

# Orexin receptor expression in human adipose tissue: effects of orexin-A and orexin-B

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## Abstract

Orexin-A and orexin-B, via their receptors orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R) have been shown to play a role in the regulation of feeding, body weight, and energy expenditure. Adipose tissue also contributes significantly to the maintenance of body weight by interacting with a complex array of bioactive peptides; however, there are no data as yet on the expression of orexin components in adipose tissue. We, therefore, analyzed the expression of OX1R and OX2R in human adipose tissue and determined functional responses to orexin-A and orexin-B. OX1R and OX2R mRNA expression was detected in subcutaneous (s.c.) and omental adipose tissue and in isolated adipocytes. Protein for OX1R and OX2R was also detected in whole

adipose tissue sections and lysates. Treatment with orexin-A, and orexin-B (100 nM, 24 h) resulted in a significant increase in peroxisome proliferator-activated receptors  $\gamma$ -2 mRNA expression in s.c. adipose tissue ( $P < 0.05$ ). Hormone sensitive lipase mRNA was significantly reduced in omental adipose tissue with orexin-A and orexin-B treatment ( $P < 0.05$ ). Glycerol release from omental adipose tissue was also significantly reduced with orexin-A treatment ( $P < 0.05$ ).

These findings demonstrate for the first time the presence of functional orexin receptors in human adipose tissue and suggest a role for orexins in adipose tissue metabolism and adipogenesis.

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## Introduction

The regulation of body weight and energy balance is controlled by a series of intricate, and highly complicated, neural and humoral mechanisms. The orexigenic peptides, orexin-A and orexin-B, are proteolytically cleaved from a common precursor, prepro-orexin and share 46% amino acid sequence identity (de Lecea *et al.* 1998, Sakurai *et al.* 1998). The neurons producing orexins are located in the lateral and posterior hypothalamus and send their projections widely into the central nervous system, resulting in multiple physiological functions, including the control of arousal and sleep–wake cycle, regulation of cardiovascular and autonomic function, and the neuroendocrine system (Ida *et al.* 1999, Samson *et al.* 1999, Peyron *et al.* 2000). Orexins bind to and activate two G-protein-coupled receptors, orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R), which display 64% amino acid sequence identity (Sakurai *et al.* 1998). Orexin-A binds with a high affinity to OX1R, whilst OX2R binds both orexin-A and orexin-B with similar affinity.

Intracerebroventricular (i.c.v.) administration of orexin-A or orexin-B stimulates food consumption in rats (Sakurai *et al.* 1998) but does not result in weight gain in the short-term (Yamanaka *et al.* 1999). Conversely, it has been shown that although genetic ablation of orexin neurons in mice causes

hypophagia (Hara *et al.* 2001), these mice develop late-onset obesity indicating a possible further role of orexins in energy expenditure. Indeed, orexins have been shown to be involved in modulating metabolic rate via stimulation of the sympathetic nervous system (Antunes *et al.* 2001), and regulation of energy expenditure and thermogenesis (Szekely *et al.* 2002, Yasuda *et al.* 2005). Interestingly, in obese as compared with non-obese humans, orexin-A levels have been reported to be significantly lower (Adam *et al.* 2002), suggesting that orexin-A is involved in the regulation of human energy metabolism at the peripheral level or non-centrally.

Although the hypothalamus is considered as the cornerstone for the maintenance of energy homeostasis, acting in concert with peripheral signals, orexin receptors have also been demonstrated in tissues known to play a role in the integration of metabolic activity and energy balance such as the adrenal gland (reviewed by Spinazzi *et al.* 2006). However, despite *in vivo* studies demonstrating a role for centrally acting orexins on energy expenditure and metabolic signals regulating the orexin-receptor system (Beck & Richy 1999, Wortley *et al.* 2003, Karteris *et al.* 2005), there are no data available on the presence of orexin receptors in adipose tissue.

We analyzed the expression of OX1R and OX2R in human adipose tissue and isolated adipocytes from human

abdominal s.c. intra-abdominal omental (Ome) adipose tissue. We also assessed the effects of orexin-A and orexin-B on the expression of key metabolic genes in adipose tissue, including lipoprotein lipase (LPL), hormone sensitive lipase (HSL), and the nuclear hormone receptors, peroxisome proliferator-activated receptors  $\gamma$ -1 and -2 (PPAR $\gamma$ -1 and PPAR $\gamma$ -2), as well as their effect on glycerol release.

## Materials and Methods

### Subjects

Adipose tissue biopsies (s.c. and Ome) were obtained from female non-diabetic subjects undergoing elective surgery ( $n=6$ , body mass index (BMI)  $26.8 \pm 1.1$  s.d., age  $32 \pm 4.3$  years). Patients had been fasted overnight prior to surgery and were not taking contraceptive agents or hormone-replacement therapy. The study was approved by the Local Research Ethics Committee and all patients involved gave their informed consent in accordance with the guidelines in The Declaration of Helsinki.

