Role of the Lipoxygenase Pathway in Angiotensin II–Induced Vascular Smooth Muscle Cell Hypertrophy

Rama Natarajan, Noe Gonzales, Linda Lanting, Jerry Nadler

Abstract The 12-lipoxygenase pathway is a key mediator of angiotensin II (Ang II)–induced effects in the adrenal cortex. We also recently demonstrated that Ang II increases 12- and 15-lipoxygenase product levels in vascular smooth muscle cells. However, the relation between lipoygenase activation and Ang II–induced vascular smooth muscle cell hypertrophy is not known. We investigated effects of Ang II and increased lipoygenase products on both total cell protein content and the levels of the matrix protein fibronectin in quiescent porcine aortic smooth muscle cells. Ang II–induced increases in cellular protein content were attenuated by the specific 12-lipoxygenase inhibitor baicalein; in contrast, the cylooxygenase inhibitor ibuprofen had no effect. Direct addition of the 12-lipoxygenase product 12-S-hydroxyeicosatetraenoic acid increased total cell protein content. We have recently shown that porcine vascular smooth muscle cell growth is potentiated in high glucose (25 mmol/L) culture conditions. We observed that both Ang II and 12-S-hydroxyeicosatetraenoic acid induced a greater increase in protein content in cells cultured for two passages in high glucose. Furthermore, Ang II and 12-S-hydroxyeicosatetraenoic acid also markedly increased fibronectin levels in cells cultured in high glucose. These results suggest that 12-lipoxygenase activation plays a key role in Ang II–induced vascular smooth muscle cell hypertrophy. Furthermore, both Ang II and lipoygenase effects are enhanced in cells cultured under hyperglycemic conditions. (Hypertension. 1994;23[suppl I]: I-142-I-147.)

Key Words • angiotensins • lipoxygenase • muscle, smooth, vascular • hypertrophy • fibronectins

Angiotensin II (Ang II) has major effects on vascular smooth muscle cell (SMC) growth in vitro and in vivo.1-4 In particular, it has become clear that Ang II–induced SMC hypertrophy is accompanied by increases in the synthesis of key extracellular matrix proteins such as collagen and fibronectin.5

It has previously been shown that the 12-lipoxygenase pathway of arachidonate metabolism plays an important role in Ang II action on growth and steroidogenesis in the adrenal cortex.6,7 We have also recently demonstrated that a leukocyte type of 12-lipoxygenase enzyme is present in porcine aortic SMCs (PSMCs) and that Ang II can increase the formation of the 12-lipoxygenase product, 12-hydroxyeicosatetraenoic acid (12-HETE) and also increase the expression of the 12-lipoxygenase mRNA and protein.8 However, the role of this 12-lipoxygenase pathway on Ang II–induced SMC hypertrophic responses has not been studied.

Diabetes mellitus is known to be associated with a higher rate of hypertension and atherosclerotic cardiovascular disease.9 Previous evidence suggests that the vasoconstrictive effects of Ang II may be increased in diabetes.10 It has also been demonstrated recently that PSMCs cultured in elevated glucose show increased activity and expression of 12-lipoxygenase.8 However, the effects of high glucose conditions on Ang II–induced SMC hypertrophic responses have not been studied previously.

In this report, the role of the 12-lipoxygenase pathway in Ang II–induced protein and fibronectin synthesis was determined. Furthermore, these responses were compared in PSMCs cultured in normal glucose and high glucose. The results suggest that 12-lipoxygenase activation plays a key role in Ang II–induced SMC hypertrophy and that Ang II effects are enhanced in PSMCs cultured under high glucose conditions.

Methods Culture of Porcine Aortic Smooth Muscle Cells Primary cultures of PSMCs were obtained as described earlier.11 Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing normal glucose 5.5 mmol/L and 10% fetal calf serum (FCS). For studies under hyperglycemic conditions, the cells were allowed to grow through at least two passages in DMEM high glucose (25 mmol/L) before use. Control for osmolality were cells grown for at least two passages in normal glucose plus 19.5 mmol/L mannose.

