Coronary Microvascular Endothelial Cell Redox State in Left Ventricular Hypertrophy
The Role of Angiotensin II

Derek Lang, Salah I. Mosfer, Alison Shakesby, Francis Donaldson, Malcolm J. Lewis

Abstract—Left ventricular hypertrophy (LVH) is associated with elevated plasma angiotensin II (Ang II) levels and endothelial dysfunction. The relationship between Ang II and endothelial dysfunction remains unknown, however, but it may involve an alteration in endothelial cell redox state. We therefore investigated the effect of Ang II on NADH/NADPH oxidase–mediated superoxide anion (O$_2^-$) production by cultured guinea pig coronary microvascular endothelial cells (CMVEs) and CMVEs freshly isolated from a guinea pig, pressure-overload model of LVH. Lucigenin chemiluminescence was used to measure O$_2^-$ production in the particulate fraction of CMVE lysates. In cultured cells, incubation with Ang II (0.1 nmol/L to 1 μmol/L for 18 hours) resulted in significant (P<0.01) increases in both NADH- and NADPH-dependent O$_2^-$ production, with a peak effect at 1 nmol/L. The latter was significantly (P<0.01) inhibited by the AT$_1$ receptor antagonist losartan (1 μmol/L for 18 hours). In contrast, the O$_2^-$ response to Ang II (0.1 nmol/L to 1 μmol/L for 18 hours) was largely unaffected by concomitant exposure to the AT$_2$ antagonist PD 123319 (1 μmol/L). In freshly isolated CMVEs from nonoperated animals, NADH- and NADPH-dependent O$_2^-$ production was not different from that in sham-operated animals but was significantly (P<0.05) elevated in the aortic-banded animals. Plasma Ang II levels were significantly (P<0.001) elevated in the aortic-banded (1.25±0.12 μg/L, n=12) compared with sham-operated animals (0.63±0.06 μg/L, n=12). These data suggest that the endothelial dysfunction associated with LVH may be due, at least in part, to the Ang II–induced upregulation of NADH/NADPH oxidase-dependent O$_2^-$ production. (Circ Res. 2000;86:463-469.)

Key Words: NADH/NADPH oxidase ■ coronary microvascular endothelium ■ angiotensin II ■ left ventricular hypertrophy ■ superoxide anion

Raised circulating levels of angiotensin II (Ang II) are associated with a number of pathological states, such as hypertension, congestive heart failure, and nitrate tolerance. Although the vasoactive effects of Ang II have been well described for >40 years, recent evidence suggests that this peptide has many other effects on the cardiovascular system. For example, Ang II has been shown to promote vascular hypertrophy,5,6 stimulate the Janus kinase/signal transducers and activators of transcription pathway,7 activate the gene transcription of proto-oncogenes including c-fos,8–10 induce cardiac hypertrophy,11–13 and modulate cardiac remodeling.14

Another important action of Ang II is the induction of superoxide anion (O$_2^-$) production by vascular smooth muscle cells via the stimulation of the NADH/NADPH oxidase system.15 It is known that these oxygen free radicals are intimately involved in the inactivation of endothelium-derived nitric oxide (NO).16–18 The question remains, however, whether Ang II has a similar effect on this free radical–generating enzyme system in endothelial cells themselves.

It is becoming increasingly apparent that in disease states affecting the cardiovascular system in which reduced NO activity has been demonstrated, such as hypercholesterolemia,19 diabetes,20 smoking-related disorders,21 reperfusion injury,22 hypertension,23 and possibly Alzheimer’s disease,24 that this inactivation of NO is associated with increased O$_2^-$ production. The general view is now emerging that it is the balance between the release of NO and O$_2^-$ that ultimately determines the level of NO activity. It is highly likely that an imbalance in this delicate system will contribute to the concomitant alterations in vascular tone seen in atherosclerosis and hypertension.

