Expression of ATRAP in Adipocytes and Negative Regulation by β-Adrenergic Stimulation of JAK/STAT

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Key words
- adipocyte
- angiotensin II
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Abstract

Sympatho-adrenergic activity and the renin-angiotensin system are considered critical regulators of obesity and hypertension. The novel angiotensin II type 1 receptor-associated protein (ATRAP) has been demonstrated to modulate angiotensin II signalling in smooth muscle cells and cardiomyocytes. Adipose tissue expresses important renin angiotensin system components and contributes to cardiometabolic disease. However, ATRAP expression and regulation in adipocytes are unknown. We investigated expression of this novel modulator of angiotensin signalling and its regulation by β-adrenergic receptors. We found ATRAP to be expressed in differentiated brown and white adipocytes. Stimulation of β-adrenoceptors strongly suppressed ATRAP expression. We hypothesised a role for JAK/STAT signalling elements. Indeed, β3-adrenergic stimulation robustly stimulated both STAT1 and STAT3 phosphorylation in a time- and dose-dependent manner. This effect was abrogated by inhibition of PKA and JAK2 signalling. Moreover, inhibition of JAK/STAT and PKA signalling reversed the β3-adrenergic suppression of ATRAP expression. This study provides the first evidence for expression and adrenergic regulation of the angiotensin II signalling modulator ATRAP in adipocytes. Further, it indicates a novel regulatory link between β-adrenergic and JAK/STAT signalling.

Abbreviations

ATRAP	angiotensin II type 1 receptor-associated protein
JAK/STAT	Janus kinase/signal transducer and activator of transcription
PKA	protein kinase A
RAS	renin-angiotensin system
SOCS	suppressor of cytokine signalling

Introduction

The sympathetic nervous system (SNS) and the renin-angiotensin system (RAS) are considered pivotal components in the control of vascular tone. Obesity is frequently associated with increases in blood pressure [1]. Increased activity of both the SNS and the RAS is found in overweight individuals and has been postulated to play a pathogenic role in the development of obesity-associated hypertension and insulin resistance [2–4].

Adipose tissue is innervated by the SNS, with differences in degree between fat depots: brown adipose tissue is more densely innervated than white [5]. Sympatho-adrenergic stimulation of the β3-adrenoceptor in brown adipocytes induces thermogenesis and, importantly, contributes to energy homeostasis in small mammals [6]. In humans, the contribution of thermogenic brown adipose tissue to the control of energy homeostasis is a matter of debate. Brown adipocytes are found dispersed in the white fat depots of adults, and a clear distinction between "dormant" brown fat precursor cells and white preadipocytes is difficult to make [6,7]. Changes in brown fat-specific gene expression have been associated with insulin resistance [8], and the β3-adrenoceptor is reported to remain a target for anti-obesity research [9]. In rodents, selective β3-adrenoceptor stimulation induces conversion from a white to a brown fat phenotype [10].

Important RAS components are found in adipose tissue in mice and humans, including angio-

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tensinogen, its cleavage product angiotensin II (ATII), and ATII receptors [3, 11, 12]. ATII levels appear to be particularly high in brown adipose tissue [11]. The contribution of the adipose RAS to the control of systemic vascular tone is insufficiently understood. In transgenic mice, adipose angiotensinogen is secreted into the circulation [13]. However, to date it remains a matter of debate whether adipose tissue RAS contributes to the systemic regulation of vascular tone in humans. A paracrine role for adipose tissue RAS in the regulation of adipocyte proliferation and differentiation as well as in the differentiation-associated modulation of adipokine secretion emerges as a possible paradigm [11].

Recently, a novel modulator of ATII signalling has been identified, the angiotensin II type 1 (AT1) receptor-associated protein (ATRAP). ATRAP was initially found in a yeast two-hybrid system by screening for molecules that bind to the AT1 receptor [14]. ATRAP is a transmembrane protein that promotes receptor internalization of the AT1 receptor, decreases ATII-stimulated transcriptional activity, and inhibits vascular smooth muscle cell proliferation [15, 16]. Moreover, ATRAP overexpression in cardiomyocytes inhibits ATII-induced hypertrophic cell responses [17]. Overall, ATRAP appears to function as a negative regulator of ATII signalling.

Regulation of ATRAP and expression in adipose tissue are unknown. Because the RAS is present in adipose tissue, and because modulation of ATII signalling in states of SNS overactivity caused by anti-obesity drugs may importantly impact on adipose tissue RAS activity, we investigated adipose expression and β-adrenergic regulation of ATRAP in murine white and brown adipocytes, a sensitive cell model that has been extensively characterised [18–26]. Our data provide first evidence for the expression of ATRAP in adipocytes. Moreover, the findings indicate a link between β-adrenergic stimulation and JAK/STAT signalling to negatively regulate this ATII signalling modulator.

