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Original Research Communication

Arachidonic Acid Metabolites Mediate Angiotensin II-Induced NADH/NADPH Oxidase Activity and Hypertrophy in Vascular Smooth Muscle Cells

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ABSTRACT

Previously, we showed that angiotensin II stimulation of the NADH/NADPH oxidase is involved in hypertrophy of cultured vascular smooth muscle cells (VSMC). Here, we examine the pathways leading to oxidase activation, and demonstrate that arachidonic acid metabolites mediate hypertrophy by activating the p22phox-based NADH/NADPH oxidase. Angiotensin II stimulates phospholipase A_2, releasing arachidonic acid, which stimulates oxidase activity *in vitro*. When arachidonic acid metabolism is blocked with 5,8,11,14- eicosatetraynoic acid (ETYA) or nordihydroguaiaretic acid (NDGA), oxidase activity decreases by 80 ± 10%. In VSMC transfected with antisense p22phox to attenuate NADH/NADPH oxidase expression, arachidonic acid is unable to stimulate NADH/NADPH-dependent superoxide production. In these cells, or in cells in which NADH/NADPH oxidase activity is inhibited by diphenylene iodonium, angiotensin II-induced [^{3}H]leucine incorporation is also inhibited. Attenuation of oxidase activation by inhibiting arachidonic acid metabolism with ETYA, NDGA, baicalein, or SKF-525A also inhibits angiotensin II-stimulated protein synthesis (74 ± 2% and 34 ± 1%, respectively). Thus, endogenous noncyclooxygenase arachidonic acid metabolites mediate angiotensin II-stimulated protein synthesis in cultured VSMC by activating the NADH/NADPH oxidase, providing mechanistic evidence for redox control of VSMC hypertrophy. Antiox. Redox Signal. 1, 167–179, 1999.

INTRODUCTION

REGULATION OF CELL GROWTH is crucial for understanding the pathophysiology of hypertension, atherosclerosis and restenosis. It has become apparent in recent years that agents that are normally vasoconstrictors, such as angiotensin II (ang II) and vasopressin, can, in the appropriate environment, stimulate cell growth. Ang II, in particular, has been shown to produce a multitude of growth effects on vascular smooth muscle cells, including hypertrophy, hyperplasia, and remodeling of the arterial wall (Griendling *et al.*, 1994). However, the intracellular signaling pathways associated with these responses are incompletely understood.

We have shown that in cultured vascular

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smooth muscle cells (VSMC), ang II stimulates superoxide ($^{\cdot}O_2^-$) formation by activating an NADH/NADPH oxidase, and that inhibition of this enzymatic pathway inhibits hypertrophy (Ushio-Fukai *et al.*, 1996; Zafari *et al.*, 1998). It has been suggested that regulation of the redox state of the cell may be a general mechanism by which growth signals are transduced (Tsai *et al.*, 1996). However, the mechanisms that regulate the oxidative state of the cell, and in particular the pathways that lead to NADH/NADPH oxidase activation, are virtually unknown. Arachidonic acid can stimulate oxidase activity *in vitro* (Griendling *et al.*, 1994), but a role for this fatty acid in nonphagocytic cells *in vivo* has not been demonstrated. Ang II induces the release of arachidonic acid in VSMC, and it has variously been reported to be rapidly metabolized to prostaglandins (Alexander and Gimbrone, 1976) or hydroxy fatty acids (Natarajan *et al.*, 1993a, 1994) and leukotrienes via activation of the cyclooxygenase and lipoxygenase pathways, respectively. Thus, arachidonic acid and its metabolites are potentially important second messengers for oxidase activation. Because of the demonstrated relationship between NADH/NADPH oxidase activation and hypertrophy (Griendling *et al.*, 1994; Ushio-Fukai *et al.*, 1996; Zafari *et al.*, 1998), the arachidonic acid metabolic pathways may also be important in hypertrophy, as suggested by studies on 12-lipoxygenase (Natarajan *et al.*, 1994).

In the present study, we have tested this hypothesis by examining the relationship between arachidonic acid metabolism, oxidase activation, and hypertrophy in ang II-stimulated VSMC. We found that the phospholipase A_2 (PLA_2)-dependent production of arachidonic acid and the subsequent metabolism of arachidonic acid via lipoxygenase and cytochrome P450 (CYP450) monooxygenase are necessary for NADH/NADPH-dependent $^{\cdot}O_2^-$ formation, and that this sequence of events is required for the growth-promoting effects of ang II. These observations suggest that the products of PLA_2 activation ultimately contribute to regulation of the oxidative state of the cell, which in turn mediates the hypertrophic response.

MATERIALS AND METHODS

Cell culture

VSMC were isolated from rat thoracic aorta by enzymatic digestion as described previously (Griendling *et al.*, 1991). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and were passaged twice a week by harvesting with trypsin:EDTA and seeding into 80-cm^2 flasks. For experiments, cells between passage levels 6 and 20 were seeded into 35-mm and 100-mm dishes, fed every other day, and used at confluence.

In some experiments, we used rat aortic smooth muscle cells that had been stably transfected with antisense p22phox, a critical component of the oxidase, as described by Ushio-Fukai *et al.* (1996). In these cells, p22phox mRNA and protein expression are abolished and ang II-stimulated NADH/NADPH oxidase activity is markedly depressed.

