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Expression of mRNA for Phospholipase A_2, Cyclooxygenases, and Lipoxygenases in Cultured Human Umbilical Vascular Endothelial and Smooth Muscle Cells and in Biopsies from Umbilical Arteries and Veins

Key Words
Endothelium
Smooth muscle cells
Veins
Arteries
Phospholipase A_2
Lipoxygenase
Cyclooxygenase

Abstract
Arachidonic acid (AA) is released by phospholipase A_2 (PLA_2) and then converted into vasoactive and inflammatory eicosanoids by cyclooxygenases (COX) and lipoxygenases (LOX). These eicosanoids are important paracrine regulators of vascular permeability, blood flow, local pro- and anticoagulant activity and they play a major role in the local inflammatory response. We have investigated the presence of mRNAs for PLA_2 and for isoforms of COX and LOX in both human endothelial cells (EC) and in human smooth muscle cells (SMC) in culture and in vascular biopsies of human umbilical veins (HUVB) and arteries (HUAB) by using the reversed transcription-polymerase chain reaction (RT-PCR) technique. Results show detectable levels of PLA_2 type IV ($cPLA_2$) in cultured EC and SMC and in vascular wall biopsies from HUAB and HUVB. The cultured EC and SMC demonstrate higher levels of both COX-1 and COX-2 with PCR analyses than do vascular wall biopsies from HUAB and HUVB. This indicates a difference in the native expression of COX-1 and COX-2 in cultures of EC and SMC compared to that in biopsies from intact vessel walls. The EC and SMC in culture do not express mRNA for 5-LOX, that was, however, expressed in the vascular wall biopsies. This speaks in favour of a constitutive, i.e. in vivo expression of 5-LOX in SMC in the vascular wall of both umbilical vein and arteries. Thus results from in vitro studies of constitutive COX and LOX expression in EC and vascular SMC in culture cannot simply be extrapolated to represent in vivo conditions.

Introduction

Phospholipase A_2 (PLA_2) catalyses the hydrolysis of the *sn*-2 fatty acyl chain of many different phospholipids releasing arachidonic acid (AA), which serves as a precursor for the synthesis of eicosanoids. The inflammatory and vasoactive eicosanoids are converted from AA either by the cyclooxygenase (COX) pathway into prostaglandins and thromboxane or by the lipoxygenase (LOX) pathway into hydroperoxy-6,8,11,14 eicosatetraenoic

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acids (HPETEs) and leucotrienes. An additional potent inflammatory mediator, platelet-activating factor may be released if PLA_2 acts on a specific phospholipid alkylacyl glycerophosphocholine [1].

Several structurally different isoforms of PLA_2 have been defined in mammals, a 14-kD type I, type II, and a 85-kD PLA_2 type IV, which preferentially hydrolyse AA-containing phospholipids [2]. The human type I PLA_2 is found in pancreas, and probably acts as a digestive proenzyme [2]. Pancreatic-like PLA_2 has also been found in lung and kidney tissues, but its biological function(s) in these tissues is not yet fully elucidated. The type II PLA_2 ($sPLA_2$) is released as a response to inflammatory mediators such as IL-1β and tumour necrosis factor-α [3, 4]. This isozyme, $sPLA_2$, is found in human synovial fluid [5], mammalian platelets, human intestinal tissue, monocytic cell lines [6] and in vascular smooth muscle cells (SMC) [7]. The 85-kD PLA_2 type IV ($cPLA_2$) [8] is another structurally distinct, cell-associated PLA_2. This enzyme is also active at neutral pH, Ca^{2+} dependent, but in contrast, it is very specific for the release of AA. These characteristics make the 85-kD PLA_2 the major liberator of AA from phospholipids for subsequent metabolism to paracrine lipid mediators.

In the vasculature, the synthesis of prostaglandins is important in maintaining normal homeostasis and vascular tone. The best-known endothelium-derived anti-platelet and vasodilator factor is PGI_2 (prostacyclin), which is the major AA metabolite found in most endothelial cells (EC) [9]. COX is thought to be the rate-limiting step in the synthesis of prostaglandins by EC: two isoforms have been identified so far, COX-1 and the recently discovered enzyme COX-2 [10]. COX-1 was first cloned from sheep seminal vesicles [11] and subsequently from human EC [10]. It is considered to be constitutively expressed in many cells [12], whereas COX-2 is highly inducible [10, 13].