### Primary explants culture and isolation of adipocytes

Adipose tissue organ explants were cultured using methods described by Fried & Moustaid-Moussa (2001). Briefly, 1–3 g adipose tissue was obtained during the first 30 min after the induction of anesthesia and placed directly into 50 ml plastic tubes containing 20 ml Media 199 (Gibco-BRL) supplemented with 50  $\mu$ g/ml gentamycin and 1% fetal bovine serum (FBS), and coarsely minced using scissors to prevent tissue hypoxia. Tissue was then immediately transported and utilized for tissue culture within 1 h of excision. Prior to culture, tissue was further minced into approximately 1 mm<sup>3</sup> fragments and washed by pouring through a screen cup fitted with a 230  $\mu$ m mesh (Sigma, filter no. 60) and rinsing with sterile PBS warmed to 37 °C. Tissue fragments were weighed and transferred to six-well plates (100 mg/well) each well containing 3 ml Media 199 (Gibco-BRL) supplemented with 50  $\mu$ g/ml gentamycin and 1% FBS, and cultured for 24 h with or without the addition of orexin-A, orexin-B ( $10^{-7}$  M), or isoproterenol ( $10^{-7}$  M), in a 37 °C incubator under an atmosphere of 5% CO<sub>2</sub>/95% air. A single concentration of orexins was chosen due to ethical considerations resulting in a limited amount of adipose tissue being available for these studies. The concentration of  $10^{-7}$  M was used as this was shown to be maximally effective in stimulating cortisol release from freshly dispersed adrenocortical adenoma cells (Spinazzi *et al.* 2005).

An additional incubation with insulin (7 nM) and dexamethasone (25 nM) was carried out as a control for tissue hypoxia, which was to ensure that the explants demonstrated an expected increase in leptin mRNA in the first 24 h of culture (Fried & Moustaid-Moussa 2001).

After 24 h, tissue fragments were removed and placed in 1 ml ice-cold Qiazol (Qiagen) for RNA extraction, or

radioimmunoprecipitation (RIPA) lysis buffer (Upstate, Lake Placid, NY, USA) for protein extraction. Media were collected and stored at  $-80$  °C before glycerol assay.

Primary adipocytes were isolated by a method modified from that of Rodbell (1964). Adipose tissue biopsies were chopped finely and adipocytes isolated by collagenase digest (Hanks balanced salt solution, containing 3 mg/ml collagenase (type II) and 1.5% BSA) in a shaking water-bath at 37 °C for 60 min. After collagenase digest, the cell suspensions were passed through a 230  $\mu$ m screen cup Filter mesh no. 60 (Sigma). Mature adipocytes were separated from the stromal vascular cells through an inert oil, bis (3,5,5 trimethylhexyl) phthalate, specific gravity 0.98 (Fluka Chemicals, Gillingham, Dorset, UK) by the method of Gliemann *et al.* (1972). To the filtered suspension, 1 ml bis (3,5,5 trimethylhexyl) phthalate was added, which was then centrifuged for 5 min at 1500 r.p.m. The adipocytes form a layer on top of the oil, which has a lower density than the collagenase-digestion medium and a higher density than the adipocytes. The stromal vascular cells were treated with an erythrocyte lysis buffer and processed along with the isolated adipocytes for RNA extraction as described for adipose tissue explants.

### RT-PCR

Total RNA was extracted using the Qiagen RNeasy Lipid Tissue Mini Kit and reverse-transcribed into cDNA as previously described (Randevara *et al.* 2001).

OX1R and OX2R expression was measured by RT-PCR, using 1  $\mu$ g RNA and oligo (dT)<sub>15</sub> as reverse transcription primers. A control reaction which omitted reverse transcriptase was included to check for the presence of genomic DNA. OX1R and OX2R were amplified using a Hybaid Thermal Cycler in 50  $\mu$ l reaction medium containing 1 unit Platinum Taq polymerase (Invitrogen Life Technologies), 20 pmol of each sense and anti-sense primer and dNTP (10 mmol/l each), using the following cycling conditions: 94 °C for 1 min, then 38 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s, followed by a 7 min extension at 72 °C. The sequences for the sense and anti-sense primers are shown in Table 1. PCR products were stained with ethidium bromide and visualized by electrophoresis through 1.5% agarose gels. Direct sequencing of the PCR products confirmed the sequence identities.

### Quantification of mRNA

The concentrations of target mRNAs were measured by reverse transcription followed by real-time PCR performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). Table 1 describes the primers used for this study. PCRs were carried out using 2  $\mu$ l cDNA in 5  $\mu$ l PCR SYBR Green-1 Light Cycler 'Master Mix' (Biogene, Kimbolton, Cambridgeshire, UK) and 1  $\mu$ l sense and anti-sense primers. A series of three dilutions for each cDNA was used to ensure linear amplification. Protocol conditions consisted of denaturation

**Table 1** Primer sequences and resulting product sizes

	Sense	Anti-sense	Product size (bp)
<b>Primer</b>			
HSL	tgcaaatcccgcctat	ctcgttgcgttttagt	338
LPL	tgtggcccggttatca	tgcaatcacgcgatag	358
PPAR $\gamma$ -2	gatacactgtctgcaaacatcacaa	ccacggagctgatcccaa	72
OX1R	ccctcctggctgaagtgaag	agtgggagaaggtgaagcag	189
OX2R	gtcgcaactggctcatctgct	cgctcctatgggtggtct	227
Prepro-orexin	caattgacagcctcaaggttc	cagaggcctgggccaggacag	519

HSL, hormone sensitive lipase; LPL, lipoprotein lipase; PPAR $\gamma$ -2, peroxisome proliferator-activated receptor  $\gamma$ -2; OX1R, orexin-1 receptor; OX2R, orexin-2 receptor.

of 95 °C for 60 s, followed by 40 cycles of 94 °C for 1 s, 60 °C for 7 s, and 72 °C for 12 s, followed by melting-curve analysis. For analysis, quantitative amounts of the genes of interest were standardized against the housekeeping gene  $\beta$ -actin. Negative controls for all the reactions included preparations lacking cDNA or RNA-lacking reverse transcriptase in place of the cDNA. The relative mRNA levels were expressed as a ratio using 'Delta-delta method' for comparing relative expression results between treatments in real-time PCR (Pfaffl 2001).

