

Melanocortin crosstalk with adipose functions: ACTH directly induces insulin resistance, promotes a pro-inflammatory adipokine profile and stimulates UCP-1 in adipocytes

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Abstract

The melanocortin (MC) system is a pivotal component of the hypothalamo-pituitary-adrenal (HPA) stress axis and plays an important role in the pathogenesis of obesity and the metabolic syndrome. Adipose dysfunction is implicated in the pathogenesis of these disorders. We investigated direct ACTH effects on adipose functions in immortalised murine white and brown adipocytes. MC receptor types 2 and 5 were expressed at the mRNA and protein levels and were strongly up-regulated during differentiation. Chronic ACTH stimulation did not affect adipogenesis. Insulin-induced glucose uptake in white adipocytes was acutely and transiently reduced by 45% upon ACTH treatment. Visfatin and adiponectin gene expression was reduced by about 50% in response to ACTH, while interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) mRNA levels were acutely up-regulated by 2100 and 60% respectively. Moreover, IL-6 secretion was

increased by 1450% within 4 h of ACTH treatment. In brown adipocytes, stimulation with ACTH caused a 690% increase in uncoupling protein (UCP)-1 mRNA levels within 8 h, followed by a 470% increase in UCP-1 protein concentrations after 24 h. Consistently, p38 mitogen-activated protein kinase (MAPK) phosphorylation was acutely increased by 1800% in response to ACTH stimulation, and selective inhibition of p38 MAPK abolished the ACTH-mediated UCP-1 protein increase. Taken together, ACTH acutely promotes an insulin-resistant, pro-inflammatory state and transiently enhances energy combustion. In conditions characterised by a dysregulation of the HPA stress axis such as the metabolic syndrome, direct MC interaction with adipocytes may contribute to dysregulated energy balance, insulin resistance and cardiometabolic complications.

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Introduction

Obesity and insulin resistance are at the centre of the metabolic syndrome which is a major risk factor for the development of cardiovascular disease. There is growing evidence for an implication of adipose dysfunction critically promoting the development of this syndrome and its complications (Despres & Lemieux 2006).

The melanocortin (MC) system plays an important role in the regulation of body weight (Butler 2006, Farooqi & O'Rahilly 2006). Moreover, MCs are also pivotal components of the hypothalamo-pituitary-adrenal (HPA) stress axis, and dysregulation of this axis is associated with human obesity (Pasquali *et al.* 1993, Vicennati & Pasquali 2000, Rosmond 2003).

The MC system consists of the agonistic peptides α , β and γ melanocyte-stimulating hormone (MSH) as well as the adrenocorticotrophic hormone (ACTH) which are cleavage products of pro-opiomelanocortin. MCs bind to and activate

five MC receptor subtypes (MC1 through 5R). These seven transmembrane G-protein-coupled receptors are distributed in a species- and tissue-specific manner. Furthermore, the system also includes endogenous MC receptor antagonists, agouti and agouti-related protein (Gantz & Fong 2003).

Few reports on direct interactions of MCs with adipocytes have been published. Stimulation of 3T3-L1 cells with MCs has been reported to cause an increase in cAMP concentrations (Boston & Cone 1996, Norman *et al.* 2003). ACTH and α MSH inhibit leptin expression and secretion in 3T3-L1 cells and primary rat adipocytes (Norman *et al.* 2003, Hoggard *et al.* 2004), and induce lipolysis (Boston 1999).

Given a potential direct implication of MCs in the induction of adipose dysfunction, we examined direct actions of ACTH on key metabolic and endocrine adipose responses in white and brown adipocytes respectively. This study demonstrates a direct ACTH-mediated induction of transient insulin resistance, a pro-inflammatory adipokine profile and

a p38 mitogen-activated protein kinase (MAPK)-mediated induction of energy combustion.

Materials and Methods

Materials

All materials were obtained from Sigma–Aldrich unless stated differently.

