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Arginine–vasopressin directly promotes a thermogenic and pro-inflammatory adipokine expression profile in brown adipocytes

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ABSTRACT

Arginine–vasopressin (AVP) — via activation of the hypothalamic-pituitary-adrenal (HPA) axis — may play a role in the regulation of energy homeostasis and related cardiovascular complications. Brown adipose tissue (BAT) — via dissipation of energy in the form of heat — contributes to whole body energy balance. BAT expresses vasopressin receptors. We investigated direct effects of AVP on brown adipose endocrine and metabolic functions. UCP-1 protein expression in differentiated brown adipocytes was induced after acute exposure of adipocytes to AVP. This effect was time-dependent with a maximum increase after 8 h. AVP also induced a time- and dose-dependent increase in p38 MAP kinase phosphorylation. Pharmacological inhibition of p38 MAP kinase with SB 202190 abolished the induction of UCP-1 protein expression. Furthermore, while acute AVP treatment enhanced mRNA expression of MCP-1 and IL-6, adiponectin mRNA expression was reduced. Yet, on the level of intracellular glucose uptake, there was no AVP-induced change of adipose insulin-induced glucose uptake. Finally, there was no difference in lipid accumulation between control and AVP-treated cells.
Taken together, our data demonstrate direct effects of AVP on thermogenic, inflammatory, and glucoregulatory gene expression in brown adipocytes, thus expanding the hitherto known spectrum of this neuropeptides's biological effects and suggesting a direct adipotropic role as a stress-promoting factor.

1. Introduction

Arginine–vasopressin (AVP), synthesized in the hypothalamus, is a posterior pituitary hormone. Along with its carrier protein, neurophysin II, AVP is packaged into neurosecretory vesicles and transported axonally in the neurohypophysis where it is either stored or secreted into the bloodstream. AVP is involved in various functions, including regulation of water excretion by direct antidiuretic action on the kidney, vasoconstriction of the peripheral vessels, involvement in cardiomyocyte remodeling, and energy homeostasis. It is also involved in cognition, tolerance, maternal behavior, and in the modulation of adrenocorticotropic hormone (ACTH) release from the pituitary in response to stress [1]. Interestingly, ACTH directly induces insulin resistance, promotes a pro-inflammatory adipokine profile and stimulates UCP-1 in adipocytes [2]. However, direct effects of AVP on adipocyte metabolism and function are unknown. AVP's physiological effects are mediated through the binding to specific membrane receptors of the target cells. The AVP receptors have been classified into at least three types: V1a, V1b (V3), and V2. V1a receptors are expressed in vascular smooth muscle cells [3,4], cardiomyocytes [5], hepatocytes [6], and platelets [7]. Recently, V1a receptors were identified in white (WAT) and brown (BAT) adipose tissue [8]. V1b receptors are expressed in pituitary cells as well as in WAT [8]. V1a AVP receptor knockout mice exhibit higher levels of ketone bodies and glycerol, increased catabolism of triacylglycerides and free fatty acids [8] and display decreased sympathetic nerve and renin angiotensin activity [9], whereas V1b AVP receptor knockout mice show a higher insulin sensitivity [10]. In mice lacking both V1 receptors an impaired glucose tolerance in response to a high-fat diet could be observed [11]. V2 receptor is found primarily in the kidney and is linked to adenylate cyclase and the production of cAMP, in association with antidiuresis [12]. AVP appears to play a role in the regulation of euglycemia since plasma AVP levels are increased in humans suffering from type 1 and type 2 diabetes [13,14]. Moreover, insulin- or sulfonylurea-therapy reduced not only plasma glucose level, but also AVP concentration [15–17]. Infusions with AVP are associated with increased circulating glucose levels in rats and humans [18,19]. These effects seem to be mediated via stimulation of glucagon release from pancreatic islet cells [20] and direct promotion of glycogenolysis and gluconeogenesis in heptatocytes [21]. Furthermore, previous work showed that AVP is involved in lipid metabolism. Circulating ketone bodies [18] were reduced and free fatty acid release was enhanced in starved rats while treating with AVP [22]. This lends support to an anitilipolytic effect of AVP on adipocytes.

Obesity and insulin resistance are core components 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

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dysfunction critically promoting the development of the metabolic syndrome and its complications [23,24]. Recently, the importance of BAT for energy disposal in obese and overweight subjects has been demonstrated in a number of major human studies [25–27]. Dysfunction of BAT may include insulin resistance, reduced thermogenesis in response to cold and food intake, as well as alterations in the accurate time- and dose-dependent secretion of adipokines. Here, we reveal direct effects of AVP on thermogenic and endocrine brown adipose functions, thus suggesting a role of this neuropeptide as an adipotropic, endocrine stress-mediating factor.

2. Materials and methods

2.1. Materials

Phospho-specific p44/p42 MAP kinase, phospho-specific and total p38 MAP kinase, and PKB/Akt antibodies were purchased from Cell Signaling (Beverly, MA, USA). UCP-1 antibody was purchased from Millipore (Billerica, MA, USA). Glucose uptake assays were performed with 2-deoxy-[^{3}H] glucose from NEN Life Technologies (Boston, MA, USA). Primers for gene expression analysis were ordered from Biometra (Goettingen, Germany). Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The specific inhibitors p38-MAP kinase inhibitor SB 202190, and Jak2 inhibitor AG490 were purchased from Calbiochem (Darmstadt, Germany). The MEK 1/2 inhibitor PD98059 was purchased from New England Biolabs (Ispwich, MA, USA).

