

## Peroxisome proliferator-activated receptor- $\gamma$ stimulates 11 $\beta$ -hydroxysteroid dehydrogenase type 1 in rat vascular smooth muscle cells

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### ABSTRACT

Glucocorticoids are metabolized in vascular tissue by two types of 11 $\beta$ -hydroxysteroid dehydrogenases (11HSD1, 11HSD2) and thus these enzymes are considered to be important factors that modulate the diverse and complex effects of glucocorticoids on cardiovascular function. The present study evaluated the effect of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) agonist pioglitazone on 11HSD1 vascular smooth muscle cells (VSMC) and compared the effect with that of corticosterone. Using primary cultures of VSMC derived from rat aorta, we showed that pioglitazone significantly increases 11HSD1 activity and mRNA expression in a dose-dependent manner with EC<sub>50</sub> 243 nM and that this effect is not blocked by RU 486, an antagonist of the glucocorticoid receptor. In contrast, corticosterone had no effect on 11HSD1. Pioglitazone positively regulated transcription of two CCAAT/enhancer-binding proteins (C/EBPs), specifically C/EBP $\alpha$  a potent activator of 11HSD1 gene transcription in some cells types, and C/EBP $\zeta$ , whereas C/EBP $\beta$  and C/EBP $\delta$  were not changed. In contrast, corticosterone stimulated the expression of C/EBP $\beta$  and C/EBP $\delta$ , but the levels of C/EBP $\alpha$  and C/EBP $\zeta$  were not changed. In conclusion, activation of PPAR $\gamma$  in VSMC up-regulates vascular 11HSD1 and thus reactivates 11-oxo metabolites to biologically active glucocorticoids through a mechanism that seems to involve C/EBP $\alpha$  and C/EBP $\zeta$ . Our data provide one of the possible explanations for PPAR $\gamma$  agonists' effects on the cardiovascular system.

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### 1. Introduction

Glucocorticoids are known to have significant and diverse effects on the cardiovascular system. They play a role in the regulation of vascular tone and exert anti-inflammatory, anti-atherogenic and anti-proliferative effects, though these effects in animal models sometimes contrast with those found in clinical investigations [1,2]. The activity of glucocorticoids on glucocorticoid receptors is regulated by 11 $\beta$ -hydroxysteroid dehydrogenase (11HSD), an enzyme expressed as two different types that inter-convert active and inactive glucocorticoids. 11HSD type 1 (11HSD1) is a bi-directional enzyme that is present in intact cells and acts largely as a reductase, generating biologically active glucocorticoids, cortisol and corticosterone, from their inactive 11-oxo metabolites, cortisone and 11-dehydrocorticosterone. In contrast, 11HSD1 type 2 (11HSD2) acts only as an oxidase, converting active glucocorticoids to their inactive 11-oxo metabolites [3]. Both 11HSDs are expressed in blood vessels, though there is some controversy regarding their distribution in endothelial and vascular smooth

muscle cells (VSMC) that seems to reflect differences among species and anatomical origin [4–7].

The agonists of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) exert similar effects as glucocorticoids. This receptor is a member of the nuclear receptor superfamily that forms a heterodimer with nuclear receptor X and binds to specific DNA sequences in the regulatory regions of target genes. Alternatively, PPAR $\gamma$  interacts with other transcription factors which may not involve direct DNA binding to regulate gene transcription. For example, PPAR $\gamma$  has been shown to interact with nuclear factor  $\kappa$ B and CCAAT/enhancer-binding proteins (C/EBP), all of which are transcription factors that regulate gene transcription [8]. PPAR $\gamma$  is expressed in various cells including endothelium and VSMC. PPAR $\gamma$  agonists reduce the activation and inflammation of endothelial cells by inhibition of gene expression of chemokines and pro-inflammatory adhesion molecules, leading to decreased adherence of monocytes to the activated endothelial cells. In addition, a variety of PPAR $\gamma$  agonists have been shown to inhibit differentiation of endothelial cells, vascular endothelial growth factor-induced angiogenesis and VSMC proliferation. PPAR $\gamma$  agonists also suppress the expression and secretion of endothelin-1, a potent vasoconstrictor and regulator of smooth muscle cell proliferation [8,9]. Evidence is also emerging that PPAR $\gamma$  agonists act as modulators of 11HSD1 gene transcription in certain types of cells [10–12].