Materials and Methods

Materials

Phosphospecific antibodies against STAT1 (Tyr701) and STAT3 (Tyr705) were purchased from Cell Signaling Technology (Beverly, MA, USA). H89 and AG490 were from Calbiochem (La Jolla, CA, USA). The β3-receptor agonist CL316,243 was kindly provided by Dr. Kurt Steiner (Wyeth Ayerst Research, Princeton, NJ, USA). Unless stated otherwise, all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Methods

Cell culture

Immortalised adipocyte cell lines were cultured and differentiated as previously described [18–20, 22, 23, 26]. In brief, preadipocytes were isolated from newborn FVB mice and infected with a puromycin resistance-conferring retroviral vector encoding the SV40T antigen. After selection with puromycin, cells were grown to confluence on 10-cm culture plates (Sarstedt, Nümbrecht, Germany) in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, Scotland, UK) and supplemented with 20% foetal bovine serum, 4.5 g/l glucose, 20 nmol/l insulin, and 1 nM T3 ("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 hours when confluence was reached. After this period, cells were changed back to differentiation medium. Cell culture was continued for five more days, before cells were serum-deprived for 24 hours prior to carrying out the experiments. Differentiated adipocytes were used between passages 10 and 25.

Western blotting

Cells were lysed using whole-cell lysis buffer containing 2 mM vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM PMSF. Protein content of the lysates was determined by using the Bradford assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Lysates were submitted 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 hours in the appropriate dilutions. Protein bands were visualised with the chemiluminescence kit from Roche Molecular Biochemicals (Mannheim, Germany).

Analysis of ATRAP and SOCS3 gene expression

Quantitative real-time RT-PCR was preformed with 36B4 as a housekeeping gene control. Cells were starved in serum-free medium for 24 hours prior to total RNA isolation with TRIzol reagent (Invitrogen, Karlsruhe, Germany). To optimise RNA quality, a cleanup and DNase digestion were performed with the RNeasy kit and RNase-Free Dnase Set (Qiagen, Hilden, Germany). The quality of RNA was tested by photometric analysis and agarose gel electrophoresis. Two micrograms of total RNA was reverse transcribed by using Superscript II (Invitrogen) and an oligo p(DT)15 primer (Roche Molecular Biochemicals) in the presence of RNase inhibitor (Roche Molecular Biochemicals) in a 20-μl reaction. One microliter of each RT reaction was amplified to 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 following primers were used: 1) ATRAP (acc. no. NM009642) TGC TTG GTG TTC TCA AGC TCC (sense) and AAG CCA CCA AGA AAC ATG CC (antisense); 2) 36B4 (acc. no. NM007475) AAG CGC GTC CTG GCA TTG TCT (sense) and CCG CAG GGG CAG CAG TGG T-(antisense); and 3) SOCS3 (acc. no. NM007707) CCC TGC ACA GCC CTC CTT TCT CAC (sense) and GCC CCA CCC AGC CCC ATA CC (antisense). PCR for all targets was performed as follows: initial denaturation at 95 °C for 900 seconds, 40 cycles with 95 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds. The identity of the amplified product was confirmed by producing melting curve profiles and by subjecting the amplicon to agarose gel electrophoresis. Optimised relative quantification was done with Relative Expression Software (REST) [27]. The amplified quantitative real-time PCR product of ATRAP was sequenced with the BigDye terminator sequencing kit (Applied Biosystems) to confirm its identity.

Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for measurement of the activity of living cells [28]. This assay is based on the capacity of cellular mitochondrial dehydrogenase enzymes to convert the yellow water-soluble substrate MTT into an insoluble, intracellular, purple formazan product. Subsequently, the optical density (OD) of this product was measured by spectrophotometer (test wavelength 570 nm and reference wavelength 630 nm).

Statistical analysis

Data are presented as mean ± SEM. SigmaStat software (Systat, San Jose, CA, USA) was employed for statistical analysis of all data. Statistical significance was determined using the unpaired Student's *t*-test; p-values <0.05 were considered significant, and those <0.01 were considered highly significant.