In all of these diseases, there are obviously many “candidate” cell types that could be involved in the overproduction of O$_2^-$, eg, vascular smooth muscle cells.15 More recently, vascular smooth muscle cells from animal models of hypertension,25 coronary artery endothelial cells,26 and rabbit aorta...
adventitial tissues\(^2\), have also been shown to possess the \(O_2^-\)-generating NADH/NADPH oxidase system, thus providing a potential source of \(O_2^-\).

Given what is known about the marked effect that coronary microvascular endothelium–derived NO has on cardiac function,\(^2\) the generation of \(O_2^-\) by these cells may have an important role in the pathogenesis of left ventricular hypertrophy (LVH) and could contribute significantly to the endothelial dysfunction associated with this and other cardiovascular diseases.

The aim of this study, therefore, was to investigate the relationship between Ang II and NADH/NADPH oxidase–mediated \(O_2^-\) production, firstly in vitro and secondly ex vivo in an experimental model of LVH. We used cultured guinea pig coronary microvascular endothelial cells (CMVEs) for the in vitro part of this study and freshly isolated CMVEs from a pressure-overload model of LVH in the guinea pig for the ex vivo experiments.

**Materials and Methods**

**Aortic Banding**
Pressure-overload LVH was induced in Dunkin Hartley guinea pigs (200 to 250 g), as previously described.\(^3\)\(^0\) All experiments involving aortic-banded and sham-operated animals were carried out 6 weeks after operation when optimum LVH had developed. Control animals for these experiments were matched for both age and weight, but with no surgical intervention.

**Isolation and Characterization of Coronary Microvascular Endothelium**
Guinea pig CMVEs were isolated and characterized as described previously.\(^3\)\(^1\)

**NADH/NADPH Oxidase Assay**
NADH/NADPH oxidase activity was measured essentially as described previously.\(^1\)\(^9\) The protein content of an aliquot of the appropriate cellular fraction was assayed as described previously.\(^3\)\(^2\)

A custom-built luminometer (see Reference 33) was used to detect changes in chemiluminescence, and the output (in volts) was then displayed on a Macintosh computer via a Maclab apparatus. The integral for the first 10 minutes of the reaction represents the total \(O_2^-\) produced over this time and was normalized to fraction protein content, and data were then expressed as volts/s/mg protein (V/s/mg protein in Figures).

Even though CMVEs were isolated and cultured under identical conditions, both basal (control) and Ang II–stimulated NADH- and NADPH-dependent responses showed considerable interbatch variability. Each separate experiment was therefore performed on cells from a single culture preparation.

**Measurement of Plasma Ang II Levels**
Blood (5 mL) from anesthetized guinea pigs (control, sham-operated, and aortic-banded) was collected by cardiac puncture into prechilled tubes containing 1.52 mg/mL EDTA and 500 kallikrein inhibitor units/mL aprotinin immediately before removal of the heart for CMVE isolation. It was then centrifuged at 1200 \(\times\) g for 15 minutes at 4°C and the resulting platelet-free plasma stored in fresh plastic tubes at −70°C. After extraction, Ang II concentrations were measured as described by a commercially available radioimmunoassay kit (Nichols Institute Diagnostics Ltd).

**Ferricytochrome c Reduction**
It has recently become apparent that lucigenin, under certain circumstances, can generate its own \(O_2^-\)\(^2\),\(^4\)\(^5\) and its suitability for the detection of these free radicals has been questioned.\(^3\)\(^6\) Therefore, to validate our findings and confirm that the \(O_2^-\) production measured in this study is via NADH/NADPH oxidase activity and not from lucigenin itself, ferricytochrome \(c\) reduction was also used.