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The similarity of the vascular effects between glucocorticoids and PPAR $\gamma$  agonists led to the hypothesis that this nuclear receptor might regulate VSMC in part by targeting 11HSD1. Despite the intensive investigation on the role of intra-vascular glucocorticoid metabolism on the modulation of vascular structure and function [13], the effect of PPAR $\gamma$  agonists on 11HSD1 expression and activity in vascular tissue has not been addressed to date. Therefore, we studied the effect of thiazolidinedione pioglitazone, a PPAR $\gamma$  synthetic ligand, on 11HSD1 mRNA expression and activity in rat VSMC. To eliminate the possibility that the effect of pioglitazone depends on activation of glucocorticoid receptors [14] rather than the PPAR $\gamma$  regulatory pathway, similar experiments have been undertaken with corticosterone.

## 2. Experimental

### 2.1. Primary smooth muscle cell culture

VSMC were isolated from the aortas of adult male Wistar rats weighing 280–350 g. The rats were killed by decapitation; thoracic aortas were dissected and cleaned of connective tissue and fat. After mechanical removal of adventitia and endothelium, smooth muscle strips were minced and digested in solution containing collagenase (2 mg/ml; Sigma), elastase (0.12 mg/ml; Sigma) and bovine serum albumin (2 mg/ml; Sigma) at 37 °C for 60 min [15]. Isolated cells were centrifuged and the pellet was resuspended in DMEM containing 20% fetal calf serum and gentamycin (Krka, Ljubljana, Slovenia), seeded onto Petri dishes and placed in an incubator. Cells were incubated in humidified 5% CO<sub>2</sub>/95% air atmosphere for 6–7 days to reach confluence. The identity of the cultured cells as VSMC was confirmed by histologic examination. Confluent cells exhibited “a hill and valley pattern” typical for VSMC and were stained positively for smooth muscle  $\alpha$ -actin. Quiescence was induced by incubating cells in serum-free medium for 24 h prior to the study. The cells were then exposed to various concentrations of pioglitazone or corticosterone for next 24 h. Control dishes received an equivalent volume of vehicle (dimethyl sulfoxide, ethanol). Pioglitazone was kindly provided by Takeda Pharmaceuticals (Osaka, Japan). The protocol used was approved by the Institutional Animal Care and Use Committee.

### 2.2. Radiometric conversion assay of 11HSD1 and 2

11HSD1 reductase activity was determined in intact cells by measuring the conversion of [<sup>3</sup>H<sub>4</sub>]11-dehydrocorticosterone to [<sup>3</sup>H<sub>4</sub>]corticosterone. The cells were incubated for 6 h in serum-free DMEM containing 100 nM [<sup>3</sup>H<sub>4</sub>]11-dehydrocorticosterone. The incubation time was determined in preliminary experiments to establish the optimal conditions, in order to work in the linear portion of the enzyme reaction. At the end of the incubation, the medium was collected and steroids were extracted from the growth medium using Strata-X C<sub>18</sub> cartridges (Phenomenex, Torrance, CA, USA), eluted with methanol and dried under nitrogen. [<sup>3</sup>H<sub>4</sub>]11-dehydrocorticosterone was synthesized in-house from 1,2,6,7-[<sup>3</sup>H<sub>4</sub>]corticosterone (PerkinElmer NEN: specific activity 70 Ci/mmol) by using guinea pig renal microsomes. 11HSD2 dehydrogenase activity was determined by adding 1,2,6,7-[<sup>3</sup>H<sub>4</sub>]corticosterone to reach the final concentration 15 nM and incubation the cells in the presence of the steroid for next 6 h. [<sup>3</sup>H<sub>4</sub>]corticosterone and [<sup>3</sup>H<sub>4</sub>]11-dehydrocorticosterone were separated by HPLC and quantified by on-line liquid scintillation counting as mentioned earlier [16]. The percentage conversion was calculated by dividing the radioactive counts identified as reaction product by the total counts associated with corticosterone plus 11-dehydrocorticosterone.

