

Prostacyclin production in rat aortic smooth muscle cells: role of protein kinase C, phospholipase D and cyclooxygenase-2 expression

Miguel A. Frias, Frédérique Dubouloz, Michela C. Rebsamen, Ursula Lang*

Division of Endocrinology and Diabetology, University Hospital, 24 rue Micheli-du-Crest, CH-1211 Geneva 14, Switzerland

Received 28 February 2003; received in revised form 18 July 2003; accepted 11 August 2003

Time for primary review 35 days

Abstract

Objective: The present study was designed to investigate the role of protein kinase C (PKC) and phospholipase D (PLD) in angiotensin II (AngII)- and phorbol ester (PMA)-induced cyclooxygenase-2 (COX-2) expression and prostacyclin (PGI₂) production in rat aortic smooth muscle cells (VSMC). **Methods:** Prostacyclin production in cultured VSMC was determined by radioimmunoassay. PKC activity was examined by measuring the transfer of ³²P from (γ-³²P)ATP to histone III-S. COX-2 expression was determined by Western blotting. To measure PLD activity, thin layer chromatography was used. **Results:** AngII (50 nM) and PMA (100 nM) promoted the translocation of PKC activity from the cytosol to the membranes within 30 min, followed by a strong increase in PLD activity as well as COX-2 expression and PGI₂ production. After 48 h exposure to PMA, PKC was downregulated resulting in a complete suppression of its activity. PKC-downregulation and the PKC inhibitor CGP41251 abolished PMA- and AngII-induced PLD activation, suppressed the stimulatory effect of PMA on COX-2 expression and PGI₂ production and strongly inhibited that of AngII. Furthermore, AngII- and PMA-induced PGI₂ production depended on protein synthesis and COX-2 but not COX-1 activity. Inhibition of PLD-mediated phosphatidic acid (PA) formation by 1% 1-butanol abolished AngII-induced COX-2 expression and PGI₂ secretion, while dioctanoyl PA increased COX-2 expression and PGI₂ production in a time- and concentration-dependent manner. **Conclusion:** Our results indicate that in VSMC, AngII promotes PGI₂ production to a large extent through a rise in COX-2 expression which is mediated by PA generated from increased PKC-dependent PLD activity.

© 2003 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Cell culture/isolation; Hormones; Signal transduction; Prostaglandins

1. Introduction

Prostacyclin (PGI₂), a potent vasodilator and inhibitor of platelet aggregation, contributes to the maintenance of vascular homeostasis. The formation of PGI₂ is regulated at two rate-limiting steps: release of arachidonic acid (AA) from membrane phospholipids by phospholipase A₂ (PLA₂) and conversion of AA to a prostanoid precursor by the enzyme cyclooxygenase (COX). Two types of COX, COX-1 and COX-2 have been identified [1]. COX-1 is constitutively

expressed in most cell types and appears to be responsible for the synthesis of prostaglandins that are important for homeostatic functions, while COX-2 is rapidly induced by cytokines and growth factors [1].

The vasoactive hormone angiotensin II (AngII) is known to promote prostaglandins and prostacyclin production in various cells including vascular smooth muscle cells (VSMC). Ohnaka et al. [2] have shown that AngII induces COX-2 expression and prostaglandin E₂ (PGE₂) production, and that COX-2 is regulated by p42/44 and p38 MAPK in aortic smooth muscle cells. Recently, AngII has been found to activate cytosolic cPLA₂ generating the metabolite 20-HETE which in turn stimulates PLD [3] leading to AA release in VSMC. Moreover, activation of PLD occurs through interaction with the

* Corresponding author. Tel.: +41-22-372-93-18; fax: +41-22-372-93-29.

E-mail address: ursula.lang@medecine.unige.ch (U. Lang).

small G-proteins of the ADP-ribosylation factor [4,5] and Rac/Rho families [6] as well as with protein kinase C [7]. However, the contribution of these factors to the activation of PLD depends on the cell type and signaling model investigated. Both, PKC-dependent and -independent activation of PLD has been reported in various agonist-stimulated cell types [8–11].

Two studies provide evidence that PGI₂ synthesis is limited by the expression levels of COX-2, and that there is a link between PLD and interleukin-1 β - and PMA-stimulated PGI₂ production in amniotic WISH cells [12,13], but there is no information concerning this link in VSMC. In this study, we investigated the possible role of PKC and PLD activation in AngII- and PMA-induced COX-2 expression and PGI₂ secretion in VSMC.

2. Materials and methods

2.1. Materials

[Ile⁵]Angiotensin II (AngII) was from Bachem (Bubendorf, Switzerland). ³H-labeled 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}) and [γ -³²P]-ATP were obtained from Amersham International (Bucks, UK). [³H]-arachidonic acid and [³H]-oleic acid were from Du Pont de Nemours International (Regensdorf, Switzerland). Valeryl salicylate and NS-398 were from Cayman Chemical (Ann Arbor, MI). Collagenase and elastase were from Sigma (St Louis, MO).

2.2. Preparation of rat aortic smooth muscle cells

Rat aortic smooth muscle cells (VSMC) were isolated by enzymatic dispersion of the thoracic aortas of 40–50 days old Wistar rats as previously described [14,15]. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1966). Briefly, the aorta was opened longitudinally on the dorsal part. The endothelium was gently scraped using a surgical blade and the media was carefully dissected from the underlying adventitia. Smooth muscle cells were isolated from the media by enzymatic digestion. Following dispersion, cells were plated in Petri dishes or in six-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Confluent cultures were passaged, and cells of the third to the sixth passage were used for all experiments described in this study. By using antibodies recognizing alpha-smooth muscle actin (specific of smooth muscle cells) and factor VIII (specific of endothelial cells), it has been shown that the contamination of smooth muscle cells by endothelial cells in primary culture was negligible [14].

2.3. Determination of prostacyclin production

VSMC, seeded into six-well plates, were washed and incubated for 20 min at 37 °C with 1 ml of a Krebs–Ringer buffer [15], containing 0.2% bovine serum albumin and 0.2% glucose. The supernatant was replaced by fresh buffer, and cells were stimulated at 37 °C. PGI₂ production was determined by radioimmunoassay of 50 μ l aliquots of the incubation medium for 6-keto-PGF_{1 α} , the stable metabolite of PGI₂. Relative affinity of the anti-6-keto-PGF_{1 α} (Oxford Biomedical Research, Oxford USA) for eicosanoids was given as follows: 100% for 6-keto-PGF_{1 α} , <1% for PGE₂, PGF_{2 α} , PGD₂, PGE₁, PGF_{1 α} and thromboxane B₂. Non-specific binding was estimated at 2.9 \pm 0.11% (n = 10). The detection limit for 6-keto-PGF_{1 α} determinations was 3 pg/ml of incubation medium, while the intra- and interassay coefficients of variation were evaluated at 4% and 5%, respectively (n = 10).

