Elevated glucose and angiotensin II increase 12-lipoxygenase activity and expression in porcine aortic smooth muscle cells

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ABSTRACT The lipoxygenase (LO) pathway of arachidonate metabolism has been suggested to play a key role in atherosclerosis and in mediating several actions of angiotensin II (AII). However, the relationship between LO activation and factors linked to accelerated diabetic vascular disease such as hyperglycemia and AII is not known. We have investigated the effect of high glucose (HG; 25 mM) and AII on LO activity as well as LO protein and mRNA expression in porcine aortic vascular smooth muscle cells (PVSMCs). We observed that cells cultured in HG had significantly higher levels of the cell-associated LO products 12- and 15-hydroxyeicosatetraenoic acids (HETEs). AII added to cells grown in HG specifically further increased only cell-associated 12-HETE levels. Using immunoblot analysis and reverse transcriptase PCRs, we demonstrated the presence in PVSMCs of porcine leukocyte-type 12-LO protein and mRNA, respectively. Furthermore, the levels of both were markedly upregulated by AII as well as by HG. These studies suggest that enhanced 12-LO activity and expression are mechanisms for accelerated vascular disease produced by HG and AII in diabetes mellitus.

The mechanisms underlying the accelerated atherosclerotic disease associated with diabetes mellitus are not very clear. Prolonged hyperglycemia is considered to be a key factor in leading to diabetic vascular complications since it can alter key biochemical pathways related to glucose metabolism (1). Elucidation of the mechanisms that regulate vascular smooth muscle cell (VSMC) proliferation is crucial to understanding the increased risk of cardiovascular disease in diabetes. We have recently shown that porcine VSMCs (PVSMCs) that were cultured in high glucose (HG; 25 mM) proliferate at a significantly faster rate and with increased cell-associated levels of the 12-lipoxygenase (12-LO) product of arachidonic acid, 12-hydroxyeicosatetraenoic acid (12-HETE), than cells cultured in normal glucose (NG; 5.5 mM) (2). Recent studies have implicated the LO enzymes and their products—namely, HETEs and hydroxyoctadecadienoic acids (HODEs)—in the pathogenesis of atherosclerosis (3). It was observed that the 15-LO mRNA and protein colocalize with epitopes of oxidized low density lipoprotein (LDL) (4) and cellular oxidation of LDL can be attenuated by LO inhibitors (5). Furthermore, increased levels of HETEs and HODEs were observed in the aortas of atherosclerotic rabbits (6). HETEs have also been suggested to play a key role in the pathogenesis of diabetic vascular disease, since vessels from infants of diabetic mothers had significantly elevated levels of 15-HETE and decreased formation of vasodilatory prostacyclin (7) and endothelial cells cultured under hyperglycemic conditions produced increased amounts of HETEs (8). Studies also suggest that HETEs have mitogenic properties (9) and can cause SMC migration at concentrations as low as 1 pM (10).

It has been demonstrated that the vasopressor effects of angiotensin II (AII) are enhanced in the diabetic state (11). Increasing evidence also indicates that AII is an important local factor in leading to VSMC hypertrophy and neointimal SMC proliferation (12, 13). We have previously shown (14, 15) that the 12-LO pathway of arachidonic acid plays a key role in AII-induced aldosterone synthesis and adrenal cell proliferation. Furthermore, inhibition of the LO pathway can reduce vasopressor responses to AII (16). In the present study, we have investigated the effects of HG and AII on LO pathway activation in cultured PVSMCs.

A leukocyte type of 12-LO has been purified and cloned from porcine leukocytes (17, 18). However, no studies have addressed whether this form of 12-LO is also present in VSMCs. Furthermore, the factor(s) regulating the expression of 12-LO is not known. In the present study, we determined whether the 12-LO enzyme is expressed in PVSMCs. We also investigated whether the 12-LO protein and mRNA levels were regulated by glucose and AII to determine whether the increases in 12-LO activity induced by HG or AII were at the translational and/or the transcriptional level.