NADH/NADPH oxidase assay

NADH/NADPH oxidase activity was measured as described previously (Griendling *et al.*, 1994). Briefly, control cultures of VSMC or cultures that had been exposed to ang II for 4 hr were washed five times with 5 ml of ice-cold phosphate-buffered saline (PBS), and cells were scraped from the plate in 5 ml of this same solution. Samples were transferred to a 50-ml centrifuge tube, and the plate was washed twice with an additional 5 ml of PBS. Cells were then centrifuged at 740 $\times$ *g* at 4°C for 10 min. The supernatant was discarded, and the pellet was resuspended (0.5–1.0 ml per dish) in lysis buffer containing protease inhibitors, 20 m*M* monobasic potassium phosphate, pH 7.0, 1 m*M* EGTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF). The cell suspension was then dounced 100 times on ice, and the homogenate was stored on ice until use. Protein content was measured in an aliquot of the homogenate by the method of Lowry *et al.* (1951).

NADH/NADPH oxidase activity was measured by a luminescence assay in a 50 m*M* phosphate buffer, pH 7.0, containing 1 m*M* EGTA, 150 m*M* sucrose, 500 μM lucigenin as the electron acceptor, and either 100 μM NADH or 100 μM NADPH as the substrate (final volume, 0.9 ml). The reaction was started by the addition of 100 μl of homogenate (50–300 μg protein). Luminescence was monitored as described previously (Griendling *et al.*, 1994).

In cell homogenates, superoxide dismutase (120 U/ml) partially inhibited the ang II-induced NADH/NADPH signal as reported previously (Griendling *et al.*, 1994; Pagano *et al.*, 1995), and Tiron (10 m*M*), a cell-permeant chelator of $\cdot O_2^-$ (Devlin *et al.*, 1981; Ledenev *et al.*, 1986) inhibited ang II-stimulated NADH and NADPH oxidase assay signals by 74.5 ± 1.9 and 73.8 ± 2.9%, respectively ($n = 3$), thus demonstrating the specificity of the lucigenin signal for $\cdot O_2^-$. Although lucigenin has recently been criticized for its ability to undergo redox cycling in certain *in vitro* conditions, we have found minimal differences in measurements made using the above lucigenin protocol and measurements made using electron spin resonance (unpublished observations) or cytochrome *c* reduction (De Keulenaer *et al.*, 1998). Other laboratories have also demonstrated a close relationship between lucigenin measurements and other methods (Mohazzab-H. and Wolin, 1994).

Measurement of NADH and NADPH consumption

Measurements of NADH and NADPH consumption were made using a modification of the method described by Brightman and coworkers (Brightman *et al.*, 1992). Cells were washed with DMEM without phenol red, pH 7.4, and then incubated with 250 μM NADH or NADPH in the same medium for varying time intervals. The rate of NADH or NADPH consumption was monitored by the decrease in absorbance at $\lambda = 340$ nm, using a spectrophotometer. The absorption extinction coefficient used to calculate the amount of NADH or NADPH consumed was 6.22 $mmol/L^{-1} \cdot cm^{-1}$. For measurements of specific oxidase activity, only the diphenylene iodonium (DPI)-inhibitable rate of consumption of NADPH and NADH was used. This was done by adding 10 μM DPI 30 min prior to the assays for NADH and NADPH consumption. All measurements were expressed as nanomoles of substrate/min/10^6 cells.

Cell fractionation

In some experiments, membranes and cytosol were separated by centrifugation, and NADH/NADPH oxidase activity was measured. These samples were prepared exactly as described above for the NADH/NADPH oxidase assays, except that after lysis, cell homogenates were centrifuged at 29,100 × *g* for 20 min at 4°C. The supernatant (cytosolic fraction) was removed, and the pellet containing both plasma and mitochondrial membranes was resuspended in the original volume of lysis buffer. NADH/NADPH oxidase activity was then measured in each fraction as described above.

Cell-associated [^{3}H]arachidonic acid production

VSMC were grown for 48 h in DMEM containing 10% calf serum. Cells were then labeled for 24 h with 1 μCi/ml [^{3}H]arachidonic acid in 10% calf serum/DMEM to reach confluence. To initiate the experiment, the label was aspirated and each dish was rinsed three times with balanced salt buffer (BSB) of the following composition: 130 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1.5 m*M* $CaCl_2$, 20 m*M* HEPES, buffered to pH 7.4 with Tris base. Cells were then preincubated in BSB for 20 min at 37°C, and subsequently incubated in 1 ml of BSB with or without ang II (100 n*M*) for the indicated time. The medium was aspirated, and each dish was extracted with chloroform/butylated hydroxytoluene (BHT)/methanol/HCl (1:0.0001:2:0.5). Cold arachidonic acid was added to the lipid extracts as an internal standard. Organic and aqueous phases were separated by a modified Bligh and Dyer extraction, and the organic phase was further subjected to solid-phase extraction using a Bond Elut:Aminopropyl (NH_2) column. The column was conditioned two

times with 2-ml portions of hexane. Lipid extract (up to 500 μl) was then applied to the column and aspirated under N_2 positive pressure through the sorbent. The column was eluted with 4 ml of chloroform/2-propanol (2:1) under N_2, and the eluate (containing neutral lipid fraction) was discarded. Finally, the column was eluted with 4 ml of 2% acetic acid in diethyl ether under N_2 to yield the fatty acid fraction. The fatty acid fraction was then lyophilized, and resuspended in 1 ml of solvent A for HPLC analysis. To quantify arachidonic acid, reverse-phase high-pressure liquid chromatography (HPLC)(LKB Produkter AB, Bromma, Sweden) was performed on a C18 column (Nucleosil 150 mm, 3 μm). Fatty acid products were eluted at a flow rate of 1 ml/min using a gradient from solvent A acetonitrile:water:orthophosphoric acid (50:50:0.01) to solvent B acetonitrile:water:orthophosphoric acid (80:20:0.01) as described by Haas and Buchanan (1988). Arachidonic acid eluted between 37 and 39 min, as defined by absorbance of cold standards. One-milliliter fractions of the eluate were collected and radioactivity was measured by liquid scintillation spectrophotometry.

