Dopamine directly increases mitochondrial mass and thermogenesis in brown adipocytes

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Abstract
Brown adipose tissue (BAT) is key to energy homeostasis. By virtue of its thermogenic potential, it may dissipate excessive energy, regulate body weight and increase insulin sensitivity. Catecholamines are critically involved in the regulation of BAT thermogenesis, yet research has focussed on the effects of noradrenaline and adrenaline. Some evidence suggests a role of dopamine (DA) in BAT thermogenesis, but the cellular mechanisms involved have not been addressed. We employed our extensively characterised murine brown adipocyte cells. D1-like and D2-like receptors were detectable at the protein level. Stimulation with DA caused an increase in cAMP concentrations. Oxygen consumption rates (OCR), mitochondrial membrane potential (Δψm) and uncoupling protein 1 (UCP1) levels increased after 24 h of treatment with either DA or a D1-like specific receptor agonist. A D1-like receptor antagonist abolished the DA-mediated effect on OCR, Δψm and UCP1. DA induced the release of fatty acids, which did not additionally alter DA-mediated increases of OCR. Mitochondrial mass (as determined by (i) CCCP- and oligomycin-mediated effects on OCR and (ii) immunoblot analysis of mitochondrial proteins) also increased within 24 h. This was accompanied by an increase in peroxisome proliferator-activated receptor gamma co-activator 1 alpha protein levels. Also, DA caused an increase in p38 MAPK phosphorylation and pharmacological inhibition of p38 MAPK abolished the DA-mediated effect on Δψm. In summary, our study is the first to reveal direct D1-like receptor and p38 MAPK-mediated increases of thermogenesis and mitochondrial mass in brown adipocytes. These results expand our understanding of catecholaminergic effects on BAT thermogenesis.

Key Words
- thermogenesis
- brown adipocytes
- dopamine
- mitochondrial mass
- UCP1
- p38 MAPK

Introduction
Adipose tissue is key to the control of energy and glucose homeostasis (Rosen & Spiegelman 2006, Chechi et al. 2013). Mitochondria-rich brown adipose tissue (BAT) contains the thermogenesis-mediating uncoupling protein 1 (UCP1) and, by virtue of its thermogenic function, it may enhance energy dissipation and insulin sensitivity in both rodents and humans (Kozak et al. 2010, Sidossis & Kajimura 2015). Due to these features, BAT has attracted attention as a potential source and target for novel therapies to treat obesity and the metabolic syndrome (Iwen et al. 2006, Klein et al. 2006, Enerback 2010, Scale 2010). Catecholamines are key to activating...
BAT thermogenesis in rodents and humans; however, the vast majority of studies were almost exclusively restricted to noradrenaline (NA) and adrenaline (A), as described in important reviews (Collins et al. 2010, Richard et al. 2010). Catecholamines have multiple effects on thermogenesis, e.g. treatment of adipocytes causes an increase of UCP1 concentrations as well as an induction of lipolysis and subsequently a rise of free fatty acid (FFA) levels (Collins et al. 2010). FFA themselves can further increase UCP1 activity, but they may also facilitate thermogenesis independently of UCP1 (Li et al. 2014).

Dopamine (DA), the precursor of NA and A, has not received much attention in this field of research. DA exerts its cellular effects by binding to either D1-like or D2-like receptors (also referred to as D1-class and D2-class receptors) (Beaulieu & Gainetdinov 2011). Initially, dopamine receptors were categorised by their ability to induce (D1-like receptors) or inhibit (D2-like receptors) adenylate cyclase (AC) and subsequently modulate cAMP levels. Cloning of DA receptors revealed further subtypes. Dopamine 1 (Dearry et al. 1990, Monsma et al. 1990, Zhou et al. 1990) and 5 (Tiberi et al. 1991) receptors comprise the D1-like receptor group, whereas dopamine 2 (Bunzow et al. 1988), 3 (Sokoloff et al. 1990) and 4 (Sunahara et al. 1991) receptors are referred to as D2-like receptor group.

Only few studies have thus far evaluated the contribution of DA to BAT thermogenesis in vivo. Cold exposure of rats, a strong stimulus of sympathetic nervous system (SNS) activity and BAT thermogenesis, causes an increase of DA concentrations in BAT (Blouquit et al. 1996) and a rise of interscapular BAT temperature (Maxwell et al. 1985b). Diet-induced thermogenesis also increases DA release from sympathetic nerves to BAT in rats (Rothwell et al. 1982). In line with these findings, exogenous DA causes a rise of heat production in rats (Davidovic et al. 1988).