The question whether human EC contain 5-LOX, 12-LOX or 15-LOX, and thus are able to generate LTA_4, hepoxilins or lipoxins from AA, or not, is controversial. Leucotrienes have been shown to regulate vascular permeability, leucocyte adhesion, and chemotaxis, as well as smooth muscle tone. Earlier studies have shown the presence of LTB_4 in both rabbit and calf aortic EC [14, 15]. On the other hand, it has been shown that LTB_4 cannot be synthesised by endogenous LTA_4 because of lack of 5-LOX [18, 19]. LTB_4, however, can be produced by EC from exogenous LTA_4 [20]. Very little is known about the ability of human SMC to produce LOX products.

The aim of this study was to investigate the gene expression of PLA_2, COX, and LOX in cultured EC and SMC. Since the cell culture conditions may affect gene expression in EC and SMC and as these model systems are often used to assess in vivo conditions, we investigated the presence of PLA_2, COX and LOX also in native biopsies of human umbilical arteries and veins. Reversed transcription-polymerase chain reaction (RT-PCR), a very sensitive method for examining mRNA in different cell types in culture and in small tissue biopsies, was used.

Materials and Methods

Cell Preparation

Endothelial Cells. Human umbilical venous endothelial cells (HUVEC) were prepared as described previously by Jaffe et al. [19, 20]. The umbilical veins were first perfused with M199 culture medium, after which it was infused with 0.1% collagenase in M199 medium. After incubation at 37 °C for 15 min, the EC were flushed from the umbilical cord vessels by perfusion, using buffer M199. The cells were sedimented at 120 *g* for 5 min after which the supernatant was removed. They were then resuspended in M199 supplemented with gentamicin sulphate (50 $mg \cdot ml^{-1}$) fungizone (2.5 $\mu g \cdot ml^{-1}$), *L*-glutamate (100 $\mu g \cdot ml^{-1}$) and 20% human serum and seeded out in 75-cm^3 flasks. When grown to confluence, the cells were harvested with 0.25% trypsin in 0.05% EDTA solution and split in a 1:3 ratio into gelatine-coated 100-mm dishes. The dishes were incubated at 37 °C under 5% CO_2 in humidified air (Forma Scientific, HEPA filtered IR incubator). The cells were used between passage two and three.

Smooth Muscle Cells. Preparation of human umbilical smooth muscle cells was performed as described previously by Okker-Reitsma et al. [21]. The arteries from the human umbilical cord were dissected under sterile conditions and the connective tissues surrounding the tunica media was stripped off. The inner smooth muscle cell layer of tunica media and tunica intima were cut into 1- to 2-mm fragments and put into siliconized E-flasks.

The tissue biopsy was incubated in M199 at 37 °C for 30 min, supplemented with 6 mg/ml glucose, 0.1% bovine serum albumin, 0.1% collagenase (Boehringer) 2.5×10^{-3}% trypsin, gentamicin sulphate (50 $mg \cdot ml^{-1}$), fungizone (2.5 $mg \cdot ml^{-1}$), and 2 mg/ml elastase under continuous shaking (70 oscillations/min). Trypsin action was stopped by adding 15% fetal bovine serum after 60 min. Cells were harvested by centrifugation for 5 min at 200 *g* and 4 °C and seeded out in flasks. The cells were used between passage four and eight.

Biopsies from Umbilical Vessels. Directly after a normal parturition, human umbilical cord arteries and vein were rinsed with isotonic NaCl, dissected under sterile conditions, excised in 1-cm-long pieces, which were immediately frozen in liquid nitrogen and stored at –80 °C until assay.

Preparation and Isolation of RNA

The biopsies from umbilical vessels and the cultured EC and SMC were prepared using the guanidinium-thiocyanate-phenol-chloroform method [22]. The RNA concentration and purity were esti-

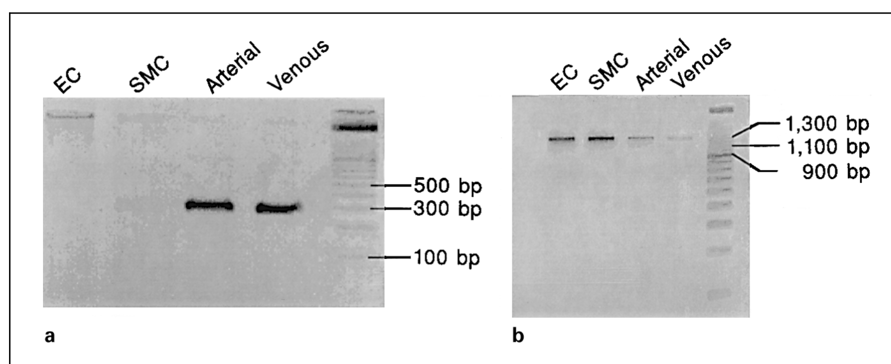

Fig. 1. Electrophoresis of $sPLA_2$ (**a**) and $cPLA_2$ (**b**) mRNA. Expression of $sPLA_2$ mRNA with a 326-bp fragment and $cPLA_2$ mRNA with a 1,228-bp fragment in EC, SMC, arterial and venous biopsies. Total RNA was isolated and analysed by the RT-PCR to measure the $sPLA_2$ and $cPLA_2$ levels as described in Materials and Methods.

