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Reduced PDE4 expression and activity contributes to enhanced catecholamine-induced cAMP accumulation in adipocytes from FOXC2 transgenic mice

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Abstract Overexpression of forkhead transcription factor FOXC2 in white adipose tissue (WAT) leads to a lean phenotype resistant to diet-induced obesity. This is due, in part, to enhanced catecholamine-induced cAMP-PKA signaling in FOXC2 transgenic mice. Here we show that rolipram treatment of adipocytes from FOXC2 transgenic mice did not increase isoproterenol-induced cAMP accumulation to the same extent as in wild type cells. Accordingly, phosphodiesterase-4 (PDE4) activity was reduced by 75% and PDE4A5 protein expression reduced by 30–50% in FOXC2 transgenic WAT compared to wild type. Thus, reduced PDE4 activity in adipocytes from FOXC2 transgenic mice contributes to amplified β-AR induced cAMP responses observed in these cells.
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Keywords: FOXC2; WAT; PDE; cAMP; Rolipram; Cilostamide; IBMX; β-AR

1. Introduction

We have previously shown that the forkhead transcription factor FOXC2, when overexpressed in white adipose tissue of mice, has a pleiotropic effect on gene expression, showing increased expression of genes involved in insulin signaling, glucose uptake and lipolysis [1]. Among the genes regulated were the β1,2,3-adrenergic receptors and the RIα regulatory subunit of PKA [1,2], leading to increased sensitivity of the βadrenergic-cAMP-PKA signaling pathway. Accordingly, we showed that catecholamine-induced cAMP accumulation is amplified in adipocytes from FOXC2 transgenic mice compared to adipocytes from wild type mice. These experiments were performed in the presence of the non-selective phosphodiesterase (PDE) inhibitor IBMX to minimize hydrolysis of cAMP by PDEs. Thus, the strongly enhanced and sustained cAMP response previously observed in FOXC2 transgenic adipocytes is most likely a result of the increased expression of β-AR receptors, since this would lead to a more profound activation of β-AR associated adenylyl cyclases (ACs) and, thus, increased generation of cAMP from ATP. However, reduced expression of cAMP-specific PDEs could also contribute to increased levels and duration of catecholamine-induced cAMP accumulation in FOXC2 transgenic WAT. The PDE enzymes belong to eleven related gene families (PDEs 1–11) [3]. Of these the PDE3 and PDE4 families provide the major route for degrading cAMP in cells such as adipocytes. In adipocytes, phosphorylation and activation of PDE3B is the major mechanism whereby insulin antagonizes catecholamine-induced lipolysis [4].

Four genes (*4A*/*4B*/*4C*/*4D*) encode the PDE4 enzyme family [5]. Each of these generate a number of isoforms that are characterized by unique N-terminal regions [5]. There is a strong body of evidence showing that PDE3 and PDE4 underpin compartmentalized cAMP signaling in cells by controlling spatially distinct pools of cAMP [6]. This is achieved by differences in intracellular targeting of these forms. The contribution of PDE3 and PDE4 to cAMP hydrolysis and cAMP signaling in cells can simply be gauged using appropriate selective inhibitors [7]. Thus, cilostamide selectivity inhibits PDE3 activity, whilst rolipram is selective for PDE4.

Interestingly, Nakamura et al. [8] have shown that PDE4 has anti-lipolytic capacity in fed rats, but not in starved rats, suggesting that PDE4 expression and/or activity in mature adipocytes is regulated according to food intake. Furthermore, in a recent study by Snyder et al. [9] PDE4 inhibition with rolipram had only moderate effect on lipolysis in murine 3T3-L1 cells and rat adipocytes, but simultaneous exposure to cilostamide and rolipram had synergistic effect on lipolysis in these cells, supporting a role for PDE4 in suppression of lipolysis. The present study was performed in order to elucidate any possible role for either PDE3 or PDE4 in underpinning the phenotypic changes seen in catecholamine-stimulated cAMP accumulation in adipocytes from FOXC2 transgenic mice. In doing this we find that PDE4A5 expression and basal PDE4 activity, but not PDE3 activity, are reduced in FOXC2 transgenic adipocytes, thereby contributing to amplified β-AR-induced cAMP responses observed in adipocytes from these transgenic animals.

