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RESEARCH PAPER

Subchronic treatment of rats with oxytocin results in improved adipocyte differentiation and increased gene expression of factors involved in adipogenesis

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BACKGROUND AND PURPOSE
Treatment with thiazolidinediones, insulin-sensitizing drugs, enhances adipogenesis, which may result in unwanted increase in adiposity. Based on the suggested metabolic effects of oxytocin, the aims of the present study were to: (i) determine whether chronic treatment with oxytocin exerts positive effects on white adipose tissue growth without increasing adiposity; (ii) investigate possible mechanisms of action of oxytocin by measuring the level of gene expression of adipogenic factors; and (iii) test the hypothesis that oxytocin's effect on adipose tissue involves specific activation of eukaryotic elongation factor 2 (eEF2).

EXPERIMENTAL APPROACH
Adult rats were subcutaneously treated with oxytocin (3.6 μg·100 g^{-1} body weight day^{-1}) via osmotic minipumps for 2 weeks. Adipocytes from epididymal adipose tissue were isolated and their size evaluated by light microscopy. Gene expression of adipogenic and angiogenic factors was determined by real-time PCR and dephosphorylation of eEF2 by immunoblotting.

KEY RESULTS
Oxytocin treatment decreased the diameter of adipocytes and increased the epididymal adipose tissue protein content without changing the adipose tissue mass. Increases in fatty acid binding protein, peroxisome proliferator-activated receptor γ, insulin-sensitive glucose transporter 4, leptin and CD31 mRNA levels were noted in the epididymal and/or retroperitoneal fat tissue of oxytocin-treated rats. Oxytocin enhanced the dephosphorylation of eEF2 in the epididymal adipose tissue.

CONCLUSIONS AND IMPLICATIONS
The present results demonstrate that subchronic treatment with oxytocin induces adipogenic and angiogenic effects and that the eEF2 signalling pathway is involved in these effects of oxytocin on adipose tissue *in vivo*. These findings are likely to motivate further research and indicate new approaches for modulating adipose tissue morphology and metabolism.

Abbreviations
CD31, platelet endothelial cell adhesion molecule; eEF2, eukaryotic elongation factor 2; FABP4, fatty acid binding protein; GLUT4, insulin-sensitive glucose transporter 4; mRNA, messenger ribonucleic acid; PPARγ, peroxisome proliferator-activated receptor γ; Pref-1, preadipocyte factor 1

Introduction

The current use of oxytocin as a therapeutic agent is limited to obstetric practice corresponding to its main physiological effects, namely the induction of labour and milk ejection. However, there is accumulating evidence indicating a significant role for oxytocin outside of pregnancy, and these actions of oxytocin may, if adequately understood, become targets for potential therapeutic strategies (Lippert *et al.*, 2003). In particular, oxytocin is nowadays considered to be a cardiovascular hormone (Gutkowska *et al.*, 2000; Gutkowska and Jankowski, 2008) and treatment with oxytocin has been shown to exert cardioprotective effects, at least under experimental conditions (Ondrejcakova *et al.*, 2009; Jankowski *et al.*, 2010). Cardiovascular dysfunction is closely related to metabolic disorders associated with insulin resistance and obesity. However, the metabolic actions of oxytocin are far from being understood.

It has been known for a long time that oxytocin exerts insulin-like effects in isolated adipocytes, for example, stimulation of glucose oxidation, lipogenesis and pyruvate dehydrogenase activity (Hanif *et al.*, 1982; Gimpl and Fahrenholz, 2001), although oxytocin has also been found to have an anti-insulin action (Stephenson and Rogol, 1984). In addition, oxytocin has been shown to stimulate glucose uptake in rat skeletal muscles and neonatal cardiomyocytes (Lee *et al.*, 2008; Florian *et al.*, 2010). Interestingly, concentrations of oxytocin in plasma were found to be significantly higher in obese men and women compared with control subjects (Stock *et al.*, 1989). Further, mice deficient in oxytocin receptors have been found to develop obesity (Takayanagi *et al.*, 2008).

Obese subjects are thought to be at a higher risk of metabolic and haemodynamic disorders if they show signs of a greater ratio of hypertrophic to hyperplastic adipose tissue growth during positive energy balance (Schling and Löffler, 2002). An increase in adipose tissue need not necessarily be a disadvantage for physically active individuals. White adipose tissue can either grow by recruiting new adipocytes from the adipose precursor cell pool (hyperplasia) or by enlarging existing adipocytes (hypertrophy). It is the hypertrophic adipose tissue that is associated with harmful metabolic and haemodynamic consequences (Imbeault *et al.*, 1999). Indeed, although treatment with the insulin-sensitizing drugs thiazolidinediones leads to an enhancement of overall adipose tissue growth, it also induces the conversion of hypertrophic to hyperplastic adipose tissue resulting in a greater number of small adipocytes and a significant decrease in large fat cells (Akazawa *et al.*, 2000). As yet, the effects of oxytocin on these aspects of fat tissue metabolism have not been investigated.

The purpose of the present study was to: (i) determine whether chronic treatment with oxytocin exerts positive effects on white adipose tissue growth without increasing adiposity; (ii) investigate the possible mechanisms of action of oxytocin at the level of gene expression of adipogenic factors; and (iii) ascertain whether the effect of oxytocin on adipose tissue involves specific activation of eukaryotic elongation factor 2 (eEF2), which has been shown to play a role in the signalling pathways involved in oxytocin's action in myometrial cells (Devost *et al.*, 2008). We also measured the levels of markers of adipogenesis, namely adipocyte fatty acid binding protein (FABP4; produced by mature adipocytes), peroxisome proliferator-activated receptor γ (PPARγ; key regulator of adipocyte differentiation), insulin-sensitive glucose transporter 4 (GLUT4; produced by mature adipocytes) and preadipocyte factor 1 (Pref-1; inhibitor of preadipocyte differentiation), as well as the gene expression of adipokines relevant to adipose tissue metabolism, namely leptin and adiponectin. To determine possible changes in angiogenesis, the gene expression of platelet endothelial cell adhesion molecule (CD31), a marker of endothelial cells, was also measured.