[^{3}H]Leucine incorporation

To measure hypertrophy of VSMC, cells were plated at low density, grown for 48 h in DMEM containing 10% calf serum, and grown for an additional 72 h in DMEM containing 0.1% calf serum. Twenty-four hours before harvest, cells were incubated with [^{3}H]leucine (1 μCi/ml) in the presence or absence of 100 n*M* ang II. Cells were then washed twice with ice-cold PBS and incubated with 5% trichloroacetic acid for 5 minutes at 4°C. After two additional washes with deionized water, cells were dissolved in 1 ml of 0.4 *N* NaOH. Duplicate aliquots (0.4 ml) were removed, acidified with 0.2 ml of 1.0 *N* NCl, and counted in 10 ml Liquiscint in a liquid scintillation spectrophotometer.

Evaluation of cytotoxicity

VSMC viability after 24 hour of exposure to DPI (10 μ*M*), 5,8,11,14-eicosatetraynoic acid (ETYA) (50 μ*M*), baicalein (1 μ*M*), nordihydroguaiaretic acid (NDGA) (10 μ*M*), indomethacin (IM) (10 μ*M*), and proadifen (SKF 525A) (25 μ*M*) was assessed by cell morphology, trypan blue exclusion, and [^{3}H]2-deoxyglucose (DOG) release (Andreoli *et al.*, 1985; Natarajan *et al.*, 1993b). For measurement of [^{3}H]DOG release, VSMC were plated at low density, grown for 48 h in DMEM containing 10% calf serum, and labeled with a 1 μCi/dish of [^{3}H]DOG for an additional 72 h in DMEM containing 0.1% calf serum. The cells were then washed twice with BSB, and were exposed to the inhibitors for additional 24 h in DMEM containing 0.1% calf serum, conditions identical to those used during measurement of hypertrophy. At the end of the incubation, an aliquot of the supernatant was centrifuged for 5 min at 1,500 × *g*, and radioactivity was measured in a liquid scintillation spectrophotometer. Each dish was then washed three times with 1 ml of BSB and radioactivity of the washes was counted. Finally, 0.4 *N* NaOH was added to each dish to lyse the cells, and cell-associated radioactivity was quantified. The percentage of [^{3}H]DOG released in treated cells was calculated using the following formula:

$$\frac{\text{Supernatant of treated cells} - \text{supernatant of control cells}}{(\text{Supernatant} + \text{washes} + \text{lysed treated cells}) - \text{supernatant of control cells}}$$

The range of spontaneous release varied from 5 to 12%.

Rate calculations and statistical analysis

Initial rates of enzyme activity in the NADH/NADPH oxidase assays were calculated by linear regression over the first 30–120 sec of exposure to lucigenin. In general, correlations (R^2 values) were ≥0.9. Rates are presented as mean ± SE.

Comparison between groups was performed by Student's paired two-tailed *t*-test or analysis of variance (ANOVA), as appropriate.

Materials

All chemicals were of analytical grade or better. Bovine serum albumin and PMSF were from Boehringer Mannheim (Indianapolis, IN). Mepacrine, dibucaine, indomethacin, and

NDGA were purchased from Sigma (St. Louis, MO), and baicalein, SKF 525A, and ETYA were from BIOMOL (Plymouth Meeting, PA). Soybean trypsin inhibitor, glutamine, penicillin, streptomycin, and trypsin:EDTA were purchased from GIBCO (Grand Island, NY). [^{3}H]Leucine (140 Ci/mmol) and [^{3}H]DOG were from Dupont NEN (Boston, MA), and Liquiscint was purchased from National Diagnostics (Atlanta, GA). Common buffer salts were obtained from Fisher (Pittsburgh, PA). All other chemicals and reagents, including DMEM with 25 m*M* HEPES and 4.5 grams/liter glucose and calf serum, were from Sigma, except chemicals for HPLC, which were purchased from Fisher.

RESULTS

Ang II-stimulated intracellular accumulation of arachidonic acid in VSMC

To verify that ang II stimulates arachidonic acid production intracellularly in these cells in a PLA_2-dependent manner, we labeled VSMC with [^{3}H]arachidonic acid and measured [^{3}H]arachidonic acid generation in the presence and absence of PLA_2 inhibitors (Sekiya and Okuda, 1982; Rao *et al.*, 1994). Ang II-induced [^{3}H]arachidonic acid production at 5 min was approximately three times the control value (control, 1,321 ± 131 dpm/dish; ang II, 4,149 ± 480 dpm/dish, $n = 5$, $p < 0.01$). This increase in [^{3}H]arachidonic acid was inhibited by 62 ± 6% in the presence of mepacrine (5 μM) and by 68 ± 4% with dibucaine (50 μM), indicating mediation by PLA_2.

Activation of the p22phox-containing NADH/NADPH oxidase by arachidonic acid

To determine whether arachidonic acid activates the p22phox-based VSMC NADH/NADPH oxidase that we have previously characterized (Griendling *et al.*, 1994; Ushio-Fukai *et al.*, 1996), we used VSMC transfected with antisense p22phox, in which ang II-stimulated NADH/NADPH oxidase activity is significantly inhibited (Ushio-Fukai *et al.*, 1996). In contrast to untransfected or vector-transfected VSMC, in which arachidonic acid (100 μM) caused a significant increase in $^{\cdot}O_2^-$ generation, arachidonic acid was unable to activate the oxidase in membrane fractions of VSMC transfected with pcDNA3/antisense-p22phox (Fig. 1), demonstrating the arachidonic acid-sensitive mode of activation of the VSMC oxidase.