Results

β-Adrenergic stimulation inhibits ATRAP expression in a time-dependent manner

We tested ATRAP expression and β-adrenergic regulation in differentiated brown and white adipocytes. Stimulation of brown adipocytes with the β3-selective adrenoceptor agonist CL 316,243 strongly suppressed basal expression levels of ATRAP. Maximal downregulation to approximately 30% of basal levels was seen after 8 hours of stimulation. This negative effect was time dependent, with an early trend towards diminished basal expression levels detectable after 1 h (**Fig. 1A**). White adipocytes were stimulated with the β-adrenoceptor agonist isoproterenol in analogous experiments. Maximal downregulation of ATRAP mRNA to approximately 35% of basal levels was observed after 2 hours and remained reduced for a further 6 hours (**Fig. 1B**). Stimulation of β-adrenoceptors classically causes an elevation in intracellular cAMP levels, as does forskolin by activating adenylate cyclase. Treatment of white adipocytes with forskolin caused analogous time-dependent reductions of ATRAP expression compared with stimulation of β-adrenoceptors (**Fig. 1C**).

β-Adrenergic stimulation acutely induces STAT1 and STAT3 phosphorylation

The JAK/STAT signalling pathway is an important mediator of angiotensin signalling. We hypothesised that JAK/STAT signalling molecules may play a role in regulating the angiotensin signalling modulator ATRAP. Treatment of differentiated brown adipocytes with the β3-selective agonist CL 316,243 induced a strong increase in STAT1 phosphorylation. This stimulatory effect was time dependent, with a peak increase of approximately 300% after 20 minutes (**Fig. 2A**). Furthermore, β3-adrenergic stimulation of STAT1 for 20 minutes was dose dependent, with a maximal increase of 300% at a 100 nM concentration of the β3-selective agonist and a significant, approximately 100% increase at a concentration of 1 nM (**Fig. 2B**). Similar time- and dose-dependent increases of approximately 150% were found for β3-adrenergic stimulation of STAT3 phosphorylation (**Fig. 2C,D**). Stimulation of differentiated white adipocytes with CL 316,243 caused a comparable time- and dose-dependent increase of STAT1 and STAT3 phosphorylation. Incubation at 100 nM of the β-adrenoceptor agonist for 20 minutes resulted in a peak increase of approximately 150% in STAT1 phosphorylation (inserts in **Fig. 2A,B**). Phosphorylation of STAT3 was also increased by approximately 150% at a 100 nM concentration of CL 316,243 (inserts in **Fig. 2C,D**). All changes occurred without alteration of STAT protein content (data not shown).

Leptin is known to stimulate STAT3 phosphorylation and activation. Treatment of differentiated brown adipocytes with leptin caused a significant reduction in ATRAP mRNA levels by approximately 50% after 4 hours (data not shown). This observation emphasises the role of STAT3 in the control of ATRAP expression.

