Circulation Research

American Heart Association®

*Learn and Live*sm

Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells

KK Griendling, CA Minieri, JD Ollerenshaw and RW Alexander

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/content/74/6/1141

Angiotensin II Stimulates NADH and NADPH Oxidase Activity in Cultured Vascular Smooth Muscle Cells

Kathy K. Griendling, Candace A. Minieri, Jeremy D. Ollerenshaw, R. Wayne Alexander

Abstract The signaling pathways involved in the long-term metabolic effects of angiotensin II (Ang II) in vascular smooth muscle cells are incompletely understood but include the generation of molecules likely to affect oxidase activity. We examined the ability of Ang II to stimulate superoxide anion formation and investigated the identity of the oxidases responsible for its production. Treatment of vascular smooth muscle cells with Ang II for 4 to 6 hours caused a 2.7 ± 0.4-fold increase in intracellular superoxide anion formation as detected by lucigenin assay. This superoxide appeared to result from activation of both the NADPH and NADH oxidases. NADPH oxidase activity increased from 3.23 ± 0.61 to 11.80 ± 1.72 nmol O_2^-/min per milligram protein after 4 hours of Ang II, whereas NADH oxidase activity increased from 16.76 ± 2.13 to 45.00 ± 4.57 nmol O_2^-/min per milligram protein. The NADPH oxidase activity was stimulated by exoge-

nous phosphatidic and arachidonic acids and was partially inhibited by the specific inhibitor diphenylene iodinium. NADH oxidase activity was increased by arachidonic and linoleic acids, was insensitive to exogenous phosphatidic acid, and was inhibited by high concentrations of quinacrine. Both of these oxidases appear to reside in the plasma membrane, on the basis of migration of the activity after cellular fractionation and their apparent insensitivity to the mitochondrial poison KCN. These observations suggest that Ang II specifically activates enzyme systems that promote superoxide generation and raise the possibility that these pathways function as second messengers for long-term responses, such as hypertrophy or hyperplasia. (*Circ Res.* 1994;74:1141-1148.)

Key Words • NADH oxidase • NADPH oxidase • vascular smooth muscle • angiotensin II • superoxide anion

Angiotensin II (Ang II) has multiple effects on vascular smooth muscle, including contraction of normal arteries and hypertrophy or hyperplasia of cultured cells or diseased vessels.[1-4] Although the signaling mechanisms used by Ang II have been well characterized, the correlation of second-messenger generation with specific physiological responses remains relatively unexplored. One of the most significant gaps in our understanding of stimulus-response coupling in this system is the role of phospholipase D (PLD) activation and the generation of phosphatidic acid (PA).

In cultured vascular smooth muscle cells (VSMCs), Ang II causes a rapid, sustained activation of PLD-mediated phosphatidylcholine hydrolysis, resulting in the formation of PA and, indirectly, diacylglycerol (DG).[5,6] Accumulation of both PA and DG is sustained for the duration of hormone exposure.[6] DG, in combination with increased intracellular Ca^{2+}, activates the serine/threonine kinase, protein kinase C.[7] In smooth muscle cells, it is apparently subsequently degraded via DG- and monoacylglycerol-lipase to free arachidonic acid.[8] Although a certain portion of the PA formed is converted to DG,[6] the role of the PA that accumulates remains unknown.

Early studies implicated PA in the regulation of Ca^{2+} influx.[9] More recently, it has been suggested that PA has a role in mitogenesis.[10-14] PAs with a fatty acid composition resembling that found in phosphatidylcholine

increase thymidine incorporation in fibroblasts[10] and human renal mesangial cells,[11] induce c-*fos* and c-*myc* mRNA expression in A431 cells,[12] increase cell number in mammary epithelial cells,[13] and inhibit activation of GTPase-activating protein.[14] In addition, PA induces the expression of platelet-derived growth factor mRNA in mesangial cells[11] and stimulates phospholipase C.[15] All of these events have been shown to have a stimulatory effect on growth, implying that activation of PLD might be an early and integral signal in the growth response. Finally, and perhaps most relevant to the present study, PA has been shown conclusively to stimulate NADPH oxidase activity in neutrophils.[16-18]

This latter observation is interesting because reactive oxygen intermediates have been proposed to act as intracellular second messengers[19] and thus represent a potential pathway by which receptor activation could be transduced to a physiological response. In this regard, it is interesting to note that oxidase activity and abnormalities in oxygen metabolism have been shown to be related to growth in tumor cells.[20] In fact, four different types of antitumor agents inhibit the plasma membrane redox system,[21] implying that the redox state of the cell may be a common step at which growth control can be achieved. In VSMCs, xanthine/xanthine oxidase (which produces H_2O_2 and O_2^-) and H_2O_2 itself stimulate proliferation.[22] Furthermore, the NADH oxidase, a plasma membrane–bound protein that, when activated, produces superoxide, has been shown to be sensitive to activation by growth factors, including epidermal growth factor and insulin.[23] This oxidase is stimulated by fatty acids,[23] one class of

second messengers produced by phospholipase A_2 or indirectly from the activation of PLD.

In the present study, we examined the ability of Ang II to activate both the NADH and NADPH oxidases in cultured VSMCs. Superoxide anion formation was measured, and oxidase activity was identified by use of known activators and inhibitors. We found that long-term treatment with Ang II causes a large increase in the activity of both of these enzymes, with the majority of superoxide anion production deriving from the NADH oxidase. These observations suggest that reactive oxygen intermediates may be part of the normal intracellular signaling mechanisms stimulated by Ang II in cultured VSMCs.

Materials and Methods

Cell Culture

VSMCs were isolated from rat thoracic aorta by enzymatic digestion as described previously.[24] Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mmol/L 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 100-mm dishes, fed every other day, and used at confluence.

Measurement of Superoxide Anion Production in Intact Cells

Lucigenin, an acridylium dinitrate compound that emits light on reduction and interaction with the superoxide anion,[25] was used to measure superoxide anion production. Control cells or cells treated with Ang II were harvested from the dish with type I collagenase (1 mg/mL, in the presence of 1 mg/mL soybean trypsin inhibitor and 2 mg/mL bovine serum albumin). Cells were pelleted by centrifugation ($200g$, 4°C, 5 minutes), the supernatant was discarded, and the pellet resuspended in a balanced salt solution of the following composition (mmol/L): NaCl 130, KCl 5, MgCl$_2$ 1, CaCl$_2$ 1.5, phosphoric acid 35, and HEPES 20, pH 7.4. After an additional centrifugation, cells were resuspended at 2×10^6 cells per milliliter in balanced salt solution containing 10 mmol/L glucose and 1 mg/mL bovine serum albumin and stored on ice until use.

