiss*): Effects of insulin and insulin like growth factor-I (IGF-I)

A. Albalat, J. Sánchez-Gurmaches, E. Capilla, J.V. Planas and I. Navarro

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Running title: Glucose uptake in rainbow trout adipocytes

Key words: Glucose uptake, adipocytes, insulin, IGF-I, rainbow trout.

Please address all correspondence to I. Navarro

Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 645, Barcelona, Spain

Fax: 34+ 93+ 4110358

Email: mnavarro@ub.edu

Abstract

In fish, information concerning glucose transport in adipose tissue and its hormonal regulation is very limited.

In the present study, we have measured glucose transport by 2-deoxyglucose uptake in isolated adipocytes from mesenteric adipose tissue of rainbow trout (*Oncorhynchus mykiss*). Glucose transport increased with cell concentration and time of incubation. Cytochalasin B was able to inhibit glucose transport in a dose-dependent manner suggesting that in adipocytes, glucose uptake is carried out by specific facilitative glucose transporters (GLUTs).

Regarding hormonal regulation, insulin (1 and 100 nM) was able to stimulate glucose transport when measured for 2 and 3 hours. Interestingly, IGF-I was more efficient than insulin in stimulating glucose transport at the same doses (1-100 nM).

Finally, in order to elucidate the intracellular mechanisms involved in the insulin-stimulated glucose transport, adipocytes were pre-incubated with wortmanin. This phosphatidylinositol 3' kinase (PI3K) inhibitor was able to block totally the stimulatory effects of insulin in glucose transport suggesting that this pathway is the main intracellular signalling mechanism involved in the observed effects.

In summary, it appears that glucose transport in rainbow trout adipocytes could be regulated by insulin and especially IGF-I and suggest that PI3K is the main intracellular mechanism involved in the insulin-stimulatory effects on glucose transport.

1. Introduction

Teleost fish, an especially carnivorous species, have a limited capacity to use carbohydrates supplied from the diet (Moon, 2001). Fish fed with high carbohydrate diets show permanent hyperglycemia and reduced growth (Cowey and Walton, 1989), although many factors (fish strain, environmental factors and the source and complexity of the carbohydrates) can affect the level of dietary carbohydrates that can be used in fish (Hemre et al., 2002). Since a deficiency in insulin secretion has proved not to be the reason for this 'glucose intolerance' in fish (Moon, 2001), it has been proposed that peripheral utilization of glucose by tissues could be a possible limiting factor in the utilization of carbohydrates by fish. Blasco et al. (1996) found an increase of 1.3–fold in the rate of glucose disappearance after a glucose load in brown trout (*Salmo trutta*), together with an elevation in insulin levels. Interestingly, there was an increase in glucose uptake in white and red muscles, while no data was presented for adipose tissue, a key tissue together with skeletal muscle in eliminating glucose load in mammals (Khan, 1996).

Transport of glucose across the cell membranes is the first step of regulation for the peripheral utilization of glucose. Glucose transport has been successfully studied in different cellular systems of fish, such as erythrocytes (Soengas and Moon, 1995), enterocytes (Soengas and Moon, 1998) and myocytes (Castillo et al., 2004). In these cases, glucose uptake was shown to be saturable, stereospecific and cytochalasin B, (specific intracellular inhibitor of facilitative glucose transporters (GLUTs)) was able to inhibit the glucose transport. Indeed, the main mechanism by which glucose enters into the cells is through facilitative glucose transporter (GLUTs). In fish, different members of GLUTs have been cloned (Teerijoki et al., 2000; Teerijoki et al., 2001; Krasnov et al., 2001) and interestingly an insulin-responsive glucose transporter (GLUT4) have been identified in skeletal muscle of brown trout (Planas et al., 2000a) and in adipose tissue of salmon (*Oncorhynchus kisutch*) (Capilla et al., 2004).

In mammals, glucose homeostasis is tightly maintained through the action of insulin, being skeletal muscle and adipose tissue the main targets for insulin action. In these tissues insulin stimulates glucose uptake through the translocation of GLUT4 into the plasma membrane (Khan, 1996). In this process, two signal transduction pathways have been identified. One involves the activation of PI3K pathway, which

phosphorylates phosphoinositides that activate downstream molecules such as PDK1, Akt and atypical protein kinase C. The other pathway involves the small GTP-binding protein TC10 in caveolae through tyrosine phosphorylation of cCbl (reviewed by Khan and Pessin, 2002). Apart for the stimulatory effects of insulin on glucose uptake, insulin growth factor-I (IGF-I) besides its mitogenic effects, has been demonstrated to act as a hypoglucemic factor and it has been shown to stimulate glucose uptake in different mammalian cell models through signalling pathways similar to insulin.

In fish, skeletal muscle and adipose tissue have shown to present insulin and IGF-I receptors, being IGF-I receptors in greater number (Parrizas et al., 1994; Planas et al., 2000b). Moreover, glucose transport measured in myocytes of rainbow trout was shown to be stimulated by insulin and specially IGF-I (Castillo et al., 2004). On the other hand, there are very few studies concerning the role of adipose tissue in glucose uptake. Christiansen et al. (1985) showed that adipocytes from rainbow trout were rather inert and quite non-specific with respect to glucose uptake. Furthermore, in that study insulin did not stimulate glucose uptake (measured for about 4 min) in adipocytes. Nevertheless, more recently, preliminary results indicated a stimulation of glucose uptake by insulin in rainbow trout adipocytes and besides a GLUT4 has been characterised in adipose tissue of salmon (Capilla et al., 2004).

For these reasons the objectives of the present study were to establish a method for the study of glucose transport in adipose tissue cells of rainbow trout and evaluate the role of insulin and IGF-I on glucose uptake in order to better understand how peripheral glucose clearance is regulated in fish. Besides, in order to elucidate the intracellular mechanisms that could be involved in the insulin effect on glucose transport we performed experiments using wortmanin (specific inhibitor of PI3K in mammals).

2. Materials and methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss*) of an average weight of 208.67 ± 15.71 g were obtained from the fish farm 'Truites del Segre' (Lleida, Spain) and maintained at the facilities of the University of Barcelona (Spain) in a closed-water flow system at 15

± 1 ºC of temperature. Animals were fed *ad libitum* by hand once a day with a standard commercial diet and were acclimated for 10 days before any experiment was conducted. Fish were kept under natural conditions of light, latitude (40º 5' N; 0º 10' E).

The day of the experiment, 24-h fasted fish were killed by a cranial blow, animals were weighed and adipose tissue was immediately extracted to perform the adipocyte isolation.

Experiments were conducted according to the Catalan government's 'Departament de Medi Ambient i Habitatge; Generalitat de Catalunya' regulations concerning treatment of experimental animals (No. 2215).

2.2. Adipocyte isolation

Adipocytes were isolated by the method of Vianen et al. (2002) with some minor modifications. Fat tissue was cut into thin pieces and incubated for 60 min in polypropylene tubes with Krebs-Hepes buffer pre-gassed with 5% CO_2 in O_2 (pH 7,4) containing collagenase type II (130 U/ml) and 1% bovine albumin serum (BSA), without glucose, in a water bath under gently shaking at 15°C. The cell suspension was filtered through a double layer of nylon cloth and then washed three times by flotation. Finally, cells were carefully resuspended at the desired concentration in Krebs-Hepes buffer containing BSA 2% using a Fuchs-Rosenthal counting chamber.

2.3. Glucose transport protocol

Glucose transport was determined by modification of the method described by Olefsky (1978). Trout adipocytes (2.5×10^5 cells/ml) were incubated in Krebs buffer in the absence or presence of insulin or insulin growth factor I (IGF-I) at different concentrations for 30 min (or the time stated) at 15ºC in a shaking bath. Subsequently, radiolabeled 2-deoxi-D-(^{3}H) glucose (0.8µCi) was added, and transport was stopped after 2 hours with cytochalasin B. The transport assay was terminated by transferring a 200-µl aliquot of cell suspension into small polyethylene microcentrifuge tubes containing 150 µl di-'isononyl' phthalate (density 980 g/l). Cells and buffer were separated by centrifugation at 16,000g for 2 min. The upper phase, which contained the adipocytes, was collected and subjected to liquid scintillation counting. Radioactivity

was measured with a β-counter (Packard Bioscience, Meriden, CT). Non-specific uptake was measured in cells pre-treated with cytochalasin B and all uptake data were subtracted with these values to correct for extracellularly trapped isotope and cell-associated radioactivity. Previous studies showed that porcine insulin had very similar effects to fish insulin when used *in vitro* to study glucose transport of adipocytes (data not shown) and muscle cells (Castillo et al., 2004) of rainbow trout.