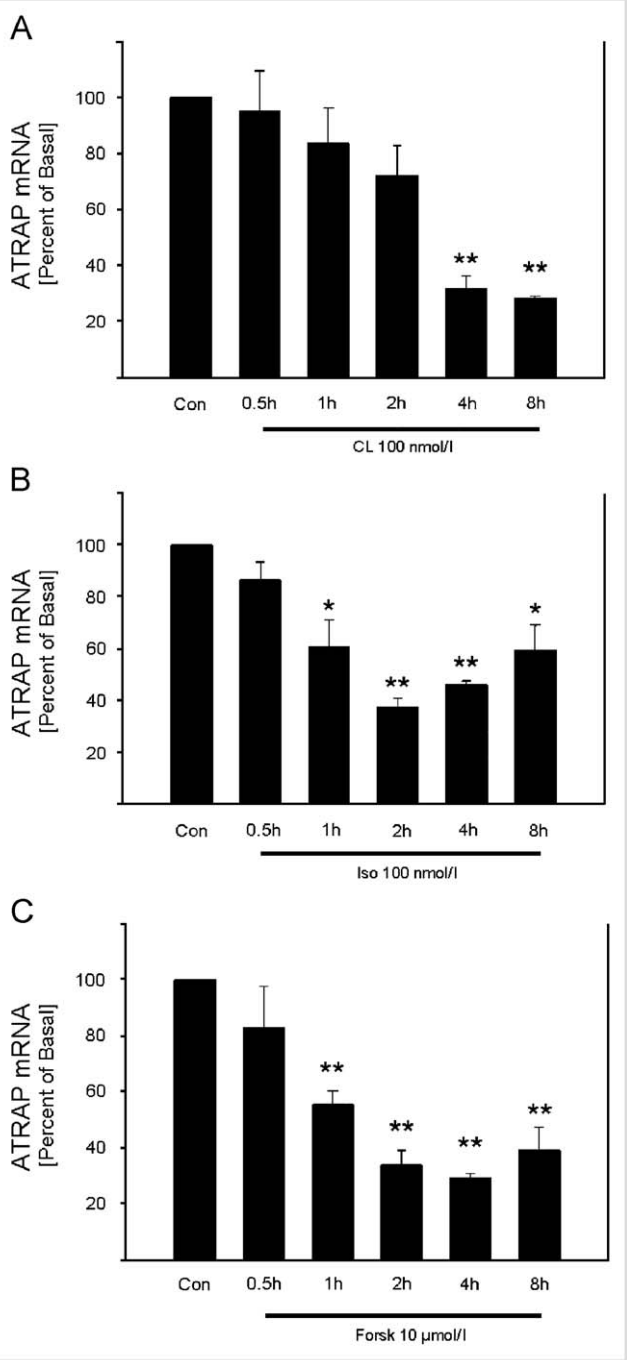

Fig. 1 β-Adrenergic stimulation suppresses expression of ATRAP. Brown adipocytes were stimulated with the β3-adrenoceptor agonist CL 316,243 (**A**), and white adipocytes were stimulated with the β-adrenoceptor agonist isoproterenol (**B**) or with forskolin (**C**) for the indicated periods of time (hours). ATRAP mRNA quantification was analysed as described in Materials and Methods. A bar graph analysis including the SEM of three independent experiments is shown. **p<0.01 comparing untreated (Con) with CL-treated cells.

Inhibition of JAK2 and PKA abolishes the β3-adrenergic stimulation of STAT1 and STAT3

G protein-coupled β-adrenergic receptors classically stimulate the protein kinase A (PKA) signalling pathway. Stimulation of differentiated brown adipocytes for 20 minutes with the β3-selective agonist (100 nM) again induced a robust stimulation of STAT1 and STAT3 phosphorylation. This effect was abolished by

