A Novel Form of Platelet-Type 12-Lipoxygenase mRNA in Human Vascular Smooth Muscle Cells


Abstract—The lipoxygenase pathway has been implicated in the growth, migration, and contraction of vascular smooth muscle cells (VSMCs). However, the precise type of lipoxygenase present in the vascular wall has not been characterized. In this study, we used a specific reverse-transcriptase polymerase chain reaction method with 2 sets of specific primers on total RNA and polyA (+)RNA of normal human VSMCs prepared from umbilical artery. Two forms of platelet-type 12-lipoxygenase mRNA were present in human VSMCs: the already published form cloned from human erythroleukemia cells and a variant form of platelet-type 12-lipoxygenase, which includes 2 additional sequences consistent with the 2 introns (D and E). This novel form of 12-lipoxygenase poly A (+)RNA was downregulated by lipopolysaccharide (10 mg/mL) and upregulated by epidermal growth factor (100 ng/mL) but was not affected by angiotensin II (10^{-7} mol/L). We developed a rabbit anti-human platelet-type 12-lipoxygenase polyclonal antibody directed against a 24–amino acid peptide encoded within exon 4. Western immunoblotting of protein extracted from VSMCs and umbilical artery and platelet extract with this antibody showed a coordinate 110-kDa protein and the already-described 70-kDa band detected in platelets and cord homogenate. Another 120-kDa protein was consistently detected in cord extracts but not in platelet or VSMC homogenates. The immunohistochemistry study performed with the same antibody showed extensive cytoplasmic staining of VSMCs. The specific role of these different forms of platelet-type 12-lipoxygenase is subject to further investigation. (Hypertension. 2001;38:864-871.)

Key Words: lipoxygenase ■ muscle, smooth, vascular ■ atherosclerosis

Lipoxygenases (LOs) are dioxygenase enzymes that incorporate a molecular oxygen into unsaturated fatty acids such as arachidonic acid and linoleic acid. These enzymes are named according to the carbon position (5, 12, or 15) at which they introduce oxygen.1 Arachidonate LOs and their products play an important role in mediating growth factor–induced tumor cell proliferation and appear to enhance the growth and migration of vascular smooth muscle cells (VSMCs).2 Some LO forms are also involved in LDL oxidation.3 Because VSMC proliferation is a key process in vascular wall function and plaque formation, it appears important to characterize the type of LO present in human VSMCs. To date, several distinct LO genes have been structurally characterized. Three murine 12-LOs are currently recognized, including platelet 12(S)-LO,4 leukocyte type,4 and an epidermal LO.5 Another LO, a porcine leukocyte type, has been cloned from porcine leukocytes6,7 porcine pituitary cells,8 and bovine tracheal cells.9,10 The human platelet-type 12-LO has been cloned from human erythroleukemia cells and found primarily in human platelets.11,12 There is also evidence for the presence of a leukocyte type of 12-LO in human adrenal glomerulosa cells.13 Additionally, an epidermal 12(R)-LO has recently been cloned from human skin.14 However, there is very limited information on the smooth muscle cell 12-LO gene expression within the arterial wall. We have previously reported that VSMCs generate 12-HETE, the production of which can be blocked by 12-LO inhibitors.15 In human placental perfusion studies, angiotensin (Ang) II increased the release of 12-HETE, whereas LO blockers inhibited both Ang II–induced 12-HETE formation and vasoconstriction.15 Furthermore, 12-LO blockade resulted in diminished blood pressure, as well as reduced vascular reactivity and arterial tone.15–18 In the present study, we investigated the expression of 12-LO in human VSMCs obtained from the human umbilical artery. A previously unrecognized form of platelet-type 12-LO was identified in these cells. This should facilitate further studies of the role of 12-LO and related peptides in the maintenance of arterial tone.

Methods

Cells and Culture

VSMCs were prepared as previously described with minor modifications.15,19 Cell migration was detected within 10 to 20 days. The
cells were trypsinized, transferred to 10-cm tissue culture dishes, and cultured to subconfluence, at which time they were used for RNA and protein preparation. In some experiments, cells were first incubated with Ang II (10−7 mol/L), lipopolysaccharide (LPS, 10 μg/mL), or epidermal growth factor (EGF, 100 ng/mL).

**Total RNA and Poly A (+) mRNA Preparation**

Before RNA preparation, cultured cells were incubated for 24 hours in 199 medium containing 0.4% fetal calf serum. Total RNA and poly A (+) mRNA from cultured VSMCs were extracted by commercial methods (TRIZOL Reagent, Gibco; Total Isolation System and Poly Tract mRNA Isolation System, Promega Corp, respectively).