2.4. Western blot analysis

After stimulation, VSMC were washed twice with cold PBS (137 mM NaCl, 1.47 mM KH₂PO₄, 8.9 mM Na₂HPO₄, pH 7.4) and lysed with 500 μ l of the following buffer: Tris/HCl (50 mM, pH 7.4), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 40 mM β -glycerophosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 0.3 mM leupeptin, 1 μ M pepstatin A and 1 mM PMSF. Cell lysates (20 μ g) were analysed by SDS-PAGE at 150 V for 1 h. After transfer, the nitrocellulose membranes were incubated for 2 h at room temperature with a polyclonal antibody raised against type II cyclooxygenase (Cayman Chemical).

The membranes were washed and incubated for 1 h with horseradish peroxidase (HRP)-labeled goat anti-rabbit (CovalAb, Oullins, France) or goat anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Immunoreactive bands were visualised by a chemiluminescent kit (Amersham, Zürich, Switzerland) and quantified by densitometry.

2.5. Preparation of cytosolic and particulate fractions

After stimulation, cells seeded in 90 mm Petri dishes were washed with Tris/HCl buffer (20 mM, pH 7.5), 2 mM EDTA, 10 mM EGTA, 10 mM dithiothreitol, 0.25 M sucrose and 30 μ M leupeptin (buffer A), and homogenized in 1 ml of the same buffer. The homogenate was centrifuged at 100,000 \times g for 1 h at 4 °C. The supernatant was used as the cytosolic fraction. The pellet was incubated for 30 min at 20 °C with Triton X-100 (1% in buffer A), diluted to the original volume of the homogenate with buffer A (final Triton concentration 0.2%), and centrifuged at 100,000 \times g for 1 h. The supernatant obtained was used as the solubilized particulate fraction.

2.6. DEAE anion-exchange chromatography and PKC activity assay

PKC activity was almost undetectable in crude cell fractions, but was expressed after DEAE-cellulose chromatography.

Cytosolic and membrane fractions were applied to a DEAE-cellulose column (2.5 × 0.9 cm), equilibrated at 4 °C in buffer B (20 mM Tris/HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA and 10 mM dithiothreitol). The kinase activity was eluted with a linear NaCl gradient (0–0.3 M) in buffer B.

One hundred microliters of each fraction from the column was assayed for PKC activity as previously described [15]. Briefly, protein kinase activity was determined by measuring the transfer of ³²P from (γ-³²P)ATP to histone III-S. The reaction mixture (240 μl) contained (0.7–1.2) × 10⁶ c.p.m. of (γ-³²P)ATP, 0.15 μM ATP, 0.46 mM CaCl₂, 6.9 mM MgCl₂ and 30 μg of histone III-S. Each fraction was assayed in the absence and presence of phosphatidylserine (80 μg) and diolein (4 μg). After 10 min at 30 °C, the reaction was stopped with 2 ml of 12% (w/v) trichloroacetic acid in the presence of γ-globulin (600 μg). After centrifugation (3000 × g, 2 min), the pellet was dissolved in 0.5 ml of 1 M NaOH and precipitated again with 12% trichloroacetic acid. The precipitate was dissolved in 0.5 ml of 1 M NaOH, and ³²P incorporation was measured by scintillation counting.

2.7. PLD activity assay

VSMC were seeded into 90 mm Petri dishes. Confluent cells were labelled for 15 h with [³H]-oleic acid (10 μCi; 10 Ci/mmol) and then washed to remove unincorporated label. These labelled cells were incubated for 30 min at 37 °C with 5 ml of a Krebs–Ringer buffer [15], containing 0.2% fatty acid free bovine serum albumin and 0.2% glucose at 37 °C, in the presence and absence of PMA or AngII. Propanol (0.5%) was added to the cells 5 min prior to the addition of PMA or AngII. After incubation, the supernatant was collected and the cells were washed before adding methanol (4 ml/Petri). After harvesting the cells, lipids were extracted according to Bligh and Dyer [16]. Radioactivity in aliquots of medium, aqueous phase and chloroform phase was determined. Lipid extracts were separated by thin layer chromatography, with either the top phase of ethyl acetate/2,2,4-trimethyl pentan/acetic acid/water (13:2:3:10) for separation of phosphatidylpropanol and phosphatidic acid or with chloroform/methanol/acetic acid/water (100:50:16:3) for separation of phospholipids.

2.8. Statistical analysis

Values were compared by analysis of variance (ANOVA) using the Scheffe *F*-test criterion for unbalanced groups and the Fisher's protected least significant difference test when

appropriate. Results represent the means ± S.E.M. of at least three independent experiments.

3. Results

3.1. AngII- and PMA stimulate COX-2 expression and PGI₂ secretion in VSMC

Incubation of rat VSMC with AngII or PMA induced a marked increase in cellular PGI₂ secretion, as assessed by the determination of its stable metabolite 6-keto-PGF_{1α} (Fig. 1, *n* = 3–4). Indeed, incubation of VSMC with 50 nM AngII or 100 nM PMA for 1 h increased prostacyclin production 8.8 and 4 times, respectively. In order to study whether type 1 or type 2 cyclooxygenase (COX-1, COX-2) are involved in this response we used their respective inhibitors, valeryl salicylate [17] and NS-398 [18]. As shown in Fig. 1, 2 h preincubation with 5 μM of valeryl salicylate did neither significantly affect agonist-stimulated nor basal PGI₂ production. Similar results were obtained with 50 μM of valeryl salicylate (data not shown). In contrast, NS-398 (1 μM) abolished AngII- and PMA-induced PGI₂ secretion, but had no effect on basal production. Our results indicate that in VSMC, AngII- and PMA-induced PGI₂ secretion is mediated by the activity of COX-2, through a mechanism independent of COX-1.