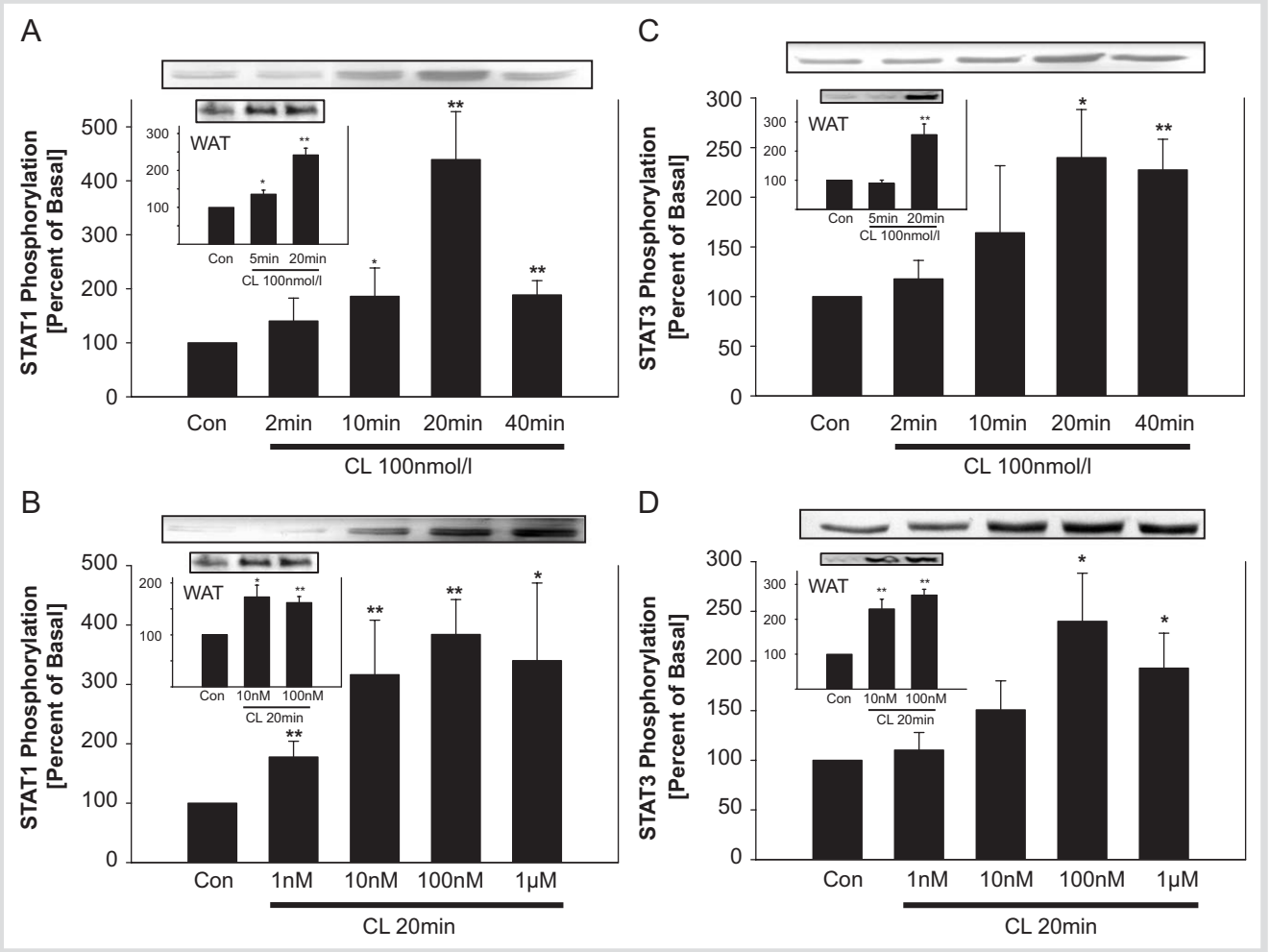

Fig. 2 β-Adrenergic stimulation acutely activates STAT1 and STAT3. Brown and white (inserts) adipocytes were stimulated with the β3-adrenoceptor agonist CL 316,243 (CL) for the times and concentrations indicated. (**A**,**C)** Time in minutes at a CL concentration of 100 nmol/l. (**B**,**D)** CL stimulation for 20 minutes. Cell lysates and immunoblots using phosphospecific antibodies were prepared as described in Material and Methods. A bar graph analysis of ≥5 independent experiments with the SEM and representative immunoblots is shown. $^{*}p<0.05$, $^{**}p<0.01$ comparing untreated (Con) with CL-treated cells.

pretreating the cells with the pharmacologic PKA inhibitor H89 (**Fig. 3A,B**). As expected, inhibition of JAK2 using the pharmacologic inhibitor AG490 also prevented the β-adrenergic stimulation of STAT phosphorylation. Incubation of differentiated cells with the inhibitors alone tended to increase STAT1, but not STAT3, phosphorylation compared with nontreated control cells (**Fig. 3A,B**). However, this effect did not reach the level of statistical significance. Furthermore, inhibition of protein kinase C, phosphatidylinositol 3-kinase, and src kinase signalling pathways did not affect β-adrenergic activation of STAT1 and STAT3 (data not shown).

Downregulation of ATRAP by β3-adrenergic stimulation is PKA and JAK/STAT dependent

Given both the strong suppression of ATRAP expression and an acute activation of STAT1 and STAT3 by β-adrenergic stimulation, we tested the involvement of PKA and JAK/STAT signalling pathways in the β-adrenergic control of ATRAP expression. Exposure of differentiated brown cells to the β3-adrenergic agonist for 8 hours again inhibited basal ATRAP expression levels by approximately 60% (**Fig. 4**). Interestingly, pretreatment with either the PKA or the JAK2 inhibitor alone prior to β3-adrenergic stimulation did not reverse this negative effect. However, combined inhibition of both PKA and JAK/STAT signalling pathways completely reversed the inhibitory β-adrenergic effect on ATRAP expression (**Fig. 4**).

β3-Adrenergic stimulation increases SOCS3 gene expression, which is abolished by inhibition of JAK2 and PKA

Suppressor of cytokine signalling (SOCS) 3 is a known downstream target of the JAK/STAT signalling pathway [29, 30]. SOCS3 mRNA increased by 315% after stimulation of adipocytes with CL 316,243 for 2 hours. Further pretreatment of the cells with AG490 for 30 minutes reversed this effect. Addition of AG490 alone did not change SOCS3 mRNA concentrations (**Fig. 5A**). Analogous experiments were carried out with the PKA inhibitor H89. Again, treatment of adipocytes with CL 316,243 increased SOCS3 mRNA concentrations. This effect was also reversed by pretreatment with H89 for 30 minutes. The inhibitor alone did not change SOCS3 levels (**Fig. 5B**)

Kinase inhibitors do not change cell viability

Because the toxicity of kinase inhibitors is a concern, we assessed cell viability by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Incubation of adi-