Cultured CMVE lysates were prepared as above. \(O_2^-\) production was then measured using a reaction buffer of the following composition (in mmol/L): phosphate buffer (pH 7.4) 10, NaCl 100, KCl 4, MgCl\(_2\) 1.3, glucose 2, CaCl\(_2\) 1, and ferricytochrome \(c\) 0.07. An aliquot of the appropriate fraction was added to the reaction buffer in the presence and absence of either NADH or NADPH (both 1 mmol/L) and allowed to incubate for 30 minutes at 37°C. These additions were repeated in the presence of superoxide dismutase (SOD, 350 U/mL). Tiron produced a nonspecific color change when added to the reaction buffer, so it could not be used in these experiments. The reduction of ferricytochrome \(c\) in the supernatant was measured at 550 nm by a standard spectrophotometer.

**Statistics**
All data are expressed as mean±SEM and are compared by ANOVA followed by either the Dunnett or Student-Newmann-Keul multiple-range test where appropriate. Significant differences are identified at the \(P<0.05\) level.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Characterization of Guinea Pig CMVEs**
The endothelial nature of the freshly isolated and cultured CMVEs used in this study was confirmed as described previously.\(^3\)\(^1\) They selectively took up fluorescein isothiocyanate–labeled, acetylated LDL, stained positively with the microvascular endothelium-specific fluorescein isothiocyanate–labeled lectin from *Lycopersicon esculentum*, quickly formed tubes (as compared with a slower rate for large-vessel endothelial cells) when grown on Matrigel, stained negatively for smooth muscle \(\alpha\)-actin, and grew normally in d-valine–containing culture medium (data not shown).

**NADH/NADPH Oxidase–Dependent \(O_2^-\) Production in Cultured CMVEs**
The cytosolic fraction of the cultured (first-passage) cells (and the freshly isolated cells) failed to produce a chemiluminescent response, either in the absence or presence of NADH or NADPH (both 1 mmol/L). All of the following data describe the responses of the particulate fraction of both cell types.

Incubation of cultured cells with Ang II (0.1 mmol/L to 1 mmol/L) for 6 hours had no effect on control levels of \(O_2^-\) production (data not shown). However, incubation with Ang II for 18 hours resulted in significant (\(P<0.01\)) increases in both NADH and NADPH oxidase–mediated \(O_2^-\) production, with the peak effect occurring at an Ang II concentration of 1 mmol/L (Figure 1). In a separate experiment, the NADH and NADPH oxidase–mediated increases in \(O_2^-\) production in the presence of Ang II (1 mmol/L for 18 hours) were completely inhibited (\(P<0.01\)) by the AT\(_1\) receptor antagonist losartan (1 \(\mu\)mol/L for 18 hours) (Figure 2). In a further experiment, cultured CMVEs were incubated for 18 hours with Ang II (0.1 mmol/L to 1 \(\mu\)mol/L) in the absence or presence of the AT\(_2\) antagonist PD 123319 (1 \(\mu\)mol/L). NADH and NADPH oxidase–mediated increases in \(O_2^-\) production in the presence of Ang II were largely unaffected by this antagonist, although
a significant \((P<0.05)\) inhibition of the \(O_2^-\) response to 10 nmol/L Ang II was observed (Figure 3).

The production of \(O_2^-\) by both control and Ang II (1 nmol/L for 18 hours)–stimulated cells was unaffected by preincubation (10 minutes at 37°C) of the appropriate particulate fraction with the NO synthase (NOS) inhibitor \(N^G\)-nitro-L-arginine methyl ester (L-NAME, 5 \(\mu\)mol/L), the xanthine oxidase inhibitor oxypurinol (1 mmol/L), or the cyclooxygenase inhibitor indo-methacin (10 \(\mu\)mol/L) (Figure 4). In a separate experiment, \(O_2^-\) production by both control and Ang II (1 nmol/L)–stimulated CMVEs was significantly \((P<0.001)\) inhibited by \(>90\%\) in the presence of the specific \(O_2^-\) scavenger tiron\(^{37,38}\) (10 mmol/L) (Figure 5).