In order to better understand the glucose transport in rainbow trout adipocytes two inhibitors were tested: Cytochalasin B, a specific inhibitor of the facilitative glucose transporters and wortmanin, an inhibitor of the PI3K-Akt pathway. In these experiments, adipocytes were pre-incubated in the absence or presence of the inhibitors cytochalasin (at different concentrations) and wortmanin (1 μM) for 15 minutes.

2.4. Chemicals

2-Deoxy-D-[2,6-^{3}H]glucose with a specific activity of 50 Ci/mmol was obtained from Amersham Pharmacia Biotech Europe (Barcelona, Spain). Porcine insulin from Sigma Aldrich Química (Madrid, Spain) and recombinant trout IGF-I was purchased from GroPep (Adelaide, Australia). Di-'isononyl' phthalate was obtained from Fluka Chemika and other reagents, such as collagenase type II, cytochalasin B and wortmanin were obtained from Sigma Aldrich Química (Madrid, Spain).

2.5. Statistical Analysis

Data are presented as the mean ± standard error of mean (S.E.M.). In the time course experiments, for each time studied, basal and insulin glucose transport were compared by paired t-test. The rest of the experiments were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed using SPSS 11.5 for Windows.

3. Results

3.1. Glucose uptake characterization in rainbow trout adipocytes

2-DG uptake measured for 20 min increased with cell concentration from 1.2 to 6.2×10^5 cells/ml. For subsequent experiments the concentration of $2.5\text{x}10^5$ cells/ml was selected in order to have suitable measurements of glucose uptake.

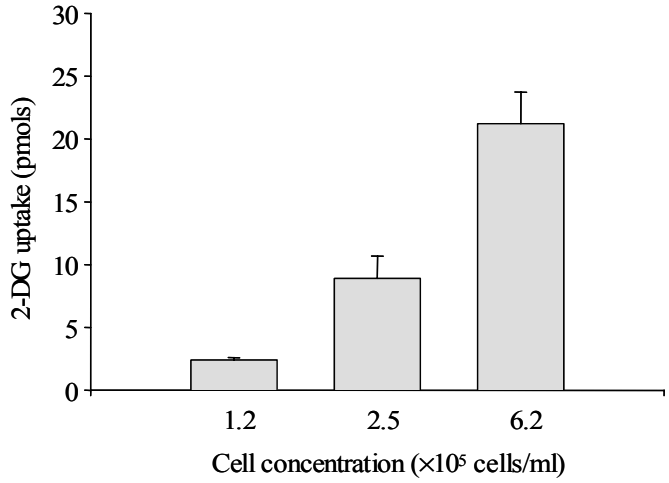

Figure 1. Effect of increasing concentrations of adipocytes on glucose uptake measured for 20 min. Values are the mean (n=3) ± S.E.M. of a representative experiment.

Furthermore, in order to ensure that the 2-DG uptake measured was due to facilitative glucose transporters, cytochalasin B was pre-incubated for 15 min and 2-DG uptake was measured for 2 hours. As shown in Figure 2 cytochalasin B, a specific intracellular inhibitor of facilitative glucose transporters (GLUTs), inhibited 2-DG uptake in rainbow trout adipocytes in a concentration-dependent manner. Maximum inhibitory effect by cytochalasin was obtained at the concentration of 2 μM (10.13 ± 3.30 % of 2-DG uptake over control).

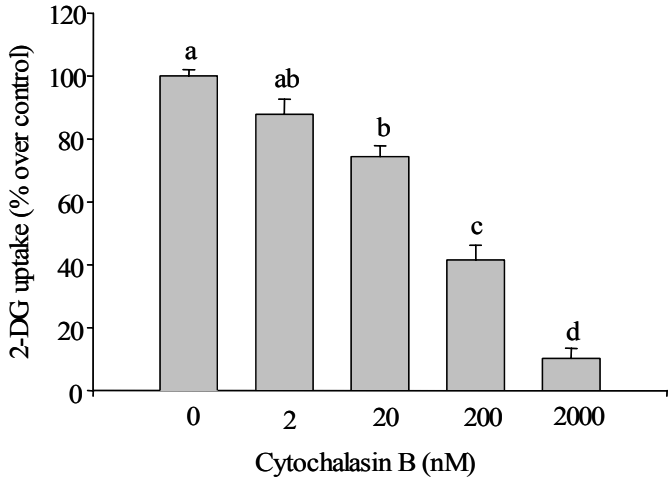

Figure 2. Inhibition of 2-DG uptake by increasing concentrations of cytochalasin B in rainbow trout adipocytes. Results are expressed as % over control group (adipocytes incubated with no cytochalasin B), which was set at (100%) (36.59 ± 0.77 pmols glucose/10^5 cells). Values are the mean (n=3) ± S.E.M. from a representative experiment from a total of 2 independent experiments. Values not sharing a common letter are significantly different ($P<0.05$).

3.2. Time-course response of insulin stimulation on glucose uptake in rainbow trout adipocytes

In order to study the possible effects of insulin on adipocyte glucose uptake insulin (100 nM) was pre-incubated for 30 min and after 2-DG uptake was measured at different times. Insulin caused a significant stimulation of glucose uptake after 2 and 3 hours of 2-DG uptake while no effect was observed at shorter periods of time as shown in Figure 3.

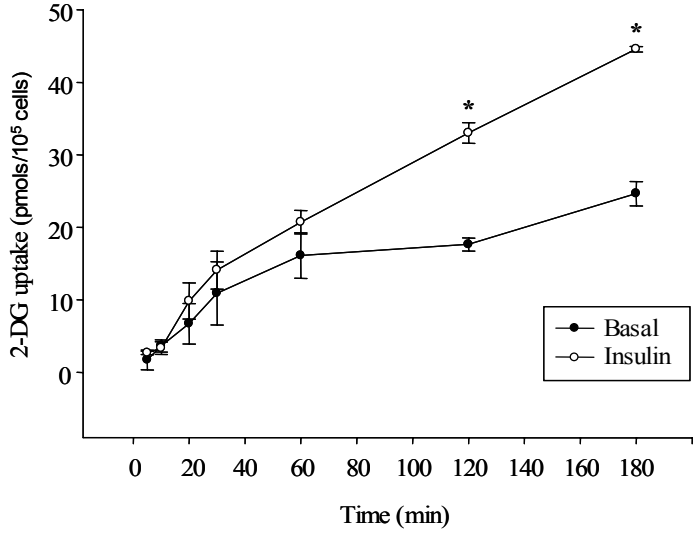

Figure 3. Time-course response of insulin (100 nM) pre-incubated for 30 min on 2-DG uptake in rainbow trout adipocytes. Values are the mean (n=3) ± S.E.M. of a representative experiment from a total of 2 independent experiments. Asterisk (*) indicates significant differences between control and insulin-treated groups for each time period ($P < 0.05$).

3.3. Effect of different pre-incubation times of insulin on glucose uptake in rainbow trout adipocytes

Time-course experiments showed that insulin pre-incubated for 30 min was able to stimulate glucose uptake when measured for 2 hours. In the present experiment we assessed whether insulin pre-incubated for a longer period of time (60 min) would affect glucose uptake measured at shorter periods of time (60 min). The results presented in Figure 4 showed that insulin pre-incubated for 60 min had the same effect (no significant positive action) that insulin pre-incubated for 30 min confirming that insulin does not affect glucose uptake when measured at shorter periods than 2 hours. For this reason in the next experiments, insulin (or IGF-I) were pre-incubated for 30 min and glucose uptake was measured for 2 hours.

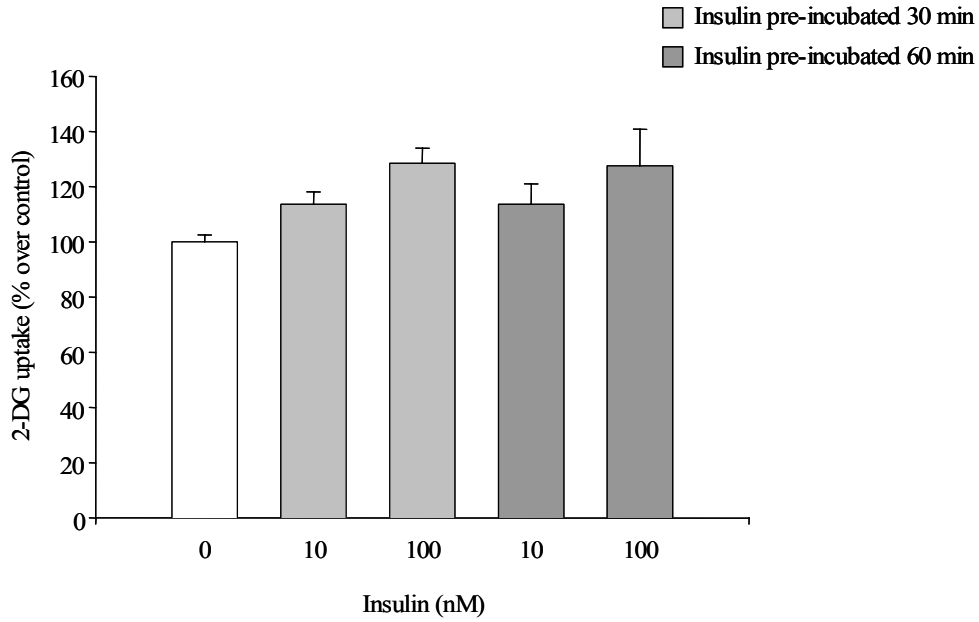

Figure 4. Effects of insulin pre-incubated at different periods of time (30 and 60 min) on 2-DG uptake measured for 60 min in rainbow trout adipocytes. Results are expressed as % over control group (adipocytes incubated with no insulin), which was set at (100%) (29.62 ± 0.76 pmols glucose/10^5 cells). Values are the mean (n=3) ± S.E.M. of a representative experiment from a total of 2 independent experiments.