We next investigated whether the synthesis of inducible COX-2 was increased following 0.5–4 h incubation of cells

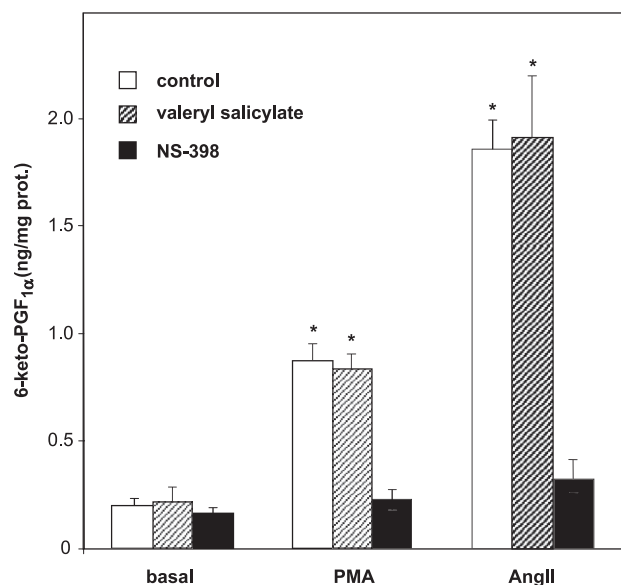


Fig. 1. Effect of valeryl salicylate and NS-398 on AngII or PMA-induced 6-keto-PGF_{1α} secretion in VSMC. Cells were stimulated for 1 h with 50 nM AngII or 100 nM PMA. Prior to stimulation, cells were left untreated (□) or were preincubated for 2 h with 5 μM of valeryl salicylate (▨) or for 45 min with 1 μM NS-398 (■), respectively. 6-keto-PGF_{1α} secretion was determined as described in Section 2. Results represent the means ± S.E.M. of three to four experiments performed in triplicate determinations. **p* < 0.05 vs. respective basal values.

with PMA or AngII. As shown in Fig. 2, AngII and PMA induced a rapid significant increase in COX-2 amount, which remained sustained during 4 h of agonist stimulation. Thus, our results demonstrate that in the signaling pathway leading to prostacyclin formation in VSMC, AngII and PMA increase both the expression and the activity of cyclooxygenase-2.

In parallel, we also investigated the role of cytosolic phospholipase A₂ (cPLA₂) by using the specific cPLA₂ inhibitor arachidonyl trifluoromethyl ketone (AACOCF₃) [19]. As shown in Fig. 3, preincubation with 30 μM AACOCF₃ for 30 min had no effect on basal PGI₂ production, but abolished the AngII-induced response. By contrast, AACOCF₃ did not affect COX-2 expression, neither in control nor in AngII-stimulated cells, indicating that in VSMC, cPLA₂ activation is a necessary step for AngII-induced PGI₂ secretion, independent of COX-2 synthesis.

In order to determine whether protein synthesis is involved in AngII- and PMA-induced COX-2 expression as well as prostacyclin secretion, we tested the effect of cycloheximide on these two parameters in VSMC. Fig. 4

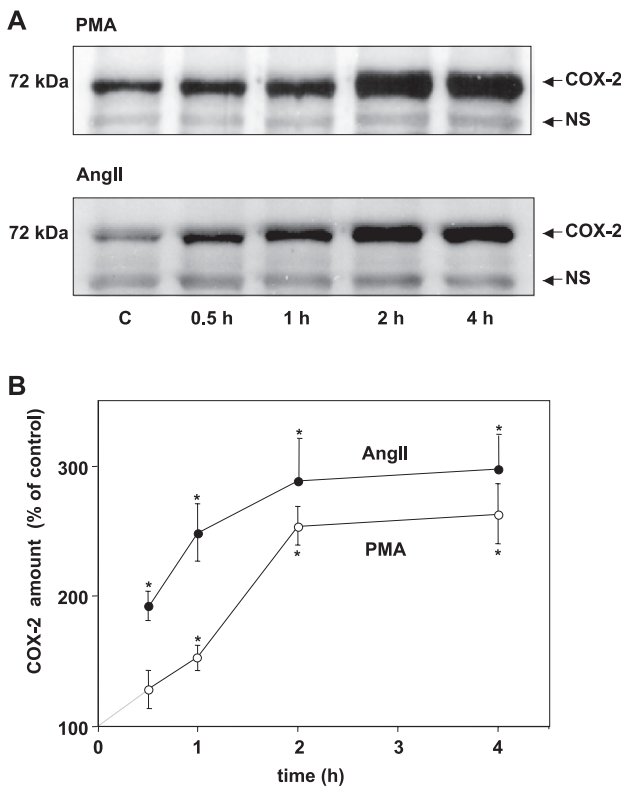


Fig. 2. Agonist-induced increase in COX-2 expression in VSMC. Cells were incubated with 50 nM AngII or 100 nM PMA for the indicated periods of time. (A) Cells were lysed and Western blotting was performed using an anti-COX-2 antibody. Equal protein loading was confirmed by non-specific bands (NS). (B) Specific bands corresponding to COX-2 amount were quantified by densitometry and expressed as percentage of control. Results represent means ± S.E.M. of values obtained in three to four experiments. **p* < 0.05 vs. control values.

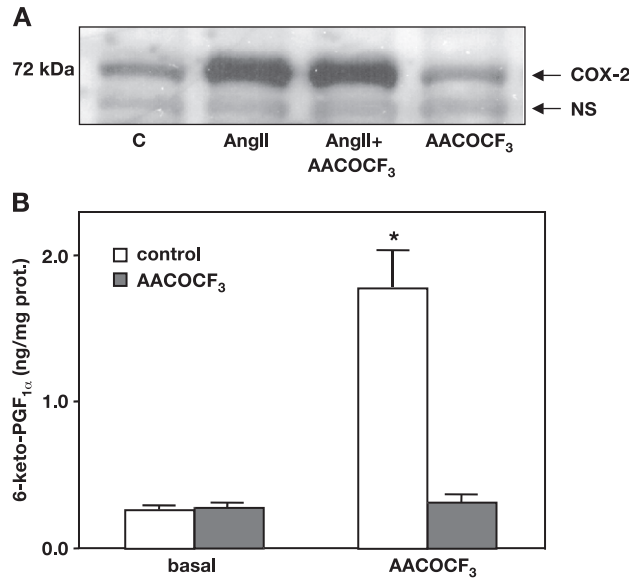


Fig. 3. Effect of AACOCF₃ on AngII-induced COX-2 expression and PGI₂ production in VSMC. (A) Western blot showing COX-2 expression: similar results were obtained in three separate experiments. (B) Cells were stimulated for 1 h with 50 nM AngII, and 6-keto-PGF_{1α} secretion was determined. Prior to stimulation, cells were left untreated (□) or were preincubated for 30 min with 30 μM of AACOCF₃ (■). Results represent the means ± S.E.M. of three experiments performed in triplicate determinations. **p* < 0.05 vs. basal control values.

