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β -Adrenoceptors, but not α -adrenoceptors, stimulate AMP-activated protein kinase in brown adipocytes independently of uncoupling protein-1

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Abstract *Aims/hypothesis:* Brown adipocytes provide a potentially important model system for understanding AMP-activated protein kinase (AMPK) regulation, where adrenergic stimulation leads to mitochondrial uncoupling through uncoupling protein-1 (UCP1) activity. AMPK is a sensor of energy homeostasis and has been implicated in glucose and lipid metabolism in several insulin-sensitive tissues. The aim of this study was to characterise the potential role of AMPK in adrenergically mediated glucose uptake and to find out whether UCP1 is involved in the adrenergic activation of AMPK. *Methods:* We used primary brown adipocytes differentiated in culture and measured AMPK phosphorylation and glucose uptake following adrenergic activation. *Results:* Treatment of adipocytes with noradrenaline (norepinephrine) caused phosphorylation of AMPK via β -adrenoceptors and not α_1 - or α_2 -adrenoceptors. This effect was not β_3 -adrenoceptor specific, since responses remained intact in adipocytes from β_3 -adrenoceptor knock-out mice. These effects were also mimicked by forskolin and cAMP analogues. Treatment of cells with adenine 8- β -D-arabinofuranoside, an AMPK inhibitor, partially blocked β -adrenoceptor-mediated increases in glucose uptake. Brown adipocytes are characterised by the production of UCP1, which can uncouple the mitochondria. Using adipocytes from *Ucp1*^{+/+} and *Ucp1*^{-/-} mice, we showed that noradrenaline-mediated phosphorylation of AMPK does not require the presence or activity of UCP1. *Conclusions/interpretation:* These results suggest a pathway where increases in cAMP mediated by β -adrenoceptors leads to activation of AMPK in brown adipocytes, which contributes in part to β -adrenoceptor-mediated

increases in glucose uptake, an effect independent of the presence or function of UCP1.

Keywords α -Adrenoceptor · β -Adrenoceptor · AMP-activated protein kinase · Primary brown adipocytes · Uncoupling protein-1

Abbreviations ACC: acetyl CoA carboxylase · AICAR: 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside · AMPK: AMP-activated protein kinase · Ara-A: adenine 8- β -D-arabinofuranoside · PI3K: phosphatidylinositol 3-kinase · PK: protein kinase · UCP1: uncoupling protein-1

Introduction

Brown adipose tissue plays an important role in body temperature regulation owing to its ability to generate heat by uncoupling mitochondrial respiration, a process that is mediated by uncoupling protein-1 (UCP1) (for review, see [1]). It is an important organ in terms of increasing our knowledge of energy regulation and, in rodents, where UCP1 is present in significant amounts in adults, its energy-utilising capacity gives it the potential to influence energy homeostasis. Brown adipose tissue has been shown to play an important role in the regulation of glucose homeostasis and insulin secretion [2]. Glucose uptake is significantly increased in brown adipose tissue in vivo by activation of the sympathetic nervous system independently of insulin [3, 4] and by adrenergic agonists in brown adipocytes in vitro [5–8].

There has recently been much interest in AMP-activated protein kinase (AMPK), which has been suggested to act as a sensor of energy homeostasis. AMPK consists of a catalytic α and β subunit and γ regulatory subunits, and is activated by phosphorylation at Thr172 on the catalytic subunit, while allosteric binding of AMP increases the activity of AMPK and the stability of the phosphorylated state (for review, see [9]). AMPK is widely produced, with high levels produced in tissues that are involved in energy homeostasis, such as liver, heart, skeletal muscle and

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adipose tissue. While the consensus is that AMPK is important in liver and skeletal muscle, its importance in adipose tissue is uncertain. Recently, the fat-derived hormones leptin and adiponectin, which regulate energy homeostasis, were shown to activate AMPK in skeletal muscle [10, 11], liver [12] and adipose tissue [13]. AMPK has extensively been shown to increase glucose uptake in skeletal muscle (for review, see [14]), but its role in glucose uptake in brown adipose tissue remains unknown.

Since AMPK activation is allosterically mediated by increases in AMP, cultured brown adipocytes are a particularly appropriate model in which to study such activation, since activation of UCP1 may elevate endogenous levels of this nucleotide at the expense of ATP, as has been reported for suspensions of mature brown adipocytes [15]. In this study, we have used brown adipocytes in primary culture that have previously been shown to possess intact adrenergic- and insulin-signalling pathways [7, 16–18]. The aim of this study was to investigate the adrenergic phosphorylation of AMPK, focussing on delineating which adrenergic subtypes are involved in the noradrenaline (norepinephrine)-mediated AMPK phosphorylation response, and whether AMPK is involved in mediating the glucose uptake response to adrenergic agonists.