#### Immunohistochemistry

Adipose tissue sections were cut at 3  $\mu$ m and floated onto 3-aminopropyltriethoxy-silane-coated slides. Heat-induced antigen retrieval was used in pH 7.8 Tris-EDTA buffer. Following a serum block (10% BSA in PBS) of 30 min at room temperature, sections were incubated with primary antibodies, 1:200; OX1R, OX2R, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, and AbCam, Cambridge, UK respectively) for 1 h at room temperature, then with the secondary antibody (biotinylated universal antibody, Universal Elite kit, Vector Laboratories, Peterborough, UK) for 30 min. Avidin-biotin complex solution was applied to the sections, and incubated for 30 min at room temperature (VECTASTAIN Elite ABC reagent, Vector Laboratories). Diaminobenzidin solution was applied to the sections for 5 min (Menarini Concentrated Substrate Cat. HK153-5K). The sections were then rinsed with deionized water, and counterstained for 1 min in Mayer's hematoxylin and blued in Scott's tap water, dehydrated, cleared, and mounted.

In relation to the primary antibodies used, OX1R antibody, a goat polyclonal antibody, was raised against the C-terminus of the human OX1R, whilst the OX2R antibody, a monoclonal antibody, was raised against the full-length fusion protein and has been validated by the supplier to ensure that it is specific for OX2R.

#### Western blotting

Protein lysates were prepared by homogenizing adipose tissue in RIPA lysis buffer (Santa Cruz Biotechnology Inc.) with the

addition of the manufacturer's protease inhibitor cocktail containing, AEBSF, aprotinin, leupeptin, bestatin, pepstatin, E-64, sodium orthovanadate, and phenylmethylsulphonyl fluoride. For protein measurement using the Bradford method (Bradford 1976), 100  $\mu$ l aliquots were taken. For sample preparation, equal amounts of Laemmli buffer (5 M urea, 0.17 M SDS, 0.4 M dithiothreitol, and 50 mM Tris-HCl, pH 8.0) were added, and samples denatured by sonication and boiling. Samples were separated by SDS-PAGE (10% resolving gel) and transferred to polyvinylidene difluoride (PVDF) membranes at 100 V for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The PVDF membranes were incubated with primary antibody for OX1R (Santa Cruz Biotechnology, Inc.) and a monoclonal OX2R antibody (Abcam) at a 1:1000 dilution in tris buffered saline (TBS)-0.1% Tween (TBST), and 5% BSA overnight at 4 °C. The membranes were washed, incubated with a secondary anti-goat (OX1R), anti-mouse (OX2R) horseradish peroxidase-conjugated antibody (1:2000) for 1 h at room temperature, and washed for 60 min with TBST. Antibody complexes were visualized using chemiluminescence. The densities were measured using a scanning densitometer coupled to Scion Image scanning software (Scion Corporation, Frederick, MD, USA).

#### Glycerol release

Media from the adipose tissue explants and isolated adipocytes were collected at the end of the incubation periods, stored at -80 °C and defrosted on ice immediately prior to assay. Free glycerol release was used as a measure of lipolysis (micromoles per milliliter) using a commercially available colorimetric kit (Randox Laboratories, Crumlin, Co Antrim, UK), which utilizes a quinoneimine chromogen system in the presence of glycerol kinase, peroxidase, and glycerol phosphate oxidase.

#### Statistical analysis

Data are shown as the mean  $\pm$  S.E.M. of each measurement. Student's *t*-test was employed to calculate the significance of differences in the means between the different groups. A *P*-value < 0.05 was considered to be significant.

## Results

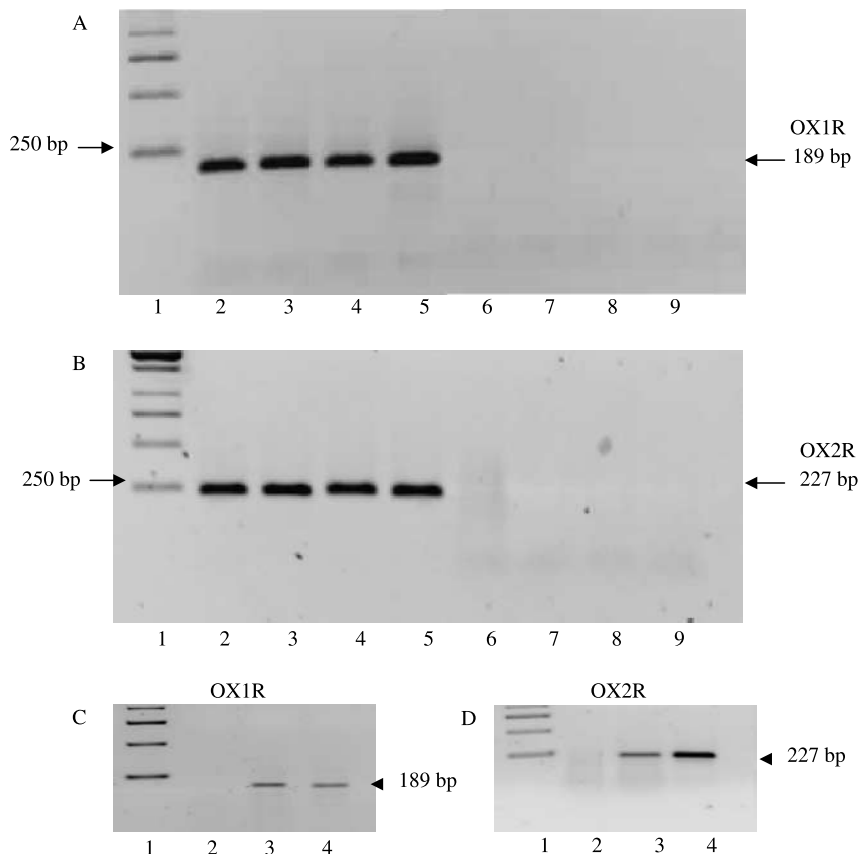
### *mRNA and protein expression of orexin receptors in human adipose tissue*