No chemiluminescent response was demonstrated by any CMVE fraction in the absence of NADH or NADPH.

Development of LVH
To assess the development of LVH, combined left/right ventricle–to–body weight (LRV/BW) ratios were measured in a sample (age-matched) population of control, sham-operated, and aortic-banded animals at a time point that coincided with CMVE isolation from the rest of the animals in the study. Combined LRV/BW ratios are shown in the Table and demonstrate a significant \((P<0.01)\) 25% increase in the aortic-banded animals compared with the sham-operated group. There was no difference in LRV/BW ratio between control and sham-operated animals.

Plasma Ang II Concentrations
Plasma Ang II levels were unaltered in the sham-operated animals compared with controls (no operation) \((0.63\pm0.06 \text{ versus } 0.68\pm0.06 \mu\text{g/L}, n=12 \text{ and } 10, \text{ respectively})\), but were significantly \((P<0.001)\) elevated in the aortic-banded animals \((1.25\pm0.12 \mu\text{g/L}, n=12)\).
NADH/NADPH Oxidase–Dependent O$_2$ Production in Freshly Isolated CMVEs

NADH and NADPH oxidase activity in CMVEs from control animals (no operation) was not different from that in sham-operated animals. However, this activity was significantly (P, 0.05) elevated in the aortic-banded animals (Figure 6).

Again, O$_2$ production in CMVE lysates from both the sham-operated and aortic-banded animals was unaffected by preincubation of the lysate (10 minutes at 37°C) with L-NAME (5 μmol/L), oxypurinol (1 mmol/L), or indomethacin (10 μmol/L) (data not shown).

**Figure 4.** Effect of the NOS inhibitor L-NAME (5 μmol/L), the xanthine oxidase inhibitor oxypurinol (OXYP, 1 mmol/L), or the cyclooxygenase inhibitor indomethacin (INDO, 10 μmol/L) (all pre-incubated for 10 minutes at 37°C) on control (A) and Ang II (1 nmol/L for 18 hours)-stimulated (B) NADH (1 mmol/L, open columns)– or NADPH (1 mmol/L, filled columns)–dependent O$_2$ production in cultured guinea pig CMVEs (particulate fraction) (all n=4).

NADH/NADPH Oxidase–Dependent O$_2^-$ Production in Freshly Isolated CMVEs

NADH and NADPH oxidase activity in CMVEs from control animals (no operation) was different from that in sham-operated animals. However, this activity was significantly (P<0.05) elevated in the aortic-banded animals (Figure 6). Again, O$_2^-$ production in CMVE lysates from both the sham-operated and aortic-banded animals was unaffected by preincubation of the lysate (10 minutes at 37°C) with L-NAME (5 μmol/L), oxypurinol (1 mmol/L), or indomethacin (10 μmol/L) (data not shown).

**Figure 5.** Effect of the O$_2^-$ scavenger tiron (100 mmol/L) on control and Ang II (1 nmol/L for 18 hours)–stimulated NADH (1 mmol/L, open columns)– or NADPH (1 mmol/L, filled columns)–dependent O$_2^-$ production in cultured guinea pig CMVEs (particulate fraction). **P<0.01, ***P<0.001 compared with control; ++ + P<0.001 compared with Ang II (all n=4).

Ferricytochrome c Reduction

NADH- and NADPH-dependent reduction in ferricytochrome c absorbance measured at 550 nm was significantly (P<0.001) greater in cultured CMVE lysates (particulate fraction) from Ang II–treated (1 nmol/L for 18 hours) compared with control (Figure 7) CMVEs. The presence of SOD (350 U/mL) caused a significant (P<0.05) inhibition in absorbance seen with both NADH and NADPH.