### 2.3. RT-PCR

Total RNA was extracted from cultured VSMC using TRIZOL reagent (Invitrogen) according to manufacturer instructions, then the isolated RNA was treated with AMP-D1 DNase (Sigma) to remove potential contamination by genomic DNA and finally, first-strand cDNA was prepared as previously reported [17]. The genes analyzed in this study were 11HSD1, 11HSD2, PPAR $\gamma$  and CCAAT/enhancer-binding proteins, C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\zeta$  (CHOP/GADD153). Levels of C/EBPs and PPAR $\gamma$  were measured with the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, USA). The probes and primers used for these experiments were developed as TaqMan gene expression assays by Applied Biosystems, specifically: PPAR $\gamma$ , Rn00440945-m1; 11HSD2, Rn00492539-m1; C/EBP $\alpha$ , Rn00560963-s1; C/EBP $\beta$ , Rn01423818-m1; C/EBP $\delta$ , Rn02532069-m1; and C/EBP $\zeta$ , Rn01499036-m1. The reaction was carried out in a final volume of 30  $\mu$ l using TaqMan Gene Expression Master Mix with AmpErase UNG (Applied Biosystems) and expression assay. For 11HSD1, amplification of the target cDNA was performed in the LightCycler 1.0 (Roche) with LightCycler Fast Start DNA Master SYBR green I as previously reported [17]. Gene-specific calibration curves were generated from serial dilutions of standard cDNA and mRNA levels of all genes of interest were normalized to  $\beta$ -actin.

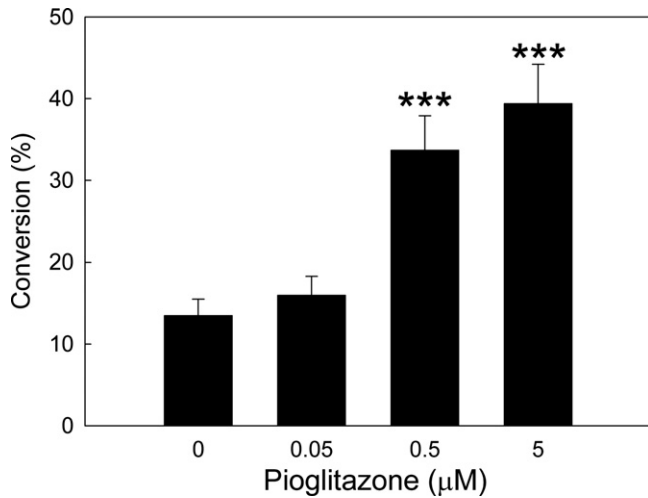
### 2.4. Statistical analysis

Results were expressed as mean  $\pm$  SEM and analyzed by an unpaired Student's *t*-test or by analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) test using Statistica 6.1 (StatSoft Inc., Tulsa, OK, USA). Pioglitazone concentration response curve was constructed from the data by fitting the sigmoid equation and EC<sub>50</sub>, the molar concentration of the agonist that produces a half-maximum response, was determined from pooled data. In all cases, a probability level of *P* < 0.05 was considered significant.