Materials and methods

Materials and reagents Drugs and reagents were purchased as follows: insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark); 2-deoxy-D-[1-³H] glucose (specific activity 35×10^4 – 44×10^4 MBq/mmol; Amersham Biosciences, Little Chalfont, UK); H89 (Calbiochem, La Jolla, CA, USA); ADP, AMP, adenine 8- β -D-arabinofuranoside (Ara-A), ATP, 8-bromo-cAMP, cirazoline, CL316243, clonidine, CoA, forskolin, (–)-isoprenaline, luciferase, D-luciferin, myokinase, nucleoside-5'-diphosphate-kinase, (–)-noradrenaline, SR59230A (Sigma Chemical Company, St Louis, MO, USA); 5-aminoimidazole-4-carboxamide 1- β -D-ribose nucleoside (AICAR) (Toronto Research Chemicals, North York, ON, Canada). ICI89406 was a gift from Imperial Chemical Industries (AstraZeneca, Södertälje, Sweden).

All cell culture media and supplements were obtained from Gibco-BRL Life Technologies (Gaithersburg, MD, USA). All antibodies, except those against UCP1, were obtained from Cell Signaling Technology (Beverly, MA, USA). All other drugs and reagents used were of analytical grade.

Animals Three-week-old FVB, *Adrb3*^{–/–} (which lack the gene encoding the β_3 -adrenoceptor), *Ucp1*^{+/+} or *Ucp1*^{–/–} mice of either sex were bred at the institute. *Ucp1*^{–/–} mice were progeny of those described previously [19], backcrossed to at least ten generations to a pure C57BL/6J background (*UCP1*^{+/+}). *Adrb3*^{–/–} mice and their controls (FVB) were the offspring of a previously described strain [20]. Experiments were conducted with permission from the North Stockholm Animal Ethics Committee.

Cell isolation and cell culture of mouse brown adipocytes Brown fat precursor cells were isolated essentially as described previously [21, 22]. Cells were grown in DMEM (4.5 g glucose/l) containing newborn calf serum (10%, v/v), insulin (2.4 nmol/l), HEPES (10 mmol/l), L-glutamine (4 mmol/l), penicillin (50 IU/ml), streptomycin (50 μ g/ml) and sodium ascorbate (25 μ g/ml) under 8% CO₂ at 37°C. After 5 days in culture, the brown adipocyte precursor cells spontaneously convert from displaying a fibroblast-like morphology to acquiring the typical multi-locular lipid droplets seen in mature brown adipocytes; this conversion occurs at the time of cellular confluence [21, 22]. In these cells, spontaneous induction of *Adrb3* mRNA reaches a steady-state level at day 5 that corresponds with the ability of noradrenaline to induce the expression of *Ucp1*, the gene encoding the most specific brown adipocyte differentiation marker, UCP1 [17].

Cells were used for all experiments following 7 days of differentiation. On day 6, cells were serum-starved overnight in DMEM/Nutrient Mix F12 (1:1) containing L-glutamine (4 mmol/l), BSA (0.5%, v/v), insulin (2.4 nmol/l), HEPES (10 mmol/l), penicillin (50 IU/ml), streptomycin (50 μ g/ml) and sodium ascorbate (25 μ g/ml).

Immunoblotting Cells were serum-starved overnight before each of the experiments performed on day 7 and were exposed to the drugs for the times and at the concentrations indicated. Extraction of cells for AMPK or acetyl CoA carboxylase (ACC) immunoblotting was performed as previously described [23], except that samples were electrotransferred to Hybond-P polyvinylidene difluoride membranes (pore size 0.45 μ m; Amersham Biosciences, Arlington Heights, IL, USA). For UCP1 immunoblotting or measurement of total AMPK levels in primary brown adipocytes during differentiation, cells were washed twice in PBS and harvested in lysis buffer (Tris [62.5 mmol/l, pH 6.8], SDS [2%, v/v], glycerol [10%, v/v]). The lysate was sonicated for a few seconds and the concentration of the soluble proteins determined [24]. Following determination of protein concentration, the lysis buffer was supplemented with dithiothreitol (50 mmol/l) and bromophenol blue (1%, v/v) in each sample at a ratio of 1:9. Proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and electrotransferred to Hybond-P membranes (Amersham Biosciences). The primary antibodies used were phospho-AMPK (Thr172), total AMPK, phospho-ACC (Ser79) or total ACC diluted 1:1,000, or UCP1 primary polyclonal antibody (produced in rabbits by NeoMPS [Strasbourg, France] from a 12 amino acid peptide sequence at the C-terminus of mouse UCP1) used at a dilution of 1:2,000. Primary antibodies were detected using a secondary antibody (1:2,000 dilution; horseradish peroxidase-linked anti-rabbit IgG) and enhanced chemiluminescence (Amersham Biosciences). A positive control (brown adipose tissue mitochondria from a cold-exposed C57BL/6J mouse; kindly donated by I. Shabalina, Stockholm University) was used in these studies and 0.2 μ g protein run on the gels to elucidate the band representing UCP1 from the primary brown adipocytes.