**Discussion**

The microvascular endothelial nature of the cells isolated from the perfused hearts was confirmed by techniques de-
effect to be concentration dependent. The present findings, however, clearly demonstrate an inverse effect of Ang II concentration on $O_2^-$ generation by CMVEs. The pattern of activation is similar to that demonstrated in neutrophils in response to Ang II.\cite{40} Chemotactic migration in these leukocytes is increased at concentrations up to 0.1 nmol/L but inhibited at those of 10 nmol/L and above. The mechanism of this effect is unclear, and a direct comparison between these 2 cell types is clearly difficult. Nevertheless, it is perhaps not surprising that because both leukocytes and endothelial cells are frequently involved in cell-cell interactions, they should both respond in a similar way to vasoactive agents such as Ang II.

The inverse effect of Ang II concentrations outlined above may be explained by Ang II coinducing an inhibitory process at the same time as upregulating $O_2^-$ generation. For instance, an inhibitory role for AT$_2$ on AT$_1$-mediated responses\cite{41,42} may exist. Such a role seems unlikely in the present study, because the responses to the highest concentrations of Ang II were not potentiated by the AT$_2$ antagonist PD 123319. Indeed, the inhibitory effect of PD 123319 on the NADH-dependent response to 10 nmol/L Ang II and the general pattern of $O_2^-$ generation in the presence of this antagonist suggests that the AT$_2$ may be involved in Ang II–dependent oxygen free radical production by this cell type. However, a direct and nonspecific inhibitory effect of PD 123319 on the AT$_1$ receptor cannot be ruled out.

It is also possible that at the higher concentrations of Ang II, the AT$_1$s become desensitized. Furthermore, Ang II has been shown to stimulate the synthesis of both the endothelial\cite{43} and inducible\cite{44} isoforms of NOS. Clearly, increased NO production in the presence of the higher concentrations of Ang II may result in decreased detection of $O_2^-$ anions, because the latter will combine readily with NO to form peroxynitrite. The upregulation, at least of endothelial NOS, is unlikely in the present study, because these CMVEs lose the ability to express both endothelial NOS protein and mRNA after culture.\cite{31} Increased expression of inducible NOS, however, cannot be excluded. Resolution of the exact mechanism(s) involved would, however, require further extensive experimentation outside the scope of the present study.

Plasma levels of Ang II provide an approximation only of concentrations in the region of the endothelial cells in the microvasculature. Levels of Ang II in the control and sham-operated animals were $\approx 0.65$ nmol/L. These were elevated to $\approx 1.3$ nmol/L in the LVH animals and were accompanied by a 2-fold increase in NADH-dependent oxidase activity and a 1.5-fold increase in NADPH-dependent oxidase activity. A small increase in plasma Ang II is therefore associated with a significant increase in $O_2^-$ production. In the cultured cells, an increase in the Ang II concentration from 0.1 to 1 nmol/L again produced a 2-fold increase in NADH-dependent oxidase activity, but an almost 4-fold increase in NADPH-dependent oxidase activity. Thus, the data from the in vitro experiments closely approximated that from the ex vivo experiments.

The development of LVH is likely to be multifactorial, with the oxidant stress generated in this condition likely to have many determinants. Superoxide anions are probably the most important oxygen free radical generated in vivo, and it is highly likely that they are derived from more than one source, not just from the Ang II–sensitive NADH/NADPH system.
oxidase system. One such example is xanthine oxidase, an enzyme that resides within endothelial cells but also exists in the bloodstream of patients in some clinical settings. Moreover, leakage of electrons from the electron transport system within mitochondria, the cycloxygenase pathway, and the auto-oxidation of catecholamines are all potential sources of oxygen free radicals. Indeed, evidence suggests that in cerebral arterioles, an acute increase in blood pressure can cause excessive activation of arachidonic acid production via the cycloxygenase pathway with the subsequent overproduction of O$_2^-$ by endothelial cells. Even the activation of NOS, albeit in the presence of suboptimal concentrations of the substrate L-arginine or the cofactor tetrahydrobiopterin, can lead to the production of O$_2^-$ from molecular oxygen. Furthermore, Ang II itself has been shown to induce the release of NO in resistance vascular beds in certain pathophysiological conditions. As mentioned above, we have previously demonstrated that the cultured CMVEs do not possess NOS activity; the ability to express this enzyme, although present in freshly isolated cells, is lost in culture. This finding provides further evidence that in the in vitro experiments, NOS is an unlikely source of the O$_2^-$. We have therefore demonstrated that neither NOS, cycloxygenase, nor xanthine oxidase is involved in O$_2^-$ production by the CMVE lysates. However, care must be taken in extrapolating from the in vitro to the in vivo situation, and it is quite feasible that the aforementioned enzymatic pathways could be involved in the generation of reactive oxygen species in the intact animal.