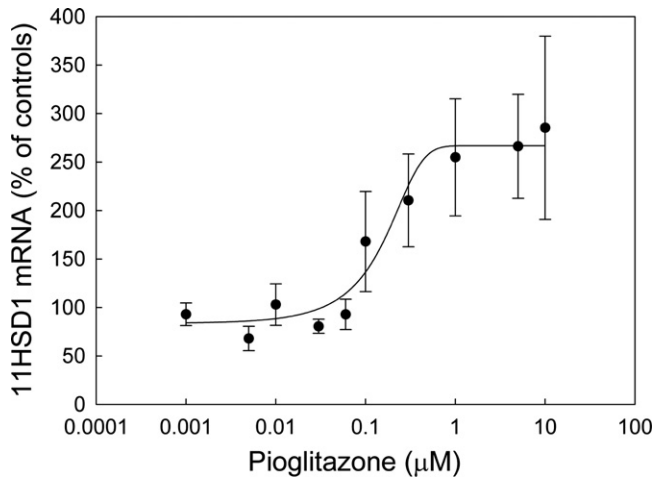
## 3. Results

To study the effect of PPAR $\gamma$  activation on 11HSD1 in VSMC, the cells were incubated for 24 h in either the presence or absence of PPAR $\gamma$  agonist pioglitazone. As the data presented in Fig. 1 demonstrate, pioglitazone dose-dependently increased 11-reductase activity. This finding correlates well with the positive regulation of 11HSD1 mRNA expression by pioglitazone in a concentration-dependent manner (Fig. 2), in which EC<sub>50</sub> was 243 nM. This suggests that PPAR $\gamma$  activation in VSMC is associated with an opposite effect on 11HSD1 than in metabolically active cells such as adipocytes [10,11] and hepatocytes [12], where PPAR $\gamma$  agonists significantly inhibited the transcription activity of 11HSD1. To eliminate the possibility that the effect of pioglitazone might be due to partial agonism of the glucocorticoid receptors [14], VSMC were also treated with corticosterone, which had no effect on 11HSD1 mRNA expression (Table 1). Similarly, RU 486, a glucocorticoid receptor antagonist, did not inhibit the effect of pioglitazone on 11HSD1 (Fig. 3). In contrast to 11-reductase, 11 $\beta$ -oxidase activity was not detected in VSMC in either control or pioglitazone treated cells even if we have found weak signal for 11HSD2 mRNA expression (average cycle threshold: 11HSD2, 38.01  $\pm$  0.25; 11HSD1, 24.23  $\pm$  0.20).

PPAR $\gamma$  agonists modulate VSMC gene transcription via C/EBPs [18], which also regulate the transcriptional activity of 11HSD1 gene [19,20]. Therefore, experiments were conducted to determine whether the members of C/EBP family in VSMC are regulated by pioglitazone and corticosterone. As shown in Fig. 4, treatment of



**Fig. 1.** Effect of pioglitazone on 11-reductase activity in VSMC. Primary cultures of VSMC were treated for 24 h with PPAR $\gamma$  agonist pioglitazone or vehicle and 11HSD1 activity was determined by a radiometric assay as described in Section 2. Data are shown as means  $\pm$  SEM from 5 independent experiments. ANOVA proved significant effect of pioglitazone on 11-reductase activity ( $P=0.0001$ ); significant difference from untreated controls is indicated (\*\* $P<0.001$ ).



**Fig. 2.** Pioglitazone-mediated activation of 11HSD1 mRNA in VSMC. Primary cultures of VSMC were treated for 24 h with various concentrations of PPAR $\gamma$  agonist pioglitazone. Abundance of 11HSD1 mRNA was determined by quantitative real-time RT-PCR and the data are expressed relative to the amount of untreated cells. EC $_{50}$  was determined as mentioned in Section 2 and reached the value of 243 nM. Each bar represents the mean  $\pm$  SEM of 5–8 independent experiments. ANOVA proved significant effect of pioglitazone on 11HSD1 mRNA expression ( $P=0.0001$ ).

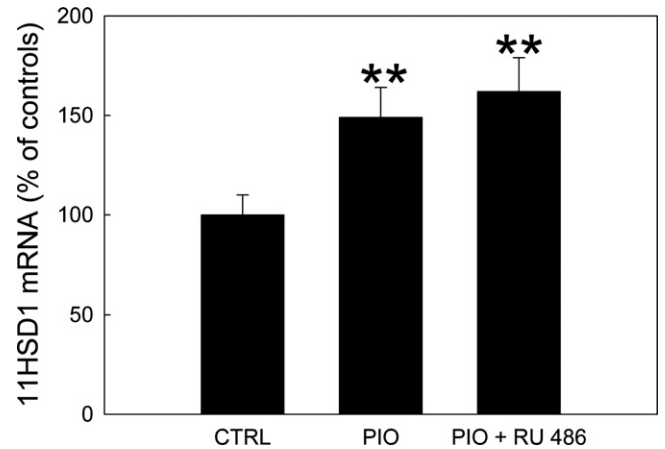
VSMC with pioglitazone or corticosterone for 24 h differentially regulated the expression of C/EBPs. C/EBP $\alpha$ , a potent activator of 11HSD1 promoter [20], increased 2.5-fold when treated with pioglitazone but was not up-regulated by corticosterone. In contrast, corticosterone significantly stimulated the expression of C/EBP $\beta$  and C/EBP $\delta$ , while pioglitazone did not change their lev-