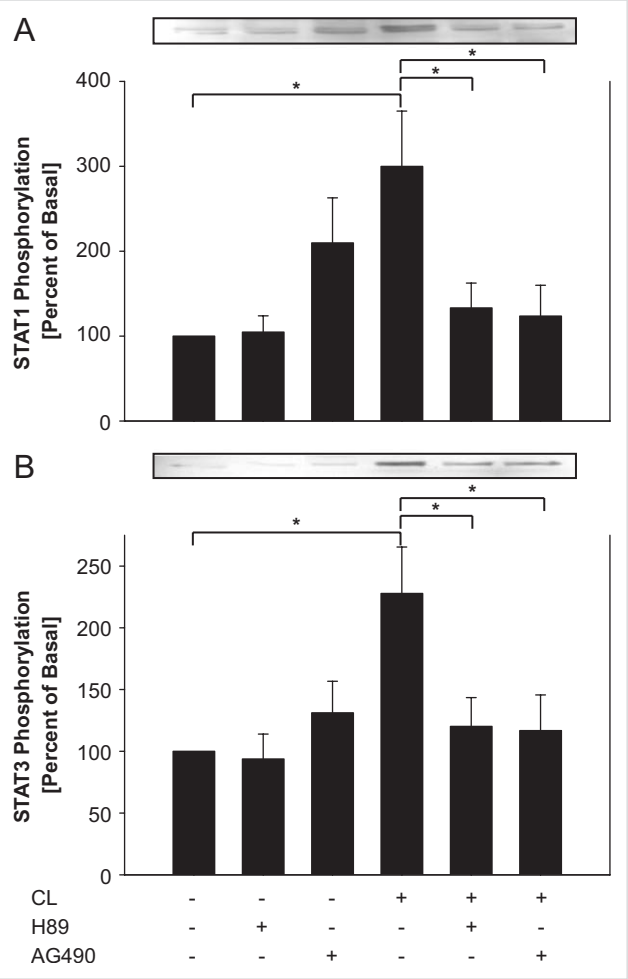

Fig. 3 β3-Adrenergic activation of (**A**) STAT1 and (**B**) STAT3 is prevented by inhibition of PKA and JAK2. Brown adipocytes were stimulated with the β3-adrenoceptor agonist CL 316,243 (CL, 100 nmol/l, 20 min) with or without PKA (H89, 10 μmol/l) and JAK2 (AG490, 50 μmol/l) inhibitors (30 min pretreatment) as indicated. Cell lysates and immunoblots using phosphospecific antibodies were prepared as described in Material and Methods. A bar graph analysis of 5 independent experiments with the SEM and representative immunoblots is shown. *$p<0.05$ comparing untreated (control) with CL-treated cells and CL-treated cells with CL- plus inhibitor-treated cells, respectively.

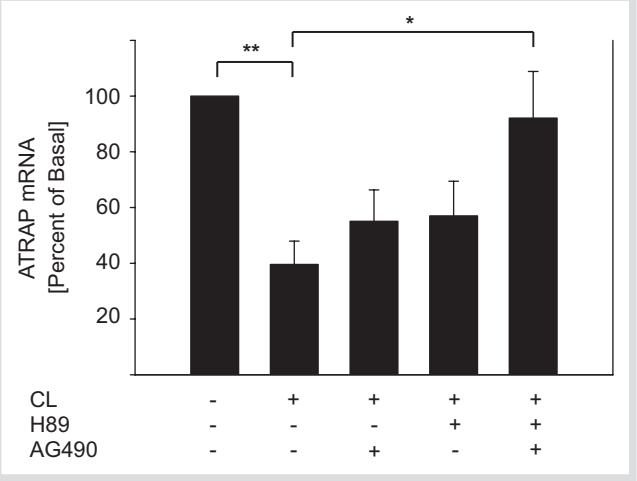

Fig. 4 β3-Adrenergic downregulation of ATRAP expression is reversed by combined inhibition of PKA and JAK2. Brown adipocytes were stimulated with the β3-adrenoceptor agonist CL 316,243 (CL, 100 nmol/l) for 8 hours with or without PKA (H89, 10 μmol/l) and JAK2 (AG490, 50 μmol/l) inhibitors (30 min pretreatment). ATRAP mRNA quantification was analysed as described in Materials and Methods. A bar graph analysis including the SEM of 8 independent experiments is shown. *$p<0.05$, **$p<0.01$ comparing nontreated (control) with CL-treated cells and CL-treated cells with CL- plus inhibitor-treated cells, respectively.

pocytes for 8 hours with H89 and AG490 alone and in combination revealed no change in cell viability (data not shown).

Discussion and Conclusions

This study is the first to demonstrate expression of the recently identified angiotensin II signalling modulator ATRAP in adipocytes. Further, it shows inhibition of ATRAP expression by β-adrenergic stimulation. Finally, this negative regulation reveals a novel link between β-adrenergic and JAK/STAT signalling pathways.