mated from the optical density at 260 and 280 nm (Perkin-Elmer, lambda 2 UV/Vis spectrophotometer).

Reversed Transcription-Polymerase Chain Reaction

Two micrograms of total RNA from each sample was reversibly transcribed into cDNA by incubation with 1 μ*M* 15 primer, 3 m*M* $MgCl_2$, 400 U murine Moloney leukaemia virus reverse transcriptase, 500 μ*M* dNTP, 0.01 m*M* dithiothreitiol, 75 m*M* KCl and 50 m*M* Tris-HCl, pH 8.3 in a final volume of 20 μl at 37 °C for 1 h.

cDNA was amplified by polymerase chain reaction (PCR) in a 40-μl reaction containing 100 ng of transcribed total RNA, 1.25 U of Taq DNA polymerase (Promega, Madison, Wisc., USA), 0.5 μ*M* of specific primers and 1.5 m*M* $MgCl_2$ in 1 × reaction buffer provided by the supplier (Promega). Amplification was processed in a Perkin-Elmer Thermal Cycler; initial heating for 2 min at 94 °C, followed by 25–32 cycles of 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C and a final extension for 7 min at 72 °C.

Ten microlitres of the reaction mixture were mixed with loading buffer and separated by electrophoresis on 1.2% agarose gels containing ethidium bromide (0.5 μg/ml) and visualised by UV transillumination.

PCR Primers

cDNA from each sample was amplified with specific primers for $sPLA_2$, $cPLA_2$ COX-1, COX-2, 5-LOX, 12-LOX and 15-LOX in separate tubes. The forward and reverse primers for $sPLA_2$ were: 5′-GACGACAGGAAAGGAAGCC-3′ and 5′-TCCCTCTGCAGT-GTTTATTGG-3′, respectively, and gave a single band corresponding to a 326-bp fragment in human $sPLA_2$ cDNA [23]. The forward and reverse primers for $cPLA_2$ were: 5′-CCTGATATGGAGAA-AGATTGCC-3′ and 5′-AGGGAAACAGAGCAACG-AGA-3′, respectively, and gave a single band corresponding to a 1,228-bp fragment in human $cPLA_2$ cDNA [24].

The forward and reverse primers for COX-1 were: 5′-TGCCCA-GCTCCTGGCCCGCCGCTT-3′ and 5′-GTGCATCAACACAGG-CGCCTCTTC-3′, respectively, and gave a single band corresponding to a 306-bp fragment in human COX-1 cDNA [10].

The recently described forward and reverse primers for COX-2 were: 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′, respectively, and gave a single band corresponding to a 307-bp fragment in human COX-2 cDNA [10]. The forward and reverse primers for 5-LOX were: 5′-TGCTTTCCCGAGGCCATCAAGG-3′ and 5′-GCTTCCTTC-ACAGGCTTCTCGATA-3′, respectively. These yielded a single PCR product corresponding to a 557-bp fragment in human cDNA [25].

The forward and reverse primers for 12-LOX were: 5′-CCACCT-CTTCTACCAAAGGGATG-3′ and 5′-AATCTGTTCGGAATTG-GTTTAGCAC-3′, respectively. These yielded a single PCR product corresponding to a 459-bp fragment from human cDNA [26].

The forward and reverse primers for 15-LOX were: 5′-AAT-CGTGAGTCTCCACTATAAGAC-3′ and 5′-AGCCAGCTCCTC-CCTGAACTTCT-3′, respectively. These yielded a single PCR product corresponding to a 466-bp fragment in human cDNA [27].

Results

In this study we used RT-PCR to examine gene expression of mRNA for $cPLA_2$, $sPLA_2$, COX-1, COX-2, 5-LOX, 12-LOX and 15-LOX in EC, SMC and in biopsies from umbilical arteries and veins. This technology is extremely sensitive and allows the monitoring of mRNA levels from small amounts of total RNA. RNA samples that were not processed with reversed transcriptase gave no detectable PCR product (result not shown).