Results are expressed as the ratio of phosphorylated: total protein, with the ratio normalised in each experiment to that of control samples, unless stated otherwise. All

experiments were performed singly or in duplicate, with 'n' referring to the number of independent experiments performed. In all experiments, basal phosphorylated

Fig. 1 Total (t-) AMPK content increases in FVB brown adipocytes during differentiation (a). Cells were harvested following 3, 5 or 7 days differentiation. The effect of the AMPK activator AICAR (2 mmol/l) (b), insulin (1 μ mol/l; $n=4$) (c), noradrenaline (general adrenergic agonist; 1 μ mol/l, $n=7$) (d), isoprenaline (β -adrenoceptor agonist; 1 μ mol/l, $n=4$) (e), CL316243 (selective β_3 -adrenoceptor agonist; 1 μ mol/l, $n=4$) (f), cirazoline (α_1 -adrenoceptor agonist; 1 μ mol/l, $n=4$) (g) or clonidine (α_2 -adrenoceptor agonist; 1 μ mol/l, $n=4$) (h) on the phosphorylation of AMPK in FVB brown adipocytes. Values represent means $\pm$ SEM of n independent experiments. Data are expressed as a percentage of the phosphorylated (p-) AMPK: AMPK ratio (normalised to 100%) of unstimulated cells

AMPK:total AMPK levels were unchanged over the time periods examined (data not shown) and have been omitted from diagrams for clarity.

2-Deoxy-D-[1-³H]glucose uptake Glucose uptake experiments were performed as described previously [7, 8].

Measurement of the AMP:ATP ratio Cells were serum-starved overnight before each experiment performed on day 7. On day 7, 1 ml of boiling water was added to each well of a six-well plate, and the cells scraped and boiled (3 min). Samples were centrifuged (12,000 g, 4°C, 10 min) to pellet cell debris, and the supernatant fractions were used for further analysis. Each sample was diluted 1:10 and three aliquots (20 µl) transferred to a white 96-well plate and 30 µl of either reagent A (aqueous tricine buffer [40 mol/l, pH 7.8], MgSO₄ [8 mmol/l], EDTA [0.17 mmol/l], which measures total cellular ATP), reagent B (reagent A buffer supplemented with dCTP [100 µmol/l], nucleoside-5'-diphosphate-kinase [10 U/ml], which converts the ADP in a given sample to ATP) or reagent C (reagent B supplemented with myokinase [10 U/ml], which converts the AMP and ADP in a given sample to ATP) added for measurement of ATP, ADP and AMP, respectively. Samples were incubated for 12 h at 37°C, after which the reactions were terminated by boiling for 5 min, and then 50 µl of luciferin–luciferase reagent (aqueous tricine buffer [25 mmol/l, pH 7.8], MgSO₄ [5 mmol/l], EDTA [0.1 mmol/l], D-luciferin [500 µmol/l], luciferase [10 µg/ml], dithiothreitol [2 mmol/l], CoA [0.5 mmol/l]) were added to each well. Light emission was measured with a Fujifilm LAS-1000 CCD camera (Fujifilm, Tokyo, Japan) and analysed with Image-QuantNT (Molecular Dynamics, Sunnyvale, CA, USA). AMP:ATP ratios were calculated using the following equation: (reagent C values–reagent B values)/reagent A values.

Statistical analysis Results are presented as the mean values±SEM. Paired or unpaired *t*-tests were performed (where indicated) to test for significance between different treatments and/or controls. A *p* value less than or equal to 0.05 was considered significant.