MATERIALS AND METHODS

Culture of PVSMCs. Primary cultures of PVSMCs were obtained as described (2). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing NG (5.5 mM) and 10% fetal calf serum (FCS). For studies under hyperglycemic conditions, the cells were cultured for at least two passages in DMEM HG (25 mM) before use. Controls for osmolality were cells grown for at least two passages in NG/19.5 mM mannose.

Measurement of LO Products 12- and 15-HETE. Serum-starved (24 hr) confluent cells in NG or HG in 100-mm dishes were placed in medium containing 0.2% bovine serum albumin (BSA) and preincubated for 20 min at 37°C, with LO or cyclooxygenase inhibitors added during the last 10 min. AII (Peninsula Laboratories) was then added and cells were incubated for an additional 10 min. The reaction was terminated by cooling on ice. The HETE(s) in the supernatants was extracted as described (19). Cell pellets were first deacylated to release the cell-associated esterified HETEs by treating them with 1.5 ml of 0.2 M NaOH in methanol and 50 μ M *n*-propyl gallate [an inhibitor of nonspecific fatty acid oxidation (6)] under N₂ for 45 min. The solution was then diluted to 15% methanol and extracted (19). The LO products in the supernatants as well as cell extracts were quantitated by a specific RIA (14). The 12-HETE RIA is specific for 12(S)-HETE with <0.1% crossreactivity with 12(R)-HETE. The identity of the HETEs was confirmed by comigration

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Abbreviations: VSMC, vascular smooth muscle cell; PVSMC, porcine VSMC; HG, high glucose; NG, normal glucose; LO, lipoxygenase; HETE, hydroxyeicosatetraenoic acid; AII, angiotensin II; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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with authentic cold 12(*S*)- and 15(*S*)-HETE using our gradient reverse-phase HPLC system (14).

Measurement of LO Activity. Confluent PVSMCs in NG or HG medium were harvested, suspended in 1 ml of Tris-HCl buffer (25 mM; pH 7.7), and then sonicated on ice. The assay mixture contained in 1.0 ml, 450 μ l of enzyme (sonicate), 400 μ l of Tris buffer, and 100 μ l of glutathione (0.5 mM). An enzyme blank was run simultaneously. The reaction was started at 37°C with 50 μ l of sodium arachidonate (160 μ M; Nu Check Prep, Elysian, MN). After a 10-min incubation, the reaction was stopped with 2 ml of isopropanol/1.2% acetic acid followed by 2 ml of chloroform. The lower organic layer was filtered and subjected to HPLC to detect HETEs. Peak heights were quantitated with a Shimadzu CR5A integrator.

Incubations for 12-LO Protein or mRNA Expression. Serum-starved PVSMCs in NG or HG in 150-mm dishes were placed in DMEM/Hepes NG or HG containing 0.2% BSA and 0.4% FCS without antibiotics, alone, or with AII. At the end of the incubation, cell pellets were processed for Western blotting or RNA extraction as described below.

Electrophoresis and Western Immunoblotting. Cell pellets were lysed in buffer containing phosphate-buffered saline (pH 7.4), 1% Triton X-100, 50 μ M leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% SDS. Lysates were centrifuged at 10,000 $\times$ *g* for 10 min and supernatants were subjected to electrophoresis and Western blotting as described (15). A polyclonal antibody against porcine leukocyte 12-LO (20) (generous gift from T. Yoshimoto, Tokushima University, Tokushima, Japan) was used at 1:600 dilution. Western blots were quantitated with a computerized video densitometer and values were expressed as arbitrary optical density (OD) units. For antibody blocking studies, the 12-LO antibody (4 μ l) was incubated with or without 30 μ l (16 μ g of protein) of partially purified 12-LO enzyme [25–50% ammonium sulfate fraction of porcine leukocyte cytosol (21); gift from T. Yoshimoto] in 500 μ l of Tris-buffered saline (pH 7.4) containing 1 mg of BSA per ml for 2.5 hr at room temperature. The blocked and free antibodies were then diluted 1:600 and used to incubate the blots containing the PVSMC cytosol samples.