Identification of DARPP-32, an intracellular third messenger for DA, in BAT of pigs suggests a direct role of DA in brown adipocytes (Meister et al. 1988). DA receptors appear to be present in BAT homogenates obtained from rats, as suggested by the response of AC to various DA receptor agonists and antagonists (Nisoli et al. 1992). Further, DA causes an increase in oxygen consumption in brown adipocytes (Maxwell et al. 1985a) and depolarisation of BAT obtained from rats (Fink & Williams 1976). Notably, most of this research was completed before the cloning of DA receptor subtypes and analysis of subsequent intracellular signalling.

In summary, current evidence suggests dopaminergic effects on BAT thermogenesis, yet the exact contribution of direct DA effects on BAT on thermogenesis has not been elucidated. Specific dopamine receptor subtypes and subsequent intracellular signalling in BAT thermogenesis have not been identified so far. Also, in vitro analyses of DA effects on brown adipocytes were restricted to either oxygen consumption or depolarisation.

The aim of this study was thus to comprehensively characterise direct effects of DA on mitochondrial functions and mass in brown adipocytes and to dissect cellular signalling pathways that mediate these effects. We employed our murine adipose cell line model which has been extensively characterised by our (Klein et al. 1999, 2000, Iwen et al. 2008, Hoppmann et al. 2010) and other (Ueki et al. 2003, Kovsan et al. 2009, Zhang et al. 2009) groups. To our knowledge, this is the first study demonstrating a direct D1-like receptor and p38 MAPK-mediated effect of DA on mitochondrial thermogenesis in brown adipocytes.

Materials and methods

Materials

Antibodies against peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1α) and the p38 mitogen-activated protein kinase (MAPK) inhibitor SB 202190 were purchased from Calbiochem. Phospho-specific p38 MAPK, total p38 MAPK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Cell Signalling. TOMM20 antibodies were purchased from Abnova (Taipei, Taiwan). ATP synthase beta antibodies and the specific D1-like agonist SKF 38393 were from Abcam. Specific UCP1 and D1-like receptor antibodies were purchased from Chemicon International. Specific D2-like receptor antibodies and the specific D2-like antagonist raclopride were from Santa Cruz Biotechnology. The specific D1-like antagonist SCH 23390 and D2-like agonist bromocriptine were purchased from Tocris Bioscience (Bristol, UK). Secondary antibodies were from Life Technologies. Fatty acid-free bovine serum albumin (BSA) was from Serva (Heidelberg, Germany). All other materials were obtained from Sigma-Aldrich.

Cell culture

Cells used in all experiments were SV-40 T immortalised brown adipocytes generated as previously described
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In brief, preadipocytes were seeded on 10 cm culture plates (Sarstedt, Nümbrecht, Germany) and grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) with 4.5 g/L glucose, supplemented with 20 nmol/L insulin, 1 nmol/L triiodothyronine, 20% foetal bovine serum (Life Technologies) and penicillin/streptomycin (Life Technologies) (‘differentiation medium’). Upon confluence, differentiation was induced for 24 h with 500 µmol/L isobutylmethylxanthine, 250 µmol/L indomethacin and 2 µg/mL dexamethasone (‘induction medium’). After that they were maintained in differentiation medium for 6 days until exhibiting a fully differentiated phenotype. Prior to the experiments, cells were serum starved overnight. Cells between passages 13 and 30 were used.

Immunoblotting

Cells were differentiated and treated with as indicated. After washing the cells with ice-cold PBS, protein of cells was extracted by RIPA buffer (supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate and 1 mmol/L sodium fluoride per 10 mL of 1× RIPA buffer). Protein concentrations were evaluated using the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific). Proteins were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore). Membranes were blocked using a ‘blocking solution’ (5% dry milk in Tris-buffered saline with 0.05% Tween20) for 1 h, incubated overnight with primary antibodies, washed and then incubated with the appropriate secondary antibodies. All protein bands, except for UCP1, were visualised and analysed using the gel documentation system Fusion SL by Vilber Lourmat (Eberhardzell, Germany). GAPDH served as loading control.