The expression of orexin receptors in human adipose tissue was analyzed by RT-PCR. OX1R and OX2R were expressed in human adipose tissue, and in isolated human adipocytes ( $n=4$ ). Figure 1A shows a representative ethidium bromide-stained gel giving a 189 bp PCR product for the OX1R, and Fig. 1B shows a 227 bp PCR product for the OX2R. Bands were excised and purified using QIAquick Gel extraction kit (Qiagen) and sequenced to confirm their identity. Using primers described in

Table 1, this study was unable to detect prepro-orexin mRNA in adipose tissue from either site (data not shown).

To ensure that mRNA expression of OX1R and OX2R was exclusively attributable to the adipocytes, RNA was also extracted from the stromal vascular cells as well as the isolated adipocytes in the same sample. RT-PCR analysis demonstrated no detectable expression for either receptor in the stromal vascular cells. A representative ethidium bromide-stained gel is shown in Fig. 1C.

Analysis of protein by western blotting revealed a band of 48 kDa for OX1R in both s.c. and Ome adipose tissue ( $n=4$ ). Furthermore, a band of 50 kDa was detected for OX2R, which is in agreement with the size predicted by the



**Figure 1** (A) The detection of OX1R in adipose tissue and isolated adipocytes. Representative results showing ethidium bromide-stained electrophoresis gel of PCR products from reverse transcribed RNA using primers for OX1R in human s.c. adipose tissue (lane 2), Ome adipose tissue (lane 3), s.c. adipocytes (lane 4), Ome adipocytes (lane 5), lanes 6–9 show corresponding RT-negative controls. (B) The detection of OX2R in adipose tissue and isolated adipocytes. Representative results showing ethidium bromide-stained electrophoresis gel of PCR products from reverse transcribed RNA using primers for OX2R in human s.c. adipose tissue (lane 2), Ome adipose tissue (lane 3), s.c. adipocytes (lane 4), Ome adipocytes (lane 5), lanes 6 to 9 show corresponding RT-negative controls. (C) Representative electrophoresis gel showing no expression of OX1R in human s.c. stromal vascular cells (lane 2) compared with s.c. adipocytes (lanes 3 and 4). (D) Representative electrophoresis gel showing no expression of OX2R in human s.c. stromal vascular cells (lane 2) compared with s.c. adipocytes (lanes 3 and 4).

Universal Protein resource (UNIPROT). Figure 2A shows a representative western blot.

#### Immunohistochemical analysis of orexin receptors in human adipose tissue

In order to investigate further the cellular distribution of orexin receptors in human adipose tissue, we used specific non-crossreactive antibodies for each receptor. Intense membrane staining demonstrated the presence of both OX1R and OX2R in human s.c. adipose tissue (Fig. 2B). No apparent expression of either of the receptors was evident in the negative controls, thus confirming staining specificity.

#### Effects of orexins on gene expression in adipose tissue

Leptin mRNA expression in adipose tissue explants treated with insulin (7 nM) and dexamethasone (25 nM) was compared with non-treated to ensure that explants were responsive in terms of mRNA expression. Explants incubated for 24 h with insulin and dexamethasone demonstrated a twofold increase in leptin mRNA (data not shown), which is in agreement with the results of Russell *et al.* (1998). Treatment of adipocyte explants with orexin-A (100 nM) for 24 h, resulted in a significant increase in PPAR $\gamma$ -2 mRNA expression in s.c. adipose tissue by 1.5-fold and with orexin-B by threefold ( $P < 0.05$ ) (Fig. 2A). Neither orexin-A nor orexin-B had any significant effect on PPAR $\gamma$ -2 expression in Ome adipose tissue (Fig. 3A). Treatment with both orexin-A

and orexin-B resulted in approximately a 30-fold decrease in HSL mRNA in Ome adipose tissue,  $P < 0.05$  (Fig. 3B).