cDNAs. Recombinant Bluescript plasmid containing the cDNA for human reticulocyte 15-LO was kindly provided by E. Sigal (University of California, San Francisco). pUC19 plasmid containing the cDNA for porcine leukocyte 12-LO (18) was a generous gift from T. Yoshimoto. The full-length 15-LO cDNA and cDNA for porcine leukocyte 12-LO were prepared by *Eco*RI and *Sal* I digestion of the plasmids, respectively.

Oligonucleotide Primers and Probes for PCR. All the oligonucleotides including human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by polyacrylamide gel electrophoresis. The sequences of oligonucleotides (Table 1) were designed based on known gene sequences (18, 22, 23) and were selected from regions dis-

playing most divergence between porcine 12-LO and human 15-LO sequences (24) because of their close homology.

Amplification of Reverse-Transcribed RNA by PCR. PVSMC total RNA was extracted with guanidium thiocyanate/phenol/chloroform using RNeasy (Cinna/Biotecx Laboratories, Friendswood, TX). Poly(A)⁺ RNA was purified by oligo(dT)-cellulose column chromatography (5 Prime $\rightarrow$ 3 Prime, Inc.). One microgram of total RNA or 0.5 μ g of mRNA was mixed with the PCR buffer (10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM MgCl₂/0.001% gelatin), 200 μ M each dNTP, 25 pmol of each 5' and 3' primer, 2 units of avian myeloblastosis virus reverse transcriptase (20 units/ μ l; Life Sciences, St. Petersburg, FL), and 2.5 units of *Taq* polymerase (Perkin-Elmer/Cetus) in a final vol of 50 μ l. In all reactions, 5 pmol of each 5' and 3' primer of GAPDH was added as an internal standard to control for RNA quantity and amplification efficiency. The samples were placed in a thermal cycler at 37°C for 8 min for the reverse transcriptase reaction to proceed. Then conditions used for PCR were a denaturation step at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min for 25 cycles. Blank reactions with no RNA template or with no reverse transcriptase were carried out through the reverse transcription and PCR steps. RNA samples from human erythroleukemia (HEL) cells, which do not express the porcine leukocyte 12-LO, were run as negative controls. For positive controls, the human 15-LO cDNA and porcine 12-LO cDNA amplifications were carried out.

Gel Analysis and Blot Hybridization. The PCR products were electrophoresed on 1.8% agarose gels followed by capillary blotting onto Zeta-Probe (Bio-Rad) membrane. Porcine 12-LO, human 15-LO, and human GAPDH oligonucleotides used as probes were labeled at the 5' end with [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs) and hybridized with membrane overnight in 6 $\times$ standard saline citrate (SSC)/0.5% non-fat dry milk/7% SDS at 42°C. Membranes were washed once in 6 $\times$ SSC at room temperature for 15 min and then once at 60°C for 15 min. These washing conditions were developed to distinguish between the PCR products of human 15-LO from those of porcine leukocyte 12-LO. After autoradiography, blots were quantitated by using a computerized video densitometer.

RESULTS

Effect of Glucose on Immunoreactive Cell-Associated and Released HETE Levels. Fig. 1 shows that PVSMCs cultured in HG (25 mM) had significantly greater levels of cell-associated 12- as well as 15-HETE than those cultured in NG (5.5 mM). In contrast, cell associated 12-HETE levels in mannose-grown cells were not significantly different from those in cells cultured in NG (1493 $\pm$ 281 pg per 10⁶ cells in NG vs. 1386 $\pm$ 232 pg in mannose). HG did not have any significant effect on the levels of released HETEs (data not shown).