Cell culture

Immortalised murine adipocyte cell lines were cultured and differentiated as previously described (Klein *et al.* 1999, 2000, 2002, Jost *et al.* 2002, Kraus *et al.* 2002, Perwitz *et al.* 2006). In brief, the cells were grown to confluence on 10-cm culture plates (Sarstedt, Nümbrecht, Germany) in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 20% foetal bovine serum, 4.5 g/l glucose, 20 nmol/l insulin and 1 nM tri-iodothyronine ('differentiation medium'). Adipocyte differentiation was induced by complementing the medium further with 250 µmol/l indomethacin, 500 µmol/l isobutylmethylxanthine and 2 µg/ml dexamethasone for 24 h when confluence was reached. After this period, the cells were changed back to differentiation medium. Cell culture was continued for 5 more days, before cells were serum deprived for 24 h prior to carrying out the experiments. Differentiated adipocytes were used between passages 15 and 30. The final concentration of ACTH (Bachem, Bubendorf, Switzerland) was 100 nM. The p38 MAPK inhibitor SB 202190 (Calbiochem, San Diego, CA, USA) was added 1 h prior to ACTH treatment of cells at a final concentration of 10 µM in the respective experiments.

Immunoblotting

Cells were lysed using whole cell lysis buffer containing 2 mM vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 2 mM phenylmethylsulphonyl fluoride. Protein content of the lysates was determined using the Bradford assay according to the manufacturer's instructions (Bio-Rad). Lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Keane, NH, USA). The membranes were incubated overnight with rinsing buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.2) containing 3% BSA ('blocking solution'). Antibodies were added for 1–2 h in the appropriate dilutions. Protein bands were visualised using a chemiluminescence kit (Roche Molecular Biochemicals). UCP-1 antibody, β-actin antibody and specific MC receptor antibodies were purchased from Chemicon (Temecula, CA, USA). Phospho-specific and total p38 MAPK antibodies were obtained from Cell Signaling (Beverly, MA, USA). Specific p2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Densitometry was performed using the programme Quantity One version 4.2.3 (Bio-Rad).

Quantitative real-time reverse transcription PCR

Quantitative real-time RT-PCR was performed, with 36B4 as a housekeeping gene. Total RNA isolation was carried out using TRIzol reagent (Invitrogen). To optimise RNA quality, clean-up and DNase digestion were performed using the RNeasy kit and RNase-Free DNase Set (Qiagen). Quality of RNA was tested by photometric analysis and agarose gel electrophoresis. Two microgram of total RNA were reverse transcribed using Superscript II (Invitrogen) and oligo p(dT)15 primers (Roche Molecular Biochemicals) in the presence of RNase inhibitor (Roche Molecular Biochemicals) in a 20 µl reaction. One microlitre of each RT reaction was amplified in a total volume of 25 µl containing 1 × QuantiTect SYBR Green PCR-Mix (Qiagen) and 250 nmol/l of each primer, using the GeneAmp 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primer sequences are available on request. 36B4 served as housekeeping gene. Identity of the amplified product was confirmed by melting curve analysis and agarose gel electrophoresis. Optimised relative quantification was done using the Relative Expression Software (Pfaffl *et al.* 2002).

PCR

Qualitative PCRs for MC receptors 1 through 5 were carried out in a Cyclon gradient cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany). Three microlitre of the RT reaction were amplified in a total volume of 30 µl containing 10 × Reaction Buffer S (Peqlab Biotechnologie GmbH), 5 U of Taq DNA Polymerase (Peqlab Biotechnologie GmbH), 166 nmol/l of each primer (Biometra, Göttingen, Germany) and 300 µmol/l of each dNTP (Fermentas, St Leon-Rot, Germany). The primer sequences are available on request. The PCR for all targets was performed as follows: initial denaturation at 95 °C for 300 s, 33 cycles with 95 °C for 60 s, 55 °C for 45 s, 72 °C for 60 s and a final elongation step at 72 °C for 420 s.

Determination of interleukin-6 (IL-6) concentrations

IL-6 concentrations were determined using the RayBio Mouse IL-6 ELISA Kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's manual.