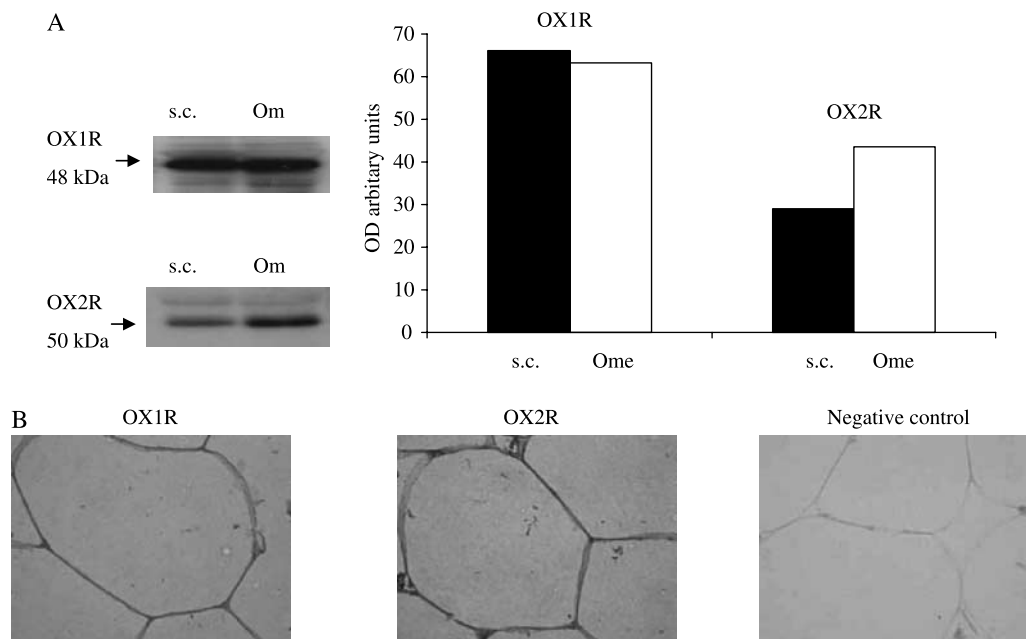
Analysis of LPL mRNA expression following treatments with orexin-A and orexin-B showed a trend (non-significant) for an increase in s.c. adipose tissue and a decrease in Ome adipose tissue (Fig. 3C).

#### Effect of orexins on adipose tissue lipolysis

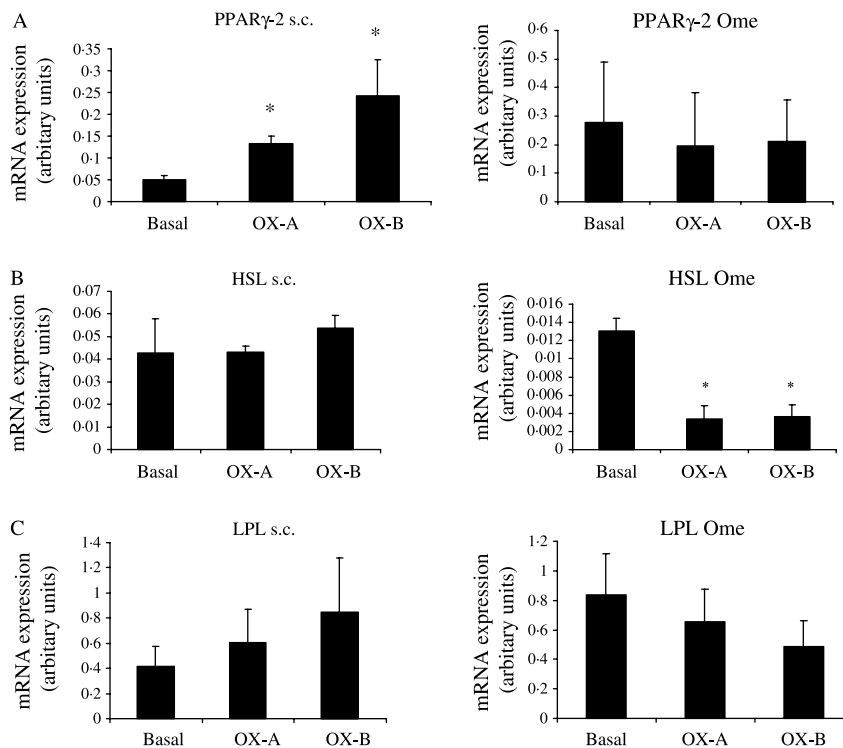
Adipose tissue explants from Ome and s.c. adipose depots were treated with orexin-A and orexin-B ( $10^{-7}$  M), or with the lipolytic agent, isoproterenol ( $10^{-6}$  M), and the assay was terminated at 24 h. As expected, incubation of adipose explants from both sites with isoproterenol significantly stimulated lipolysis ( $P < 0.01$ , Fig. 4A and B). Glycerol release was significantly reduced in Ome adipose tissue, with orexin-A ( $P < 0.05$ ). Although there was also a trend towards a reduction in glycerol release with orexin-B treatment, this was not significant ( $P < 0.09$ , Fig. 4B). However, there was no apparent effect of glycerol release in the s.c. adipose tissue, when treated with either orexin-A or orexin-B (Fig. 4A).

## Discussion

The present study demonstrates for the first time the expression of both OX1R and OX2R mRNA in human isolated adipocytes and protein in whole adipose tissue. We have previously reported detection of OX2R in human



**Figure 2** (A) Representative western blot detection of OX1R and OX2R in s.c. and omental (Ome) human adipose tissue. (B) Immunohistochemical analysis of OX1R and OX2R in human s.c. adipose tissue (1000 $\times$ ), negative control, serum only.