Methods

Drug and molecular target nomenclature conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2009).

Animals

Adult male Wistar (350–480 g) rats (AnLab s.r.o., Prague, Czech Republic) were used. The animals were kept four per cage under standard housing conditions with a constant 12:12 h light/dark cycle (lights on at 06.00 h) and temperature (22°C ± 2°C). Food and water were available *ad libitum*. Principes of laboratory animal care and all procedures were approved by the Animal Health Welfare Division of the State Veterinary and Food Administration of the Slovak Republic.

Treatment with oxytocin

The animals (n = 16) were randomly assigned to vehicle- (n = 8), and oxytocin-treated groups (n = 8). Oxytocin (Oxytocin, H-2510, Bachem, Switzerland) or vehicle (saline) was continuously administered via osmotic minipumps (Model 2002, Alzet, Durect Corp., Cupertino, CA, USA) for 2 weeks. Osmotic

minipumps were implanted subcutaneously (Hlavacova and Jezova, 2008). The concentration of oxytocin used to fill the pumps was calculated based on the mean pump infusion rate provided by the manufacturer (0.5 μL·h^{-1}, 14 days), the body weight of animals, and the dose intended. The minipumps delivered oxytocin at a rate of 3.6 μg·100 g^{-1} body weight day^{-1}. Oxytocin was dissolved in isotonic saline. A total of 220 μL was loaded into each minipump. Control animals were implanted with minipumps that contained vehicle only. After the minipumps had been implanted, the rats were housed individually.

On the 14th day following the implantation of minipumps, vehicle- and oxytocin-treated rats were killed by decapitation. Adipose tissue was quickly removed and immediately weighed. A sample of fresh epididymal adipose tissue was used immediately for adipocyte isolation and determination of protein content. The rest of the tissue including whole retroperitoneal fat was kept frozen at –70°C until use. In additional groups of rats treated with vehicle or oxytocin the water consumption and food intake were measured daily at 10.00 h.

Measurement of plasma oxytocin

Oxytocin concentrations in plasma were determined by specific radioimmunoassays, as described previously (Jezova and Michajlovski, 1992; Bakos *et al.*, 2007).

Determination of protein content in adipose tissue

Samples of epididymal adipose tissue weighing 150 mg were hydrolysed in 15 mL NaOH (1 mol·L^{-1}) by boiling for 20 min. Protein content in the hydrolysate was determined in a 0.5 mL aliquot using Folin phenol reagent (Lowry *et al.*, 1951) by measuring absorbance at 500 nm. Alternatively, the hydrolysate was diluted 1:2 in deionized water and after the addition of Folin phenol reagent the absorbance was measured at 690 nm. L[-] – Tyrosine (UCB, Belgium) dissolved in 0.1 mol·L^{-1} HCl (Merck, Darmstadt, Germany) and diluted in 1 mol·L^{-1} NaOH was used as a standard. Results are expressed as an average value from measurements at 500 and 690 nm, both run in duplicate, and expressed as mg tyrosine g^{-1} adipose tissue.

Preparation of adipocytes and evaluation of their size

Rat adipocytes from epididymal adipose tissue were isolated by collagenase digestion (Pinterova *et al.*, 2001; Zorad *et al.*, 2006). The tissue was minced in Krebs-Ringer bicarbonate-HEPES buffer, pH 7.4 supplemented with 1.5% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA), 5 mM glucose and 1 mg·mL^{-1} collagenase II (Sigma). After collagenase digestion, performed at 37°C under an O_2 : CO_2 (95:5) atmosphere for 30 min, the digested tissue was filtered through 200 μm nylon mesh to remove the vascular tissue fraction. Adipocytes were washed twice in Krebs-Ringer bicarbonate-HEPES buffer by centrifugation at 300× *g* for 5 min. The cell suspension was placed on a Bürker cell chamber and examined by light microscopy (Ukropec *et al.*, 2008) using a Leica DMLS microscope equipped with a Power Shot (Powershot S40, Canon USA, Inc., New York, NY, USA). At least 10 random visual fields were photographed for every rat adipocyte suspension. The semidiameter of the cells was calculated from the image of their planar surface. At least 100 cells from each adipocyte suspension were evaluated.

Isolation of total RNA and real-time PCR (qPCR)

Epididymal and retroperitoneal white adipose tissues were excised, weighed, rapidly frozen in liquid nitrogen and stored at –70°C until assayed. Total RNA was isolated from 100 mg of frozen epididymal and retroperitoneal adipose tissue using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's directions. Two micrograms of total RNA were reversely transcribed using a Ready-To-Go You-Prime First-Strand Beads kit and pd(N)$_6$ random hexamer primers (Amersham Biosciences, Buckinghamshire, UK). RNA and cDNA were prepared as described by Pinterova *et al.* (2000) and Zorad *et al.* (2006). qPCR was performed in a 20 μL reaction mixture consisting of 10 μL SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1.5 μL cDNA and 4 pmol·L^{-1} of each primer. The specific primers for qPCR are listed in Table 1. The initial step in the reaction was 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 51°C for PPARγ, 54°C for CD31, 55°C for FABP4, 59°C for adiponectin, 60°C for GLUT4 and 61°C for leptin and Pref-1 for 45 s. Polymerization (72°C/20 s) was the final step of qPCR and it was the same for all reactions. Reaction without cDNA as a template was used as a negative control. The relative amount of the target was normalized with the housekeeping gene GAPDH. The qPCR was performed using the RotorGene 2000 real-time cycler (Corbett Research, Sydney, Australia).