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Abbreviations: WAT, white adipose tissue; PDE, phosphodiesterase; cAMP, cyclic adenosine-3′,5′-monophosphate; IBMX, isobutylmethylxanthine; β-AR, beta-adrenergic receptor; ADA, adenosine deaminase; CHA, cyclohexyladenosine

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2. Materials and methods

2.1. Materials

The following chemicals were from Sigma: adenosine (A-9251), adenosine deaminase (A-5168) CHA (C-9901), BSA (A-4503), fatty acid free BSA (A-6003), IBMX (I-5879), and isoproterenol (I-2760). Rolipram (557330) and cilostamide (231085) were from Calbiochem and Collagenase A (103586) was from Roche. *Mice*-wild type and FOXC2 transgenic (1) C57BL/6J mice were fed a standard chow with 4% (w/w) fat content.

2.2. Adipocyte preparation

Adipocytes were isolated from intraabdominal fat pads according to the method of Rodbell [10]. All processing of cells were performed in Krebs–Ringer solution buffered with 25 mM Hepes at pH 7.5, containing 2.5 mM $CaCl_2$ (KRH medium), 500 nM adenosine and 2 mM glucose according to Honnor et al. [11].

2.3. cAMP assay

Adipocytes isolated from wild type and FOXC2 transgenic mice (2×10^7 cells/ml) were incubated in KRH medium containing 4% fatty acid free BSA, 1 U/ml adenosine deaminase and 100 nM CHA, in the presence or absence of 1 μM isoproterenol, IBMX (100 μM), rolipram (10 μM), cilostamide (1 μM) under constant agitation at 37 °C. Adenosine deaminase was added in order to remove exogenous adenosine and since this stimulates cAMP production, PKA activity and lipolysis, CHA, a specific A1 receptor agonist was added to obtain cells at basal conditions [12]. Equal amounts of cells (50 μl; 10^6 cells) were withdrawn at different time points, mixed with stop buffer containing 100 μM IBMX, and flash-frozen. Intracellular cAMP levels were determined by radioimmunoassay (cAMP kit from NEN, Boston, MA, Catalogue No. SMP004) according to the manufacturers instructions.

2.4. Immunoblotting

Intra-abdominal adipose tissues from wild type and FOXC2 transgenic mice were treated by a polytron tissue homogenizer (3×15 s) and sonicated on ice in a buffer containing 10 mM potassium phosphate (pH 6.8), 150 mM sodium chloride, 5 mM $MgCl_2$, 10 mM CHAPS, Complete™ protease inhibitor mix (Roche), 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 50 mM sodium fluoride (CHAPS/PE buffer), and centrifuged $15000 \times g$. Twenty micrograms of total protein were separated by electrophoresis, and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by semi-dry blotting. The membranes were blotted with primary antibodies over night at 4 °C in blocking solution. Here, as previously described, we used specific polyclonal antibodies to detect rodent PDE4A (AC55) [13] and PDEA5 [14]. Polyclonal antibodies were also used to detect PDE3B [15], β-arrestin-1 (RDI) and β-arrestin-2 (Santa Cruz Biotech).

2.5. PDE activity assay

Wild type and FOXC2 transgenic intra-abdominal adipose tissue lysates were prepared in CHAPS/PE buffer as described above and assayed, in duplicate, for total PDE activity as described before using 1 μM cAMP as substrate [16]. PDE3 activity was expressed as the difference between activity with and without cilostamide, and PDE4 activity was expressed as the difference between activity with and without rolipram. Protein concentrations were quantified and activities were normalized for protein content.

3. Results

3.1. Inhibition of PDE4 is essential for obtaining rapid catecholamine-induced cAMP formation in adipocytes

In the absence of PDE inhibitors, challenge of adipocytes with isoproterenol resulted in relatively small increases in intracellular cAMP in both wild type and FOXC2 transgenic cells, although 2 min after isoproterenol challenge a moderate cAMP response was observed in the FOXC2 transgenic cells (Fig. 1A). However, in marked contrast to this, challenge with isoproterenol in combination with IBMX, a non-selective inhibitor that is able to inhibit all cAMP hydrolyzing PDEs except PDE8 [17], led to a rapid and sustained (10 min) cAMP response in FOXC2 transgenic cells (Fig. 1B). In wild type cells, a similarly rapid increase in cAMP was noted, although both the baseline cAMP level and amplitude of the response were lower than in FOXC2 transgenic cells and the cAMP levels returned to baseline after 10 min following stimulation (Fig. 1B).