Results

AMPK content increases during differentiation of primary brown adipocytes To assess whether total AMPK protein levels change during adipocyte differentiation, we measured total AMPK protein in FVB cells at days 3, 5 and 7 of differentiation. AMPK levels were higher in the fully differentiated cells (day 7) than in the cells undergoing differentiation (day 3) (Fig. 1a). We therefore performed all further experiments after 7 days of differentiation.

Insulin fails to phosphorylate AMPK in primary brown adipocytes The AMPK activator AICAR was able to phosphorylate AMPK in primary brown adipocytes

(approximately four-fold increase relative to basal levels; Fig. 1b). In contrast, insulin, a potent activator of glucose uptake in these cells [7], was unable to phosphorylate AMPK (Fig. 1c).

β-Adrenoceptor, but not α₁- or α₂-adrenoceptor, activation stimulates AMPK phosphorylation in primary brown adipocytes Noradrenaline caused phosphorylation of AMPK in brown adipocytes, an effect that was sustained over the time points measured (Fig. 1d). To delineate which adrenergic receptor subtype(s) mediated the noradrenaline effect, brown adipocytes were stimulated with different adrenergic agonists. The non-selective β-adrenoceptor agonist isoprenaline and the selective β₃-adrenoceptor agonist CL316243 phosphorylated AMPK and this effect, like that of noradrenaline, was sustained over the time points examined (Fig. 1e,f). Neither the α₁-adrenoceptor agonist cirazoline nor the α₂-adrenoceptor agonist clonidine phosphorylated AMPK (Fig. 1g,h). AMPK activation results in the phosphorylation of ACC at Ser79. Protein kinase A (PKA) does not phosphorylate ACC at this site (reviewed in [25]), whereas isoprenaline does (approximately three-fold increase relative to basal phosphorylation values; *n*=3 in duplicate; Fig. 2).

Brown adipose tissue produces all three β-adrenoceptor subtypes, although it is likely that the β₂-adrenoceptor is limited to the vascular system in the tissue [8, 26–28]. Fully differentiated brown adipocytes have low levels of β₁-adrenoceptors and no β₂-adrenoceptors [8], and the predominant receptor is the β₃-adrenoceptor. The selective β₃-adrenoceptor agonist CL316243 phosphorylated AMPK (Fig. 1f). In addition, isoprenaline-mediated increases in AMPK phosphorylation in FVB adipocytes were significantly reduced by the β₃-adrenoceptor antagonist SR59230A, but not by the β₁-adrenoceptor antagonist IC189406, suggesting that β₃-adrenoceptors and not β₁-adrenoceptors couple to AMPK in FVB adipocytes (Fig. 3a). In adipocytes isolated from *Adrb3*^{−/−} mice, noradrenaline and isoprenaline were able to phosphorylate AMPK, indicating that β₁-adrenoceptors are also capable of phosphorylating AMPK (Fig. 3b).

Fig. 2 Effect of isoprenaline (1 µmol/l, 2 h) on phosphorylation (p-) of ACC at Ser79 in FVB primary brown adipocytes. Blot is representative of three individual experiments. t-, total

Fig. 3 Effect of the β_1 -adrenoceptor antagonist ICI89406 (100 nmol/l, pre-incubation 30 min, *hatched bars*) or the β_3 -adrenoceptor antagonist SR59230A (100 nmol/l pre-incubation 30 min, *black bars*) on isoprenaline (1 μ mol/l, 30 min, $n=2$ in duplicate; control, *white bars*)-mediated increases in AMPK phosphorylation in FVB brown adipocytes (**a**) and the effect of isoprenaline (1 μ mol/l), noradrenaline (1 μ mol/l), cirazoline (1 μ mol/l), AICAR (2 mmol/l) and forskolin (10 μ mol/l) in brown adipocytes from *Adrb3*^{-/-} mice (all treatments for 30 min and data measured in parallel with those obtained in Fig. 1 from FVB adipocytes; $n=3$ in duplicate) (**b**). Values represent means $\pm$ SEM of n independent experiments. Statistical difference (unpaired *t*-test) between agonist in the presence/absence of antagonist ($^{***}p<0.001$). Data are expressed as a percentage of the phosphorylated (p-) AMPK:AMPK ratio (normalised to 100%) of unstimulated cells

Forskolin and cAMP analogues mimic the phosphorylation of AMPK by the β -adrenoceptor activation β -Adrenoceptors are Gs-coupled receptors and their activation leads to the activation of adenylate cyclase, resulting in increased intracellular cAMP levels and subsequent activation of PKA. Forskolin, a direct activator of adenylate cyclase, phosphorylated AMPK in adipocytes from FVB (Fig. 4a) and *Adrb3*^{-/-} mice (Fig. 3b). A cAMP mimicking agent, 8-bromo-cAMP was also able to phosphorylate AMPK (data not shown; $n=2$). The phosphorylation of AMPK in FVB adipocytes by isoprenaline was inhibited the PKA inhibitor H89 (Fig. 4b).