To measure superoxide anion production, 9×10^5 cells were incubated at room temperature for 5 minutes. To start the assay, cells were added to a cuvette containing dark-adapted lucigenin (final concentration, 500 μmol/L) in balanced salt solution. Photon emission was measured every 15 seconds for 10 to 15 minutes in a luminometer (model 20, Turner Designs). A buffer blank (<5% of the cell signal) was subtracted from each reading before transformation of the data. The amount of superoxide produced at each time point was calculated by comparison with a standard curve generated using xanthine/xanthine oxidase as described by Ohara et al.[26]

NADH/NADPH Oxidase Assay

Control cultures or cultures that had been exposed to Ang II for the indicated times were washed five times with 5 mL ice-cold phosphate-buffered saline, 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 phosphate-buffered saline. Cells were then centrifuged at $750g$ at 4°C for 10 minutes. The supernatant was discarded, and the pellet was resuspended (0.5 to 1.0 mL per dish) in lysis buffer containing protease inhibitors (20 mmol/L monobasic potassium phosphate [pH 7.0], 1 mmol/L EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.5 mmol/L phe-

nylmethylsulfonyl fluoride). 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.[27]

NADH or NADPH oxidase activity was measured by a luminescence assay in a 50-mmol/L phosphate buffer, pH 7.0, containing 1 mmol/L EGTA, 150 mmol/L sucrose, 500 μmol/L lucigenin as the electron acceptor, and either 100 μmol/L NADH or 100 μmol/L NADPH as the substrate (final volume, 0.9 mL). This concentration fell well within the linear range of the assay (1 μmol/L to 10 mmol/L for NADPH and 1 μmol/L to 1 mmol/L for NADH), and neither NADH nor NADPH was rate limiting over the initial course of the assay. No activity could be measured in the absence of NADH and NADPH. In some experiments, inhibitors (diphenylene iodinium [DPI, 100 μmol/L] added 5 minutes before readings, KCN [1 mmol/L], quinacrine [6 mmol/L], Tiron [10 mmol/L], or superoxide dismutase [120 U/mL]) or activators (fatty acids [100 μmol/L] or PA [100 μg/mL]) were added to the cuvette. The reaction was started by the addition of 100 μL of homogenate (50 to 300 μg protein). Luminescence was monitored as described above for intact cells.

Cell Fractionation

In some experiments, membranes and cytosol were separated by centrifugation, and NADH and NADPH oxidase activity were measured. These samples were prepared exactly as described above for the NADPH/NADH oxidase assays, except that after lysis, cell homogenates were centrifuged at 29 100g for 20 minutes 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. NADPH and NADH oxidase activities were then measured as described above.

[^{3}H]Leucine Incorporation

To measure hypertrophy of VSMCs, cells were plated at low density, grown for 48 hours in Dulbecco's modified Eagle's medium containing 10% calf serum, and grown for an additional 72 hours in Dulbecco's modified Eagle's medium containing 1% platelet-poor plasma. Twenty-four hours before harvest, cells were incubated with [^{3}H]leucine (2 μCi/mL) in the presence or absence of 100 nmol/L Ang II. Cells were then washed twice with ice-cold phosphate-buffered saline and incubated with 5% trichloroacetic acid for 5 minutes at 4°C. After two additional washes, cells were dissolved in 1 mL 0.4N NaOH. Duplicate aliquots (0.4 mL) were removed, acidified with 0.2 mL of 1.0N HCl, and counted in 10 mL Liquiscint in a liquid scintillation spectrophotometer.

Rate Calculations and Statistical Analysis

Initial rates of enzyme activity were calculated by linear regression over the first 30 to 120 seconds of exposure to lucigenin. In general, correlations (R^2 values) were ≥ 0.9. Rates are presented as mean±SEM, and comparison between groups was performed by Student's paired two-tailed t test.

Chemicals

All chemicals were of analytical grade or better. Bovine serum albumin, catalase, and phenylmethylsulfonyl fluoride were from Boehringer Mannheim. DPI was purchased from Toronto Research Chemicals, and histidine was from Eastman Kodak. Collagenase was obtained from Worthington. Soybean trypsin inhibitor, glutamine, penicillin, streptomycin, and trypsin/EDTA were purchased from GIBCO. [^{3}H]Leucine (140 Ci/mmol) was from Dupont NEN, and Liquiscint was purchased from National Diagnostics. Common buffer salts were obtained from Fisher. All other chemicals and reagents, including Dulbecco's modified Eagle's medium with 25 mmol/L HEPES and 4.5 g/L glucose and calf serum, were from Sigma.
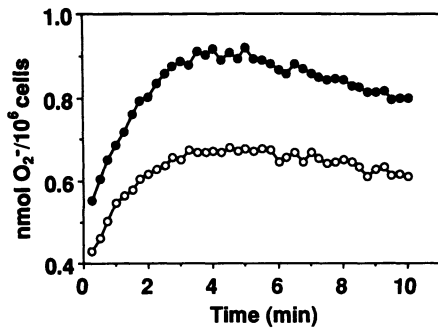
Fig 1. Graph showing angiotensin II–induced superoxide production in intact vascular smooth muscle cells. Vascular smooth muscle cells were exposed to angiotensin II (100 nmol/L) (●) or media alone (○) for 4 hours and removed from the culture dish with collagenase. Cells were added to a cuvette containing dark-adapted lucigenin in balanced salt solution, and photon emission was measured in a luminometer. Tracings are from a representative experiment repeated 15 times.