**Table 1**

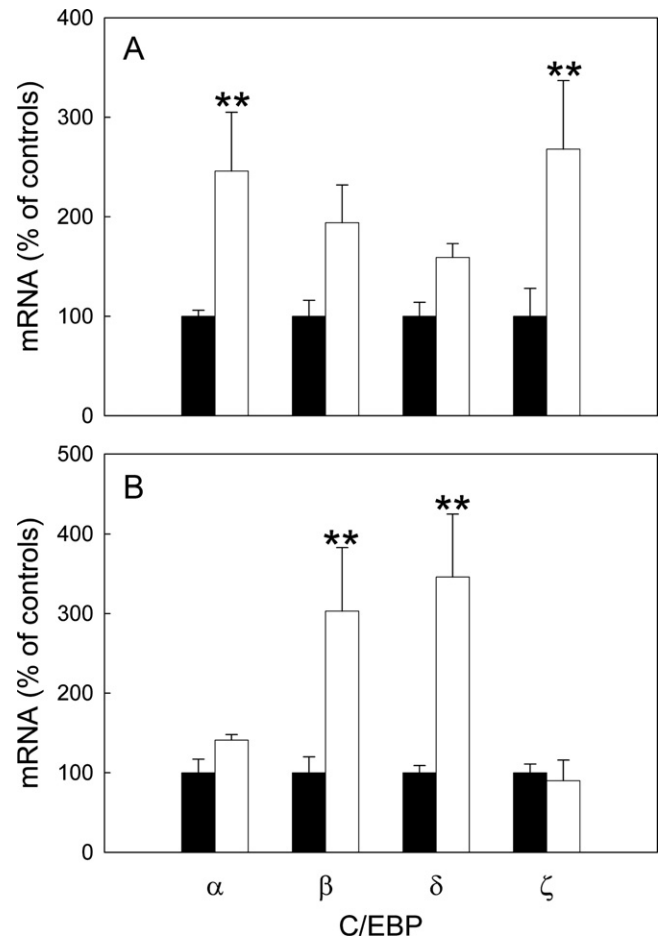
Effect of corticosterone on expression of 11HSD1 mRNA in VSMC.

Corticosterone ( $\mu$ M)	0.0	0.01	0.1	1.0	10.0	100.0
% of controls	100 $\pm$ 17	145 $\pm$ 27	125 $\pm$ 22	183 $\pm$ 41	143 $\pm$ 29	93 $\pm$ 14

Primary cultures of VSMC were treated with various concentrations of corticosterone for 24 h and abundance of 11HSD1 mRNA was determined by quantitative real-time RT-PCR. The data are expressed relative to the amount of untreated cells as means  $\pm$  SEM from 4 independent experiments. ANOVA did not prove any significant effect of corticosterone ( $P=0.357$ ).



**Fig. 3.** Expression of 11HSD1 mRNA in the presence of pioglitazone (PIO; 0.1  $\mu$ M) and RU 486 (1.0  $\mu$ M). The data are expressed relative to the amount of controls (CTRL) that received only vehicle and are shown as mean  $\pm$  SEM from 5 independent experiments. ANOVA ( $P=0.006$ ) proved significant effect of pioglitazone but there was no difference between pioglitazone (PIO) and PIO + RU486 group. Significant difference from untreated controls is indicated (\*\* $P<0.01$ ).



**Fig. 4.** Effect of pioglitazone (A) and corticosterone (B) on the expression of C/EBP transcription factors in VSMC. The mRNA levels of C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\zeta$  are expressed as a percentage of the amount of mRNA in cells treated only with vehicle (full columns). The cells were treated with pioglitazone (5  $\mu$ M) or corticosterone (10  $\mu$ M) for 24 h. Data are mean  $\pm$  SEM of 4–5 independent experiments. ANOVA proved the effect of pioglitazone ( $P=0.008$ ) and corticosterone ( $P=0.004$ ) on expression of C/EBPs. Significant difference from untreated controls is indicated (\*\* $P<0.01$ ).

els. Corticosterone also did not affect the level of C/EBP $\zeta$ ; however, it was increased by pioglitazone.