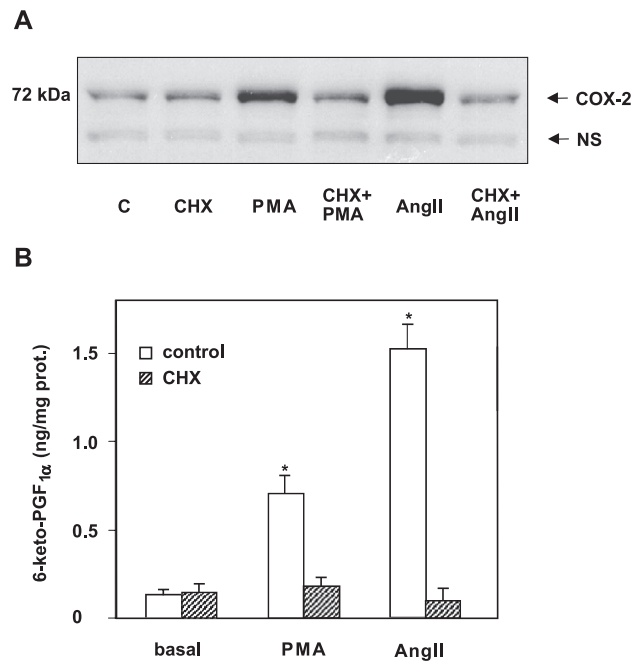


Fig. 4. Effect of cycloheximide on agonist-induced COX-2 expression and 6-keto-PGF_{1α} formation in VSMC. Cells were stimulated for 1 h with 50 nM AngII or 100 nM PMA. Prior to stimulation, cells were left untreated (C, □) or were preincubated for 15 min with 10 μg/ml of cycloheximide (CHX, ■). (A) Western blot showing COX-2 expression: similar results were obtained in three separate experiments. (B) Production of 6-keto-PGF_{1α}: results represent the means ± S.E.M. of three to four experiments performed in triplicate determinations. **p* < 0.05 vs. basal control values.

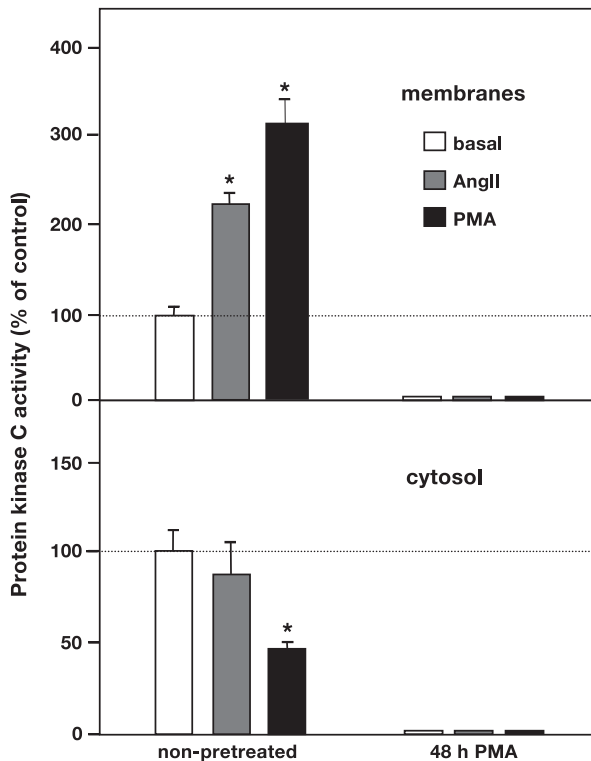


Fig. 5. Effect of AngII and PMA stimulation on subcellular location of PKC activity in VSMC. Cells were stimulated with 50 nM AngII or 100 nM PMA for 30 min or they were, prior to stimulation, pretreated for 48 h with 100 nM PMA. Membrane and cytosolic cell fractions were assayed for PKC activity as described in Section 2. The figure shows data pooled from four independent experiments. Mean values \pm S.E.M. are expressed as percentages of control values. * $p < 0.05$ vs. respective basal values.

illustrates that both COX-2 expression and PGI₂ release induced by AngII or PMA were abolished in cells preincubated for 15 min with 10 μ g/ml cycloheximide. These data indicate that in VSMC, newly synthesized proteins are necessary not only for AngII- and PMA-induced COX-2 expression but also for the PGI₂ secretion stimulated by these agonists.

3.2. Effects of AngII and PMA stimulation on PKC activity in VSMC

We studied the changes in PKC activity occurring in cytosolic and membranous fractions of VSMC incubated with AngII or PMA. As is shown in Fig. 5, exposure of these cells to 50 nM AngII or 100 nM PMA for 30 min increased basal membranous PKC activity by $120 \pm 13\%$ and by $209 \pm 29\%$, respectively. By contrast, basal cytosolic PKC activity was decreased by $55 \pm 5\%$ in PMA-treated cells, while it was not significantly changed in AngII-incubated cells.

Following 48 h of exposure to PMA, PKC activity was abolished in both the membranes and the cytosol and could no longer be stimulated neither by AngII nor by PMA, indicating a complete PKC downregulation.

3.3. Role of PKC in AngII- and PMA-induced COX-2 expression and PGI₂ secretion in VSMC

To answer the question whether PKC activation is necessary for AngII and PMA-induced COX-2 expression and PGI₂ production, we used the PKC inhibitor CGP41251 [20] as well as PKC-depleted cells (48 h exposure to 100 nM PMA). Fig. 6A shows that the rapid increase in COX-2 expression, induced by PMA, decreased following prolonged incubation with PMA, finally reaching control levels after 48 h.

Densitometric analysis of three Western blots, similar to that shown in Fig. 6B, revealed that in PKC-depleted cells (48 h PMA) AngII- and PMA-induced COX-2 expression was reduced by $75 \pm 6\%$ and $94 \pm 6\%$, respectively, when compared to non-treated cells. Likewise, as shown in Fig. 6C, the presence of the PKC inhibitor CGP41251 (1 μ M) abolished PMA-induced COX-2 expression and inhibited AngII-promoted COX-2 increase by $72 \pm 9\%$ ($n = 3$).