ATRAP has been identified in a yeast two-hybrid system by employing angiotensin type 1 receptor as a bait and screening a mouse kidney cDNA library [14]. A human homologue of ATRAP has recently been described [31]. ATRAP is considered a negative regulator of angiotensin II signalling. It promotes angiotensin II type 1 receptor internalization, prevents angiotensin II–stimulated transcriptional activity, and inhibits vascular smooth muscle cell growth and cardiomyocyte hypertrophic responses [15–17]. Important components of the RAS are present in adipose tissue, and angiotensin signalling plays a role in adipocyte proliferation and differentiation [11]. ATRAP expression in adipocytes may serve to enhance the regulation of the adipose RAS system and further indicates a functional role of its components in fat cells.

We have found a strong inhibition of ATRAP expression by β-adrenergic stimulation. This appears to be mediated via cAMP, since forskolin mimics this negative effect. Downregulation of this negative regulator of angiotensin II signalling might enhance angiotensin II effects on adipocytes and, thereby, influence proliferation and differentiation in adipocytes [11]. This, in turn, could impact on endocrine functions of adipose tissue, since many of the adipocyte-derived hormones are expressed and secreted in a differentiation-dependent manner. Brown adipose tissue and the β3-adrenergic receptor have been considered as drug targets for anti-obesity treatment approaches [7,9,32]. Despite disappointing results with a number of low-affinity receptor agonists, novel β3-adrenoceptor agonists are still reported to be in clinical trials [9]. Our data suggest that in addition to documented cardiac side effects of these compounds, alteration of adipose tissue RAS activity also must be considered.

Our data implicate JAK/STAT signalling pathways in the β-adrenergic control of ATRAP expression. We found a robust time- and dose-dependent activation of STAT1 and STAT3 by β-adrenergic stimulation. Furthermore, SOCS3 mRNA levels, a known target gene of JAK/STAT signalling pathways, are increased after β-adrenoceptor agonist stimulation. Few reports on direct adrenergic coupling to JAK/STAT signalling exist. In vascular smooth muscle cells, direct α-adrenergic stimulation of JAK/STAT signalling has been described [33]. In contrast, nonselective β-adrenergic stimulation of cardiomyocytes with isoproterenol *in vitro* failed to activate JAK/STAT signalling, while a delayed stimulation was observed in mouse heart after *in vivo* treatment via an

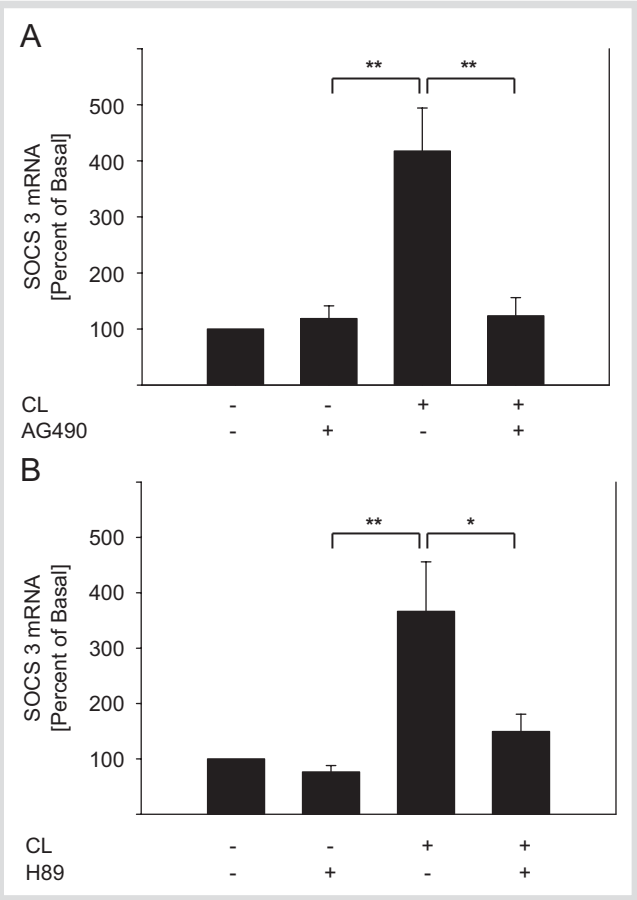

Fig. 5 β3-Adrenergic stimulation increases SOCS3 gene expression, which is abolished by inhibition of JAK2 and PKA. Brown adipocytes were treated with the β3-adrenoceptor agonist CL 316,243 (CL, 100 nmol/l) for 2 hours and/or with the (**A**) JAK2 inhibitor (AG490, 50 μmol/l, 30 min pretreatment) and (**B**) PKA inhibitor (H89, 10 μmol/l, 30 min pretreatment). SOCS3 mRNA quantification was analysed as described in Materials and Methods. A bar graph analysis including the SEM of ≥4 independent experiments is shown. $^{*}p<0.05$, $^{**}p<0.01$ comparing CL-treated cells with inhibitor-treated cells (with or without CL 316,243), respectively.