3.4. Dose-response of insulin and IGF-I on glucose uptake in rainbow trout adipocytes

The effects of insulin on 2-DG uptake at 2 hours were further studied performing a dose-response of insulin and comparing these results with a dose response of IGF-I. As

shown in Figure 5, insulin stimulated glucose uptake although not in a concentration-dependent manner at 1 and 100 nM. On the other hand, IGF-I stimulated glucose uptake with highest effect at the highest concentration tested (100 nM).

IGF-I was more potent peptide stimulating glucose uptake when compared to insulin being significant at two of the three concentrations tested (10 and 100 nM).

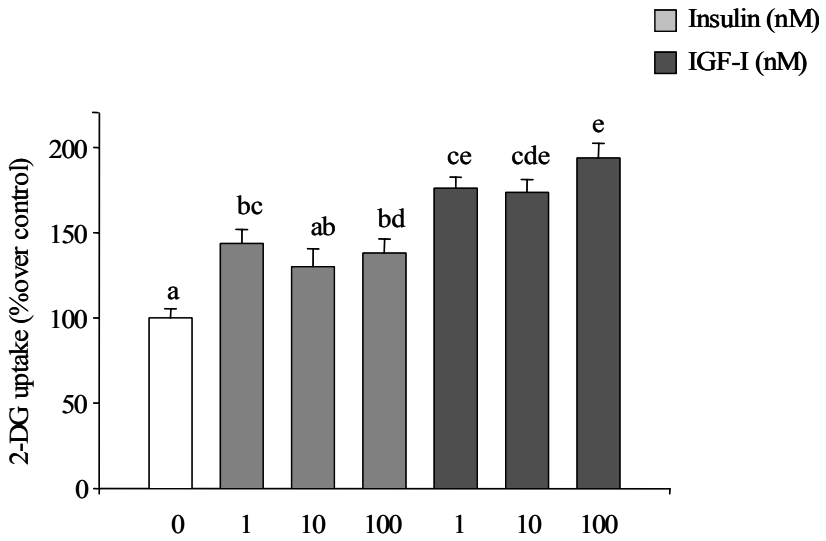

Figure 5. Dose response of insulin and IGF-I (both pre-incubated for 30 min) on 2-DG uptake measured for 2 h in rainbow trout adipocytes. Results are expressed as % over control group (adipocytes incubated without hormones), which was set at (100%) (41.41 ± 2.22 pmols glucose/10^5 cells). Values are the mean (n=3) ± S.E.M. of a representative experiment. Values not sharing a common letter are significantly different ($P < 0.05$).

3.5. Insulin-stimulated glucose uptake is inhibited by PI3K inhibitor

In order to elucidate the intracellular pathway involved in the insulin-stimulated glucose uptake we analyzed the effect of wortmanin, a specific inhibitor of the PI3K pathway. As shown in Figure 6, wortmanin (1 μM) completely blocked the stimulatory effect of insulin in glucose uptake while no effect of this inhibitor was observed on basal glucose uptake (data not shown).

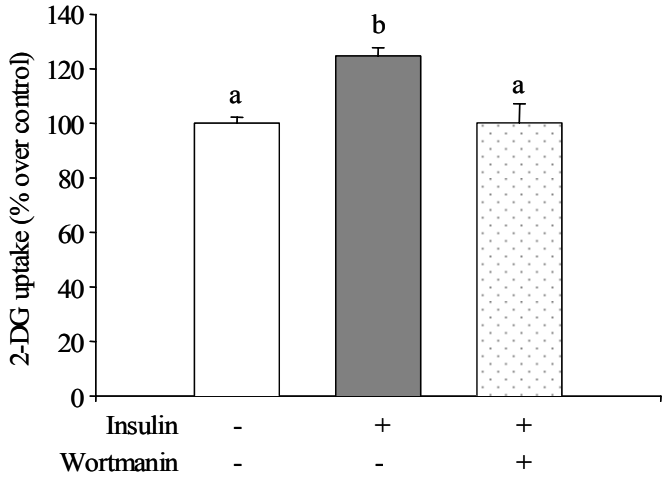

Figure 6. Effect of wortmanin (1 μM) on insulin-stimulated 2-DG uptake in rainbow trout adipocytes. Wortmanin was pre-incubated for 15 min before the addition of the hormone (30 min) and glucose uptake was measured for 2 h. Data are expressed as % change with respect to control group, which was set at 100% (control: 32 pmols glucose/10^5 cells). Values are the mean (n=3) ± S.E.M. of a representative experiment from a total of 2 independent experiments. Values not sharing a common letter are significantly different ($P < 0.05$).

4. Discussion

Glucose transport has been reported in several fish cell types in earlier studies (Soengas and Moon, 1995; Soengas and Moon, 1998; Castillo et al., 2004). In the present work, we studied glucose uptake in rainbow trout adipocytes using 2-deoxiglucose.

Glucose transport increased with cell concentration and time of incubation. In fact, the incubation times used in the present study are longer than those previously used for the study of glucose transport in mammalian adipocytes and in the only report on rainbow trout adipocytes (Christiansen et al., 1985). One of the reasons why longer incubation times were used is because experiments were performed at lower temperature (15 ºC) compared to mammals (37 ºC) according to the optimal temperature for rainbow trout. Furthermore, fish GLUTs have shown to have a lower affinity (higher K_m) for glucose than mammalian GLUTs (Burant and Bell, 1992; Teerijoki et al., 2001; Capilla et al., 2004) in agreement with the lower capacity of fish to clear a glucose load. Similarly, studies performed in tilapia (*Oreochromis mossambicus*) (Vianen et al., 2002) and seabream adipocytes (Albalat et al., 2005) to

study lipolysis and its hormonal regulation were conducted for longer times of incubation (5-6 hours) compared to those normally used in mammals (Tebar et al., 1993). Glucose transport was inhibited in a dose-dependent manner by cytochalasin B, indicating that glucose uptake in adipocytes is carried out by specific facilitative glucose transporters (GLUTs). Similar results were reported in fish erythrocytes (Tse and Young, 1990; Young et al., 1994; Soengas and Moon, 1995) and enterocytes (Soengas and Moon, 1998) were cytochalasin B inhibited glucose transport.

In mammals, it is well established that insulin stimulates glucose transport in skeletal muscle and adipose tissue (Bryant et al., 2002), while this affirmation is still under revision in fish (Wright et al., 1998). However, it is clear that exposure to insulin in fish produces hypoglycemia in the majority of species (reviewed by Navarro et al., 2002). Interestingly, Blasco et al. (1996) showed how a glucose load increased the rate of glucose uptake in white and red muscles of brown trout concomitant with high levels of plasma insulin. Furthermore, *in vitro* studies have shown that insulin stimulates glucose uptake in myocytes of rainbow trout (Castillo et al., 2004). In adipose tissue, the role of insulin is still not clear and scarcely investigated. In fact, a direct effect of insulin in the regulation of lipogenesis in adipose tissue of fish has not yet been described. Nevertheless, antilipolitic effects of insulin have been described in slices of adipose tissue (Harmon and Sheridan, 1992) and in adipocytes of rainbow trout (Albalat et al., 2002) while no such effect was found in seabream adipocytes (Albalat et al., 2005). In the present study, we wanted to know whether glucose transport in rainbow trout adipocytes could be modulated by insulin. Insulin (pre-incubation of 30 min) increased glucose uptake in adipocytes after 2 and 3 hours of incubation. At shorter times of incubation, insulin had no significant effect. This would be in agreement with the results obtained by Christiansen et al. (1985) that found no effect of insulin on glucose uptake in rainbow trout adipocytes when measured for 4 min. Interestingly, in the present work, if glucose uptake was measured at shorter periods of 2 hours no effect of insulin was found even if insulin was pre-incubated for a longer period of time (60 min). It appears that in fish adipocytes, more time is needed to assess the hormonal effects on glucose uptake.