Treatment of cells with the PDE4 selective inhibitor, rolipram rescued the acute isoproterenol-induced cAMP response in wild type cells, as also seen in the presence of IBMX, and slightly enhanced the acute cAMP response in FOXC2 transgenic adipocytes (Fig. 1C). The cAMP level in rolipram-treated wild type adipocytes was similar to that of FOXC2 transgenic cells stimulated with ispoproterenol in the absence of PDE inhibitors (2.8 versus 2.9 pmol cAMP/10^6 cells), when comparing isoproterenol-induced cAMP accumulation after 2 min of stimulation (Fig. 1C, wild type versus Fig. 1A, FOXC2 transgene). Furthermore, rolipram treatment increased isoproterenol-induced cAMP accumulation more in wild type than in FOXC2 cells (0.9 versus 0.5 pmol cAMP/10^6 cells). Treatment of wild type cells with the PDE3 inhibitor, cilostamide enhanced basal and isoproterenol-stimulated cAMP levels, but had no effect in FOXC2 transgenic adipocytes (Fig. 1D).

3.2. PDE4 activity is reduced in WAT from FOXC2 transgenic mice

Based on the apparent differences in PDE-sensitive cAMP accumulation between wild type and FOXC2 transgenic adipocytes, we set out to investigate whether there were differences in PDE activities between wild type and FOXC2 transgenic WAT by performing PDE assays on WAT lysates. Interestingly, an approximately 30% reduction in total cAMP-sepcific PDE activity compared to wild type (Fig. 2A) was observed. Basal PDE4 activity in WAT from FOXC2 transgenic mice was reduced by approximately 75% as compared to wild type mice (Fig. 2B). No differences in PDE3 activity was observed, whose levels remained similar in FOXC2 transgenic and wild-type WAT (Fig. 2C).

3.3. Reduced PDE4A expression in adipocytes from FOXC2 transgenic mice

Prompted by the reduced PDE4 activity in FOXC2 transgenic WAT, we investigated whether PDE4 expression was reduced in FOXC2 transgenic WAT. Immunoblotting using a pan-PDE4 antibody (K116) [13] showed that an immunoreactive band migrating at approximately 110–120 kDa was downregulated in FOXC2 transgenic WAT (data not shown). Based on the size, we speculated that this could be a long PDE4A isoform; PDE4A5, A10 or A11. Immunoblotting with a PDE4A specific antibody (Ac55), revealed that expression of a PDE4A isoform migrating at approximately 110 kDa was reduced by some 50% in FOXC2 transgenic WAT relative to wild type (Fig. 3A and B). We were unable to detect expression of PDE4A11 and detected only low levels of expression of PDE4A10 with no difference in expression between wild type and transgenic WAT (data not shown). Using a PDE4A5 specific antibody, we detected 30% lower expression level of this isoform in FOXC2 transgenic WAT relative to wild type