IL-6 family of cytokine-mediated pathway [34]. A functional link between G protein-coupled receptors and STAT3 activation was also investigated in human embryonic kidney 293 cells [35]: Gαs-mediated activation of STAT3 was abolished by inhibition of PKA and JAK2, with PKA acting upstream of JAK2. These findings are in accordance with those reported in our study. Although we cannot exclude an indirect effect, the acute response in our study also suggests a direct activation of STAT molecules in adipocytes by β-adrenergic stimulation. This signalling cascade appears to require activation of protein kinase A, as pharmacologic inhibition of this kinase abolished the effect. Further, protein kinase C, phosphatidylinositol 3-kinase, and src kinase signalling pathways do not appear to be involved because pharmacologic inhibition of these pathways did not affect β-adrenergic STAT phosphorylation (data not shown).

JAK/STAT signalling is key to mediating inflammatory responses [36]. Adrenergic activation of this signalling pathway might provide a molecular link between SNS activation, RAS, and the induction of inflammatory processes, which are considered a pathophysiological basis for the development of insulin resistance/diabetes and associated cardiovascular complications [37–39].

Interestingly, only combined inhibition of PKA and JAK/STAT signalling was able to reverse the β-adrenergic inhibition of ATRAP expression. This result indicates an additional PKA-dependent signalling pathway via an as yet undetermined factor, with JAK/STAT signalling being a critical component for fully controlling ATRAP expression (**Fig. 6**).

In summary, this study demonstrates expression of the novel angiotensin signalling modulator ATRAP in adipocytes and provides evidence for a link between adrenergic and JAK/STAT signalling pathways negatively regulating adipose ATRAP expression. Sympatho-adrenergic stimulation of inflammatory signalling pathways and modulation of adipose tissue angiotensin signalling might impact on obesity-associated complications.

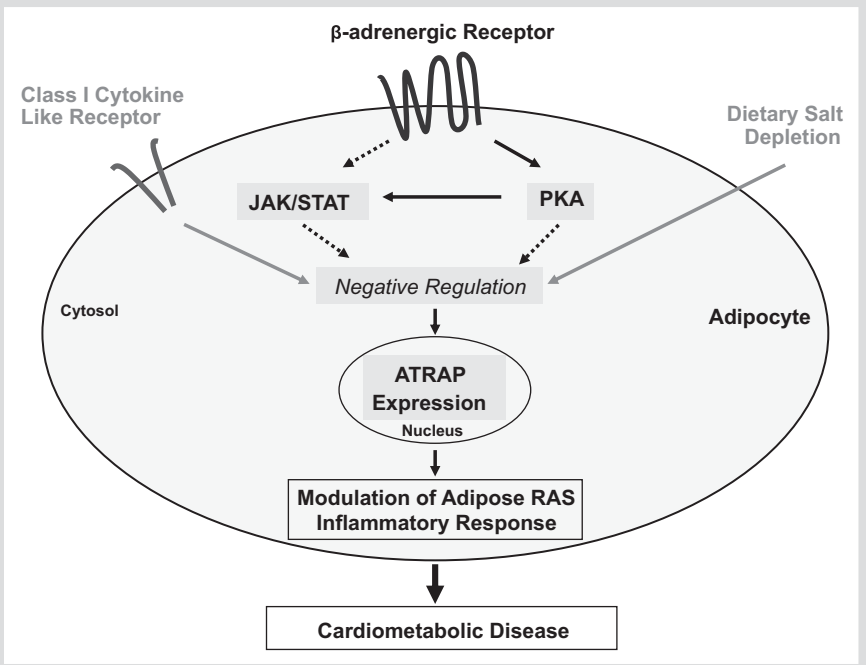

Fig. 6 Model of β-adrenergic signalling pathways regulating adipose ATRAP expression. β-adrenergic stimulation negatively regulates ATRAP expression and stimulates STAT1 and STAT3. Activation of these STAT molecules is PKA dependent, as it is prevented by inhibition of PKA. Both JAK/STAT and PKA signalling pathways are negative regulators of ATRAP. Only combined pharmacologic inhibition reverses the β-adrenergic downregulation. This finding suggests that additional PKA- and JAK/STAT-dependent signalling pathways (dashed arrows) are critical components for fully controlling ATRAP expression. Other potential pathways for ATRAP expression not originating from the β-adrenergic receptor are indicated by grey arrows (e.g., class I cytokine-like receptors [leptin receptor] or dietary salt depletion as described by Tsurumi et al. [40]).

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