*AMPK phosphorylation is not impaired in *Ucp1*^{-/-} adipocytes* UCP1 is responsible for heat production in brown adipocytes following noradrenaline stimulation: it can decrease ATP levels and elevate AMP levels [15]. In order to ascertain the influence of UCP1 in brown adipocytes on noradrenaline-mediated AMPK phosphorylation, primary brown adipocytes from either *Ucp1*^{+/+} or *Ucp1*^{-/-} mice were exposed to AICAR, noradrenaline or CL316243. AICAR, noradrenaline and CL31243 all phosphorylated AMPK in *Ucp1*^{-/-} cells (~2.5-fold over

Fig. 4 The effect of the adenylate cyclase activator forskolin (10 μ mol/l, $n=4$) on the phosphorylation of AMPK (**a**) and the effect of the PKA inhibitor H89 (10 μ mol/l, 30 min pre-incubation) (H89, *black bars*; control, *white bars*) (**b**) on isoprenaline (1 μ mol/l, 30 min, $n=3$)-mediated increases in AMPK phosphorylation in FVB brown adipocytes. Values represent means $\pm$ SEM of n independent experiments. Statistical difference (unpaired *t*-test) between agonist in the presence/absence of inhibitor ($^{*}p<0.05$). Data are expressed as a percentage of the phosphorylated (p-) AMPK:AMPK ratio (normalised to 100%) of unstimulated cells

basal phosphorylation levels), comparable with the fold increase observed in *Ucp1*^{+/+} adipocytes (Fig. 5b,d). While the fold-increase with any of these drugs was the same in *Ucp1*^{-/-} or *Ucp1*^{+/+} adipocytes (Fig. 5b,d), the basal level of AMPK phosphorylation was significantly reduced in *Ucp1*^{-/-} adipocytes compared with *Ucp1*^{+/+} adipocytes (approximately 54 $\pm$ 8% of wild type *Ucp1*^{+/+}; $n=9$; Fig. 5a,c), with no changes in the amount of total AMPK protein. However, this difference cannot be the result of UCP1 under these circumstances, since *Ucp1* is essentially not expressed prior to noradrenaline treatment (Fig. 5e). To increase the level of UCP1 in adipocytes, cells were exposed to noradrenaline (0.1 μ mol/l) for 36 h (Fig. 5e). Long-term treatment of adipocytes with noradrenaline increased levels of UCP1 protein markedly in *Ucp1*^{+/+} adipocytes, and stimulation of phosphorylation of AMPK was comparable in the two genotypes. Further addition of noradrenaline for 15 min following long-term stimulation had no additional effect (Fig. 5f). These results indicate that UCP1 is not involved in the adrenergic phosphorylation of AMPK.

*AMP:ATP ratios in *Ucp1*^{+/+} and *Ucp1*^{-/-} adipocytes are identical* Since the basal level of phosphorylated AMPK was significantly reduced in *Ucp1*^{-/-} adipocytes compared with *Ucp1*^{+/+} adipocytes, we measured the basal AMP:ATP ratios in unstimulated cells using a luciferin-luciferase-based assay. The AMP:ATP ratios were comparable in the two genotypes (AMP:ATP ratio 0.61 $\pm$ 0.03 for *Ucp1*^{+/+} vs. 0.74 $\pm$ 0.5 for *Ucp1*^{-/-}; $n=2$ in triplicate), suggesting that the observed decrease in basal AMPK phosphorylation

Fig. 5 Effect of noradrenaline (10 $\mu\text{mol/l}$; $n=4-5$) (a, b) and CL316243 (10 $\mu\text{mol/l}$; $n=5$) (c, d) on phosphorylation of AMPK in adipocytes from *Ucp1*^{+/+} and *Ucp1*^{-/-} mice on a C57/BL6J strain. Effects of noradrenaline (0.1 $\mu\text{mol/l}$, 36 h) on UCP1 (~32 kDa) protein content in *Ucp1*^{+/+} and *Ucp1*^{-/-} primary brown adipocytes and brown adipose tissue mitochondria (Mit.) isolated from a cold-exposed C57BL/6J mouse (e). Effect of long-term (0.1 $\mu\text{mol/l}$, 36 h) and acute (0.1 $\mu\text{mol/l}$, 2 h) noradrenaline treatment on AMPK phosphorylation in both *Ucp1*^{+/+} and *Ucp1*^{-/-} adipocytes ($n=3$ in duplicate) (f). Values represent means $\pm$ SEM of n independent experiments. Data in (a), (c) and (f) are expressed as a percentage of the phosphorylated (p-) AMPK:AMPK ratio (normalised to 100%) of unstimulated *Ucp1*^{+/+} cells. Data in (b) and (d) are expressed as a fold-increase over unstimulated cells from either *Ucp1*^{+/+} or *Ucp1*^{-/-} adipocytes