Measurement of Total Cellular Protein Content PSMCs in normal glucose or high glucose (approximately 90% confluent) in 12-well plates were serum depleted for 24 hours by placing in medium containing 2% bovine serum albumin (BSA) and 0.4% FCS. This medium was then freshly added along with agonists such as Ang II (Peninsula Laboratories, Belmont, Calif), 12-HETE, or 15-HETE (BIOMOL Research Laboratories Inc, Plymouth Meeting, Pa), and cells were incubated for 40 hours at 37°C. HETEs were added from 1000-fold concentrates in dimethyl sulfoxide (DMSO). The vehicle, DMSO, was added to the controls. In some experiments, before Ang II addition, cells were preincubated for 15 minutes with Ang II receptor antagonists losartan (subtype 1 [AT1]) or PD123177 (subtype 2 [AT2]) (generous gifts from Du Pont Merck, Wilmington, Del); a specific lipoygenase inhibitor, baicalein (BIOMOL); or a cyclooxygenase inhibitor,
Iipoxygenase and Vascular Smooth Muscle Growth

**Measurement of Fibronectin**

Quiescent 80% to 90% confluent PSMCs in normal glucose or high glucose in 12-well dishes were placed in media containing 0.2% BSA and 0.4% FCS without phenol red and treated for 40 hours with agonists. The supernatants were assayed for released fibronectin, while washed cell layers were sonicated in 500 μL of 1% nonidet P-40 in PBS and assayed for cell-associated fibronectin. Fibronectin levels in all samples were determined by a double-antibody sandwich enzyme-linked immunosorbent assay using the methods provided by the manufacturer (DAKO Corp, Carpinteria, Calif) and Wolthuis et al. A polyclonal rabbit anti-human fibronectin (1:1000) was used as the coating antibody, and the detection antibody was a peroxidase-conjugated rabbit anti-human fibronectin (1:2000).

**Statistical Analysis**

Results were analyzed using the Student's *t* test. For multiple comparisons or dose responses, analysis of variance was used. Duncan's test was also used for multiple comparisons.

**Results**

**Hypertrophic Effects of Angiotensin II in PSMCs Cultured in Normal Glucose or High Glucose**

Ang II produced a dose-dependent increase in total cellular protein content in cells cultured in either normal glucose or high glucose conditions (Fig 1). Furthermore, the effects of Ang II, especially at concentrations of $10^{-7}$ and $10^{-6}$ mol/L, were clearly potentiated by culturing the PSMCs in high glucose for at least two passages (Fig 1). Ang II did not cause any significant increase in cell number in these experiments (results not shown).

To determine the Ang II receptor subtype involved in the changes in protein levels, we performed additional experiments using the selective AT1 receptor antagonist losartan (DuP 753) and the AT2 blocker PD123177. As shown in Fig 2, losartan but not PD123177 blocked Ang II-induced increases in total cellular protein content. Neither the AT1 nor AT2 antagonist alone had any effect on basal protein content in either normal glucose or high glucose (data not shown).

**Effect of Lipoxygenase and Cyclooxygenase Inhibition on Angiotensin II–Induced Increase in Cellular Protein Content**

Fig 3 demonstrates the effect of the specific lipoxygenase inhibitor baicalein and specific cyclooxygenase inhibitor ibuprofen on Ang II–induced cellular protein content. Neither inhibitor alone significantly altered basal cellular protein levels. However, baicalein, the lipoxygenase inhibitor, completely blocked Ang II–induced protein increases. In marked contrast, the cyclooxygenase inhibitor, ibuprofen, had no effect on Ang II action (Fig 3).