When insulin stimulation was compared to IGF-I the results showed that IGF-I was more potent in stimulating glucose uptake in rainbow trout adipocytes. In fact, there is a complete lack of information concerning the potential role of IGF-I in adipose tissue of fish although the presence of IGF-I specific receptors have been reported

(Planas et al., 2000b). This is the first time that the effects of IGF-I have been analysed in glucose uptake in fish adipocytes. Similar results were reported in rainbow trout myocytes (Castillo et al., 2004) and in chicken muscle cells (Duclos et al., 1993) where IGF-I was more efficient stimulating glucose transport than insulin. This higher effect of IGF-I on glucose uptake compared to insulin is consistent with the fact that IGF-I receptors are more abundant (two- to ten fold) and have higher affinity (two fold) than insulin receptors in adipose tissue of brown trout (Planas et al., 2000b).

In mammals, when plasma levels of insulin increase, GLUT4 is translocated to the cell membrane in adipocytes and muscle cells. In this process, the activation of PI3K is necessary for the insulin stimulation of GLUT4 translocation (Khan and Pessin, 2002). In our study, wortmanin (an inhibitor of PI3K), blocked completely the insulin-stimulated glucose uptake in rainbow trout adipocytes, while no effect was found on basal glucose uptake (transport measured without any hormone). These results indicate that PI3K plays a critical role in the observed insulin-stimulation of glucose transport. In fish, so far, an adipose tissue GLUT4 homolog has been reported and characterized in fish (Capilla et al., 2004). However, the signalling mechanism between the insulin receptor and GLUT4 translocation has not yet been studied in fish (Moon, 2001). The results obtained in our study suggest that the increase in glucose uptake obtained by insulin in rainbow trout adipocytes is mediated through the PI3K pathway that could indeed mediate the translocation of a GLUT4 transporter to the adipocyte membrane.

In summary, we have successfully measured glucose transport in rainbow trout adipocytes. The fact that cytochalasin B inhibited glucose uptake in a dose-dependent manner indicates that the transport is mediated by specific facilitative glucose transporters (GLUTs). Insulin and specially IGF-I were able to stimulate glucose transport of rainbow trout adipocytes. Finally, PI3K was shown to be a key intracellular mechanism involved in the insulin-stimulation of glucose uptake suggesting that the translocation of a GLUT4 protein to the adipocyte membrane could mediate the observed effect.

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RESUM DE RESULTATS I DISCUSSIÓ

I. Estudi de la lipòlisi en adipòcits de la truita irisada i l'orada

Característiques generals de les preparacions d'adipòcits aïllats de la truita irisada i l'orada

El teixit adipós està format principalment per adipòcits units entre ells per fibres de col.lagen que són alliberats després d'una digestió amb col.lagenasa. En aquest treball, s'han establert les condicions adequades de concentració de col.lagenasa, temps i temperatures d'incubació per l'obtenció de preparacions d'adipòcits de qualitat per ambdues espècies, truita i orada. La morfologia d'aquestes cèl.lules, tant en peixos com en mamífers, consta basicàment d'una gota centrada de lípid que desplaça totament el nucli a la perifèria. El tamany pot variar entre 25-200 μm, tot i que en general les poblacions d'adipòcits tenen un tamany bastant constant que en humans es centra al voltant dels 80 μm (Ashwell i col., 1976). En peixos, els estudis sobre la morfologia dels adipòcits són escassos. Tot i això, s'ha descrit que els adipòcits de truita i salmó presenten una gran variabilitat de tamanys, essent els adipòcits d'un tamany inferior a 50 μm els més abundants (Zhou i col., 1996; Fauconneau i col., 1997). Aquestes observacions, estan d'acord amb els resultats trobats en el present estudi. Els adipòcits provinents de teixit adipós visceral de truita irisada van presentar una gran variabilitat de tamanys (entre 7.12-112.96 μm) i el rang més abundant va ser el d'entre 10-20 μm. A més, tant en truita irisada com en orada la majoria dels adipòcits eren bi-nucleats, una característica típica dels adipòcits de peix ja descrita per Hadjioloff (1930). Per tal de mesurar la viabilitat, els adipòcits van ser examinats al microscopi al principi i al final de les incubacions i no es van veure diferències apreciables en l'aspecte i en el nombre de cèl.lules. De forma complementària es va mesurar l'activitat lactat dehidrogenasa (LDH) en el medi com a índex de viabilitat. Aquest és un enzim intracel.lular que s'allibera al medi quan la membrana cel.lular està danyada i s'ha utilitzat prèviament en adipòcits i altres models cel.lulars de mamífer i també de peix com a índex de viabilitat cel.lular (Kinutani i col., 1985; Stralfors, 1990; Moon i col., 1996). Els resultats van mostrar que no hi havia una alliberació significativa d'aquest enzim en el medi durant el període d'incubació. D'altra banda, els controls positius incubats amb detergent durant el mateix període de temps havien alliberat pràcticament tot l'enzim al medi (més d'un

90%). Aquests resultats, doncs van confirmar que la viabilitat dels adipòcits era correcta en els períodes d'incubació utilitzats.

Tant en truita irisada com en orada es va utilitzar l'alliberació de glicerol com a índex de la lipòlisi. En mamífers, molts estudis han utilitzat el glicerol enfront els àcids grassos lliures (AGL) donat que el glicerol no pot ser re-utilitzat per l'adipòcit mentre que els AGL poden ser re-esterificats. En truita irisada, es va observar que la proporció d'AGL/glicerol disminuïa amb el temps suggerint que en les condicions utilitzades hi havia una re-esterificació parcial dels AGL possiblement degut a que no tots els AGL van ser retinguts per l'albúmina i van ser re-utilitzats e incorportats a TAG.

L'alliberació de glicerol en el medi va augmentar proporcionalment amb el nombre de cèl.lules i amb el temps d'incubació tant en truita irisada com en orada. Aquest és un punt metodològic important, observat també en mamífers (Tebar i col., 1993), ja que a l'hora de mesurar efectes hormonals és important treballar en situacions de linialitat. Pel que fa als nivells de glicerol alliberat durant el període d'incubació es va observar que la lipòlisi basal de la truita irisada (8.9-12.8 nmol/h/10^6cel) era més alta que la de l'orada (5.71 nmol/h/10^6cel) en situacions experimentals similars. Tot i això, cal tenir en compte dos fets, per una banda els adipòcits de truita irisada van ser incubats a una temperatura de 15ºC mentre que els d'orada van ser incubats a 22ºC, i per una altra banda, els experiments en truita irisada van ser efectuats al mes de febrer mentre que els experiments en orada es van realitzar al mes de setembre. En aquest sentit, seria necessari tenir informació sobre els nivells de lipòlisi al llarg de l'any per tal de comparar més acuradament la lipòlisi basal de les dues espècies i comprovar si realment és diferent. De fet, en espècies hivernants com la marmota alpina, s'ha descrit que la lipòlisi basal dels adipòcits varia al llarg de l'any en funció de l'estat nutricional-hivernació de l'animal (Cochet i col., 1999). D'aquesta manera es podrien també esperar canvis estacionals en la lipòlisi basal dels peixos, donat que la quantitat de teixit adipós visceral pot variar en els diferents períodes dels cicles annuals. D'altra banda, en mamífers s'ha descrit que la lipòlisi basal està relacionada amb el tamany dels adipòcits, així segons l'estat metabòlic de l'animal es poden esperar variacions en el tamany dels adipòcits que presentarien nivells de lipòlisi basal diferents (Tsujita i col., 1995). Finalment, l'activitat lipolítica dels adipòcits de truita irisada i orada va ser més baixa que la descrita en mamífers, tot i que en aquest cas el factor de la temperatura d'incubació (15-22ºC versus 37ºC) s'hauria de tenir en consideració (revisat per Van den Thillart i col., 2002).

Regulació nutricional i hormonal de la lipòlisi en la truita irisada i l'orada: Paper de la insulina, el glucagó i l'hormona de creixement

Per tal d'estudiar la regulació nutricional de la lipòlisi en el teixit adipós es van realitzar experiments de dejuni en truita irisada i orada. A més en el cas de l'orada, el dejuni es va realitzar amb animals prèviament alimentats amb dues dietes diferents (FM: proteïna principalment provinent de farina de peix i PP: proteïna 100% d'origen vegetal).