Oil red O staining

Tissue culture plates were washed with PBS twice and fixed with 10% formalin for at least 15 min at room temperature. Cells were then stained for 1 h at room temperature with a filtered oil red O solution (stock solution: 0.5 g oil red O in 100 ml isopropyl alcohol, working solution: 60% (v/v) stock solution and 40% (v/v) H₂O). The staining solution was washed off the cells with distilled water twice. For densitometry, oil red O stain was eluted with isopropanol and subjected to photometric analysis at 500 nm.

Genomic DNA isolation

Genomic DNA was isolated from pre-adipocytes using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

Glucose uptake

The cells were assayed for glucose uptake essentially as described previously (Klein *et al.* 1999, Fasshauer *et al.* 2000). After a serum-free starvation period of 24 h, fully differentiated monolayers of adipocytes were treated with ACTH (100 nM) for various periods of time and then with insulin (100 nM) for 30 min. At the end of the stimulation period, cells were exposed to 2-deoxy-[3H]-D-glucose (0.5 µCi/ml final concentration) for 4 min, and the incorporated radioactivity was determined by liquid scintillation counting.

Statistical analysis

Data are presented as mean \pm S.D. 'Sigma Stat'-software (SPSS Science, Chicago, IL, USA) was employed for statistical analysis of all data. Statistical significance was determined using the unpaired student's *t*-test. The values of $P < 0.05$ were considered significant, those < 0.01 highly significant.

Results

MC 2 and 5 receptors are expressed in adipocytes and are up-regulated during adipogenesis

MC 2 and 5 receptors were expressed in both the white and brown adipocytes. By contrast, MC 1, 3 and 4 receptors were not detected at the mRNA level (Fig. 1A).

Protein analysis revealed MC 2 and 5 receptors in white as well as brown adipocytes, but not 1, 3 and 4 receptors (data not shown). Both receptors were up-regulated during adipocyte differentiation (Fig. 1B).

ACTH does not alter differentiation of adipocytes

The up-regulation of MC receptors during adipocyte differentiation prompted us to investigate a possible effect of ACTH on adipogenesis and lipid accumulation. Chronic stimulation of white pre-adipocytes with ACTH during a differentiation period of 6 days did not affect lipid accumulation, as determined microscopically and by oil red O staining with subsequent densitometric evaluation (Fig. 2A). Differentiation markers were also evaluated; PPAR γ mRNA and aP2 protein concentrations did not differ significantly between chronically ACTH-treated and control cells (Fig. 2B).

ACTH acutely and transiently induces insulin resistance

Insulin sensitivity is a hallmark of mature adipocytes. Experiments with mature white adipocytes demonstrated a