Results

Superoxide Anion Production in Intact Cells

Stimulation of intact VSMCs with Ang II led to a significant increase in superoxide anion production (Fig 1). Over the 10 minutes during which superoxide production was measured, cells exposed to Ang II for 4 hours before the start of the assay produced 2.7 ± 0.4-fold more superoxide anion than did control cells ($n=15$, $P<.0002$). This increase could not be inhibited by the addition of extracellular superoxide dismutase (50 U/mL) to the cuvette but was almost completely abolished when the cells were incubated with Tiron (10 mmol/L), a cell-permeant chelator of superoxide (data not shown). These observations suggest that Ang II stimulates an increase in intracellular superoxide anion production, since lucigenin detects both intracellular and extracellular superoxide anion accumulation.[28]

Ang II Stimulation of NADPH and NADH Oxidase Activity

To eliminate any potential barrier to lucigenin entry presented by the plasma membrane and to examine the activity of specific intracellular oxidases, we prepared cell homogenates and assayed NADH and NADPH oxidase activity. These two oxidase systems were chosen for further study because preliminary experiments with oxidase inhibitors in intact cells indicated that they were the two most important oxidase systems in smooth muscle, in agreement with data presented by Omar et al.[29] A 4-hour treatment with Ang II caused a large increase in both NADH and NADPH oxidase activity, both in terms of initial rate (Table 1) and peak response (Fig 2).

The time course of Ang II stimulation of these two oxidases is shown in Fig 3. Surprisingly, the increase in oxidase activity is not apparent until ≈1 hour and continues to increase for at least 6 hours. We were unable to determine whether this delay required new protein synthesis, since both cycloheximide (1 μmol/L, 18 hours) and actinomycin D (0.1 μg/mL, 18 hours) caused a large basal increase in superoxide anion production (3.4 ± 0.5-fold [$n=4$] and 4.0 ± 0.3-fold [$n=2$], respectively, for the NADH oxidase).

TABLE 1. Effect of Angiotensin II on NADH and NADPH Oxidase Activity

	Initial Rate, nmol O_2^-/min per milligram protein	
	NADPH	**NADH**
Control	3.23 ± 0.61 ($n=31$)	16.76 ± 2.13 ($n=44$)
Ang II	$11.80 \pm 1.72^*$ ($n=31$)	$45.00 \pm 4.57^*$ ($n=44$)

Ang II indicates angiotensin II. Values are mean±SEM.

Vascular smooth muscle cells were treated with 100 nmol/L Ang II for 4 hours before homogenization. Superoxide production was measured in the presence of either 100 μmol/L NADPH or 100 μmol/L NADH. The initial rate of enzyme activity was calculated over the first 30 to 120 seconds of exposure to substrate.

$^*P<.05$ vs control.

The Ang II–induced increase in both NADH and NADPH oxidase activity was dose dependent between 0.01 and 1000 nmol/L (Fig 4). Activation of these enzymes was receptor-mediated, since the response could be blocked by the AT_1 antagonist losartan (10 μmol/L) (NADPH, $84 \pm 8\%$ inhibition; NADH, $86 \pm 7\%$ inhibition). The shallow nature of this curve suggests that there may be cooperativity in the signaling cascade activated during a 4-hour stimulation with Ang II.

Functional Identification of the NADPH Oxidase

In other systems, the NADPH oxidase is a plasma membrane–bound enzyme that is stimulated by arachidonic acid and PA or DG and is inhibited by DPI.[16,30] For further evidence of the specificity of the VSMC NADPH oxidase, we examined the sensitivity of the enzyme to these compounds and measured the partitioning of activity between membrane and cytosol. As shown in Fig 5, PA (100 μg/mL) acutely stimulated NADPH oxidase
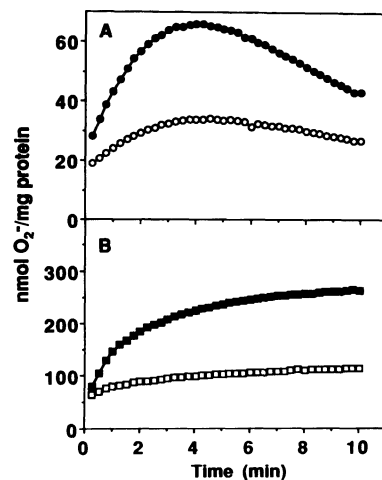

Fig 2. Graphs showing angiotensin II stimulation of NADPH and NADH oxidase activities. Vascular smooth muscle cells were exposed to angiotensin II (100 nmol/L) (filled symbols) or media alone (open symbols) for 4 hours, homogenized, and added to a cuvette containing dark-adapted lucigenin in a 50 mmol/L phosphate buffer. A, NADPH (100 μmol/L) was used as the substrate. B, NADH (100 μmol/L) was used as the substrate. Please note the difference in scale for the two graphs. Each tracing is from a representative experiment repeated 31 times for NADPH and 44 times for NADH. The mean initial rates are given in Table 1.
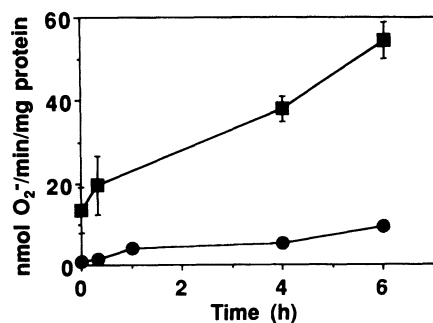
FIG 3. Time course of angiotensin II activation of the NADH and NADPH oxidases. Vascular smooth muscle cells were exposed to angiotensin II (100 nmol/L) for the indicated times, homogenized, and added to a cuvette containing dark-adapted lucigenin and NADH or NADPH in 50 mmol/L phosphate buffer. Photon emission was measured, and the initial rate of enzyme activity was calculated as described in "Materials and Methods." Data represent the mean±SEM of three experiments each for NADPH (●) and NADH (■). For NADPH, error bars were smaller than the width of the symbol.

activity (initial rate [control], 4.22±1.90 nmol O_2^-/min per milligram protein; PA, 6.87±2.80 nmol O_2^-/min per milligram protein; n=6; $P<.05$). Arachidonic acid also increased oxidase activity (Fig 5), whereas dioleoyl-DG (100 μg/mL) had no effect (data not shown). Ang II–induced NADPH oxidase activity was partially inhibited by a maximal concentration of DPI (100 μmol/L) (Fig 6). Quinacrine, an inhibitor of the NADH oxidase, actually increased NADPH oxidase activity (Fig 7).