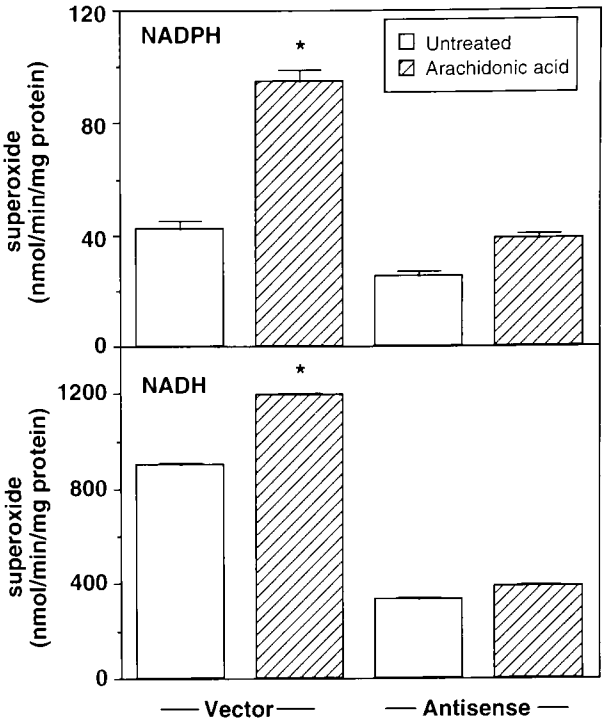

FIG. 1. Arachidonic acid stimulation of p22phox-based NADH/NADPH oxidase in VSMC. VSMC transfected with vector or antisense p22phox were grown to confluence, homogenized, and a membrane fraction prepared. Membranes were suspended in assay buffer, and $^{\cdot}O_2^-$ measured acutely in response to arachidonic acid (100 μM), as described in Materials and Methods. Each bar represents the mean ± SE of four experiments using NADPH or NADH as the substrate. (*) Value for arachidonic acid is significantly different from control ($p < 0.05$).

Role of arachidonic acid production and metabolism in angiotensin II stimulation of NADH/NADPH oxidase activity

To determine whether arachidonic acid or its metabolites mediate ang II-induced NADH/NADPH oxidase activation in intact cells, we measured the effect of arachidonic acid metabolic inhibitors on oxidase activity. Ang II (100 n*M*) induced a 2.88 ± 0.37-fold increase in NADPH-dependent oxidase activity and a 1.9 ± 0.3-fold increase in NADH-dependent

oxidase activity after 4 h (n = 3–6). Ang II also increased the rates of NADH and NADPH consumption, and this enhanced rate of consumption was completely inhibited when DPI (10 μM), a relatively specific NADH/NADPH oxidase inhibitor, was added to the cells 30 min prior to the assay (data not shown). The ang II-induced increase in NADH/NADPH oxidase activity was dramatically inhibited when the cells were incubated for 4 h with ETYA (50 μM), an inhibitor of all three enzymatic pathways of arachidonic acid metabolism, (89 ± 6% and 80 ± 10% inhibition for NADH and NADPH, respectively) or NDGA (10 μM), a dual inhibitor of lipoxygenase and CYP450 monooxygenase, (87 ± 13% and 77 ± 20% inhibition for NADH and NADPH, respectively), as demonstrated in Fig. 2. These inhibitory effects of ETYA and NDGA were also observed in the membrane fraction of VSMC (data not shown). Incubation of VSMC with indomethacin (10 μM), a cyclooxygenase inhibitor, had no effect on the ang II-induced increase in NADH/NADPH oxidase activity (data not shown). All compounds were given directly to the cells, and were washed out prior to *in vitro* measurement of oxidase activity. These observations suggest that arachidonic acid metabolites, in particular products of the CYP450 monooxygenase and lipoxygenase pathways, mediate ang II activation of the NADH/ NADPH oxidase in VSMC.

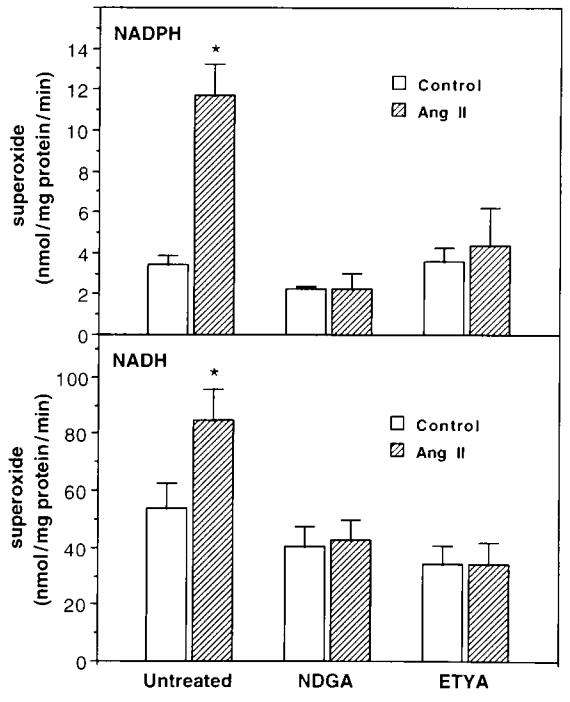

FIG. 2. Effect of ETYA and NDGA on NADH/NADPH oxidase activity in VSMC. VSMC were exposed to ang II or media in presence or absence of ETYA (50 μM) or NDGA (10 μM) for 4 hr. Cells were then homogenized and incubated with buffer for 5 min at room temperature immediately prior to measurement of $^{\cdot}O_2^-$ by chemiluminescence. Each bar represents the mean ± SE of the initial rate of enzyme activity using NADPH or NADH as the substrate. Experiments done in the presence of the inhibitors were repeated three to six times. (*) Value for ang II is significantly different from control ($p < 0.05$).