Oxygen consumption

Respiration of brown adipocytes was measured using round-bottom OxoPlates (OP 96U, 96-well microplate with integrated optical oxygen sensor; PreSens, Regensburg, Germany) according to the user’s manual. Briefly, treated adipocytes were detached from the plate by the addition of accutase (Life Technologies), 150,000 cells suspended in 280 µL medium supplemented with the substances as indicated were used per single measurement. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP; 10 µM) (10 µM) and oligomycin (2 µM) were added directly before analysis. Finally, OxoPlates were read out from the bottom side every 30 s for 90 min at 37°C in a fluorescence intensity microplate reader (FluoSTAR OPTIMA; BMG-Labtech, Ortenberg, Germany). The kinetics of fluorescence intensities was analysed with Microsoft Excel according to the manufacturer’s instruction manual. Calibration was performed with water saturated with air (100%) and with an aqueous sodium sulfite solution (0.2 g/20 mL) (0%) according to the protocol of the supplier.

cAMP determination

Mature brown adipocytes were treated as stated in the Results section and in the figures. Cells were lysed by adding 0.1 mol/L HCl and incubated for 20 min at room temperature followed by centrifugation at 1000×g for 10 min. Supernatants were analysed using an ELISA kit from Caymen Chemical, following the manuals instructions. To increase assay sensitivity, samples were acetylated according to manufacturer’s instructions. Isoproterenol served as positive control.

Fatty acid determination

Changes of fatty acid levels in cell culture medium were determined by using the free fatty acid quantification kit obtained from Abcam, following the manuals instructions. SV-40 T immortalised white epidydimal adipocytes generated as previously described (Klein et al. 1999, 2000, Perwitz et al. 2006, Iwen et al. 2008) served as control.

Mitochondrial membrane potential

Mitochondrial membrane potential was determined by using JC-10 (Biomol, Hamburg, Germany; final concentration 1µM). Fluorescence intensities of JC-10 monomers and aggregates were quantified, respectively, by FL1 (485/535 nm) and FL2 (540/590 nm) detectors of a plate reader (Tecan Austria GmbH, Groedig, Austria); the manufacturer’s instructions were followed. The JC-10 aggregate/monomer ratio is considered to be proportional to mitochondrial membrane potential.
Results

Dopamine 1- and 2-like receptors are expressed in brown adipocytes and dopamine increases cAMP levels

In the first approach, we analysed the expression of dopamine receptors. Both, D1- and D2-like receptors were expressed in mature brown adipocytes at the protein level (Fig. 1A). DA treatment of brown adipocytes for 2 min resulted in a dose-dependent increase of cAMP concentrations, reaching a maximum at 1 nM. Pre-treatment with the D1-like receptor antagonist SCH 23390 for 30 min abolished the effect of 1 nM DA on cAMP levels. Treatment of cells with SCH 23390 for 30 min alone had no significant effect on cAMP levels (Fig. 1B).

Dopamine induces mitochondrial changes that are consistent with thermogenesis via dopamine 1-like receptors

Next, brown adipocytes were stimulated with DA for 24 h, and mitochondrial functions were characterised. Oxygen consumption increased significantly after stimulation with 1 nM (Fig. 2A) and 10 nM (data not shown) DA. This was accompanied by an increase in the mitochondrial membrane potential (Δψm, Fig. 2B). Non-shivering thermogenesis is mediated by UCP1, and protein levels of UCP1 increased significantly upon treatment with DA (Fig. 2C). The D1-like receptor-specific antagonist SCH 23390 (1 nM) abolished the DA-mediated effects on oxygen consumption, Δψm, and UCP1 levels (Fig. 2). SKF 38393 (10 nM), a D1-like receptor agonist, also caused a significant increase of oxygen consumption, Δψm, and UCP1 levels after 24 h (Fig. 2). Treatment of brown adipocytes with the D2-like specific antagonist raclopride (10 nM) did not affect Δψm, it also did not abolish the DA-dependent reduction of Δψm (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Bromocriptine (1 nM) alone had no significant effect on Δψm within 24 h (Supplementary Fig. 1). Treatment of brown adipocytes with 1 nM of NA resulted in a significant increase of oxygen consumption, it also caused a slight increase of Δψm; however, this was statistically not significant (Fig. 2A and B).

Dopamine induces the release of fatty acids that have no additional effect on dopamine-induced oxygen consumption

DA (1 nM) caused a significant rise of fatty acid (FA) concentrations in cell culture medium of brown and
Research

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white adipocytes within 24 h (Fig. 3A). White epidydimal adipocytes were employed as controls. FA may alter thermogenesis independently of UCP1. FA-free bovine serum albumin (BSA) was used to bind extracellular FA, thereby excluding direct FA effects. BSA (0.4%) did not affect basal respiration rates of brown adipocytes. BSA also did not alter DA-induced increases in oxygen consumption at DA concentrations of 1 nM and 10 nM (Fig. 2B).