On fractionation of cytosol and membranes, all NADPH activity migrated with the membranes, indicating that the VSMC NADPH oxidase is membrane-associated (Table 3). The mitochondrial poison KCN did not inhibit Ang II–induced superoxide formation in the membrane fraction (response to Ang II was 187±44% of that in untreated homogenates [n=2]), suggesting that the hormone-sensitive NADPH oxidase resides in the plasma membrane rather than in the mitochondria.

Functional Identification of the NADH Oxidase

Although the NADH oxidase is less well characterized than the NADPH oxidase in hepatocytes, it has
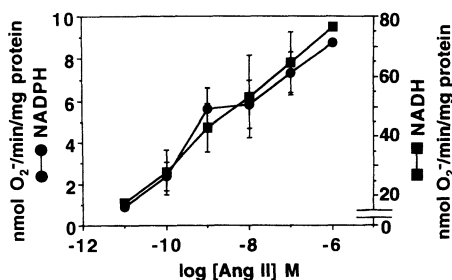

FIG 4. Graph showing dose dependence of angiotensin II (Ang II)–stimulated NADPH and NADH oxidase activity. Vascular smooth muscle cells were exposed to Ang II at the indicated concentration for 4 hours, homogenized, and added to a cuvette containing dark-adapted lucigenin in 50 mmol/L phosphate buffer. Photon emission was measured, and the initial rate of enzyme activity was calculated as described in "Materials and Methods." Data represent the mean±SEM of six experiments each for NADPH (●) and NADH (■). Control values were 1.15±0.23 nmol/min per milligram protein for NADPH and 14.63±1.99 nmol/min per milligram protein for NADH.
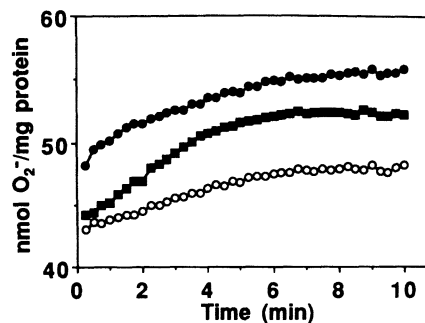

FIG 5. Graph showing stimulation of vascular smooth muscle cell NADPH oxidase by phosphatidic acid and arachidonic acid. Vascular smooth muscle cells were homogenized and added to a cuvette containing NADPH and lucigenin alone (○) or lucigenin and dioleoyl–phosphatidic acid (100 μg/mL) (■) or arachidonic acid (100 μmol/L) (●). Tracing is from one representative experiment repeated three to six times.

been established that the hormone-sensitive enzyme is stimulated by fatty acids and lysophospholipids, is inhibited by high concentrations of quinacrine, and is plasma membrane bound.[31] In addition, it is thought to be a flavin-containing enzyme and therefore should be sensitive to DPI.[31-33] We used these characteristics to further identify the VSMC NADH oxidase.

As shown in Fig 8, arachidonic acid (100 μmol/L), linoleic acid (100 μmol/L), and stearic acid (100 μmol/L) all caused an acute increase in NADH oxidase activity. PA (100 μg/mL) had almost no effect on NADH oxidase activity (data not shown). The effect of these compounds on initial rate of enzyme activity is given in Table 2. Of the fatty acids tested, arachidonic acid and linoleic acid were equally effective, whereas stearic acid was less potent an activator.

Figs 6 and 7 show the sensitivity of the VSMC NADH oxidase to DPI (100 μmol/L) and quinacrine (6 mmol/ L), respectively. This maximally effective concentration of quinacrine completely abolished the activation of the NADH oxidase by Ang II and significantly inhibited NADH oxidase activity in control cells. This was not due to nonspecific interference with the lucigenin sig-
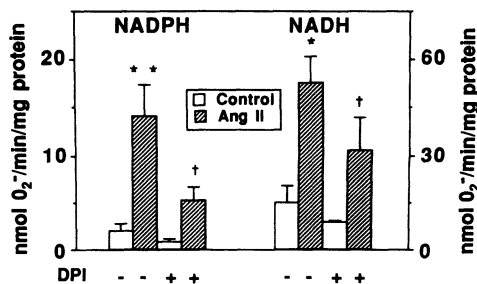

FIG 6. Bar graph showing the effect of diphenylene iodinium (DPI) on NADPH and NADH oxidase activity in vascular smooth muscle cells. Vascular smooth muscle cells were exposed to angiotensin II (Ang II) or media alone (control) for 4 hours. Cells were then homogenized and incubated with buffer (−) or 100 μmol/L DPI (+) for 5 minutes at room temperature immediately before the assay. Values for the NADPH assay are given on the left axis; values for the NADH assay are given on the right axis. Each bar represents the mean±SEM of the initial rate of enzyme activity for three (NADH) or four (NADPH) experiments. *$P<.05$ and **$P<.01$ for Ang II vs control; †$P<.05$ for the effect of DPI on the Ang II signal vs the effect of Ang II alone.
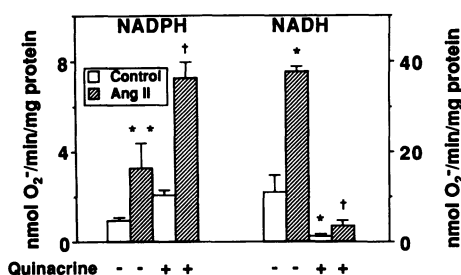
FIG 7. Bar graph showing the effect of quinacrine on NADPH and NADH oxidase activity in vascular smooth muscle cells. Vascular smooth muscle cells were exposed to angiotensin II (Ang II) or media alone (control) for 4 hours. Cells were then homogenized and added to a cuvette containing lucigenin alone (−) or lucigenin with 6 mmol/L quinacrine (+). Values for the NADPH assay are given on the left axis; values for the NADH assay are given on the right axis. Each bar represents the mean±SEM of the initial rate of enzyme activity for three (NADPH) or five (NADH) experiments. *$P<.05$ and **$P<.01$ for Ang II vs control; †$P<.05$ for quinacrine+Ang II vs Ang II alone.

nal, since quinacrine actually increased Ang II–stimulated NADPH oxidase activity (Fig 7). As expected, DPI also partially inhibited NADH oxidase activation (Fig 6).