The concentrations of inhibitors used in this study have previously been shown to inhibit the indicated enzymes effectively (Buening and Franklin, 1976; Sekiya and Okuda, 1982; Capdevilla *et al.*, 1988). To rule out nonspecific interference of these inhibitors on ang II signaling, we performed ang II binding studies and phospholipase C (PLC) activity assays in the presence of ETYA and NDGA. Neither compound had any effect on ang II binding (control, 342 fmol/mg protein; ETYA, 338 fmol/mg protein; NDGA, 302 fmol/mg protein) using [^{3}H]losartan as a ligand, or on phospholipase C activity as measured by accumulation of inositol phosphates (control, 200 ± 38% increase with ang II; ETYA, 197 ± 12% increase with ang II; NDGA, 229 ± 28% increase with ang II). These results, combined with the lack of toxicity of these compounds (see below), suggest that both are specifically inhibiting arachidonate metabolism and consequently NADH/NADPH oxidase activation.

Effect of inhibition of arachidonic acid metabolic pathways on vascular smooth muscle hypertrophy

Our previous data suggest that inhibition of the NADH/NADPH oxidase also attenuates ang II-induced hypertrophy (Griendling *et al.*, 1994; Ushio-Fukai *et al.*, 1996; Zafari *et al.*, 1998). In the culture conditions used in the present study, DPI (10 μM) attenuates the ang II-induced increase in [^{3}H]leucine incorporation by 91 ± 4% (n = 4; $p < 0.01$), emphasizing the critical importance of this pathway in cell growth. This effect of DPI is not due to toxic effects of this compound, as assessed by cell morphology (normal, <1% detached cells), trypan blue exclusion (>95%), and [^{3}H]DOG release (<12%).

More recently, we have shown that in p22phox antisense-transfected cells, ang II-induced hypertrophy was reduced by 52 ± 8% confirming the critical role of this oxidase in cell growth (Ushio-Fukai *et al.*, 1996). These data imply that inhibition of NADH/NADPH oxidase activation by inhibitors of the signaling pathway utilized by ang II to activate the oxidase should also inhibit hypertrophy.

To determine whether inhibitors of arachidonic acid metabolic pathways that are effective against NADH/NADPH oxidase activation also inhibit ang II-induced hypertrophy, we tested the effect of ETYA, NDGA and indomethacin on [^{3}H]leucine incorporation. Cells were quiesced for 72 h in 0.1% calf serum labeled with [^{3}H]leucine to measure protein synthesis and exposed to ang II (100 n*M*) in the presence or absence of indomethacin, NDGA, and ETYA for 24 hr. We also used baicalein, a selective 12-lipoxygenase inhibitor, and SKF 525A, the protypical inhibitor of CYP450 monooxygenase, to gain insight into the specific metabolic pathways involved in hypertrophy. These two compounds could not be used in the $^{\cdot}O_2^-$ assay because of color interference with the lucigenin signal. As shown in Fig. 3, ang II caused an 82 ± 4% increase in [^{3}H]leucine incorporation, as compared to an approximately 30 ± 3% increase in [^{3}H]thymidine incorporation and a <10% increase in cell number (data not shown), confirming that the major effect of ang II is hypertrophic in these cells. Similar to its effect on NADH/NADPH oxidase activation, indomethacin (10 μM) did not alter ang II-stimulated [^{3}H]leucine incorporation. In contrast, both NDGA (10 μM) and ETYA (50 μM) inhibited ang II-induced hypertrophy (34 ± 1% and 74 ± 2% inhibition, respectively, $p < 0.01$). The 12-lipoxygenase inhibitor baicalein (1 μM) had only a minimal effect (18 ± 2%), while the CYP450 monooxygenase inhibitor SKF 525A (25 μM) had an inhibitory effect of 49 ± 2%. None of these compounds was toxic, as assessed by cell morphology and trypan blue exclusion. These observations confirm that only those agents that inhibit NADH/NADPH oxidase activation also inhibit hypertrophy, and suggest that this may be one mechanism by which lipoxygenase and CYP450 monooxygenase metabolites mediate ang II-induced hypertrophy (Natarajan *et al.*, 1994).