As indicated earlier, recent evidence suggests that lucigenin, under certain circumstances, can be a source of O$_2^-$, and questions have arisen as to its suitability for the detection of these free radicals. To validate our findings and confirm that the O$_2^-$ production measured in this study was via NADH/NADPH oxidase activity and not from lucigenin itself, ferricytochrome c reduction was used in some experiments. As the data indicate, the particulate fraction from cultured cells also produces a reduction in ferricytochrome c in the presence of either NADH or NADPH. This reduction was significantly greater in lysates from cells exposed to Ang II compared with controls, an observation that mirrors the data obtained using lucigenin. Furthermore, the NADH-dependent effect is the dominant response using both techniques. The ferricytochrome c reduction is almost completely inhibited by SOD, indicating that NADH/NADPH oxidase is the likely source of O$_2^-$. SOD was used in these experiments instead of tiron, because the latter produced a nonspecific color change on addition to the reaction buffer, thus interfering with the assay.

The data presented in this study clearly demonstrate a role for Ang II in the generation of oxidant stress and endothelial dysfunction in vitro. How such changes contribute to the development of the endothelial dysfunction associated with LVH must remain a matter for speculation. Several studies have shown impairment of endothelial function in LVH in both animals and humans, the mechanism being attributed to a reduction in the expression of the constitutive NOS gene in aortic endothelial cells in one of the studies. Clearly, the increased inactivation of NO by O$_2^-$ provides an additional mechanism for the endothelial dysfunction and reduction in coronary reserve seen in LVH.

Endothelial dysfunction is an early event in many diseases affecting the cardiovascular system. Recently, Ang II has been shown to contribute to this dysfunction via the activation of endothelial cell suicide pathways leading to apoptosis, although this effect does not seem to be mediated directly by Ang II–induced O$_2^-$ production. NO has, however, been demonstrated to have an inhibitory effect on this process, suggesting that the inactivation of NO by O$_2^-$ could lead to endothelial cells being driven toward Ang II–induced apoptosis. This observation provides another possible mechanism whereby Ang II may be involved in the development of endothelial dysfunction.

NO and its intracellular second messenger cGMP are inhibitors of cardiac myocyte and fibroblast growth. Reduced NO activity or downregulation of NOS in CMVEs may therefore also contribute to increased growth of cardiac myocytes and/or fibroblasts characteristic of LVH.

In summary, the results of the present study demonstrate that Ang II upregulates an oxygen free radical–generating enzyme system in cultured guinea pig CMVEs and, furthermore, that in this model of LVH, upregulation of this oxidase in CMVEs is associated with increased plasma levels of Ang II. This study also demonstrates that the NADH/NADPH enzyme is membrane bound and is activated to a greater extent by NADH than NADPH as previously described for other cell types. Ang II–induced oxidative stress leading to the inactivation of NO and to endothelial cell injury is likely, therefore, to contribute significantly to the endothelial dysfunction associated with LVH and to play an important role in disease progression. However, it must be noted that the development of LVH is likely to be multifactorial and that Ang II may not be the only determinant in the generation of oxidant stress.

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References


29. Lang et al. LVH, Ang II, and Endothelial Superoxide


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