En les dues espècies es va observar un patró de resposta semblant enfront al dejuni, encara que en graus diferents. A nivell morfològic, les truites dejunades presentaren una disminució en el pes corporal, pes del fetge, pes del teixit adipós, índex hepatosomàtic (IHS) i factor de condició (FC). A nivell plasmàtic, la insulina va disminuïr a concentracions no detectables en les truites dejunades mentre que els AGL van augmentar pràcticament el doble. De forma similar, en orades alimentades amb dues dietes diferents, el dejuni provocà disminucions significatives en el pes del fetge i l'IHS juntament amb un augment dels AGL en plasma. Així, sembla que els períodes de dejuni escollits (21 dies per la truita irisada i 11 dies per l'orada) foren suficients per produïr canvis en l'estat metabòlic dels peixos. Pel que fa a l'orada, es va observar que la història nutricional prèvia afectava a la resposta dels animals enfront el dejuni donat que les orades alimentades amb la dieta PP presentaven uns efectes més pronunciats com a conseqüència del dejuni. Aquestes orades, van mostrar una disminució de pes corporal, FC i nivells plasmàtics d'insulina, efectes que no es van observar en les orades dejunades però prèviament alimentades amb la dieta FM. Aquesta baixada dels nivells d'insulina plasmàtics durant períodes de dejuni s'ha descrit en vàries espècies de peixos incloent els salmònids (revisat per Navarro i Gutiérrez, 1995).

Pel que fa als efectes del dejuni sobre la lipòlisi del teixit adipós, s'ha descrit que en la truita irisada el lípid s'acumula preferencialment en el teixit adipós mesentèric i és aquest lípid el que es mobilitza primer en situacions de dejuni (Jezierska i col., 1982). D'acord amb aquestes observacions, tant en truita irisada com en orada, els adipòcits aïllats d'animals dejunats presentaren un lipòlisi basal més alta que la d'animals alimentats. Aquest efecte del dejuni sobre la lipòlisi basal va ser més clar en l'orada (increment de la lipòlisi 3 cops superior en adipòcits d'animals dejunats) que en la truita irisada (increment de la lipòlisi 1.4 cops superior en adipòcits d'animals dejunats).

Aquestes dades suggereixen doncs que l'orada mobilitzaria els lípids del teixit adipós inclús de forma més accentuada que la truita irisada o bé que la resposta dels adipòcits varia al llarg del dejuni. Així, donat que els períodes de dejuni no foren exactament iguals per les dues espècies la comparació s'hauria de fer amb cautela. Segons les dades obtingudes, sembla que l'augment d'AGL mesurat en plasma podria explicar-se en part per l'augment de la lipòlisi en el teixit adipós. Tot i això, cal tenir en compte que altres teixits perifèrics com el fetge podrien jugar un paper important contribuïnt en l'augment d'AGL observat en plasma. Aquest augment de la lipòlisi dels adipòcits aïllats en situacions de dejuni s'ha descrit en mamífers (Morimoto i col., 1998; Cochet i col., 1999) però és la primera vegada que s'observa també en peixos utilitzant aquest model *in vitro*.

Pel que fa a l'efecte de la dieta sobre els paràmetres morfològics de les orades es va observar una baixada significativa del pes del fetge conjuntament amb una tendència de menor pes corporal, pes del teixit adipós i IHS en les orades adaptades a la dieta vegetal. De fet, en experiments de creixement anteriors utilitzant les mateixes dietes es va obtenir una disminució significativa de la taxa de creixement en les orades alimentades amb la dieta PP (Gómez-Requeni i col., 2004). En el nostre experiment, la disminució de pes en el grup PP no va ser significativa, possiblement degut a que la durada del experiment va ser menor (12 setmanes enfront a 6 setmanes en el present estudi). La raó d'aquesta disminució en la taxa de creixement sembla que vindria donada per una disminució progressiva en la ingesta que es dóna en animals alimentats amb dietes vegetals. Tot i que els factors responsables d'aquesta disminució en la ingesta no són clars, factors anti-nutricionals, una menor palatabilitat o canvis en la composició d'aminoàcids podrien ser factors implicats (Gómez-Requeni i col., 2004). Pel que fa a la lipòlisi del teixit adipós, els adipòcits d'orades alimentades amb la dieta PP van presentar una lipòlisi basal més alta que els adipòcits d'orades alimentades amb la dieta FM. Tot i aquest augment de la lipòlisi, els AGL en plasma van ser similars entre les dues dietes suggerint que la diferència en lipòlisi observada en teixit adipós no va ser suficient per provocar canvis significatius a nivell de plasma o bé que aquests AGL són ràpidament incorporats o utilitzats per altres teixits. Aquest resultats concorden amb estudis anteriors realitzats amb orada (Gómez-Requeni i col., 2004) o altres espècies (Dias, 1999; Kaushik i col., 1995) on peixos alimentats amb dietes amb alt contingut de proteïna vegetal mostraven una menor deposició lipídica i una disminució en la quantitat de teixit adipós mesentèric.

Per tal d'estudiar la possible regulació hormonal de la lipòlisi del teixit adipós, els adipòcits es van incubar amb diferents concentracions d'insulina, glucagó i hormona de creixement. En adipòcits de truita irisada, la insulina va disminuïr la lipòlisi significativament en les concentracions de 35 i 350 nM. Encara que cal tenir en compte que aquestes concentracions estan per sobre dels valors plasmàtics trobats normalment en truita (Moon et al., 1989). D'altra banda, en adipòcits d'orada la insulina no va tenir cap efecte significatiu sobre la lipòlisi tant si els animals havien estat alimentats com dejunats. Aquests resultats semblen indicar que o bé la resposta del teixit adipós a la insulina depèn de l'espècie o bé que els efectes de la insulina sobre el teixit adipós presenten un component estacional. De fet, en truita tant els nivells circulants d'insulina (Navarro i col., 1991) com els seus receptors (Planas i col., 2000a) varien considerablement al llarg de l'any i per tant es podria esperar una sensibilitat diferent dels adipòcits per a la insulina circulant segons l'estació de l'any. A més, tot i que en mamífers la insulina és coneguda pels seus efectes anti-lipolítics i lipogènics Morimoto i col. (1998) van veure que la insulina no tenia cap efecte sobre la lipòlisi basal en rates tant alimentades com dejunades. L'efecte anti-lipolític de la insulina, doncs quedava restringit a aquelles situacions en les que la lipòlisi havia estat prèviament estimulada per hormones lipolítiques com les catecolamines i l'ACTH. D'aquesta manera, també seria possible que en peixos els efectes anti-lipolítics de la insulina fossin aparents en situacions on la lipòlisi hagués estat prèviament estimulada hormonalment. De fet, Harmon i Sheridan (1992b) van descriure un efecte anti-lipolític de la insulina en trossos de teixit adipós incubats conjuntament amb glucagó d'acord amb aquesta hipòtesi.

Els efectes lipolítics del glucagó han estat ben estudiats en mamífers. En peixos, s'ha descrit un efecte lipolític del glucagó *in vivo* augmentant els nivells d'AGL plasmàtics en vàries espècies (revisat per Plisetskaya i Mommsen, 1996). Els estudis *in vitro* han donat resultats contradictoris. En trossos de teixit adipós, s'ha vist un efecte lipolític del glucagó (Harmon i Sheridan, 1992b) o cap efecte (Migliorini i col., 1992) mentre que Murat i col. (1985) no van observar canvis sobre l'alliberació d'AGL en adipòcits incubats amb glucagó. En el nostre estudi, aquesta hormona va estimular la lipòlisi *in vitro* en adipòcits aïllats de truita irisada (a les dosis de 10 i 100 nM) mentre que en orada els efectes no van ser tant clars. En aquesta última espècie el glucagó va ser lipolític a la concentració de 100 nM en adipòcits d'animals alimentats mentre que no va tenir cap efecte si els adipòcits provenien d'animals dejunats, possiblement degut

a que aquestes cèl.lules ja presentaven una lipòlisi basal més elevada. En qualsevol cas, els efectes són menys pronunciats que els observats en preparacions d'adipòcits de mamífer (Villanueva-Peñacarrillo i col., 2001). Tot i això, no podem descartar que les cèl.lules fossin més sensibles si s'haguessin incubat amb l'hormona homòloga.

Els resultats de l'administració *in vivo* de glucagó en truita irisada van mostrar que els adipòcits aïllats reflexen la situació hormonal *in vivo*. El glucagó, va provocar a nivell plasmàtic una hiperglicèmia i un augment dels AGL, efectes descrits en altres estudis amb peixos (Magnoni i col., 2001; Plisetskaya i Mommsen, 1996) confirmant el paper hiperglucèmic i lipolític del glucagó. A més, els nivells d'insulina van disminuïr (pràcticament a la meitat) en els animals injectats amb glucagó. Plisetskaya i col. (1989) van observar el mateix efecte del glucagó sobre els nivells d'insulina plasmàtica en dues espècies de salmó (*Oncorhynchus kisutch* i *Oncorhynchus tshawytscha*) en la majoria de les experiències que van realitzar, mentre que en anguila (*Anguilla anguilla*) no s'ha vist cap efecte del glucagó sobre la secreció d'insulina (Ince i Thorpe, 1977). El que sembla clar és, que en peixos, la secreció d'ambdues hormones és bastant independent (Plisetskaya i col., 1991; Novoa i col., 2004) i no veiem doncs la típica acció insulinotròpica del glucagó descrita en mamífers. Pel que fa a la lipòlisi del teixit adipós, els adipòcits de truites injectades amb glucagó van presentar una lipòlisi més alta que els adipòcits de truites control a les 3 hores de l'administració. Aquests resultats confirmen que el teixit adipós és un dels teixits diana del glucagó, tot i que no es pot descartar que els efectes observats en la lipòlisi no siguin indirectes, o sigui deguts en part, a la baixada d'insulina en els animals injectats amb glucagó.