Concerning prostacyclin production, we observed that after 48 h of incubation with PMA, resulting in a complete PKC downregulation, AngII- and PMA-induced PGI₂ release was inhibited by $82 \pm 5\%$ and $95 \pm 3\%$, respectively, when compared to non-pretreated cells (Fig. 7, panel A). Similar to the effect of PKC downregulation, the presence of the PKC inhibitor CGP41251 (1 μ M) reduced AngII- and PMA-stimulated PGI₂ release by $86 \pm 3\%$ and $98 \pm 1\%$, respectively ($n = 3$, Fig. 7, panel A).

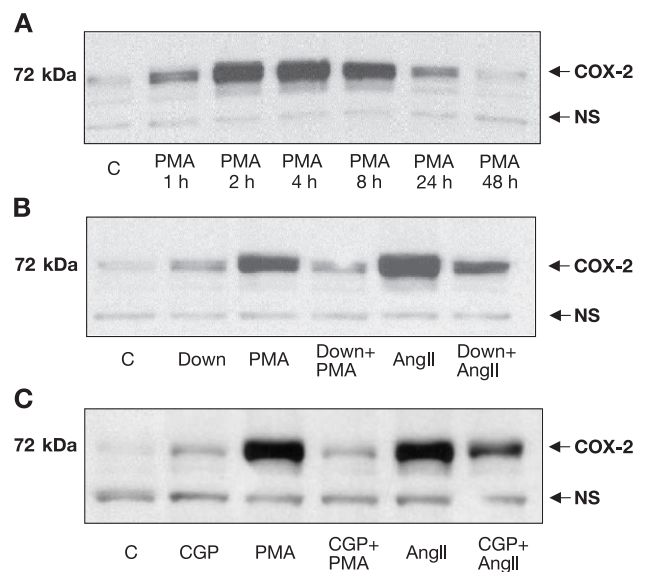


Fig. 6. Effect of PKC downregulation and PKC inhibition on COX-2 expression in VSMC. Western blot showing COX-2 expression: similar results were obtained in three separate experiments. (A) Cells were incubated for 1–48 h with 100 nM PMA. (B) VSMC were stimulated for 1 h with 50 nM AngII or 100 nM PMA. Prior to stimulation, cells were left untreated (C) or, to downregulate PKC, they were preincubated for 48 h with 100 nM PMA (Down). (C) Cells were incubated for 1 h with 50 nM AngII or 100 nM PMA in the presence or absence of 1 μ M CGP41251 (CGP).

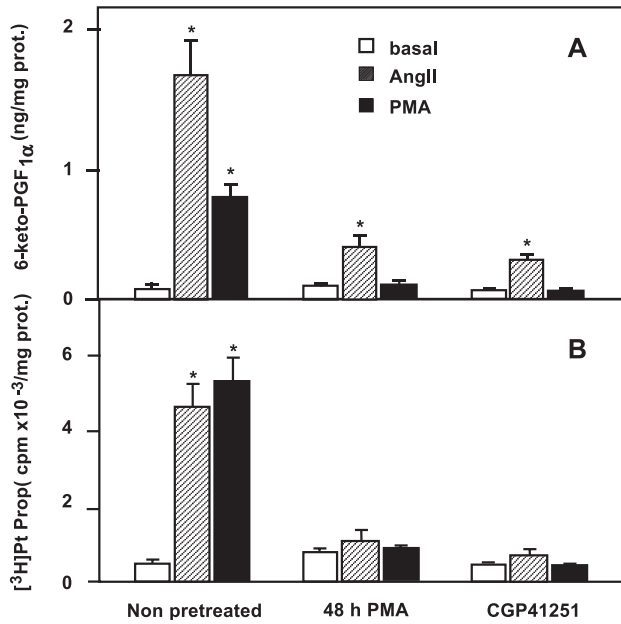


Fig. 7. Role of PKC on AngII- and PMA-induced prostacyclin production and PLD activity in VSMC. Cells were incubated for 1 h with 50 nM AngII or 100 nM PMA in the presence or absence of 1 μ M CGP41251 or prior to stimulation, they were pretreated with 100 nM PMA for 48 h. Panel A: Determination of 6-keto-PGF_{1 α} release. Panel B: PLD activity was determined by measuring [³H]-phosphatidylpropanol ([³H]-PtProp) formation in the presence of 0.5% propanol as described in section 2. The figure shows mean values \pm S.E.M. from three to five independent experiments. * p < 0.05 vs. respective basal values.

These results indicate that PKC plays a key role in AngII- and PMA-induced COX-2 expression and PGI₂ secretion in aortic smooth muscle cells.

3.4. AngII and PMA induce PLD activity in VSMC

We evaluated the formation of phosphatidic acid (PA) following stimulation with AngII and PMA. VSMC were labelled with [³H]-oleic acid for 15 h. As shown in Fig. 8 (panel A), both AngII (50 nM) and PMA (100 nM) induced a marked increase in [³H]-phosphatidic acid formation in these cells.

Indeed, AngII rapidly increased the production of [³H]-phosphatidic acid reaching a peak within 15 min, whereas PMA-induced [³H]-phosphatidic acid formation reached similar values only after 30 min of stimulation.

Since phosphatidic acid may be generated not only by the PLD-mediated hydrolysis of glycerophospholipids but also by the phosphorylation of diacylglycerol, we further assessed the contribution of PLD in agonist-induced [³H]-phosphatidic acid production measuring transphosphatidylation of primary alcohol which is a more specific marker for PLD activity [21]. In the presence of 0.5% propanol, AngII (50 nM) and PMA (100 nM) generated the production of [³H]-phosphatidylpropanol (Fig. 8, panel B), paralleled by an approximately 70% decrease in [³H]-phosphatidic acid formation (data not shown). Indeed,

AngII rapidly stimulated [³H]-phosphatidylpropanol production reaching a plateau at 15 min (Fig. 8, panel B). Initially, PMA induced lower levels of [³H]-phosphatidylpropanol compared to AngII; however, similar values were obtained after 30 min of stimulation (Fig. 8, panel B). Exposure of cells to AngII or PMA in the absence of propanol did not result in the formation of [³H]-phosphatidylpropanol (data not shown).