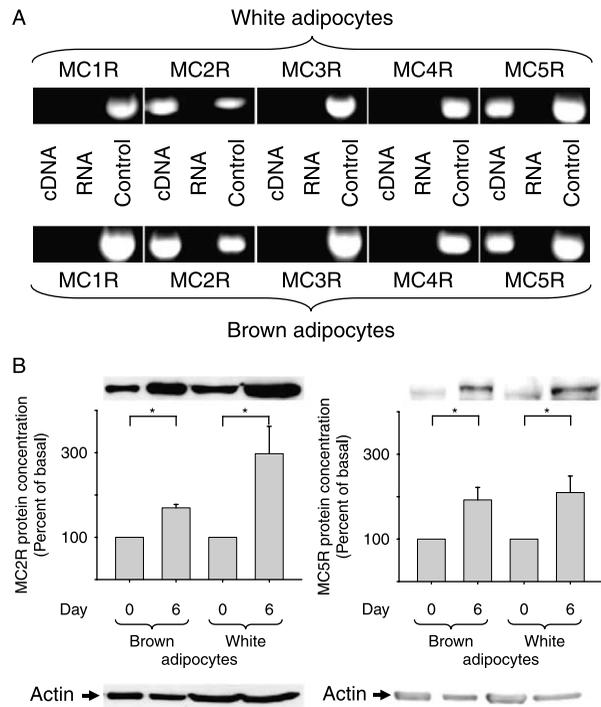


Figure 1 Melanocortin 2 and 5 receptors are expressed in white and brown adipocytes. (A) RNA was isolated from mature white and brown adipocytes, qualitative RT-PCR was performed as described in Materials and Methods ('cDNA' lane). RNA ('RNA' lane) and genomic DNA ('control' lane) were used as templates to serve as negative and positive controls respectively. (B) White and brown pre-adipocytes and mature adipocytes after 6 days of differentiation were investigated. Cell lysates and immunoblots using specific antibodies were prepared as described in Materials and Methods. A bar graph analysis of 3 (MC2R) and ≥ 4 (MC5R) independent experiments as well as representative immunoblots is shown. Actin served as loading control. MC1 through 5R denotes melanocortin 1 through 5 receptor respectively.

transient reduction in insulin-induced glucose uptake by 45% after ACTH treatment for 1 h ($P < 0.01$). This negative effect subsided after another 3 h (Fig. 3). ACTH treatment for 1 h alone increased glucose uptake by 60% ($P < 0.05$), when compared with untreated cells (Fig. 3).

ACTH induces a pro-inflammatory adipokine expression profile

Adipose insulin resistance is closely associated with endocrine dysfunction. We next examined direct effects of ACTH on pro-inflammatory and glucose-regulatory hormones in mature white adipocytes.

Visfatin gene expression was reduced by 50% after 4 h of ACTH stimulation ($P < 0.01$). This effect lasted for another 4 h (Fig. 4A). Adiponectin mRNA concentrations displayed a similar pattern, ACTH significantly decreased adiponectin mRNA levels by 22% after treatment for 2 h ($P < 0.05$). This suppression reached a maximum at 8 h with a reduction of 40% ($P < 0.001$; Fig. 4B).

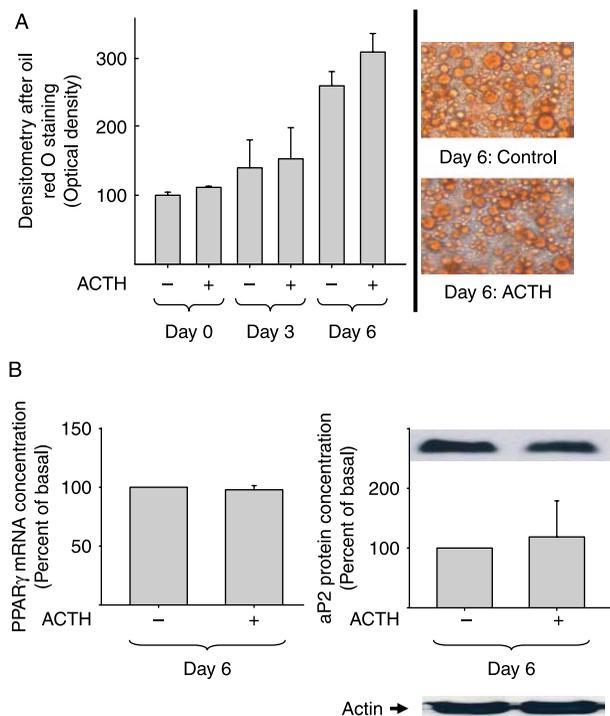


Figure 2 ACTH does not alter differentiation of adipocytes. White pre-adipocytes were chronically stimulated with ACTH over a period of 6 days during differentiation. (A) Oil red O staining with subsequent densitometric evaluation was performed as described in Materials and Methods. A bar graph analysis of three independent experiments and representative microscopic pictures is shown. (B) PPAR γ mRNA quantification was analysed as described in Materials and Methods. A bar graph analysis of three independent experiments is shown. Cell lysates and immunoblots using a specific aP2 antibody were prepared as described in Materials and Methods. A bar graph analysis of three independent experiments and a representative immunoblot is shown. Actin served as loading control.