Finalment, l'hormona de creixement va ser clarament lipolítica en adipòcits d'orada i de forma similar al cas del glucagó l'augment relatiu de glicerol en el medi (o sigui el percentatge d'estimulació respecte el seu basal) va ser superior en animals alimentats que en animals dejunats. Aquest seria un efecte contrari al descrit en humans on sembla que la sensibilitat lipolítica a l'hormona de creixement s'accentua en dejunis curts (Møller i col., 1993). De fet, amb les dades obtingudes no és possible afirmar que la sensibilitat dels adipòcits va canviar degut al dejuni. En altres models cel.lulars com els hepatòcits en peixos, sí que s'ha descrit que el dejuni afecta la capacitat de resposta de les cèl.lules enfront a l'estimulació hormonal (Harmon i Sheridan, 1992a; Pereira i col., 1995). Independentment de l'efecte del dejuni, aquests resultats estan en acord amb el paper lipolític i anti-lipogènic descrit d'aquesta hormona tant en peixos (Minick i Chavin, 1970; O'Connor i col., 1993; Vega-Rubín de Celis i col., 2003) com en

mamífers (Møller i col., 2003). L'hormona de creixement està intimament relacionada amb la promoció del creixement a través de la seva acció directa i indirecta estimulant la producció en fetge del factor de creixement tipus insulina-I (IGF-I) (revisat per Pérez-Sánchez i Le Bail, 1999) però a la vegada també té altres efectes sobre el metabolisme. Durant el dejuni, els nivells de GH augmenten en el plasma de la truita irisada i l'orada (Yao, 1993; Pérez-Sánchez i col., 1994) i a la vegada es produeix un estat de resistència a la GH per part del fetge (revisat per Pérez-Sánchez i Le Bail, 1999) que afavoreix l'acció metabòlica de la mobilització de lípids que podran ser utilitzats per altres teixits perifèrics. Així, els resultats del present estudi, confirmen que la GH estimula la lipòlisi en el teixit adipós promovent una mobilització d'energia i aquest teixit podria contribuïr a l'augment d'AGL en plasma observat durant el dejuni en orada. Finalment, la composició de la dieta no va afectar en gran mesura la resposta dels adipòcits a la GH ni a les altres hormones estudiades. Tot i això, més estudis en el futur haurien de considerar la possibilitat d'un component estacional en la resposta del teixit adipós a l'hormona de creixement donat que els seus nivells plasmàtics varien considerablement al llarg de l'any (Mingarro i col., 2002).

Regulació de la lipòlisi pel factor de necrosi tumoral-alfa (TNFα) en adipòcits de la truita irisada

El TNFα recombinant humà va estimular la lipòlisi dels adipòcits de truita irisada de forma dosi-depenent sent la concentració de 50 ng/ml la primera dosi efectiva i l'efecte lipolític màxim es va assolir després d'una hora d'incubació. Aquests resultats indiquen que de manera similar als mamífers, el TNFα estimula la lipòlisi del teixit adipós també en la truita irisada. Tot i aquesta similaritat, els efectes del TNFα en la truita irisada van ser molt més ràpids que els descrits en mamífers. Rydén i col. (2002) van obtenir una resposta similar del TNFα en adipòcits d'humans a les concentracions de 1 a 100 ng/ml però després de 48 hores d'incubació. De la mateixa manera, altres estudis han obtingut estimulacions màximes en condicions experimentals similars entre 6 i 24 hores (Hauner i col., 1995; Zhang i col., 2002). Lògicament, pel fet de no haver treballat amb la proteïna homòloga no podem assegurar que el TNFα de truita hauria actuat amb la mateixa rapidesa. Tot i això, els resultats semblen indicar, que en peixos,

el TNFα podria actuar a través de mecanismes intracel.lulars diferents als descrits en mamífers. En mamífers, les vies de transducció de senyal implicades en l'estimulació de la lipòlisi pel TNFα no estan del tot clares. El TNFα és un potent activador de les MAPKs, incloent l'ERK-1 i l'ERK-2 (p42/p44), la c-Jun NH2-kinasa terminal i la p38 (Wallach i col., 1999). Tot i això, sembla que només les ERK1/2 i la c-Jun estarien implicades en l'estimulació de la lipòlisi pel TNFα i no la p38 tant en pre-adipòcits i adipòcits diferenciats d'humans com en la línia d'adipòcits 3T3-L1 (Rydén i col., 2002; Zhang i col., 2002; Souza i col., 2003). En canvi, en el nostre estudi en truita irisada, utilitzant inhibidors de les ERK1/2 i de la p38 vam observar que totes dues vies semblen estar parcialment implicades en la resposta lipolítica del TNFα.

Els efectes lipolítics del TNFα van ser recolzats utilitzant medis condicionats de macròfag de truita estimulats amb LPS. Els adipòcits en contacte amb medis condicionats amb LPS van donar lloc a una lipòlisi més alta que la dels adipòcits en contacte amb medis condicionats no estimulats. Tot i això, en aquest experiment no podem assegurar quin(s) factors d'aquests medis condicionats van ser els responsables dels efectes lipolítics observats, tot i que sí que podem hipotetitzar que almenys un dels factors implicats pot ser el TNFα donat que l'expressió d'aquesta citoquina augmenta en medis de macròfag de truita estimulats amb LPS (MacKenzie i col., 2003).

Finalment, l'administració *in vivo* de LPS va augmentar significativament la lipòlisi basal dels adipòcits 24 hores després de la injecció mentre l'efecte ja no era aparent a les 72 hores. Tot i que en peixos no ha estat específicament estudiat, en mamífers l'administració *in vivo* de LPS estimula la secreció de TNFα cap al corrent sanguini i augmenta l'activitat HSL en teixit adipós (Kawasaki i col., 2004). És per això, que es va utilitzar aquest model enlloc de l'administració directa de TNFα donat que la injecció de LPS reflexa més adequadament una possible situació *in vivo*. A més, de forma interessant, els nivells plasmàtics d'insulina no eren diferents entre les truites injectades amb LPS i les truites control suggerint que els canvis en la lipòlisi dels adipòcits no van ser deguts a canvis en els nivells d'insulina, tot i que degut a que la insulina només es va mesurar en el moment de mostrejar (24 hores) no podem descartar canvis d'aquesta hormona entre 0 i 24 hores.

II. Regulació de l'activitat lipoproteïna lipasa (LPL) en diferents teixits de la truita irisada i l'orada

En el present treball, es va estudiar la regulació nutricional de l'activitat LPL en diversos teixits, en concret en teixit adipós, múscul vermell i múscul blanc de la truita irisada i l'orada així com el paper de la insulina com a possible modulador de l'activitat i l'expressió de la LPL. A més, en el cas de la truita irisada es va estudiar l'efecte *in vivo* d'una injecció de LPS i el paper del TNFα *in vitro* en trossos en teixit adipós.

En primer lloc, es va observar que l'activitat LPL en els diferents teixits de la truita irisada era major que els obtinguts en l'orada, d'acord amb els resultats preliminars presentats per Arantzamendi et al. (2003). En les dues espècies, el teixit adipós va ser el teixit amb l'activitat LPL més elevada seguit pel múscul vermell i pel múscul blanc, aquest últim amb nivells molt baixos, indicant la diferent habilitat dels teixits de la truita irisada i l'orada per captar els triacilglicerols (TAG) continguts en les lipoproteïnes circulants. Aquests resultats concorden amb els resultats obtinguts en truita per Black et al. (1983) on s'observaren diferències similars d'activitat LPL entre teixit adipós i múscul. El fet que el múscul vermell presenti una activitat LPL més elevada que el múscul blanc concorda amb el fet que el múscul vermell conté una major quantitat de lípids en la seva composició (del 7-20% enfront al 2-10% en el múscul blanc) així com una major capacitat per dipositar i utilitzar lípids com a font d'energia respecte al múscul blanc (Robinson i Mead, 1973).