**Amplification of Reverse-Transcribed RNA by Polymerase Chain Reaction**

All oligonucleotides were synthesized by Biotechnology General Inc and were purified by high-performance liquid chromatography (HPLC). The sequences of nucleotides (provided in the Table) were designed from the reported sequences of the various genes.

One microgram of total RNA or poly A mRNA was reversed transcribed (RT) and further amplified by polymerase chain reaction (PCR). Human GAPDH primers were used for normalization. Blank reaction controls with no RNA template or no Moloney-murine leukemia virus (MMLV)-RT were performed through the RT and PCR steps.

**Cloning and Sequencing**

The PCR products were extracted from the agarose gel with a commercial kit (Wizard Plus SV Minipreps DNA Purification System, Promega Corp), and DNA was then sequenced at the Weizman Institute Sequencing Facility (Rehovot, Israel).

**Southern Blot Hybridization**

Aliquots of the PCR products were subjected to electrophoresis, and the agarose gel was transferred to a Nytran 0.45-μm nitrocellulose (BA85; S&S), standard methods were followed. For Western transfer of protein to polyacrylamide gel (BA85; S&S), standard methods were followed.

**Immunohistochemistry of Human VSMCs and Human Umbilical Artery**

Cultured cells were washed with PBS, scraped, and lysed on ice in a lysis buffer containing PBS (7.4), 1% Triton X-100, 1 tablet of Boehringer protease inhibitors (Mannheim), and 0.1% SDS. This was followed by mild glass homogenization (10 strokes) and centrifugation at 10 000 g for 10 minutes at 4°C. Aliquots of the supernatant (cytosol) were saved for protein estimation and Western blot analysis. The SDS protein electrophoresis was performed on an 8% polyacrylamide gel. For Western transfer of protein to protean nitrocellulose (BA85; S&S), standard methods were followed.

**Platelet Preparation and Permeabilization and Measurement of 12-HETE**

Platelets were obtained on the day of the experiment as previously described. The platelet suspension was divided into 1-mL aliquots, which were used in the experiments. Platelet permeabilization was performed by incubation of platelets with 10 μg/mL digitonin for 10 minutes at 37°C in the presence of 1 mmol/L EGTA. The 12-LO antibody (against the exon 4-based peptide) was added (dilution, 1:330) for 10 minutes in the absence and presence of thrombin (0.2 U/mL). The reaction was stopped with cold ethanol (1:4 vol/vol).

For measurement of 12-HETE, we used a modification of the reverse-phase ultraviolet-HPLC method of Estra, which has been validated by radioimmunoassay, as previously described.

**Immunohistochemistry of Human VSMCs and Human Umbilical Artery**

Cultured cells were washed with PBS, fixed in situ, and enzymatically stained by the Histotain-Plus Bulk kit (Zymed Laboratories Inc) with the polyclonal 12-LO antibody generated for this study (dilution, 1:50). The human umbilical arteries were embedded in paraffin, sliced, mounted on poly-L-lysine–coated microscope slides, and stained by the same method.

**Statistical Analysis**

Results are expressed as mean±SEM. Student’s t test or 1-way ANOVA, followed by the Newman-Keuls post hoc analysis, was used as appropriate. Statistical significance was defined as P<0.05.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

**Results**

Expression of a Platelet-Type 12-LO in Cultured Human VSMCs

Two pairs of oligonucleotide nested primers, designed according to the reported sequence of the human platelet-type 12-LO mRNA, were used as indicated in the Table and Figure 1 for analysis of cultured VSMCs. Additionally, human 15-LO and porcine leukocyte 12-LO primers were applied for analysis of the same VSMC RNA preparations (Table). Figure 2 depicts the PCR-amplified products as a result of total RNA and poly A (+)mRNA using primers B

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**Table:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5-3)</th>
<th>Oligomer</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human 12-LO platelet type</td>
<td>GATGATCTACCTCTAAAAAAAAATG</td>
<td>B</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>CTGGCCCAAGAAGATCTG</td>
<td>C</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>TGGGTTGTGGACCATTGAGG</td>
<td>D</td>
<td>Sense</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>CCAATCCATCTTCAAGGAG</td>
<td>B</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>GTGTCTAGTGACTGGTCGAGG</td>
<td>C</td>
<td>Sense</td>
</tr>
<tr>
<td>Porcine leukocytes 12-LO</td>
<td>TTCATGTAGACGTGACGGAG</td>
<td>B</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>ACTGGCTGTTTGGAAAATGGAGC</td>
<td>C</td>
<td>Sense</td>
</tr>
<tr>
<td>Human 15-LO</td>
<td>AAATGAAGGGTACCTGGAG</td>
<td>B</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>ATATAGTTTTGCCAGCCCATTTCC</td>
<td>D</td>
<td>Sense</td>
</tr>
</tbody>
</table>