Table 1. PCR primers and probes for amplification and detection

		Sequence (5' to 3')	Position
Porcine leukocyte 12-LO	5' primer	TTCAGTGTAGACGTGTCCGAG	145–165
	3' primer	ATGTATGCCGGTGTCTGGCTATATTAG	451–477
	Probe	TCAGGATGCCGTGCGCCCTCCAC	301–322
Human 15-LO	5' primer	AACTCAAGGTGGAAGTACCGGAG	146–168
	3' primer	ATATAGTTTGGCCCCAGCCATATTC	453–477
	Probe	AGGCTCAGGACGCCGTTGCC	306–326
Human GAPDH	5' primer	CCCATCACCATCTTCCAGGAG	211–231
	3' primer	GTTGTCATGGATGACCTTGGC	475–495
	Probe	CTAAGCAGTTGGTGGTGCAGG	446–466

FIG. 1. Effect of HG (25 mM) on cell-associated 12- and 15-HETE levels in PVSMCs. HETEs in cell pellets were measured after a 20-min incubation in serum-free medium. Results are expressed as means $\pm$ SE from five experiments performed in duplicate. *, $P < 0.01$ vs. NG, paired.

Effect of AII on Immunoreactive Cell-Associated and Released HETEs. AII significantly increased cell-associated 12-HETE levels in cells cultured in HG only (Fig. 2). However, AII at concentrations ranging from 100 nM to 100 pM did not increase cell-associated 12-HETE levels in cells cultured in NG. Cell-associated 15-HETE levels were not significantly increased by AII either in HG [basal 15-HETE, 2722 ± 225 pg per 10^6 cells; AII (10 nM), 2928 ± 188 pg] or in NG (data not shown).

Table 2 shows that AII treatment for 10 min causes a dose-dependent increase in released 12- and 15-HETE levels in NG. AII at 2 nM selectively increased only 12-HETE. AII (100 nM) also stimulated released 12- and 15-HETE levels in HG (Table 3). The specific LO inhibitor baicalein (10 μ M) (Biomol, Plymouth Meeting, PA) significantly reduced the AII-induced release of 12- and 15-HETE in HG without altering basal HETE release (Table 3). In addition, baicalein also significantly reduced the increase in cell-associated 12-HETE formation by AII in cells grown in HG [2145 ± 90 (basal) vs. 2760 ± 160 (AII) vs. 2004 ± 114 (AII + baicalein) pg per 10^6 cells; $P < 0.05$]. The cyclooxygenase inhibitor meclofenamate (Sigma), however, attenuated AII-induced release only of 15-HETE and not of 12-HETE.

12-LO Activity in PVSMC Sonicates. Fig. 3 shows the HPLC tracings of extracts of sonicates from cells in NG and HG alone or treated with AII for 10 min. Fig. 3A shows the retention times of authentic 12- and 15-HETE. An enzyme blank (Fig. 3B) was run to control for nonspecific oxidation of arachidonic acid. A distinct peak with the same retention time as 12-HETE was seen in NG as well as in HG control cells (Fig. 3C and E), indicating that PVSMCs have 12-LO activity. A 35% increase

FIG. 2. Effect of AII (10 nM) on cell-associated 12-HETE levels in NG or HG. Results are expressed as means $\pm$ SE from four experiments performed in duplicate. *, $P < 0.01$ vs. NG control; **, $P < 0.05$ vs. HG control, paired.