As was true for the NADPH oxidase, NADH oxidase activity also partitioned with the membrane fraction on cell fractionation. In fact, there appears to be an inhibitory factor in the cytosol, since activity in isolated membranes was actually higher than that in homogenates (Table 3). KCN had no effect on the initial rate of enzyme activity in cells treated with Ang II ($126±9\%$ of that in untreated cells, n=4), suggesting that the enzyme activity that we are measuring is plasma membrane associated.

Effect of Oxidase Inhibition on VSMC Hypertrophy

To determine whether oxidase inhibition attenuated Ang II–induced hypertrophy, we quiesced cells for 72 hours in 1% platelet-poor plasma labeled with [³H]leucine to measure protein synthesis and exposed them to Ang II (100 nmol/L, 24 hours) in the presence or absence of DPI or quinacrine. In this series of experiments, the cells, by necessity, were exposed to these inhibitors for 24 hours. Both quinacrine and DPI showed a tendency to be toxic during this time course,
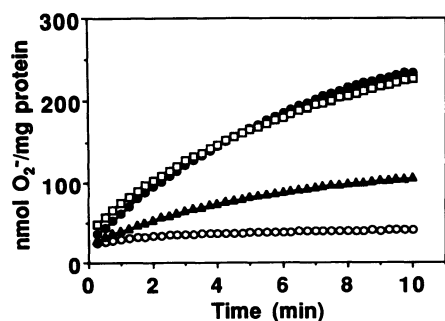

FIG 8. Graph showing fatty acid stimulation of the vascular smooth muscle cell NADH oxidase. Vascular smooth muscle cells were homogenized and added to a cuvette containing NADH and lucigenin alone (○) or lucigenin plus 100 μmol/L arachidonic acid (●), linoleic acid (□), or stearic acid (▲). Tracing is from one representative experiment repeated three or four times (Table 2).

TABLE 2. Activation of Vascular Smooth Muscle Cell NADH Oxidase by Fatty Acids

	Initial Rate, nmol O_2^-/min per milligram protein
Control	11.25±2.75 (n=4)
Arachidonic acid	32.47±7.10* (n=4)
Linoleic acid	31.75±6.74* (n=3)
Stearic acid	28.24±12.59 (n=3)

Values are mean±SEM.
Vascular smooth muscle cells were homogenized and added to a 50 mmol/L phosphate buffer containing lucigenin and 100 μmol/L of the indicated fatty acid. Enzyme activity was measured over the first 2 minutes of stimulation.
*$P<.01$ vs control.

indicating the critical importance of these pathways to cell growth and viability. As shown in Fig 9, Ang II caused approximately an 80% increase in [³H]leucine incorporation. DPI (10 μmol/L) inhibited this increase by $95±2\%$. Quinacrine (50 μmol/L) completely abolished Ang II–induced hypertrophy, to the point of interfering with viability.

Validation of the Lucigenin Assay

To verify the specificity of the lucigenin assay for superoxide anion in vascular smooth muscle cells, we examined the ability of free radicals and free radical scavengers to inhibit the lucigenin signal. Both superoxide dismutase (120 U/mL) and Tiron (10 mmol/L), which specifically scavenge superoxide anion,[28,34-37] inhibited the pure superoxide signal produced by xanthine/xanthine oxidase ($98±0.5\%$ and $99±0.01\%$ inhibition, respectively). No light emission was observed when H_2O_2 (100 μmol/L) was added to the cuvette (data not shown).

In intact cells and cell homogenates, the lucigenin signal was not inhibited by the addition of histidine (1 mmol/L) or azide (100 μmol/L) (to quench singlet oxygen), mannitol (10 mmol/L) or deferoxamine (10 mmol/L) (to prevent formation or accumulation of hydroxyl radical), or catalase (2000 U/mL). Superoxide dismutase (120 U/mL) was relatively ineffective at inhibiting superoxide accumulation. As noted above, in intact cells, no inhibition was seen with superoxide dismutase, probably because of its inability to enter cells. In cell homogenates, superoxide dismutase inhibited the Ang II–stimulated NADPH signal by $33±3\%$ (n=13) and the NADH signal by $42±4\%$ (n=12). The lack of effectiveness of superoxide dismutase seems to be a general property of smooth muscle cells,[28] possibly related to the inactivation of superoxide dismutase by H_2O_2.[38] Because of this limitation, we chose to use the nonenzymatic superoxide scavenger Tiron[28,34-37] to specifically inhibit the superoxide signal in these assays. Tiron (10 mmol/L) completely abolished the lucigenin signal in Ang II–stimulated intact cells and inhibited stimulated NADPH and NADH oxidase assay signals by $73.8±2.9$ and $74.5±1.9\%$, respectively (n=3).

Discussion

The data presented here identify a major new component of the intracellular signaling pathways stimulated by Ang II in VSMCs. Prolonged incubation with Ang II led

TABLE 3.   Fractionation of NADPH and NADH Oxidase Activity Between Vascular Smooth Muscle Cell Membranes and Cytosol

| | Initial Rate, nmol O_2^-/min per milligram protein | | | |
| | NADPH (n=5) | | NADH (n=6) | |
	Control	Ang II	Control	Ang II
Homogenate	3.91±1.20	12.43±3.93	14.15±6.74	31.80±11.82
Membranes	8.20±1.49	22.75±3.77	143.66±17.00	215.15±41.35
Cytosol	0.16±0.03	0.11±0.03	0.47±0.20	0.16±0.07

Ang II indicates angiotensin II. Values are mean±SEM.