**Figure 1.** Overall structure of the human platelet-type 12-LO mRNA. Positions of oligomers B, C, and D, which served as primers, are indicated.
and C (exons 4 and 5, 12-LO). The predicted 175-bp fragment was present. However, a dominant reproducible 356-bp band was also present. After cloning, both bands were sequenced. The 175-bp band was identical to the expected exon 4 and 5 fragments. On the other hand, the major 356-bp band showed 100% homology with exons 4 and 5 of the published 12-LO mRNA sequence with the addition of another 115-bp fragment interposed between the exon sequences. After cloning and sequencing of the genomic region (exons 4 and 5 and the intron in between) of the human platelet 12-LO DNA, it turned out that the 115-bp sequence found between the exon 4 and 5 sequences after RNA amplification was identical to the genomic sequence of intron D (Figure 3; GenBank accession number, AF222784).

Figure 2. RT-PCR analysis of platelet-type 12-LO RNA in human VSMCs. Total and poly A (+)RNA were prepared from cultured human umbilical artery smooth muscle cells after 24 hours in serum-free medium. RNA samples were RT and subjected to PCR for 30 cycles with oligomers B and C at either 2 (A) or 4 (B) mmol/L MgCl₂ at an annealing temperature of 55°C. M represents the Alu I cut pBR322 molecular weight markers. Expected species appeared at 175 bp, and a new species appeared at 356 bp.

Similar results were obtained with the second pairs of primers B and D of exons 4 and 6 (Figure 4). A 605-bp band, rather than the expected 243-bp band, was seen. On sequencing, this band was found to include, in addition to the sequences of exons 4, 5, and 6, sequences consistent with introns D (GenBank AF222784) and E (GenBank AF222785) of the human platelet 12-LO gene. (Figure 3). The possibility that this unexpected novel form of platelet-type 12-LO mRNA reflected DNA contamination was addressed in several ways. First, we prepared total RNA by 2 different methods, 1 of which included DNAase treatment (to avoid DNA contamination). Second, we prepared polyA (+)mRNA from VSMCs. These studies (shown in Figures 2 and 4) consistently yielded the same enlarged fragments described above containing the known exon sequences with the additional intron D and E sequences. Third, total RNA was further purified by a novel phenol chloroform procedure, and again,
RT-PCR amplification confirmed the presence of the variant isoform of 12-LO containing exonized introns (Figure 5). These results were therefore consistent with the existence of a previously unrecognized isoform of the platelet-type 12-LO mRNA expressed in human VSMCs containing introns D and E (Figure 3; GenBank AF222784 and AF222785).

To further confirm the existence of this variant form of platelet-type 12-LO mRNA, Southern blot analysis of the PCR products obtained with primers B and D was performed. Specific exon and intron probes were prepared by restriction digestion of the pCR600G plasmid (Figure 6). One probe was complementary to intron E (187 bp), and the second was complementary to intron D (142 bp). Hybridization of the PCR products with either of the 2 probes yielded a 605-bp band in accordance with the presence of intron D and E sequences in the 12-LO mRNA expressed in these cells (Figure 7).

In contrast to these findings with human platelet-type 12-LO–based oligonucleotides, when primers that reportedly successfully identified human 15-LO or porcine leukocyte 12-LO expression were applied, no expression could be detected in the same VSMC RNA preparations (Figure 8).

**Regulation of 12-LO mRNA in Human VSMCs**

Cultured VSMCs incubated for 24 hours in medium 199 (containing 0.4% FCS and antibiotics) were treated with LPS 10 μg/mL or Ang II 10^{-7} mol/L for 24 hours or their respective vehicle as the control. In additional studies, cells were also treated with EGF (100 ng/mL for 30 minutes). Total RNA was prepared and RT-PCR amplified. Both the 356- and 605-bp bands, representing the variant platelet-type isoform, were downregulated by LPS (Figure 9A and 9B). Results of the densitometric analysis corrected for the 283-bp amplified products of GAPDH are presented in Figure 9B and further demonstrate the overall LPS-induced suppression of 12-LO mRNA. In contrast, EGF induced a 2- to 3-fold increase in the expression of this platelet-type isoform, as shown in Figure 10. No regulation of 12-LO mRNA was found with Ang II (10^{-7} mol/L for 2 to 24 hours; data not shown).