By contrast, pro-inflammatory adipokines monocyte chemoattractant protein-1 (MCP-1) and IL-6 were up-regulated by ACTH treatment. MCP-1 gene expression maximally increased by 60% after 30 min of stimulation with ACTH ($P < 0.01$) and returned to basal level after 2 h (Fig. 4C). IL-6 mRNA concentrations were acutely increased by 2100% after 30 min ($P < 0.01$) and returned to basal concentrations after 4 h of ACTH treatment (Fig. 4D). This strong rise was paralleled by an increased secretion of IL-6. The IL-6 protein concentrations in the cell culture medium were elevated by 270% after 2 h of ACTH stimulation ($P < 0.01$) and reached a maximal increase in 1450% after 4 h (Fig. 4E).

ACTH stimulation increases UCP-1 mRNA in brown adipocytes

The thermogenic brown adipose tissue contributes to energy balance in small mammals and may be associated with insulin sensitivity in human adults (Yang *et al.* 2003).

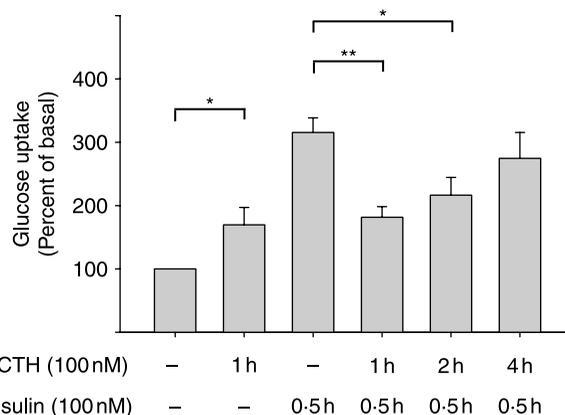


Figure 3 ACTH causes transient insulin resistance in white adipocytes. White adipocytes were stimulated with ACTH for the times indicated. Insulin-induced glucose uptake was determined as described in Materials and Methods. A bar graph analysis of ≥ 6 independent experiments is shown. * $P < 0.05$, ** $P < 0.01$.

Exposure of brown adipocytes to ACTH significantly increased UCP-1 protein concentrations by 170% after 8 h ($P < 0.01$) and maximally by 470% after 24 h ($P < 0.05$; Fig. 5B). This effect was preceded by a transient, maximally 690% rise in UCP-1 mRNA levels between 1 and 8 h of ACTH treatment ($P < 0.01$; Fig. 5A).

p38 MAPK mediates UCP-1 induction by ACTH

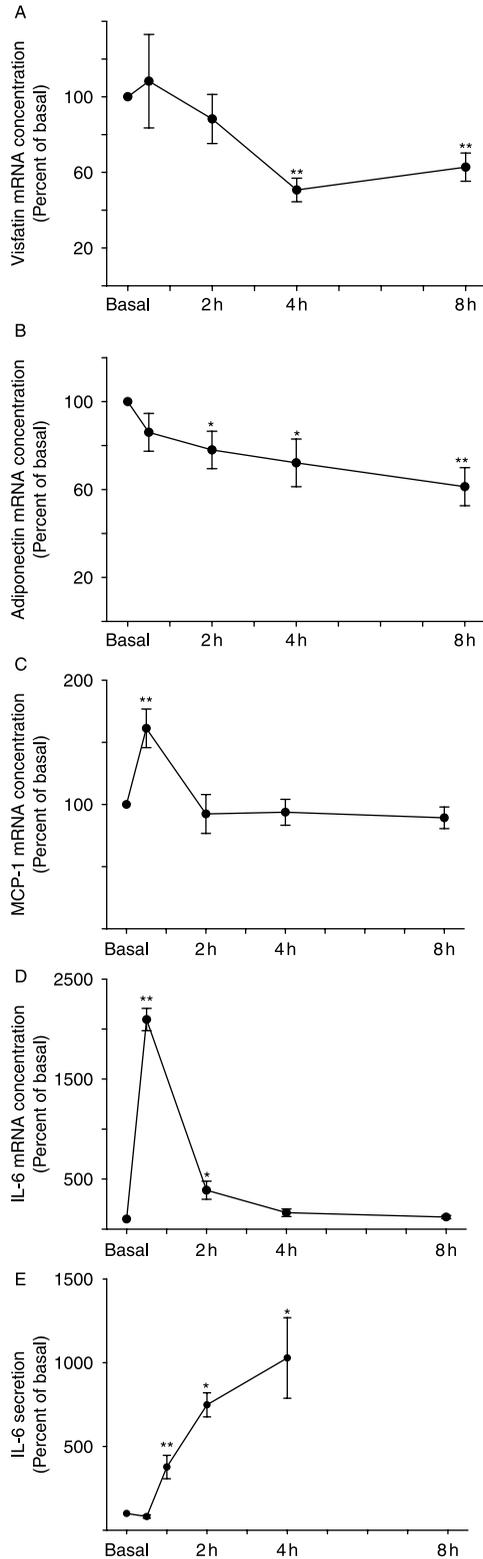
p38 MAPK pathways have been reported to mediate UCP-1 gene regulation. Using a phospho-specific antibody, an increase in p38 MAPK phosphorylation by 1800% was detectable after 10 min of ACTH treatment ($P < 0.05$; Fig. 6A). Total p38 MAPK content of the cells remained unchanged during this period (Fig. 6A, insert). The ACTH treatment of brown adipocytes for 24 h caused an increase in UCP-1 protein concentrations again, which was abolished by SB 202190, a selective inhibitor of p38 MAPK ($P < 0.01$; Fig. 6B).