Vascular smooth muscle cells were treated with 100 nmol/L Ang II for 4 hours, and membranes and cytosol were separated as described in "Materials and Methods." The initial rate of superoxide formation was measured in equivalent aliquots of each fraction, and rates were normalized to protein.

to an activation of both the NADPH and NADH oxidases, with a resultant increase in intracellular superoxide generation. Free radicals such as superoxide have numerous effects on cell function, including induction of growth,[22] regulation of kinase activity,[38,39] lipid peroxidation,[40] and inactivation of nitric oxide.[41] Thus, superoxide or its metabolites can function as intercellular and intracellular second messengers, transducing receptor stimulation into a biochemical response. The slow onset of NADH and NADPH oxidase activation in VSMCs stimulated with Ang II and the ability of oxidase inhibitors to attenuate hypertrophy make it likely that in this system, superoxide is involved in the long-term metabolic response of VSMCs to Ang II.
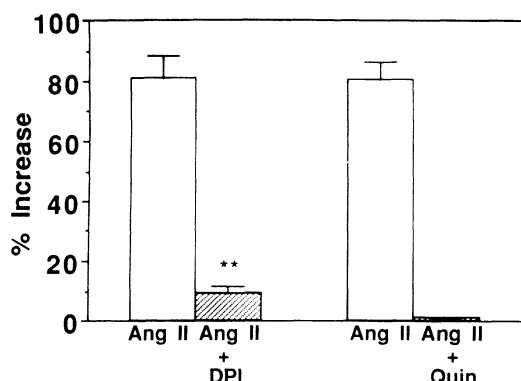
In cultured VSMCs, the largest source of hormone-sensitive superoxide anion formation appears to be the NADH oxidase. Most of the data available concerning the NADH oxidase have been gathered in plant or liver cells.[23,31] The plasma membrane NADH oxidase appears to be an intrinsic membrane protein that is stimulated by growth factors, lysophosphatidylcholine, and linoleic acid.[23,31] The best inhibitor of the NADH

FIG 9.   Bar graph showing attenuation of angiotensin II (Ang II)–induced hypertrophy by diphenylene iodinium (DPI) and quinacrine (Quin). Vascular smooth muscle cells were quiesced for 72 hours in 1% platelet-poor plasma, labeled with [³H]leucine, and exposed to Ang II (100 nmol/L) in the presence or absence of DPI (10 μmol/L) or Quin (50 μmol/L) for 24 hours. [³H]Leucine incorporation was measured as described in "Materials and Methods." Data are expressed as the percent increase in [³H]leucine incorporation induced by Ang II over the appropriate control. Each bar represents the mean of two (Quin) or three (DPI) experiments performed in triplicate. **$P<.01$ for increase in the presence of inhibitor vs increase with Ang II alone. Quin was toxic to the cells.

oxidase is quinacrine (called atebrin in the older literature),[31] but inhibition has also been seen by quinone analogues, calcitriol, and retinoic acid.[31,42]

The Ang II–sensitive VSMC NADH oxidase appears to have characteristics similar to those described in liver, except that the VSMC enzyme is insensitive to chloroquine (authors' unpublished observations). Fatty acids are potent stimuli of VSMC NADH oxidase activity, and quinacrine completely abolishes NADH-dependent superoxide generation. Preliminary data recently reported suggest that a microsomal NADH oxidase accounts for the vast majority (>94%) of the superoxide generated basally in pulmonary artery smooth muscle.[29] This agrees well with our observation that the NADH oxidase generates four to five times more superoxide anion than the NADPH oxidase, although in cultured VSMCs, the NADH oxidase activity does not migrate with the microsomal fraction (cytosolic fraction in Table 3).

The NADPH oxidase has been most extensively studied in neutrophils, where it is a plasma membrane–associated enzyme responsible for the respiratory burst essential to the microbicidal activity of these cells.[43-45] Neutrophil NADPH oxidase activity can be specifically stimulated by PA, arachidonic acid, and anionic amphophiles such as sodium dodecyl sulfate[17,18,30,46] and inhibited by DPI, p-chloromercuribenzoate, and cibacron blue.[16,33] The mechanism by which agonists activate the neutrophil NADPH oxidase is thought to involve activation of PLD, the subsequent production of PA and DG, and/or the activation of protein kinase C.[18,44,47,48]

Although many of these characteristics are shared by the VSMC NADPH oxidase (most notably, stimulation by PA and arachidonic acid, association with the plasma membrane, and sensitivity to DPI), the VSMC oxidase differs from that of neutrophils in some respects. First and foremost, the time course of stimulation of the oxidase differs dramatically in the two cell types. Superoxide anion generation by the neutrophil NADPH oxidase begins within minutes of exposure to hormones,[47,48] whereas the activation of the NADPH oxidase in VSMCs stimulated with Ang II seen in the present study occurs over a period of hours. In this regard, the VSMC NADPH oxidase is more like that identified in human fibroblasts[49] and mesangial cells.[50] Second, the superoxide generated here in response to Ang II appears to be mostly intracellular, with only a

limited amount of superoxide being released to the exterior, as occurs in phagocytes. Whether this radical is then converted to H_2O_2 or some other oxygen-derived radical remains to be determined. Finally, the physiological role of VSMC superoxide anion is almost certainly quite different from that of phagocytic cells. In phagocytic cells, superoxide generation serves an extracellular microbicidal function that is rapidly complete. In VSMCs, the delayed, prolonged NADH and NADPH oxidase activation is more likely to function as an intracellular signal transduction pathway, perhaps related to the long-term hypertrophic effects of Ang II.

The mechanism by which Ang II increases NADH and NADPH oxidase activity is unclear. In neutrophils, agonist stimulation of the NADPH oxidase is closely correlated with the activation of PLD,[18] an enzyme that is robustly stimulated by Ang II in VSMCs.[5,6] There is much debate as to whether the direct product of PLD, PA, is the molecule that activates the NADPH oxidase or whether subsequent conversion of PA to DG is required for stimulation of the oxidase.[18,30,48] Accumulation of both of these lipids occurs in response to Ang II in VSMCs,[6] so that either could serve as an endogenous stimulus of the NADPH oxidase. Alternatively, and perhaps of more importance to NADH oxidase activation, Ang II generates arachidonate formation in VSMCs[51] directly from phospholipase A_2 hydrolysis of phosphatidylcholine (authors' unpublished observations) and indirectly from conversion of accumulated DG by the action of DG and monoacylglycerol lipases.[8]

Although these signaling molecules are capable of stimulating both oxidases, the situation in VSMCs is more complex than a simple activation in response to second messengers. The 4-hour stimulation required before superoxide accumulation becomes evident could be a result of several factors. First, a superoxide-scavenging system is likely present in VSMCs. Over the prolonged stimulation with Ang II, this system may simply become overwhelmed or may undergo inactivation as a result of metabolism or as a specific response to Ang II. SOD activity remains constant for up to 6 hours of Ang II stimulation (authors' unpublished observations), so the identity of this putative scavenging system remains elusive. Alternatively, a component of the oxidase may have to be newly synthesized before full activation can be achieved. We were unable to evaluate this possibility fully, since cycloheximide and actinomycin D both caused a large basal increase in superoxide formation. However, it seems unlikely that induction of new protein is the sole explanation for the delay in response to Ang II, since PA and arachidonate were able to activate both oxidases acutely in cell homogenates. Another possibility is that a second-messenger system initially activated by Ang II desensitizes with time, allowing delayed superoxide formation to occur in response to the remaining signals. Such a desensitization occurs in the case of Ang II stimulation of protein kinase C activity, which disappears within 4 hours of stimulation with Ang II.[52] The nature of a system of this type relevant to superoxide formation is at present purely speculative.