**Expression of a Platelet-Type 12-LO Protein**

Western immunoblotting of protein extracted from smooth muscle cells performed with the exon 4–based antibody directly detected the presence of 110-kDa protein. As shown in Figure 11, the same 110-kDa protein was seen in extracts

**Figure 5.** Effect of acid phenol chloroform treatment on the resultant PCR products. Total RNA prepared from VSMCs was treated with acid phenol chloroform to rule out contaminating DNA. Total RNA underwent touchdown PCR with oligomers B and D and an annealing temperature gradient of 69°C to 55°C in the presence of 2.5 mmol/L MgCl₂. Lane 1 (M) represents the Alu I cut pBR322 molecular weight markers; lanes 2 through 4, untreated total RNA; lanes 5 through 7, treated total RNA; lanes 8 through 10, negative controls for RT reaction from untreated RNA; and lanes 11 and 12, negative controls for the RT reactions with acid phenol chloroform treated RNA. The same band (605 bp) was obtained with and without acid phenol treatment of RNA.
from human VSMCs, umbilical cord homogenate, and platelet extract. This band appeared to be the dominant form in platelet extract, as well as the cord homogenate, and was the only isoform form seen in human VSMCs. Additionally, a 70-kDa band was also detected in platelet and cord homogenates with this antibody. A 120-kDa band and a 100-kDa band were also present in cord homogenates but not in VSMC extracts. Immunoblotting with an anti-leukocyte 12-LO resulted in no detectable bands (Figure 11).

**Functional Specificity of the 12-LO Antibody**

The functional specificity of the 12-LO antibody prepared for this study was assessed in permeabilized platelets to allow internalization of the antibody and its binding to the 12-LO enzyme within the platelets. Thrombin-stimulated 12-HETE generation was reduced by 48.5±5.2% (n=6; P<0.05) in the presence of this polyclonal 12-LO antibody (compared with nonimmune serum).

**Immunohistochemistry of 12-LO**

Representative immunohistochemistry studies of cultured human VSMCs obtained with our antibody against the exon 4 domain of platelet-type 12-LO are shown in Figure 12A through 12D. In either primary cultures (not shown) or intact umbilical artery cross sections, VSMCs showed extensive cytoplasmic staining with this antibody (Figure 12D). In contrast, the endothelial lining of the arteries remained stain free (Figure 12B).

**Substrate Specificity of the Human VSMC Platelet-Type 12-LO**

VSMCs were exposed to either arachidonic acid or linoleic acid (100 μmol/L each) for 1 hour. Cells were then washed twice and subsequently incubated for 10 minutes, after which eicosanoid and fatty acid content was determined. Both the supernatant and cells were harvested and prepared for HPLC analysis as described above. The mean uptake of arachidonic acid and linoleic acid was 15 and 37 μg/mg protein, respectively. As shown in Figure 13, after preincubation with arachidonic acid, the 12-, 11-, and 15-HETE could be easily detected (Figure 13B). Notably, however, the leukocyte-type 12-LO product of linoleic acid oxidation, 13-
hydroxyoctadienoic acid (13-HODE) acid, was not detected in cells preincubated with linoleic acid (Figure 13C).

**Discussion**

The present study demonstrates the presence of a novel platelet 12-LO isoform in human VSMCs derived from umbilical arteries. The detection of 12-LO mRNA in these cells was undertaken by use of a RT-PCR protocol with multiple primers designed to detect the reported human platelet-type 12-LO mRNA. Additionally, when primers were used to detect either porcine leukocyte 12-LO or human 15-LO with the same VSMC RNA preparations, no expression of these mRNAs was seen. Both of these leukocyte 12-LO and 15-LO sets of primers have been used successfully to identify these mRNAs in other systems. In previous studies, we have shown that cultured human VSMCs, human umbilical artery, and rat aortic strips generate 12-HETE, the production of which can be blocked by different LO inhibitors. The presence of platelet-type 12-LO in human VSMCs may confer substrate specificity with respect to lipid oxidation. There is evidence that platelet 12-LO is much more active with C20 acids such as arachidonic acid than with C18 and C22 acids. In contrast, the leukocyte type 12-LO preferentially oxygenates C18 and C22 polyenoic acids. That human VSMCs in the present study used arachidonic acid to produce 12-HETE but did not generate 13-HODE when exposed to linoleic acid is more consistent with the expression of a human platelet-type 12-LO in these cells.