**Direct Effect of 12- and 15-Lipoxygenase Products on Cellular Protein Content**

Ang II has been shown to directly increase 12- and 15-HETE levels in PSMCs cultured in normal glucose and high glucose. We therefore evaluated the direct effect of addition of these lipoxygenase products on cellular protein levels in PSMCs cultured in normal glucose and high glucose. As shown in Fig 4A, in normal glucose, 12-HETE at $10^{-6}$ and $10^{-7}$ mol/L had similar stimulatory effects as Ang II ($10^{-7}$ mol/L) on total cell protein levels. However, the 15-lipoxygenase product 15-HETE at concentrations of $10^{-6}$ through $10^{-4}$ mol/L had no effect on protein content (Fig 4A). Fig 4B shows the dose-dependent effects of the lipoxygenase products on cellular protein content in PSMCs cultured in high glucose. As in the normal glucose condition, 12-HETE at $10^{-6}$ and $10^{-7}$ mol/L showed similar stimulatory responses as Ang II. The effects of Ang II and also 12-HETE were enhanced in high glucose, with 12-HETE at $10^{-4}$ mol/L showing a significant response (Fig 4B). The effects of 15-HETE were also potentiated
by high glucose culture conditions, with concentrations of 15-HETE at $10^{-6}$ to $10^{-8}$ mol/L showing stimulatory effects. However, the effects of 15-HETE even at $10^{-5}$ mol/L were less than the responses to Ang II (Fig 4B). Neither 12- nor 15-HETE changed the cell number (results not shown).
by guest on July 12, 2017 http://hyper.ahajournals.org/ Downloaded from

Ang II and 12-HETE led to a dose-dependent stimulation of cellular fibronectin levels in cells cultured in high glucose. In contrast, Ang II at all concentrations tested stimulated released fibronectin levels in cells cultured in normal glucose. The maximal effect of Ang II was at $10^{-7}$ mol/L. In normal glucose, 12-HETE stimulated fibronectin only at $10^{-7}$ mol/L. However, in high glucose, 12-HETE produced a greater stimulation, with a dose-dependent effect from $10^{-7}$ to $10^{-9}$ mol/L.

The changes in cell-associated fibronectin levels are shown in Fig 5B. Neither Ang II nor 12-HETE at $10^{-7}$ to $10^{-9}$ mol/L significantly changed basal cell-associated fibronectin levels in normal glucose. In contrast, both Ang II and 12-HETE led to a dose-dependent stimulation of cellular fibronectin levels in cells cultured in high glucose (Fig 5B).

**Discussion**

Clear evidence indicates that Ang II produces hypertrophic responses in cultured aortic medial SMCs. The present study suggests that Ang II–induced increases in protein synthesis in PSMCs are mediated predominantly by the AT$_1$ receptor, because losartan but not PD123177 prevents Ang II effects. These results are similar to those in a recent report on rat SMCs. The signal transduction mechanisms for Ang II–induced hypertrophic responses are not completely understood. Ang II can activate a phosphatidylinositol-specific phospholipase C leading to inositol trisphosphate, which can mobilize calcium. However, Ang II–induced activation of phospholipase C and other phospholipases including phospholipase D can generate diacylglycerols. These and other signals can then produce expression of certain oncogenes and growth factors.

We have demonstrated previously that activation of a 12-lipoxygenase enzyme plays a key role in mediating both Ang II–induced steroidogenic and mitogenic responses in the adrenal cortex. Furthermore, Ang II increases both the activity and expression of a leukocyte type of 12-lipoxygenase enzyme in PSMCs. In the current report, we show for the first time that the 12-lipoxygenase pathway is particularly involved in Ang II–induced hypertrophy in PSMCs. This is supported by results showing that a selectively 12-lipoxygenase inhibitor but not a cyclooxygenase inhibitor completely blocks Ang II responses. Furthermore, a 12-lipoxygenase product, 12-HETE, directly increases protein content to a greater extent than 15-HETE. The precise mechanism by which 12-HETE increases protein concentration was not assessed in the current report. However, previous studies have shown that 12-lipoxygenase products can increase protein kinase C activity and oncogene expression. The effects of glucose on increasing protein synthesis in PSMCs are not simply due to hyperosmolar effects, and no evidence of hyperplasia was seen. We have not fully addressed the issue of whether the increases in protein content are due to increases in protein synthesis or decreases in protein degradation. In preliminary unpublished experiments we observed that both Ang II and 12-HETE cause increases in [H]$^3$H]leucine incorporation into protein, suggesting that new protein synthesis may be involved. Further experiments will be needed to determine these parameters.