3.5. Role of PKC in AngII- and PMA-induced PLD activity in aortic smooth muscle cells

PLD activity was assessed by measuring the transphosphatidylation of primary alcohol. As shown in Fig. 7, panel B, exposure of VSMC to 50 nM AngII or 100 nM PMA for 1 h in the presence of 0.5% propanol increased [³H]-phosphatidylpropanol production from 452 \pm 31 to 4871 \pm 593 and 5516 \pm 689 cpm/mg cell protein, respectively.

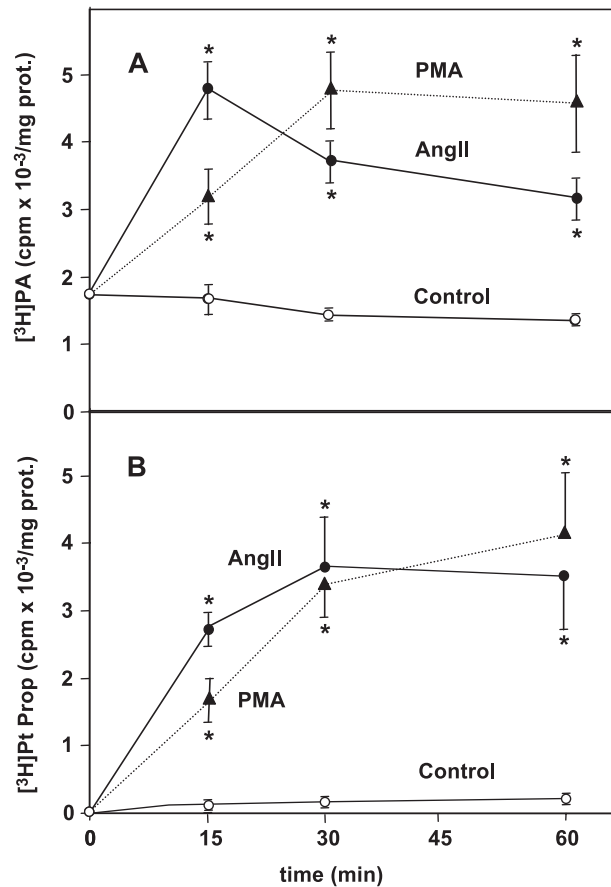


Fig. 8. Effect of AngII and PMA on PLD activity in VSMC. Cells were labeled with [³H]-oleic acid as described in section 2, washed and stimulated for the indicated periods of time with 50 nM AngII or 100 nM PMA. PLD activity was determined by measuring the production of [³H]-phosphatidic acid ([³H]-PA) in the absence of alcohol (panel A), and by measuring the formation of [³H]-phosphatidylpropanol ([³H]-PtProp) in the presence of 0.5% propanol (panel B). The figure shows mean values \pm S.E.M. pooled from three to five independent experiments. * p < 0.05 vs. control values.

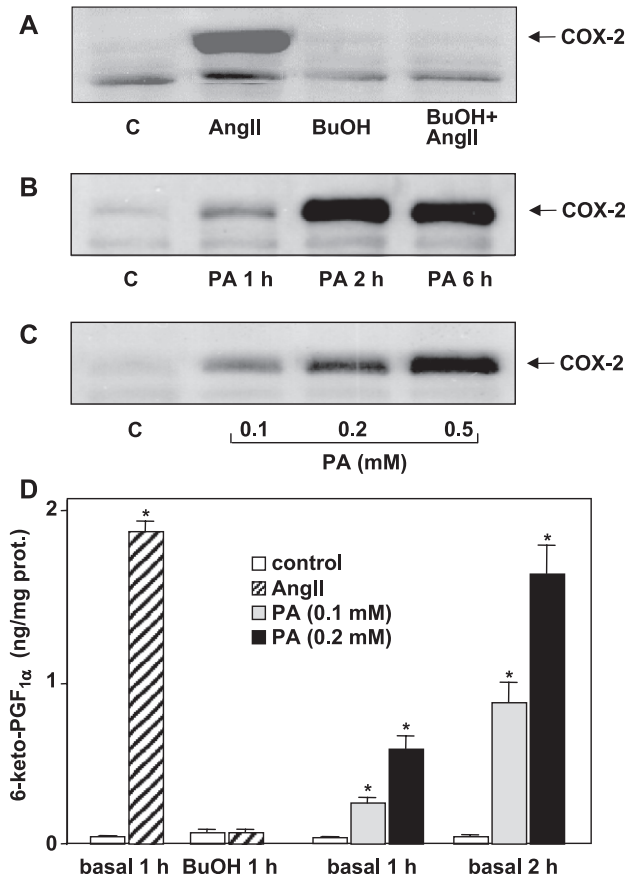


Fig. 9. Effect of 1-butanol and dioctanoyl PA on COX-2 expression and 6-keto-PGF_{1α} secretion in VSMC. Each Western blot shown is representative of three independent experiments. (A) Cells were incubated for 1 h with 50 nM AngII in the presence or in the absence of 1% 1-butanol (BuOH). (B) Cells were exposed to 0.2 mM dioctanoyl PA (PA) for the indicated periods of time. (C) Cells were incubated for 1 h with the indicated concentrations of dioctanoyl PA (PA). (D) Production of 6-keto-PGF_{1α} in cells stimulated for 1 h with 50 nM AngII in the presence or in the absence of 1% 1-butanol (BuOH) and in cells incubated for 1 or 2 h with 0.1 and 0.2 mM dioctanoyl PA (PA).

Consistent with our results of PKC activity, after 48 h of incubation with PMA, resulting in PKC downregulation, AngII and PMA no longer increased PLD activity (Fig. 7, panel B). Likewise, the presence of the PKC inhibitor CGP41251 (1 μM) abolished both the AngII- and the PMA-stimulated PLD activity.

These data indicate that PKC plays an important role in AngII- and PMA-induced PLD activation in VSMC.

3.6. Role of PLD in AngII-induced COX-2 expression and PGI₂ secretion in VSMC

To assess whether PLD activity is involved in AngII-induced COX-2 expression and PGI₂ release, we used 1-butanol (BuOH) which is known to suppress PLD action mainly by inhibiting phosphatidic acid (PA) formation. VSMC were incubated for 1 h with 50 nM AngII in the absence or in the presence of 1% BuOH. As illustrated in

Fig. 9A and D, BuOH abolished both, AngII-induced COX-2 expression and PGI₂ release, without affecting basal values. To provide further evidence that PA is involved in COX-2 expression as well as in PGI₂ production, cells were incubated for 1–6 h with 0.1–0.5 mM of a short-chained dioctanoyl PA. Fig. 9B–D shows that dioctanoyl PA induced COX-2 expression as well as PGI₂ release in a time- and concentration-dependent manner. These data indicate that PLD activation and PA formation are involved in AngII-induced COX-2 expression and PGI₂ secretion in VSMC.