levels observed in the *Ucp1*^{-/-} adipocytes cannot be due to an initial reduction in the AMP:ATP ratio.

β -Adrenergically mediated glucose uptake requires AMPK
 β -Adrenoceptors activate glucose uptake in primary brown adipocytes via several intracellular mechanisms, including cAMP, phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) [7, 8]. To investigate whether AMPK is involved in adrenergically mediated glucose uptake, glucose uptake was carried out in the presence of Ara-A, an AMPK inhibitor. The AMPK activator AICAR stimulated a two-fold increase in glucose uptake, as did isoprenaline (Fig. 6). Ara-A inhibited isoprenaline-stimulated glucose uptake by ~38%, implying that AMPK is a mediator of β -adrenergically stimulated glucose uptake (Fig. 6a). Ara-A also inhibited AICAR-mediated glucose uptake by 61% (Fig. 6b).

UCP1 is not required for β -adrenergically stimulated glucose uptake
 We examined whether UCP1 is involved in insulin- or adrenergically mediated glucose uptake using *Ucp1*^{+/+} or *Ucp1*^{-/-} mice. Basal glucose uptake was similar in cells derived from the two genotypes (data not shown). Acute exposure of adipocytes to insulin or noradrenaline stimulated glucose uptake to a similar extent in *Ucp1*^{+/+} and *Ucp1*^{-/-} mice, indicating that there is no

impairment of the insulin- or adrenergically-mediated pathways of glucose uptake (Fig. 6c). Long-term noradrenaline treatment, which, as shown above, significantly raises UCP1 protein levels, had no influence on basal glucose uptake (data not shown), and acute exposure of adipocytes to noradrenaline increased glucose uptake to the same extent in *Ucp1*^{+/+} and *Ucp1*^{-/-} adipocytes.

Discussion

In this study, we have investigated whether AMPK plays a role in adrenergically mediated glucose uptake in primary brown adipocytes. Noradrenaline phosphorylated AMPK in these cells, which are known to have intact insulin and adrenergic signalling systems. Brown adipose tissue produces α_1 -, α_2 - and β -adrenoceptor subtypes, but brown adipocytes, in addition to α_1 -adrenoceptors, predominantly produce β_1 - and β_3 -adrenoceptors, with little or no β_2 -adrenoceptors [8]. Since brown adipose tissue contains cell types other than adipocytes, such as endothelial cells, blood vessels, fibroblasts and nerves, the relative contribution of the different adrenergic subtypes in each cell type differs vastly. Endothelial cells have been used to elucidate AMPK signalling and effects by several agents [29–31], which makes it difficult to elucidate the

Fig. 6 The effect of the AMPK inhibitor Ara-A (2.5 mmol/l, 30 min pre-incubation) on isoprenaline-mediated (1 μ mol/l, 2 h) (a) and AICAR-mediated (2 mmol/l, 2 h) (b) glucose uptake in primary brown adipocytes from FVB mice ($n=5$; Ara-A, black bars; control, white bars). Glucose uptake in response to insulin, acute noradrenaline (0.1 μ mol/l, 2 h) and acute noradrenaline following long-term noradrenaline treatment (0.1 μ mol/l, 36 h) in *Ucp1*^{+/+} and *Ucp1*^{-/-} adipocytes on a C57BL/6J background ($n=3$ in duplicate) (c). Values represent means $\pm$ SEM. Basal, white boxes; insulin, black boxes; acute, upward diagonal boxes, long term+acute, downward diagonal boxes

effects of noradrenaline in brown adipocytes using brown adipose tissue, because of potential interactions with other cell types in the tissue. We therefore used primary brown adipocytes in culture to examine effects on mature brown adipocytes.