Another major finding in the present study is the demonstration that high glucose culture potentiates the stimulatory effects of both Ang II and 12-HETE on protein synthesis in PSMCs. A previous report has also revealed that high glucose can enhance Ang II–induced hypertrophic responses in murine proximal tubular cells. The effects of glucose on increasing protein synthesis in PSMCs are not simply due to hyperosmolar effects, as we have shown that similar concentrations of mannose do not alter high glucose–induced increased growth of PSMCs. The precise mechanism of how glucose potentiates Ang II growth responses will require further study. However, it has been reported that elevated glucose can lead to de novo synthesis of diacylglycerols and activate protein kinase C in vascular cells. Furthermore, high glucose can increase the activity and expression of the 12-lipoxygenase pathway. Therefore, increases in 12-HETE with subsequent increases in specific isoforms of protein kinase C may be involved in upregulation of these responses. However, many additional mechanisms are possible, including changes in G proteins, receptor binding, or calcium mobilization.

In addition to changes in total protein, the study indicates that both Ang II and 12-HETE can produce
Fig 5. Bar graphs show effects of angiotensin II (A II) and 12-hydroxyeicosatetraenoic acid (12-HETE) treatment for 40 hours on levels of released fibronectin (A) and cell-associated fibronectin (B) in porcine smooth muscle cells. Results are mean±SEM from four to five experiments performed in triplicate. Released fibronectin: **P<.001, <.01, <.05 vs basal, respectively; tP<.03 vs angiotensin II 10^{-7} mol/L; tP<.02 vs 12-HETE 10^{-7} mol/L. Cell-associated fibronectin: **P<.001, P<.01 vs basal, respectively; tP<.01 vs angiotensin II 10^{-7} mol/L. In addition, angiotensin II and 12-HETE effects were significantly greater in high glucose (P<.01). Basal fibronectin values: released, 12.1±0.7 μg/10^6 cells normal glucose and 18.6±1.1 μg high glucose; cell-associated, 2.0±0.3 μg/10^6 cells normal glucose and 3.05±0.4 μg high glucose.

dose-dependent increases in cell-associated and released fibronectin levels only in PSMCs cultured in high glucose. The effect of 12-HETE on fibronectin levels could be seen at concentrations as low as 10^{-9} mol/L. High glucose alone also produced increases in cell-associated and released fibronectin levels (data not shown). Additional studies will be needed to confirm whether the increases in fibronectin are due to changes in synthesis or reduced degradation. However, most reports have shown that glucose or Ang II-induced increases in matrix proteins are primarily produced by increases in synthesis.25,26

Diabetes mellitus is associated with a substantially increased prevalence of hypertensive and atherosclerotic cardiovascular disease.9 Prolonged hyperglycemia-induced biochemical changes have been associated with diabetes-specific vascular disease.27 Enhanced responses to the vasoressor action of Ang II have also been shown in diabetic patients.10 The present report supports the hypothesis that Ang II–induced vascular hypertrophic responses may be accentuated in diabetes by activation of a 12-lipoxygenase pathway in the vascular smooth muscle.

Acknowledgments
This work was supported by grants from the National Institutes of Health, Bethesda, Md (HL-48920, RO1-DK39721, and SCOR HL-44404). We thank Ms Elizabeth Rees for her excellent secretarial assistance.

References


Role of the lipoxygenase pathway in angiotensin II-induced vascular smooth muscle cell hypertrophy.
R Natarajan, N Gonzales, L Lanting and J Nadler

Hypertension. 1994;23:I142
doi: 10.1161/01.HYP.23.1_Suppl.I142

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/23/1_Suppl/I142

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/