2.2. Cell culture

Interscapular brown adipose tissue was isolated from newborn FVB mice. Cells were immortalized by infection with the retroviral vector pBabe, encoding SV40 T antigen [28]. Preadipocytes were seeded on tissue culture plates (Sarstedt, Nümbrecht, Germany) and grown to confluence in culture medium with Dulbecco's modified Eagle's medium, supplemented with 20% fetal bovine serum (FBS), 4.5 g/l glucose, 20 nM insulin, 1 nM triiodthyronine ('differentiation medium'), and penicillin/streptomycin. Adipocyte differentiation was induced by complementing the medium further with 250 μM indomethacin, 500 μM isobutylmethylxanthine and 2 μg/ml dexamethasone for 24 h when confluence was reached. After this induction period, cells were changed back to differentiation medium. Cell culture was continued for 5 more days before cells were starved for 24 h with serum-free medium prior to carrying out the experiments. Differentiated brown adipocytes were used between passages 10 and 30. Culturing these cells allows to investigate metabolic and endocrine characteristics of adipocyte biology [2].

2.3. 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 Bradford protein assay according to the manufacturer's instructions (Biorad, Hercules, CA, USA). Subsequent to separation by SDS-PAGE protein was transferred to nitrocellulose membranes (Schleicher and Schuell Inc., Keane, NH, USA). The membranes were incubated overnight with 10 mM Tris, 150 mM NaCl, 0.05% Tween (pH 7.2), and 3% bovine serum albumine. Membranes were exposed to the respective antibodies for 1 to 2 h in the appropriate dilutions. Protein bands were visualized using a chemiluminescence kit (Roche Molecular Biochemicals, Mannheim, Germany). Densitometry was performed using Quantity One Software version 4.5.2 (Bio-Rad, Hercules, USA).

2.4. Gene expression analysis

Reverse transcription followed by polymerase chain reaction (PCR) was performed in order to measure monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and adiponectin mRNA expression with 36B4 as a housekeeping gene control. Mature brown adipocytes were starved in serum-free medium for 24 h prior to total RNA isolation with TRIzol reagent (Invitrogen, Karlsruhe, Germany) To optimise RNA quality, a cleanup and DNase digestion were performed with a RNeasy kit and RNase-Free Dnase Set (Qiagen, Hilden, Germany). The quality of RNA was tested by photometry analysis and/or RNA visualization on an agarose gel. Up to 1 μg of total RNA was reverse-transcribed using Superscript II (Invitrogen, Karlsruhe, Germany) and an oligo p(DT)$_{15}$ primer (Roche Molecular Biochemicals, Mannheim, Germany) in the presence of RNase inhibitor (Roche Molecular Biochemicals, Mannheim, Germany) in a 20 μl reaction. 1 μl of each RT reaction was amplified to a total volume of 25 μl containing, 250 nM of each primer, and 1 × QuantiTect SYBR Green PCR-Mix (Qiagen, Hilden, Germany). PCR was performed using a Mastercycler ep realplex (Eppendorf GmbH, Hamburg, Germany). The following primers were used: 36B4 (acc. no. NM_007475) AAG CGC GTC CTG GA TTG TCT (sense) and CCG CAG GGG CAG CAG TGG T (antisense); adiponectin (acc. no. NM_009605) CTT AAT CCT GCC CAG TCA TGC (sense) and CCT TCC AAC CTG CAC AAG TTC (antisense); monocyte chemoattractant protein-1 (MCP-1) (acc. no. NM_011333) GCC CCA CTC ACC TGC TGC TAC T (sense) and CCT GCT GCT GGT GAT CCT CTT GT (antisense); interleukin-6 (IL-6) (acc. no. NM_031168) AGC CAG AGT CCT TCA GA (sense) and GGT CCT TAG CCA CTC CT (antisense). PCR was performed as follows: initial denaturation at 95 °C for 300 s, 40 cycles with 95 °C for 20 s, 60 °C for 30 s, Specific amplification was confirmed by producing melting curve profiles Optimized relative quantification was done by using the Mastercycler® ep realplex software based on the second derivative maximum method normalized to 36B4 as a housekeeping gene.

2.5. Oil red O staining

Tissue cultures were washed with phosphate-buffered saline twice and fixed with 10% formalin for at least 15 min at room temperature. Cells were stained for 1 h at room temperature with a filtered oil red O solution (0.5 g of oil red O in 100 ml of isopropanol, working solution 60% stocksolution and 40% H_2O). To remove excess stain and any precipitate that may have formed, the cells were rinsed with distilled water several times.

2.6. Glucose uptake

Analysis of intracellular glucose uptake was performed in triplicate measurements in 12-well plates as described previously [29]. Subsequent to 15 min of pre-treatment with adenosine desaminase (2 U/ml), fully differentiated monolayers of adipocytes were either treated or non-treated with AVP (1 h, 100 nM), with or without adding insulin (5 min, 100 nM). Afterwards, cells were exposed to 50 μl of 2-deoxy-[^{3}H] glucose (0.5 μCi/ml final concentration) for four additional minutes, washed in ice-cold phosphate-buffered saline, and lysed with 0.1% sodium dodecyl sulfate. The radioactivity incorporated was determined using liquid scintillation counting.

2.7. Statistical analysis

Data are presented as S.E.M. 'SigmaPlot® 9.0.1'-software (Systat Software Inc., Richmond, CA, 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 $P<0.01$ highly significant.