Different activities of the 2 types of 12-LO have been also reported with respect to the use of phospholipids as substrates. The porcine leukocyte 12-LO enzyme oxygenates phosphatidylcholine containing arachidonic acid or linoleic acid at ~30% of its rate for the free fatty acids. In comparison, human platelet 12-LO appears much less active with this substrate. Thus, the existence of a platelet-type 12-LO in human VSMCs is consistent with a role for LO-driven 12-HETE formation from arachidonic acid in VSMC physiology. Evidence is accumulating that 12-HETE generated from arachidonic acid by 12-LO may be an important intracellular signal. For example, inhibition of diacylglycerol lipase, an enzyme known to provide arachidonate for 12-LO, reportedly lowers both Ang II–induced 12-HETE formation and aldosterone synthesis in glomerulosa cells. In contrast, inhibition of diacylglycerol kinase, which increases the availability of diacylglycerol for arachidonate cleavage, subsequently results in increased 12-HETE formation and aldosterone synthesis. In human VSMCs, preferential oxygenation of arachidonic acid released during activation of the VSMC signal transduction cascade by the platelet-type 12-LO may result in selective formation of 12-HETE to promote calcium transients and VSMC contraction without more generalized fatty acid oxidation.

In the present study, 12-LO RNA was not found to be regulated by Ang II. This suggests that the observed Ang II–driven 12-HETE formation in human VSMCs, which we previously reported, probably resulted from increased signaling along the appropriate transduction cascade rather than through increased 12-LO gene expression. Furthermore, the quick action of Ang II (within 7 minutes of application) in these studies is more consistent with quick signaling transduction than new gene expression.

In the present study, in addition to the already reported form of the platelet-type 12-LO, we found a variant isoform of platelet 12-LO that appears to be the dominant isoform in VSMCs. This previously unrecognized form of platelet-type 12-LO contains new sequence regions between perfectly conserved exons 4 and 5 and between exons 5 and 6. After parallel genomic cloning and sequencing of the human 12-LO gene were performed, the previously unsequenced introns D and E were compared with the new cDNA sequences and were found to be identical. Because we confirmed this with different approaches, including DNAase treatment of prepared total RNA preparations, poly A RNA preparation (to
rule out DNA contamination of RNA preparations), and Southern analysis of amplified cDNA (probed with pure intron D and E probes), we believe that this is a truly novel 12-LO variant isoform. It is noteworthy that multiple mRNA species for platelet 12-LO have been presented in previous studies involving several different nonvascular tissues. Because various functions have been attributed to 12-LO and its products, the possibility that the different isoforms may have different activities and/or different functions requires examination.

Western immunoblotting with a polyclonal antibody generated for this study (directed against an amino acid sequence encoded by exon 4) detected, in addition to the already recognized 70-kDa 12-LO protein seen in platelets and cord homogenate, a previously unreported band of 110 kDa present in both platelets and VSMCs. Because a variant novel 12-LO mRNA isoform exists in VSMCs, the presence of an additional, larger, 12-LO protein is not surprising. Moreover, 2 additional isoforms, 100 and 120 kDa, are also evident within the whole-cord extracts but not the cultured VSMCs or platelets. These may be unique to the cells found within cord or may be downregulated in VSMCs on culture. However, because the putative variant platelet-type 12-LO in VSMCs has not been fully characterized and sequenced in this study, an exact accounting of the variant structure of this novel protein is not yet possible. Nevertheless, because the exon 4 epitope 12-LO antibody was able to inhibit the generation of 12-HETE in platelets, it appears that a platelet-type isoform is indeed responsible for the 12-LO activity found in these cells. The incomplete inhibition of platelet 12-LO activity in this study may have resulted from an incomplete inactivating capacity of the antibodies we generated. It is of interest that we found not only that this antibody stained VSMCs in culture or in slices of human umbilical cord but also that the staining appeared to be cytoplasmic in nature. This is consistent with several other reports that 12-LO is localized in the cytoplasm.

Previous reports provided evidence for the existence of other forms of LO in human vascular tissue. Yla-Herttuala and coworkers noted expression of 15-LO mRNA and the formation of 15-LO products in human atherosclerotic human arteries merely express different 12-LO forms that should prompt further research to determine whether different vascular tissues express different 12-LO forms. Taken together, our results and previous reports suggest that no major differences in in vivo differentiation, or differences in origin of the vascular tissue (aortic versus umbilical tissue), with these reports. Rather, they may reflect differences in the degree of involvement in atherosclerosis-related processes. Taken together, our results and previous reports should prompt further research to determine whether different human arteries merely express different 12-LO forms that serve a similar purpose (eg, maintaining vascular tone) or whether these different isoforms reflect variation in arterial function, adaptation, or propensity to atherosclerosis.

In conclusion, this is the first study to demonstrate the existence of a platelet-type 12-LO gene expression in human VSMCs. The function of the variant 12-LO mRNA and protein isoforms in VSMCs will be the subject of further investigation.

References


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