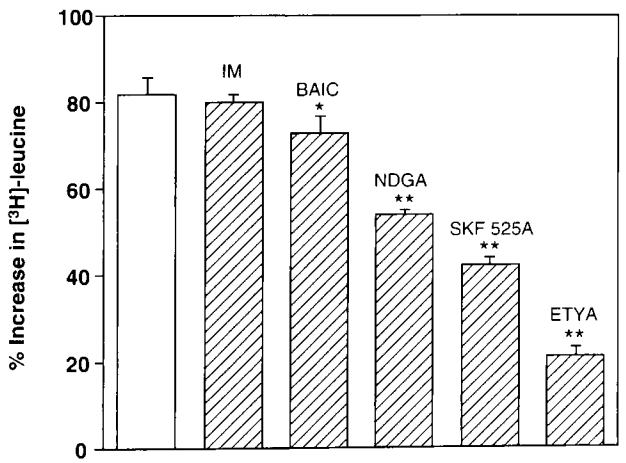

FIG. 3. Attenuation of angiotensin II-induced hypertrophy by inhibitors of cyclooxygenase, lipoxygenase, and cytochrome P450 monooxygenase. VSMC were quiesced for 72 hr in DMEM supplemented with 0.1% calf serum, labeled with [^{3}H]leucine, and exposed to ang II (100 n*M*) in the presence or absence of IM (10 μM), baicalein (BAIC) (1 μM), NDGA (10 μM), proadifen (SKF 525A) (25 μM), and ETYA (50 μM) for 24 hr. [^{3}H]Leucine incorporation was measured as described in Materials and Methods. Data are expressed as the percent increase in [^{3}H]leucine incorporation induced by ang II over the appropriate control. Each bar represents the mean of four to seven experiments performed in triplicate. (*) $p < 0.05$; (**) $p < 0.01$—for increase in the presence of inhibitor versus increase with ang II alone.

Role of PLA_2 in vascular smooth muscle hypertrophy

Because arachidonic acid is the precursor for these active metabolites, and PLA_2 appears to be the ultimate source of the arachidonic acid, we hypothesized that PLA_2 inhibitors should also inhibit hypertrophy, whereas arachidonic acid should stimulate hypertrophy. We quiesced cells labeled with [^{3}H]leucine and exposed them to ang II (100 μM) in the presence or absence of mepacrine or dibucaine for 24 h. As shown in Fig. 4, A and B, ang II caused approximately an 80% increase in [^{3}H]leucine incorporation. Mepacrine (5 μM) inhibited this increase by 47 ± 2% and dibucaine (50 μM) by 59 ± 2% ($p < 0.01$ for both inhibitors), similar to their ability to inhibit [^{3}H]arachidonic acid production (see above). Finally, VSMC treated with arachidonic acid (10 μM) alone for 24 hr,

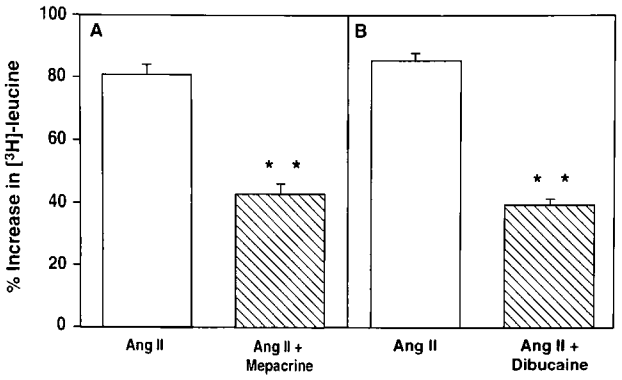

FIG. 4. Attenuation of angiotensin II-induced hypertrophy by PLA_2 inhibition. VSMC were quiesced for 72 hr in DMEM supplemented with 0.1% calf serum, labeled with [^{4}H]leucine, and exposed to ang II (100 n*M*) in the presence or absence of mepacrine (5 μM) for 24 hr **(A)**, or dibucaine (50 μM) for 24 hr **(B)**. [^{3}H]Leucine incorporation was measured as described in Materials and Methods. Data are expressed as the percent increase in [^{3}H]leucine incorporation induced by ang II over the appropriate control. Each bar represents the mean of three to four experiments performed in triplicate. (**) $p < 0.01$ for increase in the presence of inhibitor versus increase with ang II alone.

showed a 24 ± 2% increase in [^{3}H]leucine incorporation, providing further evidence for a role for this polyunsaturated fatty acid in linking oxidase activity and subsequent $\cdot O_2^-$ generation to VSMC hypertrophy.

Effect of AT_1 receptor inhibition during the development of angiotensin II-induced smooth muscle cell hypertrophy

The above data suggest that the rapid activation of PLA_2 and metabolism of arachidonic acid by lipoxygenase and CYP450 monooxygenase are important in both oxidase activation and hypertrophy. However, ang II stimulation of NADH/NADPH oxidase activity is delayed by several hours (Griendling *et al.*, 1994). This suggests that additional signals may be necessary, but it also suggests that once the signaling cascade is underway, the cells will be irreversibly committed to hypertrophy. To test this hypothesis, we quiesced cells labeled with [^{3}H]leucine and exposed them to ang II (100 n*M*, 24 hr). The AT_1 receptor antagonist losartan (10 μM) was then added to parallel cultures at the time of exposure to ang II, or 5 min to 18 hr after exposure to ang II. As shown in Fig. 5, losartan given simultaneously with ang II for 24 hr to VSMC completely inhibited ang II-induced increase in protein synthesis, as expected if hypertrophy is mediated by the AT_1 receptor. When losartan is given 5 min after ang II, a significant portion of the increase in [^{3}H]leucine still occurs (27 ± 1%). By 4 hr, the majority of the cells are committed to hypertrophy, because [^{3}H]leucine incorporation in response to ang II is still almost 66 ± 3% in the presence of losartan. Notably protein synthesis in ang II-treated rat aortic smooth muscle cells can be partially inhibited even when losartan is given 12 hr after the cells are exposed to ang II, (18 ± 4% inhibition), suggesting that continued signal generation is necessary for a full hypertrophic response.