Els estudis de regulació nutricional en truita irisada van mostrar que l'activitat LPL en teixit adipós augmentava en resposta a la ingesta a les 2 hores i significativament a les 4 hores mentre que la resposta en múscul vermell i blanc no era significativa. En mamífers, s'ha descrit una resposta similar d'aquests teixits enfront a la ingesta. Així, l'activitat LPL augmenta en teixit adipós (Eckel, 1987) mentre que en múscul l'activitat o no varia o disminueix (Lanza-Jacoby i col., 1997; Ong i col., 1994; Picard i col., 1999; Ladu i col., 1991). En mamífers, aquest augment de l'activitat LPL en teixit adipós s'ha relacionat directament amb la pujada d'insulina plasmàtica que té lloc post-prandialment (Picard i col., 1999). En el present estudi, la insulina en plasma

va augmentar després de la ingesta suggerint que aquesta hormona podria estar implicada en l'increment d'activitat LPL detectat en el teixit adipós de la truita irisada.

D'altra banda, tant la truita irisada com l'orada van respondre de forma similar al dejuni. En les dues espècies l'activitat LPL va disminuir en teixit adipós tot i que la diferència va ser més pronunciada en l'orada. A més, en el cas de l'orada, es va mesurar l'activitat LPL del múscul vermell i es va veure que no variava. Resultats similars es van presentar per Black i Skinner (1986) en truita on un dejuni llarg (8 setmanes) va provocar una disminució de l'activitat LPL en teixit adipós mentre que no es van obtenir variacions en el múscul vermell. Així, en situacions de dejuni es promou la mobilització lipídica (Navarro i Gutiérrez, 1995) i paral.lelament el teixit adipós atura l'entrada d'AGL. Pel que fa al múscul vermell, s'ha descrit que en períodes de dejuni tant les reserves d'energia (Johnston i Goldspink, 1973) com l'integritat estructural d'aquest múscul (Patterson i Goldspink, 1973) es mantenen a expenses d'altres teixits. D'aquesta manera, el fet que l'activitat LPL en múscul vermell no va variar en el present estudi recolza la idea que aquest és un teixit essencial per als peixos i per tant es manté constant l'aport de lípids a expenses d'altres teixits per tal d'assegurar un correcte funcionament del múscul vermell. Pel que fa a la possible regulació hormonal darrera dels efectes observats, es va detectar una baixada de la insulina plasmàtica en truites i orades dejunades tot i que amb aquests experiments de dejuni no es pot assegurar que la insulina reguli l'activitat LPL directament.

Per tal d'estudiar més concretament el possible efecte de la insulina sobre l'activitat LPL es van realitzar experiments d'injecció d'insulina en truita irisada; i d'arginina i d'insulina en orada. Aquesta és la primera vegada que s'estudiava el possible paper de la insulina com a regulador de la LPL en peixos. En les dues espècies, la injecció d'insulina va provocar un augment de l'activitat LPL en teixit adipós a les 3 hores, mentre que a les 6 i a les 18 hores l'efecte ja no era significatiu tant per la truita irisada com per l'orada respectivament. A més, en el cas de l'orada no es va veure cap resposta de l'activitat LPL en múscul vermell degut a la injecció d'insulina. Aquests resultats doncs presenten a la insulina com un regulador positiu de l'activitat LPL en teixit adipós mentre que sembla que aquesta hormona no té un paper directe sobre múscul vermell. Els resultats obtinguts són comparables als descrits en mamífers on la insulina administrada *in vivo* té un efecte diferent segons el teixit. En teixit adipós, l'activitat LPL augmenta (Farese i col., 1991) i en múscul esquelètic l'activitat o no es veu afectada (Tan i col., 1977) o disminueix (Cryer i col., 1976). A més, en el cas de

l'orada es va veure un paral.lelisme entre els canvis en l'activitat i els canvis en els nivells d'ARNm en teixit adipós i múscul vermell induïts per la insulina, tot i que els canvis en l'expressió de la LPL en el teixit adipós van ser més lents que els canvis obtinguts en activitat. De fet, en mamífers, la regulació de la LPL s'ha vist que és molt complexa i es pot produir a diferents nivells. En principi, tant en situacions de dejuni com d'administració d'insulina s'ha observat un correlació positiva entre expressió i activitat LPL en teixit adipós (Ong i col., 1988). En aquest sentit, sembla que la insulina actuaria afavorint l'estabilitat de l'ARNm (Raynolds i col., 1990). Tot i això, en dejunis curts en rates la disminució d'activitat LPL no va acompanyada d'una disminució en els nivells d'ARNm suggerint una regulació de l'enzim post-translacional (Bergö i col., 1996), en aquest cas la proteïna passa a una forma inactiva amb baixa afinitat per l'heparina. En el present estudi, l'activitat LPL després de l'administració d'insulina va augmentar significativament abans que s'observessin canvis d'expressió. D'aquesta manera sembla que a curt termini la regulació també seria post-translacional en l'orada. Els resultats obtinguts *in vitro* amb trossos de teixit adipós de truita irisada recolzen aquesta idea, donat que la incubació amb insulina (4 hores) va augmentar el percentatge de LPL alliberable per heparina o sigui la fracció activa de l'enzim. Això no descarta que la insulina pugui tenir un efecte positiu sobre l'expressió de la LPL a més llarg termini, com s'ha vist en l'orada, ja sigui afavorint l'estabilitat de l'ARNm o per altres mecanismes.

D'altra banda, es va veure que l'arginina no és un bon model hiperinsulinèmic en l'orada donat que aquest aminoàcid, a diferència d'altres espècies de peixos, va estimular de forma més potent la secreció de glucagó que la secreció d'insulina i sota aquestes circumstàncies no es van veure canvis significatius en l'activitat LPL del teixit adipós.

Finalment, es va estudiar l'efecte d'una injecció *in vivo* de LPS sobre l'activitat LPL en teixit adipós de la truita irisada. L'administració *in vivo* de LPS va provocar una disminució de l'activitat LPL en teixit adipós 24 hores després de la injecció. Donat que la lipòlisi dels adipòcits obtinguts de truites injectades amb LPS va ser més elevada, es confirma que el LPS no només activa la lipòlisi en teixit adipós sinó que a més inhibeix l'entrada d'AGL en aquest teixit, de forma similar a l'observat en rates (Kawasaki i col., 2004). De fet en mamífers, s'ha vist que a part del dejuni hi ha altres situacions on clarament disminueix l'activitat LPL i és en situacions de trauma/sepsis/administracions de LPS. En aquest últim cas s'ha descrit que la resposta és mediada per l'acció del

TNFα. De fet s'ha descrit un efecte directe del TNFα sobre l'activitat LPL tant *in vivo* (Semb i col., 1987; Grunfeld i col., 1989) com *in vitro* en adipòcits aïllats. Per tal de comprovar si el TNFα juga un paper directe sobre l'activitat LPL en teixit adipós de la truita irisada es van realitzar experiments *in vitro* amb trossos de teixit adipós. Els resultats obtinguts no van ser conclusius donat que després de la incubació amb TNFα es va observar una disminució de la fracció de LPL alliberable per heparina (efecte contrari a l'obtingut en incubacions amb insulina) però els canvis no foren significatius. De manera que és necessària més informació per tal de concloure si el TNFα afecta directament l'activitat LPL en el teixit adipós dels peixos.

III. El transport de glucosa en adipòcits de la truita irisada

Els adipòcits estan formats majoritàriament per TAG i per aquest motiu el volum d'aquestes cèl.lules està en funció del balanç que es dóna entre la síntesi i la degradació o lipòlisi d'aquests TAG. En la reserva de TAG intervenen l'entrada d'AGL exògens i la síntesi *de novo* d'AGL (o lipogènesi), i l'acumulació d'aquests AGL en TAG. La lipogènesi i síntesi de TAG doncs, dependrà en part de la disponibilitat d'AGL i glucosa, substrat important per a la síntesi *de novo* d'AGL i de glicerofosfats. És per això, que l'estudi del transport de glucosa dins l'adipòcit és un dels primers passos a tenir en compte a l'hora d'entendre la regulació de la lipògenesi i acumulació de TAG en el teixit adipós.

Per tal de mesurar el transport de glucosa en adipòcits de truita irisada, es van realitzar mesures de la captació de 2-deoxi-D-glucosa (2-DG) en adipòcits aïllats. La 2-DG és un anàleg no metabolitzable de la glucosa que s'utilitza de forma habitual per a la mesura del transport de glucosa. En el present treball, es va observar que la captació de 2-DG augmentava amb el nombre de cèl.lules i el temps d'incubació. A més, es va veure que el transport es donava a través de transportadors de glucosa específics de difusió facilitada o GLUTs donat que el transport era inhibible de forma dosi depenent per la citocalasina B. Resultats similars es van obtenir en eritròcits d'anguila (*Anguilla rostrata*) (Soengas i Moon, 1995), enteròcits de peix gat (*Ictalurus melas*) (Soengas i Moon, 1998) i miòcits de truita irisada (Castillo i col., 2004) on es va veure que el transport de glucosa en aquestes cèl.lules també era inhibible per la citocalasina B.