Treatment of VSMC with 5  $\mu$ M pioglitazone for 24 h caused a down-regulation of PPAR $\gamma$  mRNA to less than 20% of untreated cells (controls: 100  $\pm$  16% vs. pioglitazone: 13  $\pm$  4%; *t*-test, *P* < 0.01), thus corroborating the findings of others that PPAR $\gamma$  expression is down-regulated by its own agonists [11,21].

#### 4. Discussion

Glucocorticoids have been shown to foster hypertension indirectly due to enhanced vascular tone by potentiation of the vasoconstrictor hormones and directly by action on VSMC that is independent of these hormones [1]. The local effect of glucocorticoids depends on intracellular 11HSD1-mediated glucocorticoid metabolism which has been shown to modulate vascular structure and function [2,13]. Expression of this enzyme is regulated by many factors, although their effects often vary from tissue to tissue. In summary, the data indicate that glucocorticoids, pro-inflammatory cytokines and C/EBP transcription factors increase 11HSD1 expression, whereas insulin-like growth factor 1 and oxysterols, the endogenous activators of liver X receptors, inhibit 11HSD1 expression [22]. Direct stimulatory effect of pro-inflammatory cytokines related to inducible transcription factors AP1 and C/EBPs was also shown in case of VSMC 11HSD1 [5,23] and the involvement of C/EBP family members has been suggested in transcriptional regulation of PPAR- $\gamma$  activated VSMC [18]. As PPAR $\gamma$  agonists have been shown to decrease 11HSD1 activity in hepatocytes and adipocytes [10–12] we can hypothesize that PPAR $\gamma$  activation is able to decrease the level of biologically active glucocorticoids in the vasculature and thus induce a vasodepressor effect.

Despite these in vitro data, PPAR $\gamma$  knock-out mice have hypotension [24] similar to animals with VSMC-selective PPAR $\gamma$  deficiency [25]. These data reflect the physiological significance of PPAR $\gamma$  in vasculature, but it does not seem plausible that activation of PPAR $\gamma$  receptors might induce a vasodepressor effect due to regulation of local glucocorticoid availability. Therefore, we assessed whether PPAR $\gamma$  agonists modulate 11HSD1 expression in VSMC. Our results indicate that in contrast to the generally accepted notion that thiazolidinediones markedly inhibit 11HSD1 [10–12], pioglitazone up-regulates 11HSD1 in VSMC with EC<sub>50</sub> value similar to *K*<sub>d</sub> of rosiglitazone binding to PPAR $\gamma$  [26]. This suggests that endogenous glucocorticoid generation from their 11-oxo metabolites provides one of the possible mechanisms of the PPAR $\gamma$ -mediated effect. Based on earlier observations [1], this local corticosterone generation could foster augmentation of vascular tone. Up-regulation of 11HSD1 by pioglitazone is in agreement with previous findings that the endogenous PPAR $\gamma$  ligand 15-deoxy- $\Delta$ 12,14-PGJ<sub>2</sub>, significantly increases 11HSD1 activity in primary cultures of human adipocytes [27]. In contrast to this acute effect of PPAR $\gamma$  activation, the chronic administration of PPAR $\gamma$  agonists to rats showed either down-regulation of 11HSD1 expression [11] or lack of any effect [28] in fat and liver cells.

Changes in glucocorticoid signaling are unlikely to contribute to the effect of pioglitazone on up-regulation of 11HSD1 even though glucocorticoids have been reported to increase 11HSD1 [29,30] and thiazolidinediones have recently been shown to be partial agonists of glucocorticoid receptors [14]. First, EC<sub>50</sub> value for pioglitazone-dependent up-regulation of 11HSD1 was one order of magnitude lower than EC<sub>50</sub> value for activation of glucocorticoid receptors by rosiglitazone [14]. Second, there was no discernible effect of corticosterone whereas pioglitazone efficiently up-regulated 11HSD1 mRNA.