DISCUSSION

The data presented here provide the first evidence concerning the molecular pathways that lead to NADH/NADPH oxidase activation in VSMC, and establish a link between arachidonate-dependent stimulation of this enzyme and

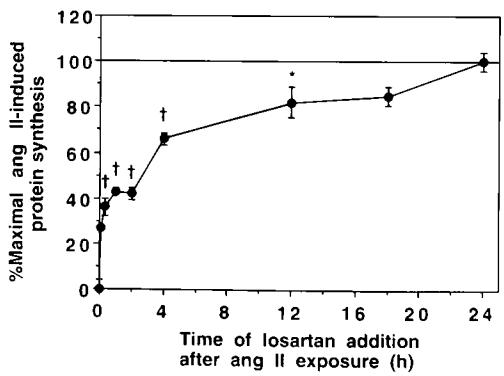

FIG. 5. Time course of AT_1 receptor inhibition in relation to angiotensin II-induced protein synthesis. VSMC were quiesced for 72 hr in DMEM supplemented with 0.1% calf serum, labeled with [^{3}H]leucine, and exposed to ang II (100 n*M*, 24 hr) in the presence of losartan (10 μM) simultaneously with ang II, and at 5 min, 20 min, 1 hr, 2 hr, 4 hr, 12 hr, and 18 hr after cells were exposed to ang II. [^{3}H]Leucine incorporation was measured as described in Materials and Methods. Data are expressed as the percent increase in [^{3}H]leucine incorporation induced by ang II over the appropriate control. This curve is representative of two experiments performed in triplicate. (*) $p < 0.05$; (†) $p < 0.001$—for increase in the presence of inhibitor versus increase with ang II alone.

ang II-induced hypertrophy. Thus, arachidonic acid stimulates NADH/NADPH oxidase activity in normal cells, but not in cells in which oxidase expression has been attenuated by antisense p22phox. Arachidonic acid is produced in response to ang II, and inhibition of arachidonic acid metabolism via the lipoxygenase/CYP450 monooxygenase pathways significantly reduces the ability of ang II to stimulate the oxidase. Taken together, these data suggest that PLA_2-dependent arachidonic acid production is necessary for oxidase activation. Furthermore, when oxidase activity is decreased either with DPI or inhibitors of arachidonic acid metabolism, or by transfection with antisense p22phox, ang II-induced hypertrophy is also decreased, establishing a link between PLA_2-activation, arachidonic acid production and metabolism, NADH/NADPH oxidase activation, and hypertrophy.

In studies on the possible involvement of metabolites of enzymatic pathways of arachidonic acid in vascular physiology and pathophysiology, experimental designs based on the use of inhibitors of the enzymatic pathways of arachidonic acid have proved invaluable. To identify the enzymatic pathways involved in oxidase activation and protein synthesis, we used several specific inhibitors. Indomethacin (10 μM), which inhibits cyclooxygenase, had no effect on ang II-induced $^{\cdot}O_2^-$ formation or hypertrophy. At this concentration, indomethacin does not inhibit the other enzymatic pathways (Capdevila *et al.*, 1988). Baicalein, a selective 12-lipoxygenase inhibitor (Sekiya and Okuda, 1982), had a minimal effect on hypertrophy, in contrast to the complete inhibition of ang II-induced protein synthesis observed with this compound in porcine aortic smooth muscle cells (Natarajan *et al.*, 1994), perhaps because of the difference in cell origin. NDGA (10 μM), a dual inhibitor of lipoxygenase and CYP450 monooxygenase (Capdevila *et al.*, 1992), had a moderate effect on $^{\cdot}O_2^-$ generation and hypertrophy. SKF 525A, the prototypical inhibitor of CYP450 monooxygenase which binds to the protein moiety of the enzyme (Buening and Franklin, 1976), attenuated ang II-induced hypertrophy by approximately 50%. Finally, ETYA (50 μM), an inhibitor of all three enzymatic pathways of arachidonic acid (Capdevila *et al.*, 1988), diminished robustly both oxidase activity and protein synthesis. The similar effects of indomethacin, NDGA, and ETYA on NADH/NADPH oxidase activity and protein synthesis suggest that multiple noncyclooxygenase pathways mediate in ang II-induced hypertrophy in cultured VSMC by activating the NADH/NADPH oxidase. This conclusion is supported by our experiments in VSMC stably transfected with antisense p22phox. These cells have a significantly decreased oxidase activity when stimulated with ang II or arachidonic acid, and a marked reduction of protein synthesis in response to ang II (Ushio-Fukai *et al.*, 1996).

The signals that increase NADH/NADPH oxidase activity in nonphagocytic cells have not been previously defined, but the current data suggest that the PLA_2/arachidonic acid pathway is a major mediator. In neutrophils, agonist stimulation of the NADPH oxidase is closely correlated with the activation of phospholipase D (Agwu *et al.*, 1991), which, in fact, is markedly stimulated by ang II in VSMC. There has been much debate as to whether the direct product of PLD, phosphatidic acid, or its subsequent conversion product, diacylglycerol (Burnham *et al.*, 1990; Bauldry *et al.*, 1992), is the molecule that activates the neutrophil NADPH oxidase. However, *in vitro* studies have shown that phorbol esters (Rotrosen and Leto, 1990), arachidonate (Henderson *et al.*, 1993), lipoxin A, and leukotriene B_4 (Serhan *et al.*, 1984, 1985) are direct activators of the NADPH oxidase. In VSMC, ang II generates arachidonic acid directly from protein kinase C (PKC)-mediated PLA_2 hydrolysis of the *sn*-2 acylester bond of membrane phospholipids (Rao *et al.*, 1994). Indirectly, arachidonic acid is also formed via the phospholipase C and D pathways, by conversion of accumulated diacylglycerol by diacylglycerol and monoglycerol lipases (Severson and Hee-Cheong, 1989). The large production of this fatty acid make it (or its metabolites) an excellent candidate for the endogenous oxidase activator. Because the ang II-induced increase in PLA_2 activity with subsequent arachidonic acid production occurs early in the signaling cascade and well before oxidase activation, metabolites of arachidonic acid are ideal second messengers to transduce

the signal by virtue of their structure, location, and temporal metabolic sequence. It is also possible that arachidonic acid metabolites initiate a cascade of biochemical events that lead to oxidase activation.