Dopamine increases mitochondrial mass

Cells were treated with DA 10 nM for 24 h, and oligomycin (to inhibit ATP synthase) or CCCP (a potent substance to uncouple oxidative phosphorylation, OXPHOS) was added directly before OCR analysis, this aimed at assessing the functional capacity of mitochondria. In cells not treated with DA, OCR did not increase in response to oligomycin, and there was only a moderate increase of OCR due to CCCP-induced uncoupling of OXPHOS. Stimulation of cells with DA caused the known significant increase of OCR as compared to basal. Treatment of these cells with both, oligomycin and CCCP, resulted in a further significant rise of OCR (Fig. 4A). These findings suggest an increase in functional mitochondrial capacity and mitochondrial mass after stimulation of brown adipocytes with DA. Also, two mitochondrial proteins, mitochondrial import receptor subunit TOM20 homolog (TOMM20) and ATP synthase beta, were analysed by immunoblotting to assess mitochondrial mass. Upon stimulation of brown adipocytes with DA for 24 h, levels of both proteins increased dose dependently (Fig. 4B and C). Peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1A) protein levels, a master regulator of mitochondrial mass and function, also increased significantly after treatment with DA for 24 h (Fig. 4D).

p38 MAPK mediates the dopaminergic effect on mitochondrial membrane potential

p38 MAPK is known to be involved in the induction of thermogenesis. Stimulation of brown adipocytes with DA for 10 min increased p38 MAPK phosphorylation in a dose-dependent fashion (Fig. 5A). Treatment of brown adipocytes with the p38 MAPK-specific inhibitor SB 202190 (10 µM) for 24 h did not affect $\Delta \psi_{m}$ but it abolished the effect of 1 nM DA (Fig. 5B).
Dopamine induces fatty acid release without additional effects on oxygen consumption. Brown and white adipocytes were treated with dopamine (DA) and/or fatty acid-free bovine serum albumin (BSA) for 24 h at concentrations indicated. (A) DA increases fatty acid concentrations in cell culture medium, as determined by using a free fatty acid quantification kit; n = 6. (B) BSA was added to exclude direct effects of fatty acids on oxygen consumption. BSA alone did not, whereas DA increased oxygen consumption, as determined by applying OxoPlates. There were no significant differences between DA-induced oxygen consumption with or without BSA; n = 7. CL-316243 served as positive control (data not shown); *P < 0.05, **P < 0.01.

Discussion

Our study reveals direct D1-like receptor and p38 MAPK-mediated alterations of mitochondrial functions in brown adipocytes.

In a first approach, we analysed the expression of dopamine receptors in brown adipocytes. Both, D1-like and D2-like receptors were present at the protein level. Treatment of brown adipocytes with DA caused an increase of cAMP levels, suggesting functional relevance of D1-like receptors. The DA-induced rise of cAMP concentrations was blocked by the specific D1-like antagonist SCH 23390. This constellation of findings demonstrates not only the presence of D1-like-receptors but also their functional relevance in brown adipocytes. In line with these findings, Nisoli and coworkers analysed the cAMP response of various DA receptor agonists and antagonists in rat BAT homogenates and dispersed brown adipocytes (Nisoli et al. 1992). Results of this pharmacological approach prompted the authors to conclude that dopaminergic receptors differing from the ‘classical’ D2R are present on the membranes of rat brown adipocytes.

Mitochondrial thermogenesis is mediated by UCP1, which uncouples OXPHOS at the inner mitochondrial membrane (IMM) and thereby combusting energy (Collins et al. 2010). Typically, this results in an increase in oxygen consumption and/or an increase in ΔΨm (i.e. depolarisation) at the IMM. As outlined in the introduction, previous studies suggest a direct role of DA in BAT thermogenesis in vivo as well as in vitro. Yet, in vivo analyses of mitochondrial functions were limited to either oxygen consumption or depolarisation of brown adipocytes. Our study does not only demonstrate the direct dopaminergic effects on these two key features of mitochondrial thermogenesis but also to our knowledge, it is the first to provide evidence for a DA-induced increase of UCP1 levels in brown adipocytes. Next, we aimed at identifying the DA receptor subtype that mediates the dopaminergic effect on mitochondrial thermogenesis. The D1-like specific antagonist abolished the DA-mediated effect on oxygen consumption, ΔΨm, and UCP1 levels, demonstrating D1-like receptor specificity. This notion was further supported by the observation that a specific D1-like receptor agonist also caused a significant increase of oxygen consumption, ΔΨm, and UCP1 levels, similar to DA alone. On the other hand, neither bromocriptine, as D2-like receptor agonist, had a significant effect on ΔΨm nor did raclopride, a D2-specific receptor antagonist, abolish the DA-mediated increase of ΔΨm.