Table 2. Dose-dependent effects of AII on released 12- and 15-HETE levels in PVSMCs grown in NG

	12-HETE, pg per 10^6 cells	15-HETE, pg per 10^6 cells
Basal	119 $\pm$ 25	126 $\pm$ 10
AII (0.2 nM)	108 $\pm$ 27	119 $\pm$ 22
AII (2 nM)	171 $\pm$ 30*	160 $\pm$ 40
AII (20 nM)	239 $\pm$ 21**	199 $\pm$ 14**
AII (200 nM)	248 $\pm$ 32**	220 $\pm$ 30**

12- and 15-HETE levels were measured by a specific RIA of cell supernatants after a 10-min treatment with or without AII. Results are expressed as means $\pm$ SE from three to six experiments run in duplicate. *, $P < 0.05$ vs. basal; **, $P < 0.01$ vs. basal.

in 12-LO activity (12-HETE formation) was consistently seen in HG control cells as compared to those in NG control cells. AII (100 nM) caused a further increase (38%) in 12-LO activity in NG (Fig. 3D) and an additional increase (60%) in HG (Fig. 3F). Under these assay conditions for LO activity, no clear production of 15-HETE was detected.

Regulation of Porcine Leukocyte-Type 12-LO Protein and mRNA by AII and HG. The 12-LO protein was identified by immunoblotting with a specific antibody to the porcine leukocyte 12-LO. A distinct band was detected with a molecular mass of nearly 72 kDa (Fig. 4A, upper band), which is the reported molecular mass of the porcine leukocyte-type 12-LO (20). In addition, antibody blocking studies were performed to confirm the identity of the band. Immunoprecipitation of the 72-kDa band obtained with authentic porcine leukocyte 12-LO enzyme (lane 1) as well as PVSMC cytosols (lanes 2 and 3) could be completely prevented by using antibody that had been pretreated with partially purified porcine leukocyte 12-LO enzyme (Fig. 4B). Furthermore, one or two lower bands previously visible in the Western blots were also eliminated in the blocking studies. The source of these bands is not clearly known at the present time.

Basal 12-LO enzyme (72 kDa) expression is increased nearly 5-fold in PVSMCs cultured in HG (Fig. 5). In addition, AII (100 nM) at 45 hr caused a marked stimulation (2- to 3-fold) of 12-LO expression in NG as well as HG (Fig. 5). The 12-LO antibody did not detect any 72-kDa bands with lysates from human platelets and HEL cells (results not shown). We also probed the blots with an antibody to human 15-LO (gift from E. Sigal) and could not detect any bands near 70 kDa, the reported molecular mass for 15-LO (data not shown).

To evaluate the expression and regulation of 12-LO mRNA in PVSMCs, we used a reverse transcriptase PCR assay because of the very low expression of this mRNA. The size of the PCR amplified fragment is 333 bp for both 12- and 15-LO. Specific conditions to distinguish leukocyte 12-LO from human 15-LO were achieved by increasing stringency and raising washing temperature of hybridized membranes to

Table 3. Effect of LO and cyclooxygenase blockers on AII-induced HETE release in PVSMCs grown in HG

Treatment	Released 12-HETE, pg per 10^6 cells	Released 15-HETE, pg per 10^6 cells
Basal	120 $\pm$ 10	138 $\pm$ 15
AII (100 nM)	190 $\pm$ 28*	196 $\pm$ 18*
AII + baicalein (10 μ M)	141 $\pm$ 26**	144 $\pm$ 11**
AII + meclofenamate (1 μ M)	170 $\pm$ 19*	149 $\pm$ 15**
Baicalein (10 μ M)	129 $\pm$ 12	127 $\pm$ 17

Results are expressed as means $\pm$ SE from three experiments. Incubations and measurement of immunoreactive released HETEs in the supernatants were performed as described. *, $P < 0.05$ vs. basal; **, $P < 0.05$ vs. AII.

FIG. 3. Reverse-phase HPLC analysis of products of cold arachidonate metabolism by sonicated PVSMCs grown in NG (C and D) or HG (E and F). Detection was at 237 nm. Arrows a and b indicate positions of 15- and 12-HETE, respectively. (A) Retention time of authentic cold 15- and 12-HETE (17.2 and 18.0 min, respectively). (B) Tracing of enzyme blank. (C and E) Basal (control) activity in NG and HG, respectively. (D and F) Activity with 10-min AII (100 nM) treatment in NG and HG, respectively. Results shown are representative of three experiments.