By contrast, pharmacological inhibition of phosphatidylinositol 3-kinase (LY 294002) and p44/p42 MAPK (PD 98059) did not affect the ACTH-induced rise in UCP-1 mRNA concentrations (data not shown).

Discussion

Our study demonstrates direct multi-level crosstalk of ACTH with key metabolic and endocrine functions of white and brown adipocytes. ACTH induces a transient insulin resistance, a pro-inflammatory adipokine expression profile and a p38 MAPK-mediated increase in UCP-1.

To our knowledge, this study is the first to profile MC receptor expression at the protein level. We find MC 2 and 5 receptors, but not 1, 3 and 4 receptors. Our findings are in accordance with previous reports on MC receptor mRNA



expression in murine adipose cells (Boston & Cone 1996, Norman *et al.* 2003). The observed up-regulation of the receptors during differentiation raised the question of an involvement of ACTH in this process. An inhibition of differentiation and adipogenesis of murine pre-adipocytes in the presence of α MSH has previously been reported (Smith *et al.* 2003). In our study, ACTH did not significantly alter adipogenesis of white adipocytes. This may be due to differences in receptor affinities of α MSH and ACTH: ACTH binds to both receptors, whereas α MSH only binds to MC5R.

Insulin resistance is a key component of the metabolic syndrome and its complications. ACTH caused transient insulin resistance as demonstrated by a decrease in insulin-induced glucose uptake. *In vivo*, this short-term effect may facilitate an increased availability of glucose to other tissues such as the brain and muscle in physiologically stressful states. An increase in total body energy demand might further be met by previously reported increases in lipolysis mediated by MCs in rodents, *in vivo* as well as *in vitro* (White & Engel 1958, Cho *et al.* 2005).

ACTH stimulation appears to induce a pro-inflammatory adipocyte response. Adiponectin and visfatin have been reported to ameliorate insulin resistance *in vivo*, and their expression is reduced in subjects with insulin resistance (Fasshauer & Paschke 2003, Hug & Lodish 2005, Klein *et al.* 2006). Our study provides evidence for an acute decrease in the expression of both adipokines, which could contribute to an overall insulin-resistant state.