In cultured EC, only high-molecular-weight cytosolic PLA_2 was expressed at detectable levels (fig. 1). Furthermore, five different oxygenases, representing key enzymes for the downstream metabolism of AA for the production of specific lipid messengers, were analysed. Clear expression of COX-1 and COX-2 mRNA was found in EC (fig. 2). However, no mRNA for the leucotriene synthesis pathway 5-LOX and 12-LOX or of 15-LOX mRNA could be detected (fig. 3).

In cultured SMC, $cPLA_2$ gene expression was evident in analogy to the EC in culture. However, small amounts of $sPLA_2$ mRNA were also consistently observed in these cells (not visible on the gel presented in fig. 1a). Similar to the findings in EC, significant levels of COX-1, COX-2 mRNA were expressed (fig. 2). The mRNA expressions of

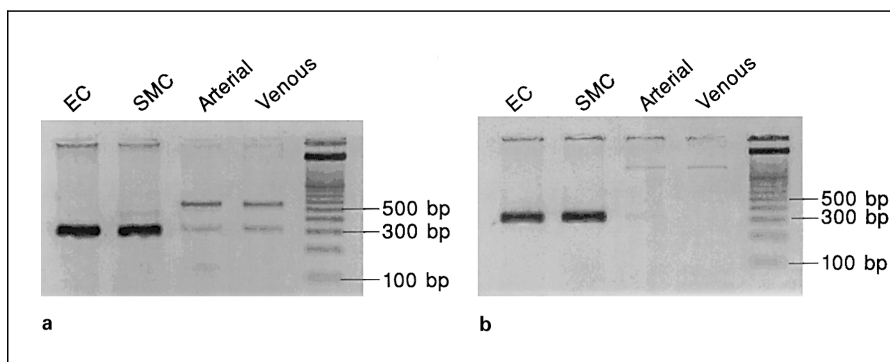

Fig. 2. Electrophoresis of COX-1 (**a**) and COX-2 (**b**) mRNA. Expression of COX-1 mRNA with 306-bp fragment and COX-2 mRNA with a 307-bp fragment in EC, SMC, arterial and venous biopsies. Total RNA was isolated and analysed by the RT-PCR to measure the COX-1 and COX-2 levels as described in Materials and Methods.

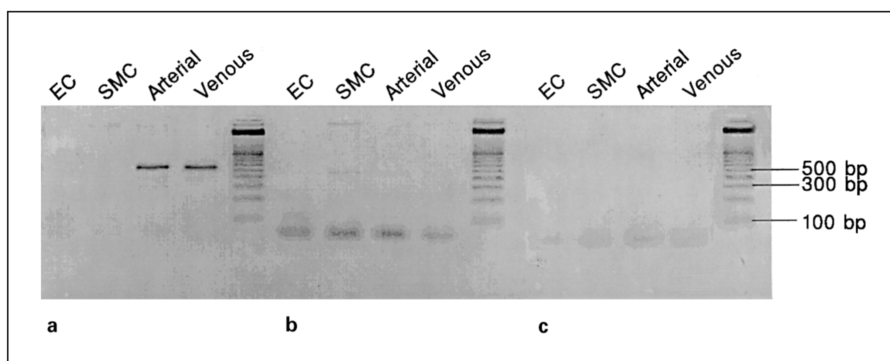

Fig. 3. Electrophoresis of 5-LOX (**a**), 12-LOX (**b**) and 15-LOX (**c**) mRNA. Expression of 5-LOX mRNA with a 557-bp fragment, 12-LOX mRNA with a 459-bp fragment and 15-LOX mRNA with 466-bp fragments in EC, SMC, arterial and venous biopsies. Total RNA was isolated from respective samples and analysed by the RT-PCR to measure the 5-LOX, 12-LOX and 15-LOX levels as described in Materials and Methods.

5-LOX and 15-LOX were missing, whereas very small amounts of 12-LOX was found in SMC (fig. 3b).

In analogy to cultured SMC and EC, we found significant levels of $cPLA_2$ mRNA in the vascular biopsies (fig. 1). In contrast to the findings in cultured cells, we also found high levels of mRNA for $sPLA_2$ in the biopsies of tunicae mediae from umbilical arteries and veins. Furthermore, very small levels of COX-1 and COX-2 mRNA were expressed in biopsies of human umbilical arteries (HUAB) and veins (HUVB) (fig. 2b).