4. Discussion

This study demonstrates that in VSMC, AngII promotes PGI₂ secretion to a large extent by a PKC-dependent induction of COX-2 via an increase in PLD activity and subsequent augmentation of PA. The signaling pathway follows the sequence AngII–PKC–PLD–PA–COX-2–PGI₂ (Fig. 10).

Moreover, the present work shows that in VSMC, AngII-induced PGI₂ secretion is mediated by the activities of both cPLA₂ and COX-2, through a mechanism independent of COX-1. The fact that the cPLA₂-specific inhibitor AACOCF₃ did neither effect basal nor agonist-induced COX-2 expression suggests that cPLA₂ activity is not involved in COX-2 synthesis, but is rather necessary for COX-2 activity by providing arachidonic acid.

AngII and PMA promoted the translocation of PKC activity from the cytosol to the membranes within 30 min, followed by a marked increase in PLD activity as well as COX-2 expression and PGI₂ production. After 48 h of exposure to PMA, membrane and cytosolic PKC activities

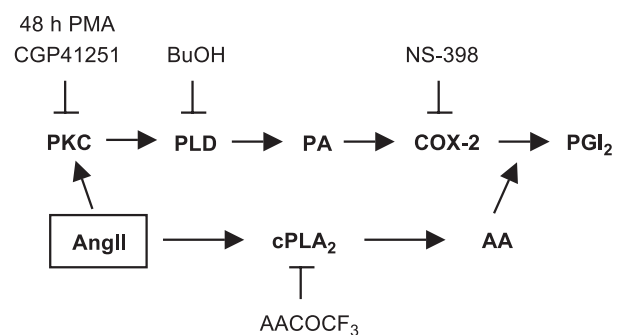


Fig. 10. Major pathways involved in AngII-induced PGI₂ production in VSMC. Angiotensin II (AngII) activates protein kinase C (PKC) leading to increased phospholipase D (PLD) activity and subsequent augmentation of phosphatidic acid (PA) which in turn induces cyclooxygenase (COX-2) expression and prostacyclin (PGI₂) secretion. On the other hand, AngII-induced cytosolic phospholipase A₂ (cPLA₂) activation, providing arachidonic acid (AA), is a necessary step for PGI₂ production. Abbreviations: CGP41251, PKC inhibitor; 48 h PMA, incubation of VSMC with PMA for 48 h leading to PKC downregulation; AACOCF₃, cPLA₂ inhibitor; BuOH (1-butanol) inhibitor of PLD activity; NS-398, COX-2 inhibitor.

were completely downregulated and could no longer be stimulated by AngII or PMA. In these PKC-depleted cells, as well as in the presence of the PKC inhibitor CGP41251 [20], both the PMA- and the AngII-induced activation of PLD was abolished. Under the same conditions, the stimulatory effect of PMA on COX-2 expression and PGI₂ production was also completely suppressed, whereas that of AngII was strongly inhibited. This difference can be explained by the fact that PMA directly activates PKC while AngII acts through phosphatidylinositol 4,5-bisphosphate hydrolysis, thus increasing not only membranous PKC activity but also intracellular free calcium concentration ($[Ca^{2+}]_i$) which contributes to the regulation of PGI₂ secretion in hormone-stimulated VSMC [15]. Moreover, our data provide indirect evidence that increased $[Ca^{2+}]_i$ is not involved in AngII-stimulated PLD activity in VSMC which is in agreement with recent work reporting that in adrenal glomerulosa cells, AngII-induced PLD activation is independent of calcium influx [8].

Our data present strong evidence that in aortic smooth muscle cells, AngII-induced PLD activation and subsequent increase in COX-2 expression and PGI₂ release occurs to a great extent via the activation of PKC. Two earlier studies [22,23] reported that following 24 h exposure of VSMC to PMA, the AngII-induced PLD activation was not affected, suggesting that PKC is not involved in this process. We obtained similar results after 24 h incubation of VSMC with PMA, but we also observed that in these cells, PKC activity was not completely suppressed and could still be tripled upon addition of AngII (unpublished results). Our results, obtained with PKC-depleted VSMC after 48 h of exposure to PMA and with cells incubated with the PKC inhibitor CGP41251, are in agreement with previous reports indicating that PKC is an important regulator of PLD activity (for review, see Ref. [7]). However, Andresen et al. [9] reported that in preglomerular microvascular smooth muscle cells, AngII-stimulated PLD activity was not affected by pharmacological inhibition of PKC. These different observations suggest a tissue-specific role for PKC in the regulation of PLD activity. Moreover, depending on the cell type, PLD activity can also be modulated positively or negatively by protein kinase A [24,25].

To assess the link between PLD activation on one hand, and COX-2 expression and PGI₂ production on the other hand, we stimulated VSMC with AngII in the presence of 1-butanol which leads to the formation of phosphatidylethanol instead of PA. We found that in these cells, 1-butanol abolished AngII-induced COX-2 expression and PGI₂ secretion. Moreover, the short-chain dioctanoyl PA increased COX-2 expression and PGI₂ production in a time- and concentration-dependent manner. These observations suggest that AngII-induced COX-2 expression and subsequent increase in PGI₂ production is to a great extent mediated by PA resulting from PLD activation. In agreement with our results, PLD was reported to be involved in the regulation of COX-2 expression in amnion-derived

WISH cells stimulated by interleukin-1 β [13] and phorbol ester [12].

Our investigations concerning the role of PKC and PLD in AngII-stimulated PGI₂ release indicate that induction of COX-2 plays a key role in this response. Concomitant with increased or decreased PGI₂ production in VSMC, we observed corresponding changes in COX-2 expression levels. The importance of increased COX-2 expression in AngII- and PMA-stimulated PGI₂ release was further confirmed by the observations that AngII- and PMA-induced PGI₂ production depended on newly synthesized proteins and COX-2 activity, while COX-1 was not involved.