Immunoblotting

Epididymal and retroperitoneal adipose tissues were homogenized on ice by sonication at a low setting in lysate buffer [25 mM HEPES, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 1% Triton X-100, mix of protease inhibitors and phosphatase

Table 1
Primers used for real-time PCR

Gene	Primer sequence	Reference
Adiponectin	F: CTCCACCCAAGGAAACTTGT	Tsuda *et al.* (2004)
	R: CTGGTCCACATTTTTTTCCT	
FABP4	F: AGCGTAGAAGGGGACTTGGT	U75581
	R: ATGGTGGTCGACTTTCCATC	
GAPDH	F: TGAACGGGAAGCTCACTGG	Inoue *et al.* (2002)
	R: TCCACCACCCTGTTGCTGTA	
GLUT4	F: TTTCCAGTATGTTGCGGATG	Hosaka *et al.* (1992)
	R: TCAGTCATTCTCATCTGGCC	
Leptin	F: CCAGGATGACACCAAAACCCTC	NM013076
	R: ATCCAGGCTCTCTGGCTTCTGC	
PPARγ	F: CATTTCTGCTCCACACTATGAA	Tsuda *et al.* (2004)
	R: CGGGAAGGACTTTATGTATGAG	
Pref-1	F: AGCCCTCCTGCGCGTCCTCTT	Cabrero *et al.* (2001)
	R: AGTCCCATTGTTGGCGCAGGG	
CD31	F: CCCAGTGACATTCACAGACA	Zhu *et al.* (2010)
	R: ACCTTGACCCTCAGGATCTC	

F: forward primer 5′-3′; R: reverse primer 5′-3′.

inhibitors (Devost *et al.*, 2005)]. The homogenates were clarified by centrifugation at 500× *g* for 10 min at 4°C to remove fat, nuclei and cell debris. The supernatant was centrifuged at 14 000× *g* for 10 min at 4°C to separate cytosol fraction from plasma membrane. The protein content was determined by the Lowry method (Lowry *et al.*, 1951). Proteins were denaturated in Laemmli buffer (125 mM Tris, 2% sodium dodecyl sulphate, 20% glycerol, 2% β-mercaptoethanol, 0.025% bromphenol blue; pH 6.8) for 15 min at 60°C. For Western blot, 50 µg of cytosolic proteins was separated by electrophoresis on 12.5% polyacrylamide gel and transferred to polyvinylidene difluoride membrane Immobilon-P (Millipore, Bedford, MA, USA) by Panther Semidry Electroblotter (Owl Separation Systems, New Hampshire, UK). The membranes were blocked for 60 min in blocking buffer (10 mM Tris, 150 mM NaCl, 0.1% Igepal, 5% BSA; pH 7.4) and incubated at 4°C overnight with rabbit polyclonal antibodies against phosphorylated and total eEF2 (1:1000, Cell Signaling Technology, Danvers, MA, USA). The membranes were washed in washing buffer (10 mM Tris, 150 mM NaCl, 0.1% Igepal; pH 7.4) and incubated for 60 min at room temperature with a horseradish peroxidase – conjugated secondary anti-rabbit IgG antibody (1:15 000, Sigma). After subsequent washing, the membranes were subjected to an Immobilon Western chemiluminiscent HRP substrate (Millipore) and exposed to X-ray film. The chemiluminiscent signal was acquired by densitometric scanning and evaluated with Scion Image Software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

The data are expressed as the mean ± SEM. Statistical comparisons were performed using one-way ANOVA. Differences in adipocyte size distributions were analysed with the Kolmogorov-Smirnov two-sample test (Fisher and van Belle, 1993). A value of $P < 0.05$ was considered statistically significant.

Results

Body weight gain was 35.9 ± 10.5 g in the oxytocin group and 16.6 ± 3.4 g in controls (non-significant; $P > 0.05$) at the end of the treatment. Neither the absolute nor the relative mass (adiposity index) of epididymal and retroperitoneal fat tissue was affected by oxytocin treatment (Figure 1).

In epididymal adipose tissue, oxytocin treatment resulted in a significant increase in protein content (oxytocin: 2.93 ± 0.17 $mg \cdot g^{-1}$ vs. control: 2.44 ± 0.098 $mg \cdot g^{-1}$, $P < 0.05$). There was a significant decrease in the semidiameter of adipocytes (Figure 2), as revealed by one-way ANOVA ($F_{(1,12)} = 28.22$, $P < 0.001$). Evaluation of adipocyte size distribution revealed a significantly ($P < 0.01$) increased