The most notable difference between cultured EC and SMC and the same cells in umbilical vessel wall biopsies was the gene expression of 5-LOX in the latter (fig. 3). This was significantly expressed in both HUAB and HUVB, but not in EC and SMC in culture, where no detectable levels of 5-LOX mRNA were expressed. No detectable levels of 12-LOX or 15-LOX were found in either HUAB or HUVB.

Discussion

Both SMC and EC are known to contain PLA_2 [28, 29]. The former have been shown to mainly contain the $sPLA_2$ [30], which is in agreement with our results. In addition, we have found significant expression of mRNA for $cPLA_2$ in both SMC and EC. Recently, it was shown that stimulation of bovine EC with bradykinin increases the activity of a $[Ca^{2+}]$-sensitive high-molecular PLA_2 [31]. Further, IL-1β and transforming growth factor-β have been shown to induce expression of $cPLA_2$ mRNA in calf pulmonary artery EC in a concentration-dependent fashion [32]. Since recombinant human tumour necrosis factor and human IL-1β rapidly increase PLA_2 activity [29], this could indicate that $cPLA_2$ is post-transcriptionally regulated by these agents and, thus, probably constitutively expressed in these cells.

Our investigations of the COX-1 and COX-2 showed significant levels of COX-1 and COX-2 in both EC and SMC, which is in agreement with former investigations [32, 33]. COX-2 is reported to be induced only after cytokine stimulation [34]. However, some recent reports found it to be highly expressed in EC without any cytokine stimulation [35, 36]. In accordance with the difference in the expressions in actively proliferating SMC and EC in in vitro cultures in mature, non-synthesising, non-cycling corresponding cell types in cells in biopsies from vessel walls in this study, such discrepancies could depend on growth conditions, i.e. serum factors and number of passages. However, in vascular biopsies no, or minute

amounts of mRNA for COX-1 or COX-2 were present. This may indicate that the isoforms of COX investigated in this study are not involved in normal, paracrine vascular regulation, but may be induced under pathophysiological conditions such as inflammation or angiogenesis.

Contradictory results concerning the presence of 5-LOX in EC have also been reported. Thus, whether or not a constitutive production of LTA_4 and the other leucotrienes occur in these cells is controversial at present. In aortic and other arterial EC from various animals, 5-, 12-, 15-HETE and LTB_4 have been identified, demonstrating a constitutive expression of 5-, 12- and 15-LOX activity in EC at these locations [14, 15, 37, 38]. However, the presence of 5-LOX in HUVEC has not been reported. Hopkins et al. [16] have demonstrated the presence of 15-HPETE and 8,15-di-HETE in HUVEC, but not 5-HETE or LTA_4. Johnsson et al. [39] have shown that EC can metabolise, but not synthesise leucotrienes. These results are in agreement with our results. While in our study we have found an expression of 5-LOX mRNA in both HUAB and HUVB, we cannot exclude the possibility that the observed differences between cultured cells and vessel biopsies could be due to contamination of mRNA from non-vascular cells, normally residing within the walls of human umbilical veins and arteries. Histologic [40] and electron-microscopic [41] studies have shown the presence of lymphocytes and monocytes within the arterial intima from other vascular locations than the umbilical vessels. The methods used in our study only reflect the mRNA levels for the specific enzymes. For COX it has been demonstrated that the mRNA level correlates with the level of protein and prostaglandin produced [34, 36]. It may then be pertinent to assume that the presence or absence of specific mRNA in a particular cell type, either under in vitro or in vivo conditions, also generally correlates with the presence or absence of the protein.

In summary our investigation indicates that there is a different expression of mRNA for COX-1, COX-2 and 5-LOX in native biopsies of blood vessel walls and in cultured vascular cells isolated from the same vessels. Even though the RT-PCR method cannot be used to quantitatively assess tissue concentrations of specific mRNA, our results do disclose not only a qualitative difference of expressions in the same cell type – ECs and SMCs – but also a quantitative difference, depending on whether they are in an actively cycling/protein synthesising mode or in the non-cycling state in the mature vessel wall. For COX-1 and COX-2, hardly any mRNA was found in the umbilical blood vessel biopsies, in contrast to results from SMC and EC from the same vessels in culture. There the cDNA fragments for both COX-1 and COX-2 were easily detected with the RT-PCR technique. mRNA for 5-LOX was not expressed at all in the cultured vascular cells, but was regularly detected in all vascular biopsies. These differences most probably reflect the difference of expressions of nuclear and other factors between the two states. However, these differences indicate the necessity to be careful when extrapolating results from in vitro experiments with EC and SMC in culture to in vivo or to clinical conditions.

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