By contrast, the pro-inflammatory adipokines IL-6 and MCP-1 are acutely increased by ACTH at the mRNA level. The elevation of IL-6 gene expression is paralleled by an increased IL-6 protein secretion. Effects of IL-6 are complex and appear to be time and tissue specific. Of note, IL-6 production in muscle fibres is increased by exercise, and IL-6 might act as a myocyte-derived 'exercise factor' that increases energy supply for the muscle by enhancing myocyte glucose uptake and β -oxidation of fatty acids (Al-Khalili *et al.* 2006, Weigert *et al.* 2006). The IL-6 secretion of adipocytes in states of acute stress might contribute to these effects. On the other hand, chronically increased IL-6 concentrations correlate with an overall insulin-resistant state (Fernandez-Real & Ricart 2003). Elevated MCP-1 levels are also associated with insulin resistance and an inflammatory state (Sartipy & Loskutoff 2003, Kamei *et al.* 2006, Kanda *et al.* 2006, Sell *et al.* 2006, Shoelson *et al.* 2006). Taken together, ACTH

Figure 4 ACTH stimulation of white adipocytes causes a pro-inflammatory adipokine profile. White adipocytes were stimulated with ACTH for the times indicated. (A–D) mRNA quantification of adipokines in white adipocytes were analysed as described in Materials and Methods. Bar graph analyses of four independent experiments are shown. (E) IL-6 concentrations in the cell culture supernatant of white adipocytes were determined as described in Materials and Methods. A bar graph analysis of four independent experiments is shown. * $P < 0.05$, ** $P < 0.01$.

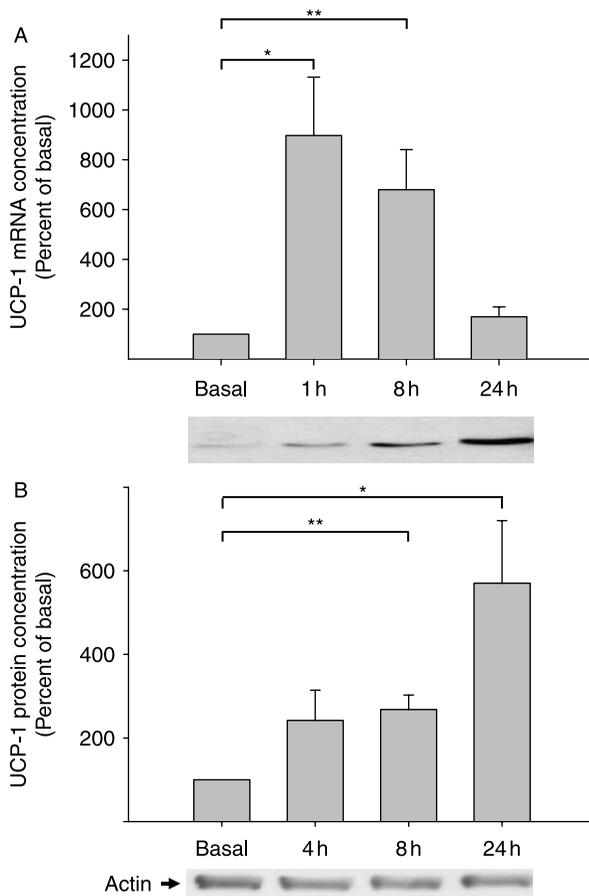


Figure 5 Up-regulation of UCP-1 in brown adipocytes caused by ACTH. Brown adipocytes were stimulated with ACTH for the times indicated. (A) UCP-1 mRNA quantification was analysed as described in Materials and Methods. A bar graph analysis of five independent experiments is shown. (B) Cell lysates and immunoblots using a specific antibody were prepared as described in Materials and Methods. A bar graph analysis of five independent experiments and a representative immunoblot is shown. Actin served as loading control. * $P < 0.05$, ** $P < 0.01$.

induces an adipokine profile that is associated with insulin resistance and inflammation.

Thermogenesis is mediated by UCP-1 in brown adipocytes and contributes to energy expenditure. Centrally administered MCs are known to increase energy expenditure and UCP-1 gene expression in brown adipocytes of rodents (Pierroz *et al.* 2002, Williams *et al.* 2003, Li *et al.* 2004). This effect is thought to be indirectly mediated via the sympathetic nervous system (Voss-Andreae *et al.* 2007). We report evidence for a direct induction of UCP-1 gene expression by MCs. This provides a complementary direct mechanism of ACTH action. This finding is further supported by a previous report; peripheral administration of a MC receptor agonist to food-restricted rats prevented the typically observed decrease in UCP-1 (Cettour-Rose & Rohner-Jeanrenaud 2002).