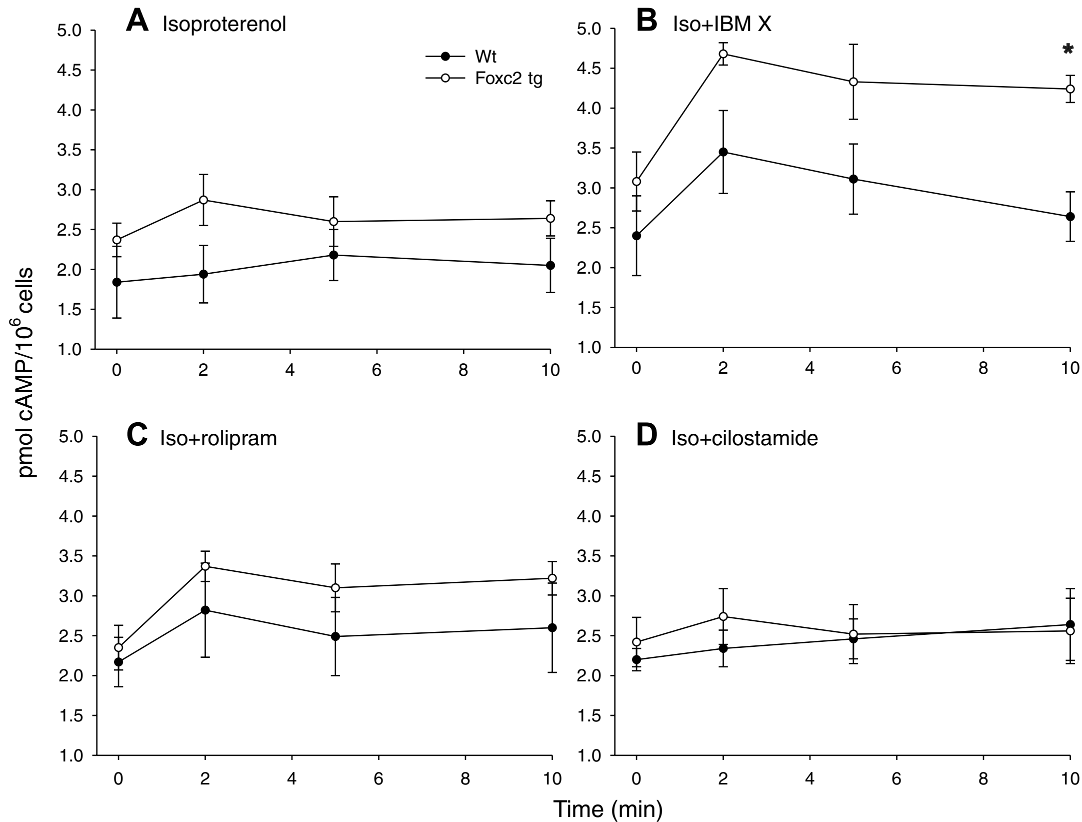

Fig. 1. Acute isoproterenol-induced cAMP accumulation in FOXC2 transgenic adipocytes is maintained in the absence of PDE inhibitors. Adipocytes isolated from wild type and FOXC2 transgenic intraabdominal fat pads were preincubated for 15 min with ADA and CHA in the absence (A) or presence of PDE inhibitors IBMX (B), cilostamide (C) and rolipram (D) (time 0) followed by stimulation with isoproterenol. Cells were harvested 0-2-5 and 10 min after stimulation and assayed for intracellular cAMP. Statistical analysis was performed using Student's unpaired *t*-test. $*$ denote significant differences where $P \leqslant 0.05$. Data represents mean ± S.E.M. from three individual experiments performed in duplicate.

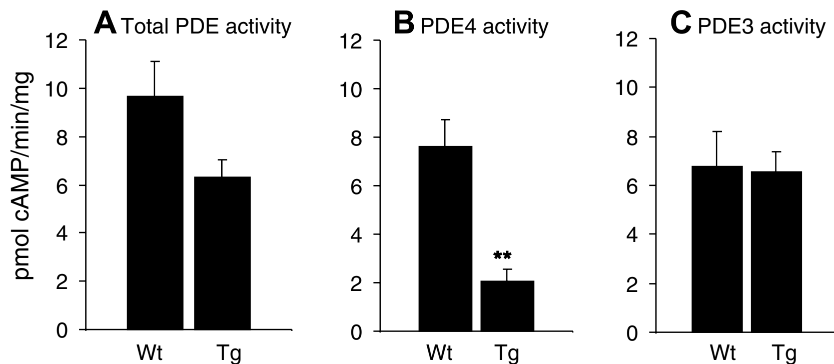

Fig. 2. PDE4 activity is reduced in FOXC2 transgenic mice. Wild type and FOXC2 transgenic WAT lysates were assayed for total PDE activity (A), PDE4 activity (B) and PDE3 activity (C). The data represents the mean ± S.E.M. from three (B and C) and six (A) individual experiments performed in duplicate. Statistical analysis was performed using Student's unpaired *t*-test. $**$ denote significant differences where $P \leqslant 0.01$.

(Fig. 3A and B). We did not detect differences in expression levels of PDE4B, 4C, 4D (not shown) or PDE3B between wild type and FOXC2 transgenic WAT (Fig. 3A).

Since various PDE4 isoforms are able to be recruited to ligand-activated β-adrenergic receptors via association with β-arrestins [18], we also examined β-arrestin levels. However, we did not detect any difference in expression levels of either β-arrestin-1 (Fig. 3A) or β-arrestin-2 (not shown) between wild type and FOXC2 transgenic WAT.

4. Discussion

Mice over-expressing FOXC2 in WAT have increased catecholamine-induced cAMP accumulation and lowered threshold for PKA activation by cAMP [1]. This increased signaling through the β-AR-cAMP-PKA pathway can, at least in part, explain the lean phenotype of FOXC2 transgenic WAT. In this study, we set out to elucidate the effect of PDE inhibitors on β-AR-induced cAMP levels in wild type