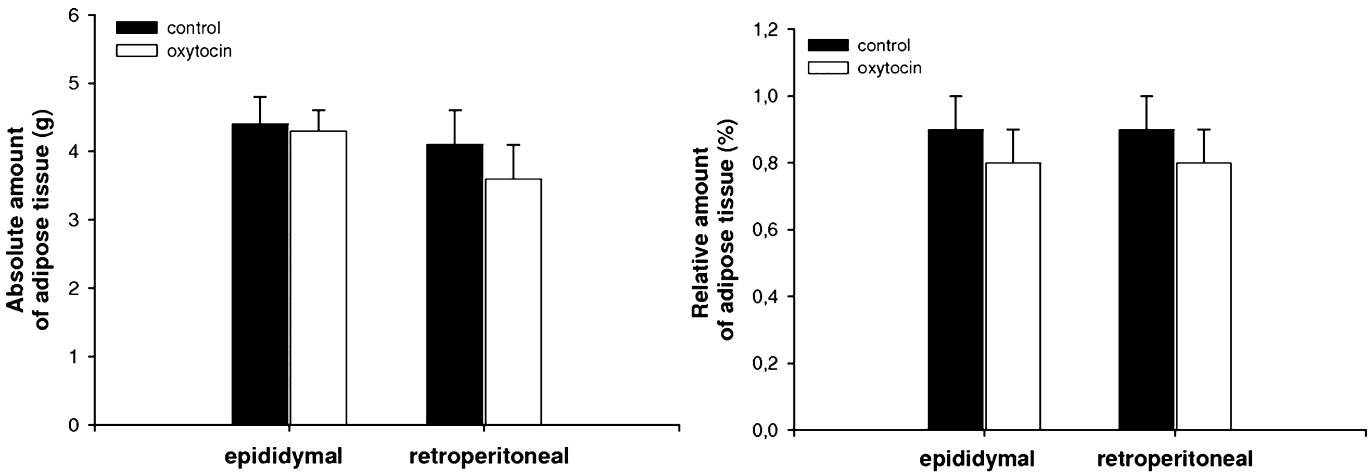

Figure 1

Effect of subchronic oxytocin treatment on absolute and relative (adiposity index) mass of white adipose tissue. Two groups of male Wistar rats were treated either with oxytocin or saline for 2 weeks and killed by decapitation at the end of the experiment. Relative amounts of epididymal and retroperitoneal adipose tissue are expressed as a % of adipose tissue weight compared with body weight. Results are expressed as means ± SEM, $n = 8$ for each group of animals.

Table 2

Effect of subchronic oxytocin treatment on the gene expression of selected parameters in retroperitoneal adipose tissue

	Control (arbitrary units)	Oxytocin (arbitrary units)
FABP4 mRNA	8.09 ± 0.70	12.61 ± 0.38***
PPARγ mRNA	0.16 ± 0.01	0.22 ± 0.01***
Leptin mRNA	0.43 ± 0.06	0.60 ± 0.07
Adiponectin mRNA	2.73 ± 0.20	3.72 ± 0.14**
GLUT4 mRNA	0.20 ± 0.02	0.18 ± 0.01
CD31 mRNA	0.15 ± 0.02	0.16 ± 0.02
Pref-1 mRNA	0.01 ± 0.00	0.01 ± 0.00

Data are shown as mean values ± SEM. **$P < 0.01$, ***$P < 0.001$, one-way ANOVA.

number of small adipocytes in epididymal adipose tissue of oxytocin-treated rats (Figure 3).

Prolonged release of oxytocin via osmotic minipumps resulted in a significant increase in oxytocin concentrations in plasma (Figure 4A), as revealed by one-way ANOVA ($F_{(1,11)} = 15.29$, $P < 0.01$). There was a significant negative correlation (Pearson correlation test, $r = –0.60$, $P < 0.05$) between concentrations of oxytocin in plasma and the adipocyte size (Figure 4B).

Treatment with oxytocin led to an increase in the expression of markers of adipogenesis, FABP4 ($F_{(1,14)} = 5.07$, $P < 0.05$) and PPARγ ($F_{(1,14)} = 4.67$, $P < 0.05$). No changes were noticed in the expression of the gene for the Pref-1. The 2 weeks treatment with oxytocin significantly increased the expression of leptin ($F_{(1,14)} = 7.96$, $P < 0.05$) and of the GLUT4 ($F_{(1,13)} = 6.68$, $P < 0.05$) in the same tissue. However, adiponectin mRNA concentrations were not significantly affected by oxytocin (Figure 5).

The oxytocin treatment increased the expression of the gene for CD31 ($F_{(1,12)} = 8.36$, $P < 0.05$) in epididymal adipose tissue (Figure 6A). In this tissue, oxytocin treatment also resulted in a significant increase ($F_{(1,8)} = 20.57$, $P < 0.01$) in dephosphorylation of eEF2 (Figure 6B).

Selected parameters were also measured in the retroperitoneal adipose tissue. The expression of the adipose markers FABP4 ($F_{(1,14)} = 27.91$, $P < 0.001$) and PPARγ ($F_{(1,14)} = 33.85$, $P < 0.001$) was found to be increased in oxytocin-treated animals compared with that in controls. We failed to observe any changes in the expression of the genes for leptin, GLUT4, CD31 and Pref-1. On the other hand, oxytocin treatment resulted in a moderate but statistically significant increase ($F_{(1,14)} = 14.66$, $P < 0.05$) in adiponectin gene expression (Table 2). Oxytocin treatment had no significant effect on the dephosphorylation of eEF2 in retroperitoneal adipose tissue (data not shown).

Discussion

The main finding of this study is that subchronic oxytocin treatment has positive metabolic effects on white adipose tissue growth and remodelling without increasing adiposity in rats. This conclusion is based on the changes in adipose tissue mass, a