**Figure 3** (A) Treatment with orexin-A (OX-A) results in a 1.5-fold increase in peroxisome proliferator-activated receptor  $\gamma$ -2 (PPAR $\gamma$ -2) mRNA in s.c. adipose tissue and Orexin-B (OX-B) treatment results in a threefold increase in PPAR $\gamma$ -2 mRNA in s.c. adipose tissue ( $n=6$ ). (B) Treatment of s.c. adipose tissue had no significant effect on hormone sensitive lipase (HSL) mRNA expression. Treatment of Ome adipose tissue with OX-A and OX-B resulted in a significant decrease in mRNA expression of HSL ( $n=6$ ). (C) Relative mRNA expression of lipoprotein lipase (LPL) in OX-A and OX-B-treated s.c. and Ome adipose tissue ( $n=6$ ).

adrenal tissue at 38 kDa, and in the male reproductive system at 40 kDa, with little or no immunodetection when a blocking peptide was used. These observations indicate probable post-translational modification of this receptor according to tissue type and further studies are required to ascertain the tissue-specific function(s) of altered OX2R.

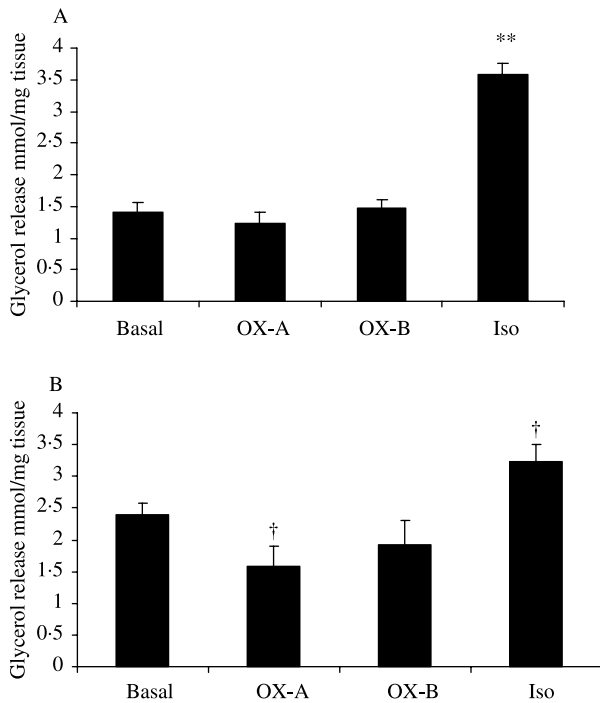
The novel finding of these orexin receptors in adipocytes significantly adds to the current known non-central tissue distribution, which includes the adrenal gland (Randeve *et al.* 2001), male reproductive system (Karteris *et al.* 2004) thyroid, lung, kidney, and jejunum (Johren *et al.* 2001).

Orexins and their receptors are influenced by nutritional status and peripheral signals, including leptin (Beck & Richy 1999, Karteris *et al.* 2005). Furthermore, circulating lipid levels rise noticeably with dietary obesity and the hypertriglyceridemia increases hypothalamic orexin gene expression (Wortley *et al.* 2003). The detection at both the mRNA and protein level of orexin receptors in our study indicates a possible direct role for orexins in adipose tissue metabolism.

The orphan nuclear hormone receptor PPAR $\gamma$ , of which there are two isoforms PPAR $\gamma$ -1 and -2, plays a crucial role in adipogenesis and modulation of adipocytes-specific genes (Kintscher & Law 2005). In human adipose tissue, PPAR $\gamma$ -2

is the predominant isoform and is more highly expressed in s.c. compared with Ome adipose tissue (Giusti *et al.* 2005). Furthermore, studies using selective PPAR $\gamma$ -2 knockout mice have shown that PPAR $\gamma$ -2, rather than PPAR $\gamma$ -1 appears to modulate insulin sensitivity, and the development of insulin resistance in high-fat diet fed mice (Medina-Gomez *et al.* 2005). In the present study, treatment with both orexin-A and orexin-B for 24 h resulted in a significant increase in PPAR $\gamma$ -2 mRNA in s.c. adipose tissue but not in Ome. The site-specific effect of orexins on PPAR $\gamma$ -2 is consistent with that of PPAR $\gamma$  agonists, the thiazolidinediones, which preferentially stimulate differentiation in human s.c. compared with Ome pre-adipocytes (Adams *et al.* 1997). As a consequence, the observed alterations in PPAR $\gamma$ -2 expression may have effects on the expression of key genes known to be upregulated such as LPL, fatty acid binding protein, acyl CoA synthetase, and perilipin (Dalen *et al.* 2004, Kintscher & Law 2005). Such alterations in PPAR $\gamma$ -2 expression give credence to the idea that orexins may play a role in adipocyte metabolism and adipogenesis.