We previously reported that NADPH-dependent $\cdot O_2^-$ generation was stimulated acutely *in vitro* by exogenous phosphatidic acid and arachidonic acid, whereas NADH-dependent $\cdot O_2^-$ generation was increased by arachidonic and linoleic acids (Griendling *et al.*, 1994). Our current data show that it may actually be arachidonic acid metabolites of the lipoxygenase and CYP450 monooxygenase pathways that are the immediate activators of these oxidases. Lipoxygenases convert arachidonic acid to unstable hydroperoxy intermediates that go on to form the leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and lipoxins. CYP450 monooxygenases catalyze the formation of epoxides (EETs), mid-chain *cis-trans* conjugated dienols, and C-19/C-20 alcohols (19-OH-AA and 20-OH-AAs). Interestingly, in human leukocytes, lipoxin A has been shown to activate the NADPH oxidase (Serhan *et al.*, 1984, 1985).

Metabolites of CYP450 and lipoxygenase pathways have been shown to have several growth-related effects, many of which may be modulated by the oxidase pathways identified here. Several CYP450 metabolites exhibit potent prohypertensive properties *in vitro* and *in vivo* (McGiff, 1991), including vasoconstriction (Escalante *et al.*, 1989), stimulation of Na^+-K^+-ATPase activity (Escalante *et al.*, 1988), increased renal production of ω-/ω-1 HETE during the developmental phase of hypertension in spontaneously hypertensive rats (SHR) (Sacerdoti *et al.*, 1988), and modulation of the growth response in rat mesangial cells (Sellmayer *et al.*, 1991). The 12-lipoxygenase pathway is involved in ang II-induced hypertrophy in porcine aortic SMC, where 12-HETE directly increased protein content to a greater extent than 15-HETE (Natarajan *et al.*, 1994). Several studies have also shown that 12-lipoxygenase products increase PKC activity and oncogene expression (Shearman *et al.*, 1989; Haliday *et al.*, 1991; Natarajan *et al.*, 1992).

The possible implications of ang II-stimulated NADH/NADPH oxidase activation on smooth muscle cell growth via noncyclooxygenase metabolites of arachidonic acid is intriguing. Ang II may be a crucial hypertrophic/hyperplastic effector or proinflammatory mediator in hypertension, restenosis post angioplasty, and atherosclerosis (Alexander, 1995). The long-term nature of the NADH/NADPH oxidase activity stimulated by ang II, combined with the known association of reactive oxygen species (ROS) with growth (Cerutti, 1985; Rao and Berk, 1992; Ushio-Fukai *et al.*, 1996; Zafari *et al.*, 1998), suggest that these enzyme systems may be an integral part of the intracellular redox system, priming the smooth muscle cell for hypertrophy and growth. Superoxide generation by the naphthoquinolinedione LY 83,583, and hydrogen peroxide (H_2O_2), one immediate product of $\cdot O_2^-$ metabolism, have been shown to stimulate mitogenesis in VSMC (Rao and Berk, 1992; Baas and Berk, 1995). Furthermore, both protein tyrosine kinases and protein tyrosine phosphatases, signaling pathways intimately involved in the growth response in many cell types, are regulated by ROS (Larsson and Cerutti, 1988; Pumiglia *et al.*, 1992). Further research will be necessary to understand fully redox control of vascular growth.

In summary, we have shown that ang II-stimulated NADH/NADPH oxidase activation in cultured VSMC is mediated by noncyclooxygenase metabolites of arachidonic acid. The arachidonic acid is derived, at least in part, from rapid activation of PLA_2, and is subsequently metabolized by lipoxygenase and CYP450 monooxygenase to yield a metabolite or metabolites that activate the NADH/NADPH oxidase and generate $\cdot O_2^-$. Because inhibitors of these enzymes reduce both oxidase activity and hypertrophy in parallel, it seems likely that noncyclooxygenase arachidonic acid products mediate ang II-induced hypertrophy by regulating NADH/NADPH oxidase activity. These observations also suggest that metabolites of arachidonic acid are growth promoting in part because of their ability to activate NADH/NADPH oxidases. Thus, our data support the notion that

regulation of the intracellular redox state of the cell may be a central mechanism by which vascular growth is regulated, and suggest that noncyclooxygenase metabolic pathways are potential therapeutic targets by virtue of their ability to stimulate the NADH/NADPH oxidase.

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ABBREVIATIONS

ang II, angiotensin II; ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BSB, balanced salt buffer; CYP450, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DOG, 2-deoxyglucose; DPI, diphenylene iodonium; EETs, epoxides; ETYA, 5,8,11,14-eicosatetraynoic acid; H_2O_2, hydrogen peroxide; HETEs, hydroxyeicosatetraenoic acids; HPLC, high-performance liquid chromatography; IM, indomethacin; NDGA, nordihydroguaiaretic acid; PBS, phosphate-buffered saline; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PMSF, phenylmethyl sulfonyl fluoride; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; SKF 525A, proadifen; VSMC, vascular smooth muscle cells.

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