Several studies have linked the regulation of 11HSD1 to C/EBP family of transcription factors [19,20] and thus the pioglitazone-

dependent increase of 11HSD1 in VSMC might be attributed to an indirect effect involving a C/EBP-dependent mechanism. Several studies have also linked PPAR $\gamma$  to c/EBP transcription factors in various cells and tissues. For example, PPAR $\gamma$  ligands attenuated cytokine-induced C/EBP $\delta$  expression in VSMC while overexpression of PPAR $\gamma$  decreased the basal promoter activity of C/EBP $\delta$  and enhanced the inhibitory effect of PPAR $\gamma$  ligands [18]. Similarly, Wu et al. [31] have demonstrated cross-regulation between PPAR $\gamma$  and C/EBP $\alpha$ , and Fukuoka et al. [32] have shown that the PPAR $\gamma$  ligands regulate C/EBP $\delta$  gene transcription presumably through an interaction between PPAR $\gamma$  and other transcription factors. In addition, it has been reported that the promoter sequence of the PPAR $\gamma$  gene has a tandem repeat of the C/EBP-binding motifs and C/EBP factors have been shown to play a role in regulation of the PPAR $\gamma$  gene [33,34]. Here we have shown that treatment with pioglitazone caused a significant up-regulation of C/EBP $\alpha$  and C/EBP $\zeta$  without any significant effect on C/EBP $\beta$  and C/EBP $\delta$ , whereas exposure to corticosterone had quite opposite effect: up-regulation of C/EBP $\beta$  and C/EBP $\delta$ , without any changes of C/EBP $\alpha$  and C/EBP $\zeta$ . The thiazolidinedione-dependent increase of C/EBP $\alpha$  and C/EBP $\zeta$  is in agreement with the findings of Choi et al. [35] and Satoh et al. [36] who reported stimulation of C/EBP $\alpha$  and C/EBP $\zeta$  by rosiglitazone and troglitazone, respectively. Similarly, our findings with corticosterone are in line with the data in a variety of cells, including muscle cells [37] and lung epithelial cells A549 [30] that showed glucocorticoid-dependent positive regulation of C/EBP $\beta$  and C/EBP $\delta$  without any change of C/EBP $\zeta$ . However, in A549 cells, the up-regulation of C/EBP $\delta$  and particularly C/EBP $\beta$  was involved in positive regulation of 11HSD1 expression [30]. In contrast, the positive regulation of vascular 11HSD1 by pioglitazone was associated with the up-regulation of C/EBP $\alpha$  a potent transcription factor of the 11HSD1 gene [19,20]. Similar C/EBP $\alpha$ -dependent up-regulation of 11HSD1 was recently observed in human amnion fibroblasts [29]. Synthesizing these findings suggests that pioglitazone could exert its effect on 11HSD1 in VSMC through C/EBP $\alpha$ . In addition, the differences between pioglitazone and corticosterone indicate that they act in a tissue-specific and context-dependent manner.

Up-regulation of C/EBP $\alpha$  by pioglitazone was associated with an increase of C/EBP $\zeta$  a transcription factor that operates either as a negative regulator that dimerizes with other C/EBPs and attenuates their DNA-binding and transcription [38] or enhances the transcriptional activity of AP-1 by tethering to the AP-1 complex without direct binding of DNA [39]. Since the putative binding site for AP-1 is located in the promoter region of the 11HSD1 gene [12], we cannot exclude that overexpression of C/EBP $\zeta$  is able to significantly enhance the promoter activity of 11HSD1. Additional studies will be required to clarify the role of C/EBP $\zeta$  in the transcriptional activity of 11HSD1 gene.

In conclusion, pioglitazone up-regulates 11HSD1 in VSMC and thus increases the local amplification of glucocorticoids. This pioglitazone-dependent positive regulation of 11HSD1 seems to be caused by a switch in the expression of transcription factors of the C/EBP family. The finding that pioglitazone up-regulates 11HSD1 in VSMC could help to explain the mechanism for the known anti-inflammatory, anti-atherogenic and circulatory effects of PPAR $\gamma$  agonists on the cardiovascular system [8,9].

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