Elucidation of the receptor subtype(s) involved in noradrenaline activation using selective adrenergic agonists revealed that β -adrenoceptors, but not α_1 - or α_2 -adrenoceptors, were responsible for the noradrenaline effect. Previous studies in recombinant CHO-K1 and L6 skeletal muscle cells indicated that Gq-coupled receptors, but not Gs- or Gi-coupled receptors, phosphorylate and activate AMPK [32]. However, our results in primary brown adipocytes, showing that Gs-coupled receptors (β -adrenoceptors) are capable of phosphorylating AMPK, are in accordance with studies in 3T3-L1 adipocytes [33] and in isolated rat adipocytes [34]. In FVB adipocytes, isoprenaline (a non-selective β -adrenergic agonist that acts primarily via β_3 -adrenoceptors and not β_1 -adrenoceptors in this study) and CL316243 (a β_3 -adrenergic agonist) phosphorylated AMPK to the same degree as noradrena-

line. In addition, isoprenaline was able to phosphorylate ACC at Ser79, which is phosphorylated in response to AMPK, and not PKA, activation (for review, see [25]), demonstrating that isoprenaline affects the AMPK cascade at another level. By comparing primary brown adipocytes from FVB and *Adrb3*^{-/-} mice, it was clear that both β_3 - and β_1 -adrenoceptors were capable of mediating AMPK phosphorylation. In mature adipocytes, the β_3 -adrenoceptor is the predominant β -adrenoceptor that is coupled to intracellular signalling events [7, 17]. The β_1 -adrenoceptor only becomes coupled when the β_3 -adrenoceptor is absent in mature adipocytes [8]. In contrast to earlier results, which indicated that α_1 -adrenoceptors activated glucose uptake in cells from *Adrb3*^{-/-} mice [8], no α_1 -adrenoceptor stimulation of AMPK was evident, irrespective of whether the β_3 -adrenoceptor was present or not. Thus, the previously reported α_1 -adrenoceptor stimulation of glucose uptake clearly does not involve activation of AMPK. Since forskolin and 8-bromo-cAMP both phosphorylated AMPK, this indicates that β -adrenoceptors phosphorylated AMPK through increases in cAMP. The PKA inhibitor H89 inhibited AMPK phosphorylation by isoprenaline in FVB adipocytes, but these results may not be reflective of the role of PKA, since H89 potentially inhibits mammalian AMPK [35]. H89 is also an antagonist of β_1 - and β_2 -adrenoceptors [36], but the action of H89 is presumably not achieved through any antagonist action at β_3 -adrenoceptors [37]. Therefore, the use of PKA inhibitors that are more selective to address this issue is under investigation.

UCP1 has been hypothesised to play a role in AMPK activation, since UCP1 activity can decrease the ATP:ADP ratio in a cell and therefore increase the AMP:ATP ratio [15]; this means that UCP1 activation could be linked to AMPK activation. It is well established that adrenergic activation in brown adipocytes significantly increases both UCP1 production and activity. Based on this it may be hypothesised that noradrenaline increases AMPK phosphorylation via a UCP1-mediated increase in AMP. An interaction between UCP1 and AMPK activities has been suggested [38], following observations that ectopic overproduction of UCP1 in white adipocytes enhanced AMPK activity, and that mitochondrial uncouplers such as dinitrophenol activate AMPK in skeletal muscle [39, 40]. In our study, in adipocytes grown in culture from *Ucp1*^{+/+} or *Ucp1*^{-/-} mice, where effects from alterations in the sympathetic drive from the animals are abolished, AICAR- and adrenergically mediated increases in AMPK phosphorylation levels (measured as a fold-increase relative to baseline for each of the respective genotypes) were identical. One difference was the observation that basal phosphorylation levels of AMPK were consistently lower (~50%) in adipocytes derived from *Ucp1*^{-/-} animals when results were expressed as a percentage of the *Ucp1*^{+/+} unstimulated state, a result not easily explainable in terms of UCP1 activity, since UCP1 production is negligible in non-stimulated +/+ cells and the basal AMP:ATP ratio is similar between genotypes. Under conditions (long-term adrenergic stimulation) where UCP1 protein levels are markedly enhanced and activated in *Ucp1*^{+/+}, but not

Ucp1^{-/-} adipocytes, stimulation of phosphorylation of AMPK was comparable in the two genotypes, suggesting that UCP1 activity is not a prerequisite for adrenergically mediated increases in AMPK.