Overall, our study resulted in new, complementary information concerning the mechanism involved in AngII-stimulated PGI₂ secretion in VSMC. The investigation of Ohnaka et al. [2] provided evidence that AngII regulates COX-2 expression and prostaglandin production through MAP kinase-mediated signaling pathways in VSMC. In A10 cells, a vascular smooth muscle cell line, the stimulation of MAPK phosphorylation by AngII is inhibited in the absence of PLD activity, and addition of phosphatidic acid restores the effect of AngII on MAPK phosphorylation [26]. Our investigation provide evidence that in VSMC, AngII-stimulated PGI₂ production occurs to a great extent through a rise in COX-2 expression which is mediated by PA generated from increased PKC-dependent PLD activity.

Acknowledgements

We thank Ms C. Gerber-Wicht, M. Klein and M. Rey for their excellent technical assistance. This study was supported by grant nos. 31-52935.97 and 31-063799.00 from the Swiss National Science Foundation.

References

- [1] Vane JR, Bakhle Y, Botting R. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 1998;38:97–120.
- [2] Ohnaka K, Numaguchi K, Yamakawa T, Inagami T. Induction of cyclooxygenase-2 by angiotensin II in cultured rat vascular smooth muscle cells. *Hypertension* 2000;35:68–75.
- [3] Parmentier J-H, Mubarak MM, Nishimoto AT, Malik KU. 20-Hydroxyeicosatetraenoic acid mediates angiotensin II-induced phospholipase D activation in vascular smooth muscle cells. *Hypertension* 2001;37:623–629.
- [4] Shome K, Nie Y, Romero G. ADP-ribosylation factor proteins mediate agonist-induced activation of phospholipase D. *J Biol Chem* 1998;273:30836–30841.
- [5] Hammond SM, Altshuler YM, Sung TC, Rose K, Engelbrecht J, Morris AJ, et al. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new, and highly conserved gene family. *J Biol Chem* 1995;270:29640–29643.
- [6] Malcolm KC, Elliott CM, Exton JH. Evidence for Rho-mediated agonist stimulation of phospholipase D in rat fibroblasts. Effects of clostridium botulinum C3 exoenzyme. *J Biol Chem* 1996;13135–13139.
- [7] Exton JH. Regulation of phospholipase D (minireview). *FEBS Lett* 2002;531:58–61.

- [8] Bollag WB, Jung EM, Calle RA. Mechanism of angiotensin II-induced phospholipase D activation in bovine adrenal glomerulosa cells. *Mol Cell Endocrinol* 2002;192:7–16.
- [9] Andresen BT, Jackson EK, Romero GG. Angiotensin II signaling to phospholipase D in renal microvascular smooth muscle cells in SHR. *Hypertension* 2001;37:635–639.
- [10] Murthy KS, Makhlof GM. Agonist-mediated activation of phosphatidylcholine-specific phospholipase C and D in intestinal smooth muscle cells. *Mol Pharmacol* 1995;48:293–304.
- [11] Sciorra VA, Daniel LW. Phospholipase D-derived products in the regulation of 12-O-tetradecanoylphorbol-13-acetate-stimulated prostaglandin synthesis in Madin-Darby canine kidney cells. *J Biol Chem* 1996;271:14226–14232.
- [12] Johnson CA, Balboa MA, Balsinde J, Dennis EA. Regulation of cyclooxygenase-2 expression by phosphatidate phosphohydrolase in human amnionic WISH cells. *J Biol Chem* 1999;274:27689–27693.
- [13] Park DW, Bae YS, Nam JO, Kim JH, Lee YG, Park YK, et al. Regulation of cyclooxygenase-2 expression by phospholipase D in human amnion-derived WISH cells. *Mol Pharmacol* 2002;61:614–619.
- [14] Bochaton-Piallat ML, Gabbiani F, Ropraz P, Gabbiani G. Cultured aortic smooth muscle cells from newborn and adult rats show distinct cytoskeletal features. *Differentiation* 1992;49:175–185.
- [15] Erbrich AC, Church DJ, Vallotton MB, Lang U. Regulation of prostacyclin production by calcium and protein kinase C in aortic smooth muscle cells. *Am J Physiol* 1992;263:E800–E806.
- [16] Bligh EC, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Biophysiol* 1959;37:911–918.
- [17] Bhattacharyya DK, Lecomte M, Dunn J, Morgans DJ, Smith WL. Selective inhibition of prostaglandin endoperoxide synthase-1 (cyclooxygenase-1) by valerylsalicylic acid. *Arch Biochem Biophys* 1995;317:19–24.
- [18] Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, Otomo S. NS-398, a new antiinflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins* 1994;47:55–59.
- [19] Street IP, Lin HK, Laliberte F, Ghomashchi F, Wang Z, Perrier H, et al. Slow- and thight-binding inhibitors of the 85-kDa human phospholipase A₂. *Biochemistry* 1993;32:5935–5940.
- [20] Meyer T, Regenass U, Fabbro D, Alteri E, Rösel J, Müller M, et al. A derivative of staurosporine (CGP41251) shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumor activity. *Int J Cancer* 1989;43:851–856.
- [21] Billah MM, Anthes JC. The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem J* 1990;269:281–291.
- [22] Lassègue B, Alexandre RW, Clark M, Griendling KK. Angiotensin II-induced phosphatidylcholine hydrolysis in cultured vascular smooth muscle cells. Regulation and localization. *Biochem J* 1991;276:19–25.
- [23] Freeman EJ, Chisolm GM, Tallant EA. Role of calcium and protein kinase C in the activation of phospholipase D by angiotensin II in vascular smooth muscle cells. *Archives Biochem Biophys* 1995;319:84–92.
- [24] Ginsberg J, Gupta S, Matowe WC, Kline L, Brindley DN. Activation of phospholipase D in FRTL-5 thyroid cells by forskolin and dibutyryl-cyclic adenosine monophosphate. *Endocrinology* 1997;138:3645–3651.
- [25] Tyagi SR, Olson SC, Burnham DN, Lambeth JD. Cyclic AMP-elevating agents block chemoattractant activation of diacylglycerol generation by inhibiting phospholipase D activation. *J Biol Chem* 1991;266:3498–3404.
- [26] Shome K, Rizzo MA, Vasudevan C, Andersen B, Romero G. The activation of phospholipase D by endothelin-1, angiotensin II, and platelet-derived growth factor in vascular smooth muscle A10 cells is mediated by small G proteins of the ADP-ribosylation factor family. *Endocrinology* 2000;141:2200–2208.