It is well known that catecholamines, in particular NA, induce lipolysis and that FA may alter thermogenesis independently of UCP1 (Li et al. 2014): (i) by serving as fuel, (ii) by directly increasing UCP1-activity and (iii) by mediating a proton leak at the inner mitochondrial membrane independently of UCP1. The increase of FA levels upon treatment of brown and white adipocytes suggests the induction of lipolysis by DA. Free FA binds to BSA that thereby serves as scavenger for FA. We added FA-free BSA to the cell culture medium, which helped us to exclude FA-mediated effects on mitochondrial respiration of brown adipocytes in response to DA.

Further, dopaminergic effects on mitochondrial mass have not been addressed to date. The clear oligomycin- and CCCP-dependent rise of OCR in cells treated with...
DA 10 nM for 24 h suggests an elevation of mitochondrial capacity and mass at the functional level. Increases of TOMM20 and ATP synthase beta concentrations, as markers of mitochondrial mass at the protein level, within 24 h of DA treatment also suggest direct dopaminergic effects on mitochondrial biogenesis. Consistent with this, PGC1A protein levels, as important regulator of mitochondrial mass (Seale 2010), also increased upon DA stimulation. These results reveal multilevel dopaminergic effects on mitochondrial functions and mass.

p38 MAPK pathways have been reported to mediate UCP1 gene expression and thermogenesis (Puigserver 2005, Collins et al. 2010). Using our brown adipocytes, we were previously able to show an ACTH-induced rise in UCP1 expression, which was abolished by p38 MAPK inhibition (Iwen et al. 2008). Dopaminergic stimulation of brown adipocytes also caused a dose-dependent increase of p38 MAPK phosphorylation. Further, we were able to demonstrate that pharmacological inhibition of p38 MAPK abolished the DA-induced effect on $\Delta \psi_m$. These findings confirm the central role of p38 MAPK in DA-induced modulation of mitochondrial function in brown adipocytes.

In a broader context, we provide comprehensive in vitro evidence for direct dopaminergic effects on brown adipose thermogenesis and mass. To our knowledge, only one study examined the effect of cold exposure, a very potent stimulus of thermogenesis, on catecholamine content of BAT in rats (Blouquit et al. 1996). DA levels increased 4.7-fold on the first day and 17.9-fold on the 30th day of cold exposure, as compared to controls. The effect on NA content of BAT was less pronounced; on day one, there was no significant increase and only a 1.3-fold rise on day 30, as compared to controls. This suggests a role of DA in cold-induced SNS activation, as not only NA but also DA appears to be released from sympathetic nerve fibres to BAT. Our current study on dopaminergic effects on thermogenesis in vitro provides very plausible evidence for the physiological relevance of the in vivo data obtained from rats. Bloquit and coworkers expressed the catecholamine content of BAT as nanograms per fat pad; hence, the exact DA concentration in BAT remains unclear (Blouquit et al. 1996). To address this question, we examined in a separate study the effects of cold exposure on BAT metabolism and plasma catecholamines in healthy males (Backhaus et al. 2016). Cold-induced BAT activation, as measured by PET/CT quantification of [18F]-FDG uptake, and significant increases in plasma NA and DA, but not A, were found. This pattern is characteristic for cold-induced activation of SNS, which does not affect the
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Adrenal medulla and therefore A concentrations (Romijn & Fliers 2005), it is likely that DA concentrations in BAT will exceed 1 nM, as used in this current study. Further, the significant increase of DA in humans in response to cold exposure as well as BAT activation suggests a role of DA in thermogenesis not only in rodents but also in humans.

Taken together, our current study provides evidence for direct stimulatory effects of DA on mitochondrial thermogenesis and mass. These effects are mediated by D1-like receptors and p38 MAPK. Targeting D1-like receptors in BAT may help to induce thermogenesis and ameliorate insulin resistance, clearly pointing towards novel potential strategies to treat obesity and the metabolic syndrome.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-16-0159.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
R K researched data, contributed to discussion and wrote the manuscript; N P researched data, contributed to discussion and reviewed the manuscript. J R researched data. S M S researched data and reviewed the manuscript; H L contributed to discussion and reviewed the manuscript. J K researched data, contributed to discussion and reviewed the manuscript.

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