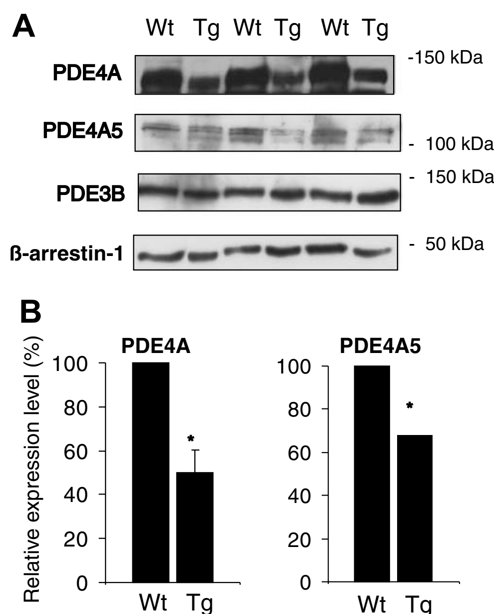

Fig. 3. PDE4A5 protein expression levels are reduced in FOXC2 transgenic WAT. (A) Wild type and FOXC2 transgenic WAT lysates were prepared and examined by immunoblotting using antibodies against PDE4A, PDE4A5, PDE3B and β-arrestin-1. Mobilities of the Precision Plus protein standard (Bio-Rad) is shown. (B) The PDE4A and PDE4A5 immunoreactive bands in A were quantified using the Quantity One software (Version 4.5; Bio-Rad). Bars represent intensities of PDE4A and PDE4A5 in FOXC2 transgenic WAT relative to that in wild type (set to 100%). Statistical analysis was performed using Student's unpaired *t*-test. * denote $P \leqslant 0.05$.

and FOXC2 transgenic adipocytes. We show that stimulation with isoproterenol together with the non-selective PDE inhibitor, IBMX resulted in higher cAMP levels in FOXC2 transgenic adipocytes compared to wild type cells.

Neither rolipram nor cilostamide increased isoproterenol-induced cAMP accumulation to the same degree as the non-selective PDE inhibitor, IBMX in wild type and FOXC2 transgenic cells suggesting that simultaneous inhibition of PDE3 and PDE4 is necessary for optimal cAMP accumulation. As PDE4 and PDE3 represent compartmentalized PDE activities, cAMP elevations after inhibition of each isoform alone will contribute to local elevations in cAMP that may be diluted when measuring whole cell cAMP levels. This may be the reason why the effect of rolipram is less pronounced than that of IBMX. A recent paper by Snyder et al. [9] shows that cilostamide and rolipram acts synergistically and in a similar way as IBMX on the rate of lipolysis in rat and mouse adipocytes, suggesting that inhibition of both PDE3 and PDE4 is required for maximal lipolysis in adipocytes. Due to the low triglyceride content of FOXC2 transgenic WAT, glycerol release after isoproterenol treatment is extremely low (1.5-fold) by 1 μM isoproterenol in the absence of PDE inhibitors for 2 h at 37 °C as compared to wild type adipocytes (10-fold) (data not shown). This confirms that only small increases in cAMP levels results in profound effects on lipolysis in wild type adipocytes. PDE4 inhibition with rolipram did not amplify isoproterenol-induced cAMP levels in FOXC2 transgenic cells to the same extent as in wild-type cells, suggesting reduced PDE4 activity in FOXC2 transgenic cells. PDE3 inhibition did not effect isoproterenol-induced cAMP accumulation in FOXC2 transgenic adipocytes with only a small increase in wild-type cells. Our finding that rolipram was able to increase isoproterenol-stimulated cAMP accumulation to a greater extent than cilostamide in wild-type cells is in agreement with previous observations [19]. Our PDE activity analyses revealed a 75% reduction in PDE4 activity in FOXC2 transgenic WAT compared to wild type WAT with no difference in PDE3 activity. Total cAMP-specific PDE activity was reduced by 30% in FOXC2 transgenic cells indicating that PDE4 is the main regulated PDE in FOXC2 transgenic adipocytes.

PDE4 isoforms, including PDE4A5, can be recruited to the plasma membrane via association with β-arrestin, leading to desensitization of ligand-activated β-adrenergic receptors and quenching of PKA activity [18]. Loss of acute (2 min) β-adrenergic cAMP activation in isoproterenol-treated wild type cells, which is simply rescued by PDE inhibition through treatment with either IBMX or rolipram, but not cilostamide, indicates that PDE4, at least in part, controls inhibition of acute β2-AR-induced cAMP formation in adipocytes.

These data together with our observation that PDE4A5 is expressed in β2-AR containing lipid rafts/caveolae of 3T3-L1 adipocytes (data not shown), supports a role for PDE4 controlling membrane/receptor associated PKA activity mediating attenuation of basal and catecholamine-induced cAMP accumulation in adipocytes. Reduced PDE4A5 expression in FOXC2 transgenic adipocytes would, consequently, contribute to the amplified cAMP levels observed in these cells.

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