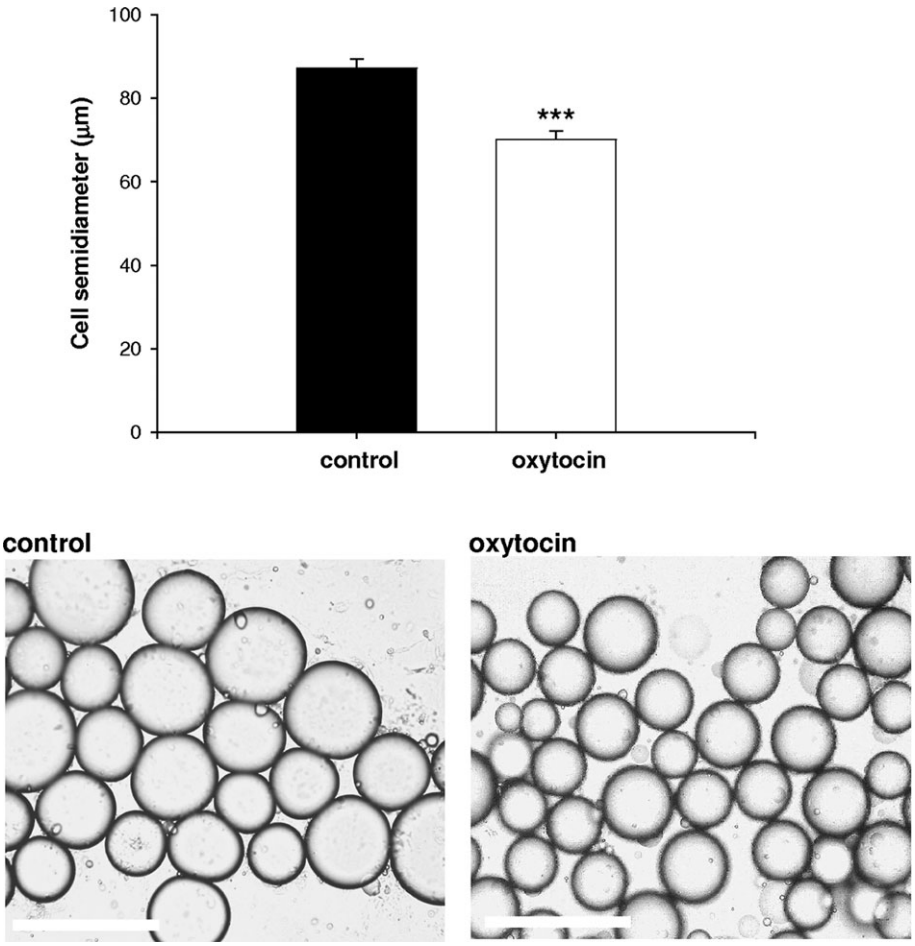

Figure 2

Effect of subchronic oxytocin treatment on the semidiameter of adipocytes from epididymal fat tissue. Two groups of male Wistar rats were treated with either oxytocin or saline for 2 weeks. At the end of the treatment the rats were killed by decapitation and adipocytes were isolated from epididymal fat. The semidiameter of at least 100 cells from each rat was used to calculate average value. Representative pictures of adipocytes isolated from control and oxytocin-treated animals. Scale bar = 200 μm. Results are expressed as means ± SEM, $n = 8$ for each group. ***$P < 0.001$.

decrease in adipocyte diameter, a higher number of small adipocytes and an elevation in gene expression of markers of adipogenesis observed in epididymal and/or retroperitoneal adipose tissue of oxytocin-treated rats. In addition, oxytocin treatment was found to have an effect on the stromal vascular fraction of the epididymal adipose tissue. Moreover, we obtained evidence that oxytocin-induced activation of the intracellular growth promoting pathway leads to increased protein synthesis in this adipose tissue *in vivo*.

We have confirmed our previous results (Ondrejcakova *et al.*, 2010) demonstrating that oxytocin treatment induced an elevation in circulating oxytocin in the range occurring during intensive stress situations. Moreover, the correlation analyses showed a negative correlation between circulating oxytocin concentrations and the size of adipocytes, which represents a type of dose–response evaluation.

The low dose of oxytocin used in the present study failed to induce any changes in food intake and induced a slight decrease (Ondrejcakova *et al.*, 2010) or no change (the present study) in the body weight of the rats used. It should be noted that a decrease in the body weight of oxytocin-treated animals is only apparent if the data from several experiments are put together (M. Ondrejcakova, unpubl. data). Thus, the results obtained in this laboratory do not support the anorexigenic effects of single i.p. injections of high doses of oxytocin (Arletti *et al.*, 1989).

The present results revealed that the adipose tissue of rats treated with oxytocin had smaller adipocytes compared with control animals. This occurred with no concomitant changes in the

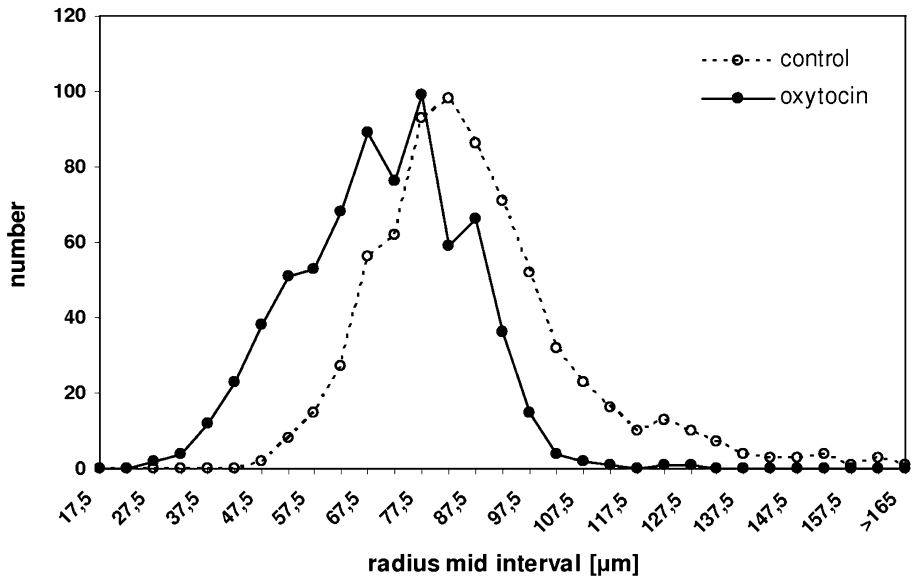

Figure 3

Cell size distribution profile for adipocytes from the epididymal adipose tissue of control animals and oxytocin-treated animals. Results are presented as mean values ± SEM, $n = 8$ for both groups of animals. The distributions are significantly different at $P < 0.01$.