60°C. PCRs were run at 25 cycles to maintain amplification in a linear range.

Fig. 6 is a Southern blot showing that the 333-bp specific amplified products of porcine leukocyte-type 12-LO are detected in PVSMCs cultured in both NG as well as HG. In addition, cells cultured in HG had a 20-fold increase in expression of the 12-LO PCR amplified product. OD of the 12-LO band in arbitrary units: HG, 3092; NG, 137. OD of GAPDH band (284 bp): HG, 1247; NG, 1115. Fig. 7 shows that 12-LO mRNA expression (333 bp) was much greater in HG (with little basal expression in NG). In addition, AII (100 nM) treatment for 24 hr caused a significant 3- to 4-fold increase in expression in both NG and HG according to the following OD readings: 12-LO band, HG control, 1072; HG AII, 3000; NG control, 40; NG AII, 303; GAPDH band, HG control, 1214; HG AII, 1293; NG control, 667; NG AII, 1168. We were unable to detect any amplified products of 15-LO with specific PCR primers and probes for 15-LO (data not shown).

DISCUSSION

The 12- and 15-LO enzymes have recently been isolated and their cDNAs were cloned. 15-LO was cloned from human reticulocytes (22). One form of 12-LO was cloned from human platelets and HEL cells (24–26). 12-LO has also been

FIG. 4. Specific expression of porcine leukocyte 12-LO protein in PVSMCs. Lanes: 1, partially purified authentic porcine leukocyte 12-LO; 2 and 3, PVSMC cytosols. (A) With porcine leukocyte 12-LO antibody. (B) With 12-LO antibody preincubated with authentic 12-LO.

FIG. 5. Expression of porcine leukocyte-type 12-LO (72 kDa) in PVSMCs cultured in NG or HG and after AII (100 nM) treatment for 45 hr. Equal amounts of protein were electrophoresed and blots were probed with a specific antibody to porcine leukocyte 12-LO. Similar results were found in three separate experiments.

identified and cloned from porcine leukocytes (17, 18) and bovine tracheal epithelial cells (27, 28). Human platelet and porcine leukocyte 12-LO enzymes share only 65% amino acid identity (24–26). In contrast, the leukocyte-type 12-LO shows nearly 87% homology with the 15-LO sequence (18). In the present study, we showed that PVSMCs express the porcine leukocyte-type 12-LO, since a specific antibody to the leukocyte 12-LO immunoprecipitated a protein with a molecular mass similar to that of the porcine leukocyte 12-LO. Furthermore, using a polyclonal human 15-LO antibody, no specific band was seen around 70 kDa. We also used a specific reverse transcriptase PCR technique to evaluate 12-LO mRNA expression in PVSMCs. Using the porcine leukocyte 12-LO primers and probe, we could amplify and detect the expected 333-bp amplified product. However, no clear product was found with the 15-LO primers and probe. It is possible that the 15-HETE made by these cells, in part, arises from the 12-LO pathway or from the cyclooxygenase pathway since our data show that cyclooxygenase inhibitors can block release of only 15-HETE. However, the results do not completely exclude the possibility that PVSMCs also express a type of 15-LO that may differ sufficiently in homology with the human 15-LO so as not to be detected with the human 15-LO antibody or probe.

We recently demonstrated that PVSMCs cultured in HG proliferate faster and have greater total cellular protein content than those in NG (2). Similar results were also observed in rat VSMCs (29). The results of the present study indicate that PVSMCs cultured chronically in HG synthesize greater amounts of the 12-LO product 12-HETE and have increased 12-LO activity as compared to cells cultured in NG. These effects were not simply due to the hyperosmolar effects of HG since equimolar concentrations of mannose did

FIG. 6. Identification of porcine leukocyte-type 12-LO mRNA in PVSMCs by reverse transcriptase PCR and its regulation by HG. One-microgram total RNA samples were amplified for 25 cycles with porcine leukocyte 12-LO primers. Positive control with 12-LO cDNA is shown in the rightmost lane. Similar results were found in three separate experiments. (A) Hybridized with 12-LO oligonucleotide probe. (B) Same membrane hybridized with GAPDH probe.