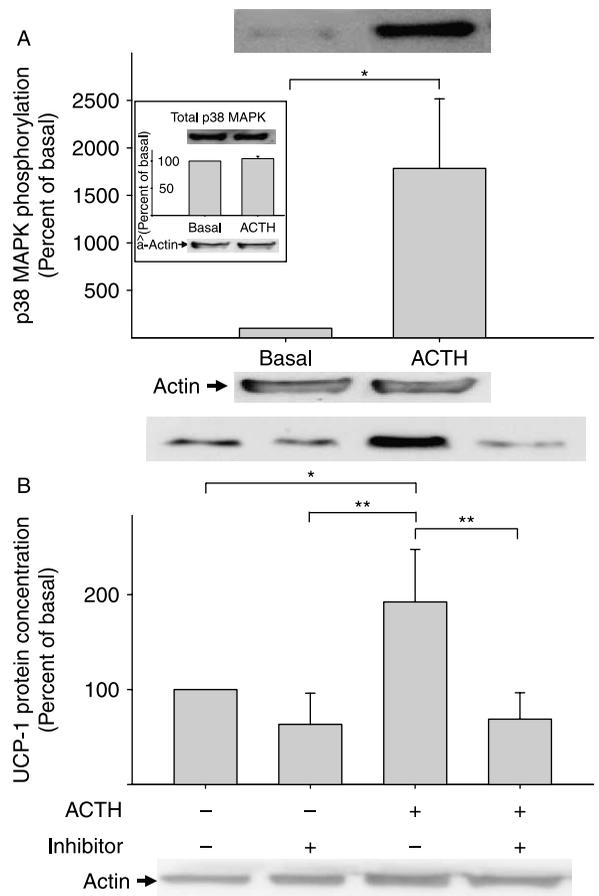


Figure 6 Involvement of p38 MAPK in regulation of UCP-1 protein concentration by ACTH. (A) Brown adipocytes were stimulated with ACTH for 10 min. Cell lysates and immunoblots using specific antibodies were prepared as described in Materials and Methods. p38 MAPK phosphorylation: A bar graph analysis of eight independent experiments with s.e.m. and a representative immunoblot is shown. Total p38 MAPK: A bar graph analysis of four independent experiments and a representative immunoblot is shown. (B) Brown adipocytes were stimulated with ACTH for 24 h with or without the specific p38 MAPK inhibitor SB 202190 was added 1 h prior to ACTH. UCP-1 protein was analysed as described in Materials and Methods. A bar graph analysis including the s.e.m. of four independent experiments and a representative immunoblot is shown. * $P < 0.05$, ** $P < 0.01$. Actin served as loading control.

The direct effect of ACTH on the up-regulation of UCP-1 gene activity is mediated by p38 MAPK. ACTH not only increased p38 MAPK phosphorylation by 1800%, but specific inhibition of p38 MAPK also abolished the ACTH-mediated elevation of UCP-1 protein concentration. These findings are in accordance with previous reports on UCP-1 activation (Valladares *et al.* 2001, Teruel *et al.* 2003, Cao *et al.* 2004). Furthermore, PI 3-K and p44/p42 MAPK do not seem to play a role in the ACTH-mediated UCP-1 gene up-regulation, since pharmacological inhibition of these kinases did not affect the ACTH-induced rise of UCP-1 mRNA concentrations.

Taken together, our data provide evidence for direct multi-level effects of ACTH on adipose insulin sensitivity, endocrine function and thermogenesis. This direct crosstalk promotes an insulin-resistant, pro-inflammatory state and transiently enhances energy combustion. In a wider context, these findings provide evidence for direct effects of neuroendocrine peptides on key adipose functions. Direct adipotropic actions of neuropeptides thereby contribute to adipose dysregulation and associated metabolic and cardiovascular disease.

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