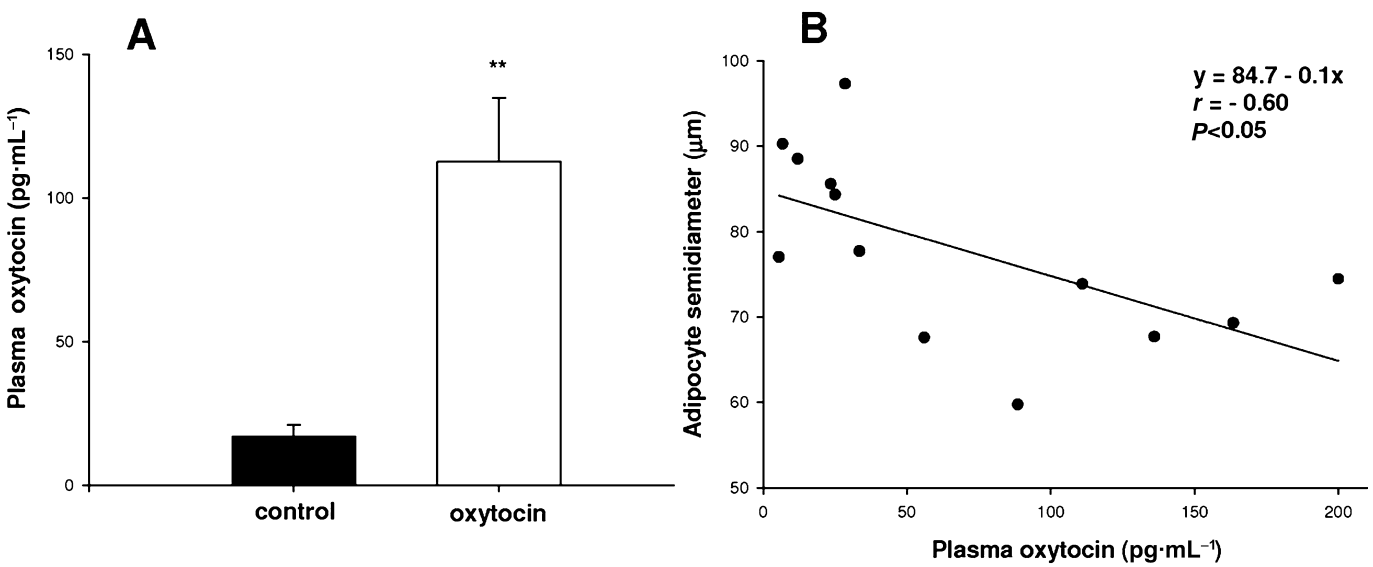

Figure 4

(A) Plasma oxytocin concentrations in male Wistar rats treated with either oxytocin ($n = 6$) or saline ($n = 7$) for 2 weeks. Data are expressed as means ± SEM, $^*P < 0.01$. (B) The correlation between plasma oxytocin concentrations and adipocyte semidiameters. Data were statistically analysed using Pearson correlation test ($n = 13$).

absolute and relative weights of epididymal and retroperitoneal adipose tissue. Adipose tissue with a higher number of small adipocytes has a greater ability to accumulate triacylglycerols and supports a redistribution of lipids from ectopic sites to adipose tissue (Zorad *et al.*, 2006). This leads to positive metabolic changes, such as increased peripheral insulin sensitivity (Zorad *et al.*, 2006; Lundgren *et al.*, 2007). A higher number of small adipocytes has repeatedly been observed following treatment with thiazolidinediones; however, in contrast to the present results, this effect was associated with an increase in adipose tissue mass (Akazawa *et al.*, 2000; Yamauchi *et al.*, 2001) resulting in undesirable obesity. Thiazolidinediones seem to have a two-phase effect, stimulating adipogenesis at the very