AMPK is extensively linked to glucose uptake in skeletal muscle (for review, see [14]), and the fat-derived hormones leptin and adiponectin activate AMPK in skeletal muscle, liver and adipose tissue [10–13]. AICAR increases glucose uptake in skeletal muscle. Results in white adipocytes are varied, with some reports showing a positive action of AICAR on glucose uptake [41, 42] and others not [13]. More confusion arises because of reports that the positive action of AICAR on glucose uptake in white adipocytes is due to actions other than AMPK, despite AICAR being able to phosphorylate AMPK [41, 42]. In this study we have shown that AICAR significantly increases glucose uptake in primary brown adipocytes and that this increase is correlated with the ability of AICAR to phosphorylate AMPK. Mitochondrial uncouplers and inhibitors of mitochondrial complexes are activators of glucose uptake [40] and AMPK activity [39, 40, 43], and it has been suggested that UCP1 activity may also promote glucose uptake as a result of AMPK phosphorylation in adipocytes. We show that UCP1 was not required for β -adrenoceptor AMPK phosphorylation or adrenergically mediated glucose uptake.

Ara-A, an intracellular competitive inhibitor of AMPK, inhibits AMPK-mediated glucose uptake in skeletal muscle [44], papillary muscle [45] and adipose tissue [13]. Its use to inhibit skeletal muscle contraction-mediated glucose uptake was 'unsatisfactory' [44], since Ara-A partially inhibited contraction-mediated glucose uptake with no inhibition of contraction-mediated increases in AMPK activity. In adipose tissue, Ara-A has proved more reliable since it inhibits AICAR-mediated increases in AMPK phosphorylation and activity and does not inhibit insulin-mediated increases in glucose uptake [13]. β -Adrenoceptor-mediated glucose uptake was partially inhibited by Ara-A, suggesting that AMPK is involved in adrenergically mediated glucose uptake. In brown adipocytes, insulin stimulation results in the translocation of vesicles containing GLUT4 (now known as SLC2A4) from intracellular compartments to the plasma membrane, resulting in increased glucose uptake [46]. Noradrenaline has been proposed to increase glucose uptake in brown adipocytes via a mechanism that is independent of GLUT4 translocation [46, 47] and instead involves increases in GLUT1 activity [47]. The involvement of AMPK in this process is currently under investigation.

It was recently suggested that sympathetically stimulated glucose utilisation in brown adipose tissue is due to the serial activation of UCP1 and AMPK [48]. However, our results indicate that β -adrenoceptors activate AMPK independently of activation of UCP1 (but that there is a basal difference between cells isolated from *Ucp1*^{+/+} and *Ucp1*^{-/-} animals that we are currently unable to explain but is clearly independent of UCP1 production). The differences between the results of Inokuma et al. [48] and the present results are most likely to be a consequence of the

different systems used. Cells in culture have not previously been exposed to a sympathetic stimulus, whereas this is probably the case in intact mice at 26°C. We have indications that sympathetic activity is enhanced to a greater degree in *Ucp1*^{-/-} animals; these are consistent with other studies [48]. Thus, we suggest that the stimulatory action of sympathetic activity (noradrenaline) is probably already maximal in the *Ucp1*^{-/-} mice, not that this activity is lost.

AMPK also has implications for fatty acid synthesis and oxidation, glycogen synthesis, fatty acid uptake and lipolysis in adipose tissue. Recently, β -adrenoceptors were shown to stimulate lipolysis partly via AMPK in 3T3-L1 adipocytes, using adenoviral expression of a gene encoding a dominant negative form of the α_2 -AMPK subunit [33]. This is in contrast to other studies where AICAR inhibited isoprenaline-mediated lipolysis in rat white adipocytes [49, 50] and 3T3-L1 adipocytes [50]. Isoprenaline-mediated lipolysis was reduced by expression of gene encoding a constitutively active form of AMPK and, conversely, increased when AMPK activity was decreased by either a dominant negative form of AMPK or in α_1 -AMPK subunit knock-out mice (the predominant subunit expressed in white adipocytes) [50]. Hence, investigation of the role of AMPK in other β -adrenoceptor effects in adipose tissue would be of interest, considering that AMPK is a candidate target for the treatment of diabetes and obesity.

In conclusion we have shown that noradrenaline, acting via β -adrenoceptors, increases AMPK phosphorylation probably via increases in cAMP levels, a response that is independent of UCP1 expression and function. This activation of AMPK is partially responsible for the noradrenaline-mediated increases in glucose uptake seen in primary brown adipocytes. Further studies are needed to ascertain the importance of AMPK in other adrenergically mediated functions in brown adipose tissue, such as lipolysis, glycogen synthesis and fatty acid metabolism.

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