It is intriguing to consider the possible implications of Ang II–stimulated NADH and NADPH oxidase activation on smooth muscle cell function. Ang II is an important hypertrophic/hyperplastic agent in certain forms of vascular disease,[4] including hypertension and restenosis after angioplasty. The intracellular signals responsible for initiation and maintenance of the growth program have not been fully delineated. The long-term nature of the NADH/NADPH oxidase response, combined with the known association of reactive oxygen species with growth,[20-22] suggests that these enzymes may be an integral part of the growth program in smooth muscle. Recent work has shown that H_2O_2, one immediate product of superoxide metabolism, stimulates mitogenesis in VSMCs.[22] Furthermore, both protein tyrosine kinase and protein tyrosine phosphatase activities are regulated by reactive oxygen species.[39,53] These signaling pathways are intimately involved in the growth response in many cell types. The growth-related pathways regulated by reactive oxygen species and the potential interactions among them remain to be determined in VSMCs.

In summary, we have shown that Ang II stimulates superoxide formation in cultured VSMCs by activating both NADPH and NADH oxidases. The long-term nature of this response suggests that it may participate in growth-related signal generation, serving to transduce information from the surface signal of Ang II binding to its receptor to the ultimate response of hypertrophy or hyperplasia of VSMCs.

Acknowledgments

References

1. Geisterfer A, Peach MJ, Owens GK. Angiotensin II induces hypertrophy, not hyperplasia of cultured rat aortic smooth muscle cells. *Circ Res.* 1988;62:749-756.
2. Berk BC, Vekshtein V, Gordon HM, Tsuda T. Angiotensin II–stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension.* 1989;13:305-314.
3. Campbell-Boswell M, Robertson A. Effects of angiotensin II and vasopressin on human smooth muscle cells in vitro. *Exp Mol Pathol.* 1981;35:265-276.
4. Daemen MJAP, Lombardi DM, Bosman FT, Schwartz SM. Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ Res.* 1991;68:450-456.
5. Lassègue B, Alexander RW, Clark M, Griendling KK. Angiotensin II-induced phosphatidylcholine hydrolysis in cultured vascular smooth muscle cells: regulation and localization. *Biochem J.* 1991; 276:19-25.
6. Lassègue B, Alexander RW, Clark M, Akers MA, Griendling KK. Phosphatidylcholine is a major source of phosphatidic acid and diacylglycerol in angiotensin II-stimulated vascular smooth muscle cells. *Biochem J.* 1993;292:509-517.
7. Tsuda T, Griendling KK, Ollerenshaw JD, Lassègue B, Alexander RW. Angiotensin II- and endothelin-induced protein phosphorylation in cultured vascular smooth muscle cells. *J Vasc Res.* 1993; 30:241-249.
8. Severson DL, Hee-Cheong M. Diacylglycerol metabolism in isolated aortic smooth muscle cells. *Am J Physiol.* 1989;256: C11-C17.
9. Putney JW, Weiss SJ, Van De Walle CM, Haddas RA. Is phosphatidic acid a calcium ionophore under neurohumoral control? *Nature.* 1980;284:345-347.
10. Yu C, Tsai M, Stacey DW. Cellular ras activity and phospholipid metabolism. *Cell.* 1988;52:63-71.
11. Knauss TC, Jaffer FE, Abboud HE. Phosphatidic acid modulates DNA synthesis, phospholipase C, and platelet-derived growth factor mRNAs in cultured mesangial cells. *J Biol Chem.* 1990;265: 14457-14463.

12. Moolenaar WH, Kruijer W, Tilly BC, Verlaan I, Bierman AJ, de Laat SW. Growth factor-like action of phosphatidic acid. *Nature.* 1986;323:171-173.

13. Imagawa W, Bandyopadhyay GK, Wallace D, Nandi S. Phospholipids containing polyunsaturated fatty acyl groups are mitogenic for normal mouse mammary epithelial cells in serum-free primary cell culture. *Proc Natl Acad Sci U S A.* 1989;86:4122-4126.

14. Tsai M, Yu C, Wei F, Stacey DW. The effect of GTPase activating protein upon ras is inhibited by mitogenically responsive lipids. *Science.* 1989;243:522-526.

15. Kroll MH, Zavoico GB, Schafer AI. Second messenger function of phosphatidic acid in platelet activation. *J Cell Physiol.* 1989;139:558-564.

16. Bellavite P, Corso F, Dusi S, Grzeskowiak M, Della-Bianca V, Rossi F. Activation of NADPH-dependent superoxide production in plasma membrane extracts of pig neutrophils by phosphatidic acid. *J Biol Chem.* 1988;263:8210-8214.

17. Ohtsuka T, Ozawa M, Okamura N, Ishibashi S. Stimulatory effects of a short-chain phosphatidate on superoxide anion production in guinea pig polymorphonuclear leukocytes. *J Biochem.* 1989;106:259-263.

18. Agwu DE, McPhail LC, Sozzani S, Bass DA, McCall CE. Phosphatidic acid as a second messenger in human polymorphonuclear leukocytes: effects on activation of NADPH oxidase. *J Clin Invest.* 1991;88:531-539.

19. Wolin MS. Activated oxygen metabolites as regulators of vascular tone. *Klin Wochenschr.* 1991;69:1046-1049.

20. Cerutti P. Prooxidant states and tumor promotion. *Science.* 1985;227:375-381.

21. Crane FL, Sun IL, Clark MG, Grebing C, Low H. Transplasma-membrane redox systems in growth and development. *Biochim Biophys Acta.* 1985;811:233-264.