Paper de la insulina i el factor de creixement tipus insulina-I (IGF-I)

En mamífers és ben conegut que la insulina estimula el transport de glucosa en múscul esquelètic i teixit adipós (Bryant i col., 2002), mentre que en peixos no està tan clar (Wright i col., 1998). Únicament en miòcits de truita irisada, Castillo i col. (2004) van determinar que la insulina estimulava la captació de glucosa. Fins el moment, no s'han descrit efectes lipogènics de la insulina en el teixit adipós dels peixos. El treball de Christiansen i col. (1985), l'únic que analitzà el transport de glucosa en adipòcits de truita, no van veure cap efecte de la insulina sobre el transport de glucosa. Tot i això, en tal estudi es van realitzar captacions de glucosa curtes (entre 4 i 5 minuts) degut a la

fragilitat dels adipòcits sota les seves condicions. Aquests resultats, doncs, contrasten amb els del present estudi donat que la insulina va estimular la captació de glucosa quan l'hormona es pre-incubava durant 30 minuts i es mesurava el transport durant 2-3 hores. De forma similar en miòcits de truita irisada les estimulacions per insulina sobre el transport de glucosa es donaven quan les cèl.lules eren pre-incubades amb l'hormona 30 minuts i es mesurava el transport durant 30 o 60 minuts (Castillo i col., 2004). Sembla doncs, que calen temps d'incubació més llargs que els habitualment utilitzats en mamífers (de l'ordre de minuts) per observar estimulacions per hormones en peixos. En aquest sentit, s'ha descrit que els GLUTs en peixos presenten una menor afinitat per la glucosa (Teerijoki i col., 2001; Capilla i col., 2004) comparat amb els mamífers (Burant i Bell, 1992); aquest fet podria ser un dels factors implicats que podria explicar, almenys en part, la necessitat de temps d'incubació més llargs.

L'IGF-I va ser més potent que la insulina estimulant la captació de glucosa en adipòcits de truita irisada. Resultats similars van ser obtinguts en miòcits de truita irisada on l'IGF-I va ser més eficient que la insulina estimulant el transport de glucosa (Castillo i col., 2004). De fet, tot i que fins el moment no s'han descrit efectes directes del IGF-I sobre el teixit adipós en peixos, Planas i col. (2000a) ja van veure que el teixit adipós de la truita comú (*Salmo trutta*), i també altres teixits, contenien més receptors d'IGF-I que d'insulina fet que suggereix un possible paper important d'aquest pèptid també en el teixit adipós.

Finalment, experiments realitzats utilitzant wortmanina van donar com a resultat un bloqueig total dels efectes estimuladors de la insulina sobre el transport de glucosa. Per aquest motiu, podem dir que en adipòcits de truita irisada sembla que la principal via intracel.lular implicada en els efectes observats és la de la PI3K/Akt. En mamífers, aquesta via ha estat implicada en els efectes estimuladors de la insulina sobre el transport de glucosa. Així sembla que la insulina, a través de la PI3K entre d'altres, estimula la translocació del GLUT4 a la membrana plasmàtica per tal d'augmentar la captació de glucosa en teixits diana per la insulina com el múscul esquelètic i el teixit adipós. En peixos, Capilla i col. (2004) van caracteritzar un transportador de glucosa en teixit adipós de salmó homòleg al GLUT4 de mamífers. El fet que l'estimulació de la insulina sobre el transport de glucosa sigui mediat a través de la via de la PI3K recolza l'existència d'un GLUT4 funcional en teixit adipós de la truita que podria translocar-se a la membrana després de l'estimulació per insulina.

CONCLUSIONS

1. S'han establert les condicions per a l'obtenció de preparacions d'adipòcits aïllats a partir de teixit adipós mesentèric de la truita irisada (*Oncorhynchus mykiss*) i l'orada (*Sparus aurata*) amb característiques morfològiques i viabilitat adequades, que han permés l'estudi dels nivells de lipòlisi en aquestes cèl.lules.

2. La lipòlisi, mesurada com l'alliberació de glicerol en el medi, augmenta en adipòcits aïllats de la truita irisada i l'orada proporcionalment amb el temps d'incubació (fins almenys les 7 hores) i amb la concentració de cèl.lules (almenys fins a $13.2x10^5$ i $12x10^5$ adipòcits/ml en el cas de la truita i l'orada respectivament).

3. La incubació amb insulina a 35 i 350 nM va disminuir la lipòlisi dels adipòcits aïllats de la truita irisada *in vitro* mentre que el glucagó va ser lipolític a les concentracions de 10 i 100 nM. A més, el dejuni, acompanyat d'una hipoinsulinèmia, així com la injecció de glucagó, van incrementar la lipòlisi mesurada en adipòcits aïllats indicant que aquest sistema *in vitro* reflexa l'estat nutricional i hormonal del peix.

4. De forma similar a l'observat en la truita irisada, la lipòlisi d'adipòcits d'orades dejunades va ser superior a la d'adipòcits d'orades alimentades. En aquest espàrid, el glucagó i especialment l'hormona de creixement incubats *in vitro* estimularen la lipòlisi dels adipòcits aïllats mentre que la insulina no va tenir cap efecte significatiu sobre la lipòlisi, fet que pot ser degut a factors estacionals o a diferències interespecífiques en l'acció hormonal.

5. Els adipòcits d'orades alimentades amb una dieta amb proteïna 100% d'origen vegetal presentaren una lipòlisi més elevada que la d'adipòcits d'orades alimentades amb una dieta amb proteïna procedent de farina de peix. D'altra banda, l'adaptació de les orades a una o altra dieta no va provocar canvis significatius ni en la resposta dels adipòcits al dejuni ni a l'efecte de les hormones estudiades (insulina, glucagó i hormona de creixement).

6. El factor de necrosi tumoral-alfa (TNFα) va estimular la lipòlisi dels adipòcits de la truita irisada de forma dosi i temps-depenent. Aquest efecte estimulador va

ser bloquejat parcialment utilitzant inhibidors de les MAPKs: ERK1/2 i p38 suggerint una implicació d'aquestes vies de senyalització en els efectes observats. A més, els adipòcits incubats amb medis condicionats de macròfags de truites irisades incubats amb lipopolisacàrid (LPS) van presentar una lipòlisi basal més alta respecte a la dels adipòcits incubats amb medis condicionats control, recolzant una possible estimulació per part del TNFα o d'altres citoquines.

7. L'administració *in vivo* de LPS en la truita irisada va provocar un augment de la lipòlisi dels adipòcits *in vitro* 24 hores després de la injecció. A més, en aquest temps simultàniament, el LPS va inhibir l'activitat lipoproteïna lipasa (LPL) del teixit adipós indicant que en situacions d'infecció per LPS no només s'activa la lipòlisi en el teixit adipós sinó que a més disminueix l'entrada d'AGL en aquest teixit.

8. En truita, l'activitat LPL va augmentar en resposta a la ingesta en el teixit adipós i no en múscul. El dejuni, tant en la truita irisada com en l'orada, va provocar una disminució en l'activitat LPL del teixit adipós paral.lelament amb una baixada de la insulina plasmàtica, mentre que a més en l'orada l'activitat en el múscul vermell va romandre constant.

9. L'administració *in vivo* d'insulina va estimular l'activitat LPL en el teixit adipós 3 hores després de la injecció en la truita irisada i l'orada. En l'orada, aquest canvi en l'activitat va anar acompanyat per un augment dels nivells d'ARNm tot i que els efectes van ser més endarrerits en el temps. D'altrabanda, en la truita irisada la incubació *in vitro* de trossos de teixit adipós amb insulina va produir un augment en la proporció d'activitat LPL alliberable per heparina, que correspon a la fracció extracel.lular activa de l'enzim.

   Aquests resultats suggereixen que la insulina pot actuar regulant l'activitat de l'enzim tant a nivell transcripcional com post-traduccional en les espècies estudiades.

10. El transport de glucosa mesurat com la captació de 2-DG en adipòcits de la truita irisada va augmentar amb la concentració de cèl.lules i el temps d'incubació. Aquest transport és inhibible per citocalasina B, indicant que el transport en aquestes cèl.lules es dóna a través de transportadors específics de glucosa de difusió facilitada o GLUTs.

11. La insulina i especialment l'IGF-I van estimular la captació de glucosa dels adipòcits aïllats de la truita irisada. L'efecte estimulador de la insulina va ser totalment bloquejat per la wortmanina suggerint que la via de la PI3K/Akt és la principal via de senyalització implicada.

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PUBLICACIONS

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