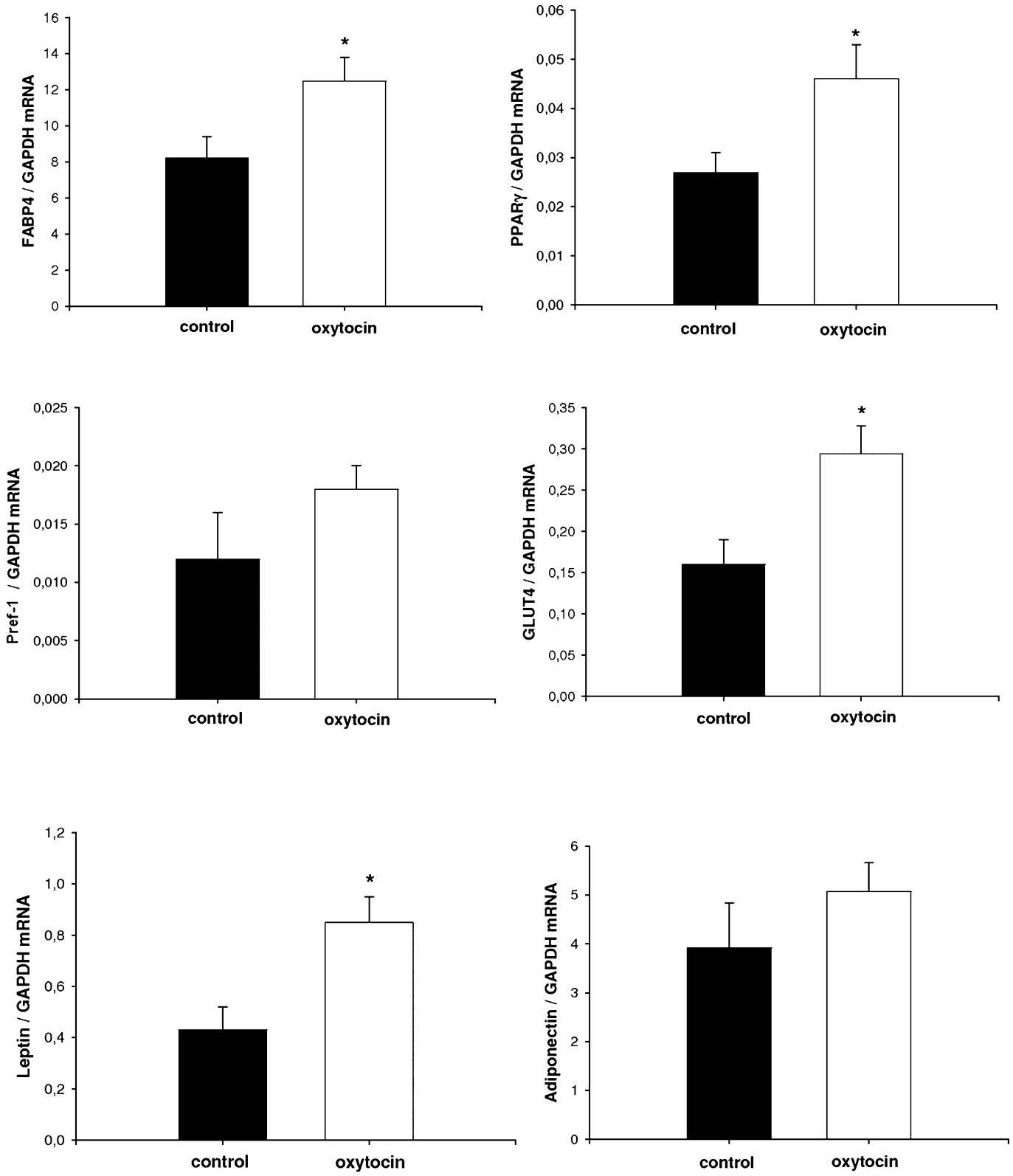

Figure 5

Effect of subchronic oxytocin treatment on gene expression of FABP4, PPARγ, Pref-1, GLUT4, leptin and adiponectin in epididymal adipose tissue. At the end of the treatment with either oxytocin or saline the rats were killed by decapitation and epididymal adipose tissue was used for RNA isolation and RT-PCR. Expression of selected genes was normalized to the level of GAPDH mRNA. Results are expressed as means ± SEM, $n = 8$ for each experimental group. *$P < 0.05$.

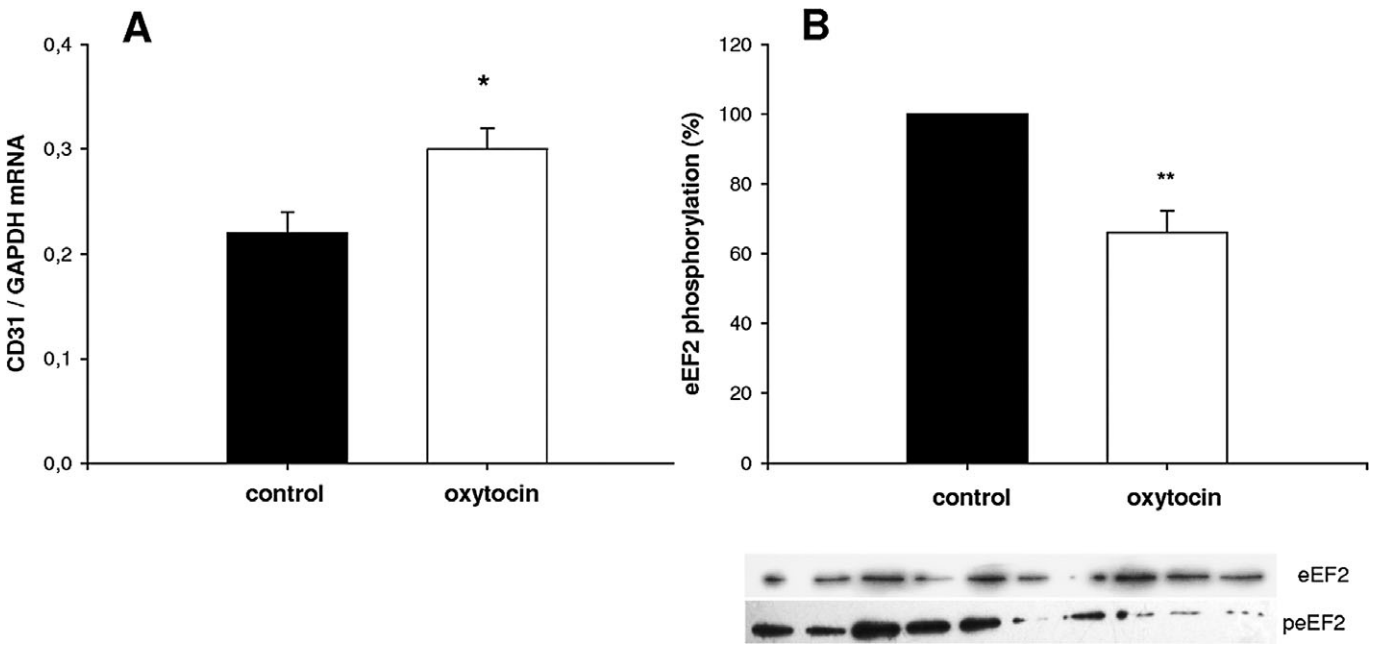

Figure 6

(A) Effect of subchronic oxytocin treatment on gene expression of CD31 in epididymal adipose tissue normalized to the level of GAPDH mRNA. Results are expressed as means ± SEM, $n = 8$ for each experimental group. *$P < 0.05$. (B) Oxytocin-induced enhancement of eEF2 dephosphorylation in epididymal adipose tissue lysates demonstrated by decreased amount of phosporylated form of the factor. Values for phosphorylated eEF2 (peEF2) were normalized with respect to total eEF2 and plotted as means ± SEM as a percentage of control ($n = 5$ for each experimental group, **$P < 0.01$). A representative blot is shown at the bottom of the figure.

beginning of the treatment followed by adipocyte hypertrophy (MacKellar *et al.*, 2009).