FIG. 7. Regulation of porcine leukocyte-type 12-LO mRNA by AII (100 nM) treatment for 24 hr in PVSMCs cultured in NG or HG. mRNA samples (0.5 μ g each) were amplified for 25 cycles with porcine leukocyte 12-LO primers. Similar results were obtained in two separate experiments. (A and B) Hybridization with 12-LO oligonucleotide and GAPDH probes, respectively.

not result in excess PVSMC cell growth (2) or increased 12-LO product formation. Although 12-HETE release was not increased in these cells cultured chronically in HG, recent evidence shows that acute HG treatment (24–48 hr) can stimulate levels of released 12-HETE (unpublished observations). Previous studies showed that glucose can increase 12-HETE levels in rat islets (30). Very few studies have evaluated changes in gene expression by HG. Very recently, HG was shown to increase transforming growth factor α and fibroblast growth factor mRNAs in rat VSMCs (31). In the current study, we have shown that HG upregulates 12-LO mRNA and protein in PVSMCs.

In the current report, we also observed that AII specifically increased cell-associated 12-HETE concentrations in cells cultured in HG. Therefore, the 12-LO pathway may be specifically involved in mediating effects of AII in VSMCs when exposed to chronically high levels of glucose as in diabetes. In addition, we have demonstrated that AII can directly increase 12-LO mRNA and protein expression in PVSMCs cultured in NG or HG. Therefore, HG and AII may produce additive effects on 12-LO activation in VSMCs. Previous studies have shown that HG can enhance AII-induced hypertrophy in renal tubular cells (32).

The effects of AII on HETE levels were measured only in short-term incubations since HETEs can be reesterified into membrane lipids and also metabolized to several products. The acute effects of AII on HETE formation are most likely via activation of phospholipases and substrate release, while the longer-term effects are also via increases in LO expression. The precise mechanism of how AII and HG can increase LO mRNA or protein expression needs to be investigated. However, the 12-LO promoter region may be sensitive to protein kinase C (33). Therefore, both AII and glucose may increase 12-LO expression by activation of protein kinase C. The mechanism of acute stimulatory effects of AII on HETEs in PVSMCs may be by providing more substrate through diacylglycerol metabolism (19).

12-LO products can be mitogenic and activate several systems linked to increased vascular disease and cell proliferation including protein kinase C (15, 34) and activity of the *ras* oncogene (35). Furthermore, 12-HETE can activate *c-fos* oncogene expression (36) and also lead to SMC migration (10). We have also observed in preliminary studies that inhibition of the LO pathway with baicalein markedly reduces excess proliferation of PVSMCs cultured in HG toward levels seen in NG and that LO inhibition can reduce AII-induced hypertrophic effects in VSMCs (unpublished observations). In the current report, we show that this same concentration of baicalein inhibits 12-HETE formation in PVSMCs. Therefore, it is possible that excess VSMC growth under hyperglycemic conditions may be due to excess 12-LO activation. However, additional studies with selected products of the 12-LO and other LO pathways may be needed to define the precise pathway or products involved. The *in vivo* relevance of these findings will need to be addressed by additional studies in diabetic animal models and in humans.

Nevertheless, the marked activation of the 12-LO pathway by HG and AII in this model cell system suggests that 12-LO activation may be a key mechanism for accelerated vascular disease produced by hyperglycemia and AII in diabetes mellitus. Support for this hypothesis is recent preliminary evidence showing marked increases in 12-HETE excretion in patients with type II diabetes compared to controls (37).

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