Both orexin-A and orexin-B treatment resulted in a significant decrease in HSL mRNA expression in Ome but not s.c. adipose tissue explants. HSL is a key enzyme involved



**Figure 4** (A) Glycerol release from s.c. adipose tissue (micromoles/milligram tissue), treated with orexin-A (OX-A), orexin-B (OX-B), and isoproterenol (Iso)  $10^{-7}$  M; \*\*significantly different from control  $P < 0.01$  ( $n = 4$ ). (B) Glycerol release from Ome adipose tissue (micromoles/milligram tissue), treated with OX-A, OX-B and Iso  $10^{-7}$  M; †significantly different from control  $P < 0.05$  ( $n = 4$ ).

in the hydrolysis of triacylglycerol into fatty acids in adipose tissue. Information on its regulation is limited; however, in 3T3-L1 adipocytes, HSL mRNA expression has been shown to be downregulated by isoproterenol, tumor necrosis factor- $\alpha$ , and insulin (Kralisch *et al.* 2005). Alterations in mRNA expression are also reflected by similar alterations in protein (Large *et al.* 1998). HSL is subject to post-translational activation by adrenergic stimulation of cyclic AMP-dependent protein kinase, via the coupling of plasma membrane receptors to several GTP-binding proteins, of which  $G_s$  is the most well known. However, of interest is an alternative pathway involving extracellular signal-regulated kinase 1/2 (ERK 1/2) activation coupled to  $G_i$ , which has been shown to inhibit lipolysis (Carmen & Victor 2006). In addition, orexin-A has been demonstrated to phosphorylate ERK1/2 via activation of OX1R (Milasta *et al.* 2005). Our group has shown that orexins activate multiple G-proteins (Randevara *et al.* 2001, Karteris *et al.* 2005), and therefore it is tempting to speculate that orexins may affect HSL expression and activity as they share a common signaling pathway through activation of ERK via  $G_q$  (Carmen & Victor 2006). Future studies, beyond the aims and scope of this investigation, are needed to dissect the OXR-mediated signaling pathway(s) in adipocytes that may lead to alterations in HSL mRNA expression.

In the present study, glycerol release from adipose tissue explants was used as an index of lipolysis in adipocytes (Lin 1977). Glycerol release was reduced in orexin-A-treated Ome adipose tissue explants. Although there was a trend with respect to the reduction with orexin-B treatment, the lack of statistical significance could be due to the number of subjects studied. These differential effects of orexins may be tissue-dependent. For example, a number of studies have shown that only orexin-A concentration dose-dependently increases basal corticosterone or cortisol secretion from dispersed or cultured rat and human purified bovine adrenal zonal fasciculata/reticularis (ZF/ZR) cells (Mazzocchi *et al.* 2001, Spinazzi *et al.* 2005). The lack of effect on glucocorticoid secretion with orexin-B treatment, which predominantly binds OX2R, indicates differential tissue-specific involvement of these receptors. It should also be noted that only a few studies have addressed the effects of both orexin-A and orexin-B in the same system. The reduction in glycerol release compared with basal values could, in part, be due to re-esterification of fatty acids, however, the concurrent reduction in the expression of HSL indicates an overall reduction in lipolysis. In rats, s.c. administration of orexin-A or orexin-B for 7 days resulted in a significant gain in body mass (Switonska *et al.* 2002), which is of interest given our findings that orexins have an anti-lipolytic effect and activate PPAR $\gamma$ -2 in s.c. adipose tissue.

In conclusion, this study has demonstrated that orexin-A and orexin-B have site-specific actions on expression of key genes involved in adipogenesis and adipocyte metabolism, indicating a role for orexins in the adipose-hypothalamic axis. While the precise role that orexins may play in energy homeostasis at the peripheral level has yet to be elucidated, low circulating orexin-A concentrations have been shown to be associated significantly with severe obesity (BMI > 40 kg/m<sup>2</sup>) (Adam *et al.* 2002), implicating a role for this peptide in the regulation of energy expenditure and body mass. A clearer understanding of the effect of orexins on adipose tissue metabolism, in particular, how such effects may be altered with obesity, is required.

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## References

- Adam JAMP, van Dielen FM, Soeters PB, Buurman WA & Greve JW 2002 Decreased plasma orexin-A levels in obese individuals. *International Journal of Obesity and Related Metabolic Disorders* **26** 274–276.

- Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK *et al.* 1997 Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *Journal of Clinical Investigation* **100** 3149–3153.
- Antunes VRBG, Kwok EH, Scruggs P & Dun NJ 2001 Orexins/hypocretins excite rat sympathetic preganglionic neurons *in vivo* and *in vitro*. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **281** R1801–R1807.
- Beck B & Richey S 1999 Hypothalamic hypocretin/orexin and neuropeptide Y: divergent interaction with energy depletion and leptin. *Biochemical and Biophysical Research Communications* **258** 119–122.
- Bradford M 1976 A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Carmen GY & Victor SM 2006 Signalling mechanisms regulating lipolysis. *Cellular Signalling* **18** 401–408.
- Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, Auwerx J & Nebb HI 2004 Adipose tissue expression of the lipid droplet-associated proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. *Diabetes* **53** 1243–1252.
- Fried SF & Moustaid-Moussa N 2001 Culture of adipose tissue and isolated adipocytes. In *Adipose Tissue Protocols*, pp 197–212. Ed. G Ailhaud. Totowa, New Jersey: Humana Press.
- Giusti V, Verdumo C, Suter M, Gaillard RC, Burckhardt P & Pralong F 2005 Expression of peroxisome proliferator-activated receptor-gamma1 and peroxisome proliferator-activated receptor-gamma2 in visceral and subcutaneous adipose tissue of obese women. *Diabetes* **52** 1673–1676.
- Gliemann J, Osterlind K, Vinten J & Gammeltoft S 1972 A procedure for measurement of distribution spaces in isolated fat cells. *Biochimica et Biophysica Acta* **286** 1–9.
- Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M *et al.* 2001 Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* **30** 345–354.
- Ida TNK, Katayama T, Murakami N & Nakazato M 1999 Effect of lateral cerebroventricular injection of the appetite-stimulating neuropeptide, orexin and neuropeptide Y, on the various behavioral activities of rats. *Brain Research* **821** 526–529.
- Johren O, Neidert SJ, Kummer M, Dendorfer A & Dominiak P 2001 Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. *Endocrinology* **142** 3324–3331.
- Karteris E, Chen J & Rande HS 2004 Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. *Journal of Clinical Endocrinology and Metabolism* **89** 1957–1962.
- Karteris E, Machado RJ, Chen J, Zervou S, Hillhouse EW & Rande HS 2005 Food deprivation differentially modulates orexin receptor expression and signaling in rat hypothalamus and adrenal cortex. *American Journal of Physiology* **288** E1089–E1100.
- Kintscher U & Law RE 2005 PPAR-gamma-mediated insulin sensitization: the importance of fat versus muscle. *American Journal of Physiology* **288** E287–E291.
- Kralisch S, Klein J, Lossner U, Blüher M, Paschke R, Stumvoll M & Fasshauer M 2005 Isoproterenol, TNFalpha, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. *Molecular and Cellular Endocrinology* **240** 43–49.
- Large V, Arner P, Reynisdottir S, Grober J, Van Harmelen V, Holm C & Langin D 1998 Hormone-sensitive lipase expression and activity in relation to lipolysis in human fat cells. *Journal of Lipid Research* **39** 1688–1695.
- de Lecea LKT, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS II, Frankel WN *et al.* 1998 The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *PNAS* **95** 322–327.
- Lin E 1977 Glycerol utilization and its regulation in mammals. *Annual Review of Biochemistry* **46** 765–795.
- Mazzocchi G, Malendowicz LK, Gottardo L, Aragona F & Nussdorfer GG 2001 Orexin A stimulates cortisol secretion from human adrenocortical cells through activation of the adenylate cyclase-dependent signaling cascade. *Journal of Clinical Endocrinology and Metabolism* **86** 778–782.
- Medina-Gomez GVS, Lelliott C, Boiani R, Campbell M, Christodoulides C, Perrin C, Jimenez-Linan M, Blount M, Dixon J, Zahn D *et al.* 2005 The link between nutritional status and insulin sensitivity is dependent on the adipocyte-specific peroxisome proliferator-activated receptor-gamma2 isoform. *Diabetes* **54** 1706–1716.
- Milasta S, Evans NA, Ormiston L, Wilson S, Lefkowitz RJ & Milligan G 2005 The sustainability of interactions between the orexin-1 receptor and beta-arrestin-2 is defined by a single C-terminal cluster of hydroxy amino acids and modulates the kinetics of ERK MAPK regulation. *Biochemical Journal* **1** 573–583.
- Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R *et al.* 2000 A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nature Medicine* **6** 991–997.
- Pfaffl M 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** e45.
- Rande HS, Karteris E, Grammatopoulos D & Hillhouse EW 2001 Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. *Journal of Clinical Endocrinology and Metabolism* **86** 4808–4813.
- Rodbell M 1964 Metabolism of isolated fat cells. *Journal of Biological Chemistry* **239** 375–380.
- Russell CD, Peterson RN, Rao SP, Ricci MR, Prasad A, Zhang Y, Brodin RE & Fried SK 1998 Leptin expression in adipose tissue from obese humans: depot-specific regulation by insulin and dexamethasone. *American Journal of Physiology* **275** E507–E515.
- Sakurai TAA, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR *et al.* 1998 Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behaviour. *Cell* **92** 573–585.
- Samson WK, Gosnell B, Chang JK, Resch ZT & Murphy TC 1999 Cardiovascular regulatory actions of the hypocretins in brain. *Brain Research* **831** 248–253.
- Spinazzi R, Rucinski M, Neri G, Malendowicz K & Nussdorfer G 2005 Preproorexin and orexin receptors are expressed in cortisol-secreting adenomas, and stimulate *in vitro* cortisol secretion and growth of tumor cells. *Journal of Clinical Endocrinology and Metabolism* **90** 3544–3549.
- Spinazzi R, Andreis PG, Rossi GP & Nussdorfer GG 2006 Orexins in the regulation of the hypothalamic–pituitary–adrenal axis. *Pharmacological Reviews* **58** 46–57.
- Switonska MM, Kaczmarek P, Malendowicz LK & Nowak KW 2002 Orexins and adipoinular axis function in the rat. *Regulatory Peptides* **104** 69–73.
- Szekely M, Petervari E, Balasko M, Hernadi I & Uzsocki B 2002 Effects of orexins on energy balance and thermoregulation. *Regulatory Peptides* **104** 47–53.
- Wortley KE, Chang GQ, Davydova Z & Leibowitz SF 2003 Peptides that regulate food intake: orexin gene expression is increased during states of hypertriglyceridemia. *American Journal of Physiology* **284** R1454–R1465.
- Yamanaka A, Sakurai T, Katsumoto T, Yanagisawa M & Goto K 1999 Chronic intracerebroventricular administration of orexin-A to rats increases food intake in daytime, but has no effect on body weight. *Brain Research* **489** 248–252.
- Yasuda T, Masaki T, Kakuma T, Hara M, Nawata T, Katsuragi I & Yoshimatsu H 2005 Dual regulatory effects of orexins on sympathetic nerve activity innervating brown adipose tissue in rats. *Endocrinology* **146** 2744–2748.

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