Several results of the present study indicate an adipogenic effect of oxytocin without any effects on adipose tissue mass. Thus, the gene expression of the FABP4 in both epididymal and retroperitoneal adipose tissue was enhanced in oxytocin-treated rats. This effect is likely to be mediated by PPARγ, as the gene expression of this nuclear receptor was triggered by oxytocin treatment in both of the adipose tissues analysed. PPARγ is the key regulator of adipocyte differentiation and has been shown to induce FABP4 gene expression (Tontonoz *et al.*, 1994; Gregoire *et al.*, 1998). The stimulation of PPARγ by its agonists, thiazolidinediones, leads to enhanced differentiation of preadipocytes into adipocytes (Boden and Zhang, 2006; Tarcin *et al.*, 2007). Therefore, we hypothesize that the increase in the number of small white adipose cells induced by oxytocin treatment, as revealed in this study, is due to a stimulant effect of oxytocin on adipocyte differentiation through PPARγ.

Oxytocin treatment failed to modify the gene expression of Pref-1. This factor is abundant in preadipocytes but absent in differentiated fat cells suggesting that expression of Pref-1 is involved in the differentiation process (Smas and Sul, 1993). The lack of changes in Pref-1 expression does not exclude the possibility that oxytocin treatment increased adipocyte differentiation. Moreover, it is likely that in white adipose tissue of oxytocin-treated rats, large adipocytes were lost by apoptosis. This hypothesis is supported by the increased expression of PPARγ, as Yamauchi *et al.* (2001) found that a PPARγ agonist stimulates adipocyte differentiation and apoptosis.

We found that the expression of the gene for GLUT4 was increased in epididymal but not retroperitoneal tissue of oxytocin-treated rats. GLUT4 is not produced in preadipocytes but its expression is markedly enhanced during adipocyte differentiation (Gregoire *et al.*, 1998). Activation of GLUT4 gene expression is under the control of PPARγ (Tamori *et al.*, 2002); therefore, it is likely that oxytocin treatment stimulated GLUT4 expression in the epididymal fat tissue through PPARγ, which was increased in the oxytocin-treated animals. In retroperitoneal fat, the oxytocin-induced increase in PPARγ expression might not have been high enough to influence the expression of GLUT4. Alternatively, it is possible that retroperitoneal fat tissue does not contain a sufficient amount of endogenous PPARγ ligands to affect GLUT4.

In the epididymal fat tissue, the oxytocin treatment elevated the gene expression of leptin. This is in contrast to the effects of thiazolidinedione-induced adipogenesis (Okuno *et al.*, 1998) and

angiotensin II inhibition-induced fat tissue mass reduction by AT_1 receptor or renin blockade (Zorad *et al.*, 2006; Stucchi *et al.*, 2009). As leptin is also produced by nonadipocyte cells, such as preadipocytes, fibroblasts, macrophages and endothelial cells (Bornstein *et al.*, 2000; Guo *et al.*, 2004), oxytocin treatment may have predominantly influenced gene expression of leptin in these cells rather than in adipocytes. Such an effect combined with the oxytocin-induced enhancement of small adipocytes may result in an increase in metabolic activity and leptin gene expression (Guo *et al.*, 2004).

One would expect that smaller adipocytes, which are more sensitive to insulin, produce substantially higher amounts of adiponectin mRNA. However, in the present experiments, the changes in adiponectin gene expression were subtle with statistically significant differences only occurring in retroperitoneal adipose tissue. We assume that the lack of a marked elevation in adiponectin mRNA is due to differences in time course of PPARγ, GLUT4 and adiponectin gene induction in different adipose tissue depots.

Oxytocin treatment increased gene expression of adipose tissue CD31, an adhesion molecule that is considered to be a marker of endothelial cell density and indirect indicator of vascular density (Cattaneo *et al.*, 2008). This effect was observed in the epididymal but not in the retroperitoneal adipose tissue. Increased expression of CD31 indicates enhanced angiogenesis. Crosstalk occurs between adipocytes and endothelial cells and if the vascular density is high enough this helps to maintain hyperplastic growth of adipose tissue (Pang *et al.*, 2008). The influence of oxytocin on markers of angiogenesis *in vivo* has not been studied previously.

As to the mechanisms involved in the effects of oxytocin, we demonstrated that it activated the dephosporylation of eEF2. This latter effect has been shown to be accompanied by an increase in the rate of peptide chain elongation, and, as a result, of protein synthesis (Devost *et al.*, 2005). *In vitro* studies in myometrial cells have shown that oxytocin stimulates the dephosphorylation of eEF2 via a novel oxytocin-specific protein kinase C-dependent signalling pathway (Devost *et al.*, 2008). This effect of oxytocin on eEF2 dephosporylation, as well as its ability to increase protein content, as revealed in the present study, supports a novel role for oxytocin as a trophic factor in adipose tissue and under *in vivo* conditions.

Oxytocin appears to induce different changes in the epididymal and retroperitoneal adipose tissue. The reasons for these differences are not clear but they may be the result of a lower sensitivity to oxytocin of retroperitoneal compared with epididymal tissue in the stromal vascular part and to a less significant involvement of eEF2 in the signalling pathway.

In conclusion, the present results demonstrate that subchronic treatment with oxytocin induces adipogenic and angiogenic effects. Moreover, to our knowledge, this is the first time evidence indicating that the eEF2 signalling pathway is involved in the effects of oxytocin in adipose tissue under *in vivo* conditions. The observed effects of oxytocin are likely to motivate further research and may open up new approaches to modulate adipose tissue morphology and metabolism.

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Conflicts of interest

The authors declare no conflicts of interest.

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