22. Rao GN, Berk BC. Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res.* 1992;70:593-599.

23. Brightman AO, Wang J, Miu RK, Sun IL, Barr R, Crane FL, Morré DJ. A growth factor- and hormone-stimulated NADH oxidase from rat liver plasma membrane. *Biochim Biophys Acta.* 1992;1105:109-117.

24. Griendling KK, Taubman MB, Akers M, Mendlowitz M, Alexander RW. Characterization of phosphatidylinositol-specific phospholipase C from cultured vascular smooth muscle cells. *J Biol Chem.* 1991;266:15498-15504.

25. Gyllenhammar H. Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J Immunol Methods.* 1987;97:209-213.

26. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest.* 1993;91:2546-2551.

27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193:265-275.

28. Paky A, Michael JR, Burke-Wolin TM, Wolin MS, Gurtner GH. Endogenous production of superoxide by rabbit lungs: effects of hypoxia or metabolic inhibitors. *J Appl Physiol.* 1993;74:2868-2874.

29. Omar HA, Mohazzab KM, Mortelliti MP, Wolin MS. O_2-dependent modulation of calf pulmonary artery tone by lactate: potential role of H_2O_2 and cGMP. *Am J Physiol.* 1993;264:L141-L145.

30. Burnham DN, Uhlinger DJ, Lambeth JD. Diradylglycerol synergizes with an anionic amphiphile to activate superoxide generation and phosphorylation of p47$_{phox}$ in a cell-free system from human neutrophils. *J Biol Chem.* 1990;265:17550-17559.

31. Morré DJ, Brightman AO. Mini-review: NADH oxidase of plasma membranes. *J Bioenerg Biomembr.* 1991;23:469-489.

32. Nisimoto Y, Tamura M, Lambeth JD. A menadione-stimulated pyridine nucleotide oxidase from resting bovine neutrophil membranes. *J Biol Chem.* 1988;263:11657-11663.

33. O'Donnell VB, Tew DG, Jones OTG, England PJ. Studies on the inhibitory mechanism of iodinium compounds with special reference to neutrophil NADPH oxidase. *Biochem J.* 1993;290:41-49.

34. Whiteside C, Hassan HM. Role of oxyradicals in the inactivation of catalase by ozone. *Free Radic Biol Med.* 1988;5:305-312.

35. Ledenev AN, Kostantinov AA, Popova E, Ruuge EK. A simple assay of the superoxide generation rate with tiron as an EPR-visible radical scavenger. *Biochem Int.* 1986;13:391-396.

36. Pagano PJ, Tornheim K, Cohen RA. Superoxide anion production by rabbit thoracic aorta: effect of endothelium-derived nitric oxide. *Am J Physiol.* 1993;265:H707-H712.

37. Devlin RG, Lin CS, Perper RJ, Dougherty H. Evaluation of free radical scavengers in studies of lymphocyte-mediated cytolysis. *Immunopharmacology.* 1981;3:147-159.

38. Hodgson EK, Fridovich I. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. *Biochemistry.* 1975;14:5294-5295.

39. Larsson R, Cerutti P. Oxidants induce phosphorylation of ribosomal protein S6. *J Biol Chem.* 1988;263:17452-17458.

40. Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. *Lab Invest.* 1982;47:412-425.

41. Mügge A, Elwell JH, Peterson TE, Hofmeyer TG, Heistad DD, Harrison DG. Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ Res.* 1991;69:1293-1300.

42. Morré DJ, Morré DM, Paulik M, Batova A, Broome A, Pirisi L, Creek KE. Retinoic acid and calcitriol inhibition of growth and NADH oxidase of normal and immortalized human keratinocytes. *Biochim Biophys Acta.* 1992;1134:217-222.

43. Ortmeyer J, Mohsenin V. Glucose suppresses superoxide generation in normal neutrophils: interference in phospholipase D activation. *Am J Physiol.* 1993;264:C402-C410.

44. Maridonneau-Parini I, Tringale SM, Tauber AI. Identification of distinct activation pathways of the human neutrophil NADPH-oxidase. *J Immunol.* 1986;137:2925-2929.

45. Dusi S, Della Bianca V, Grzeskowiak M, Rossi F. Relationship between phosphorylation and translocation to the plasma membrane of p47$_{phox}$ and p67$_{phox}$ and activation of the NADPH oxidase in normal and Ca^{2+}-depleted human neutrophils. *Biochem J.* 1993;290:173-178.

46. Cox JA, Jeng AY, Blumberg PM, Tauber AI. Comparison of subcellular activation of the human neutrophil NADPH-oxidase by arachidonic acid, sodium dodecyl sulfate (SDS), and phorbol myristate acetate (PMA). *J Immunol.* 1987;138:1884-1888.

47. Watson F, Robinson J, Edwards SW. Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils. *J Biol Chem.* 1991;266:7432-7439.

48. Bauldry SA, Elsey KL, Bass DA. Activation of NADPH oxidase and phospholipase D in permeabilized human neutrophils. *J Biol Chem.* 1992;267:25141-25152.

49. Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, Habermehl GG. Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumor necrosis factor-α. *Biochem J.* 1989;263:539-545.

50. Radeke HH, Cross AR, Hancock JT, Jones OTG, Nakamura M, Kaever V, Resch K. Functional expression of NADPH oxidase components (α- and β-subunits of cytochrome b$_{558}$ and 45-kDa flavoprotein) by intrinsic human glomerular mesangial cells. *J Biol Chem.* 1991;266:21025-21029.

51. Alexander RW, Gimbrone MA. Stimulation of prostaglandin E synthesis in cultured human umbilical vein smooth muscle. *Proc Natl Acad Sci U S A.* 1976;73:1617-1620.

52. Mitsuka M, Berk BC. Long-term regulation of Na^+-H^+ exchange in vascular smooth muscle cells: role of protein kinase C. *Am J Physiol.* 1991;260:C562-C569.

53. Pumiglia KM, Lau L-F, Huang C-K, Burroughs S, Feinstein MB. Activation of signal transduction in platelets by the tyrosine phosphatase inhibitor pervanadate (vanadyl hydroperoxide). *Biochem J.* 1992;286:441-449.