Coronary Microvascular Endothelial Stunning After Acute Pressure Overload in the Conscious Dog Is Caused by Oxidant Processes

The Role of Angiotensin II Type 1 Receptor and NAD(P)H Oxidase

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Background—Few studies have examined the effect of acute pressure overload on endothelial function in the coronary microcirculation.

Methods and Results—In instrumented conscious dogs with heart rate held constant, veratrine caused a cholinergic nitric oxide (NO)–dependent increase in coronary blood flow by 23 ± 1 mL/min (Bezold-Jarisch reflex). Ten minutes after release of constriction of the ascending aorta to increase left ventricular (LV) systolic pressure to 214 ± 5 mm Hg for 30 minutes, the veratrine-induced increase in coronary blood flow (7 ± 1 mL/min) was reduced by 66% and remained depressed for 2 hours (ie, endothelial stunning [ES]). Nitrite production from isolated coronary microvessels during ES was not different from normal. Ascorbic acid (AA), losartan, or apocynin prevented ES. Myocardial oxygen consumption (MVDO2) of LV tissue was measured in vitro in response to bradykinin with preincubation of angiotensin II for 30 minutes. Bradykinin (10−4 mol/L)–induced reduction in MVDO2 was reversed in a concentration-dependent manner by angiotensin II (38 ± 1% versus 19 ± 2% at 10−8 mol/L) and restored by coincubation of AA (37 ± 2%), tempol (33 ± 2%), losartan (34 ± 2%), or apocynin (36 ± 1%). Exogenous NO-induced reduction in MVDO2 was not altered by angiotensin II. Angiotensin II increased lucigenin-detectable superoxide anion in LV tissue in a manner that was inhibited by bradykinin, AA, tempol, losartan, or apocynin.

Conclusions—Endothelial stunning is caused by oxidant processes inhibited by ascorbate, and the activation of NAD(P)H oxidase by increased angiotensin II plays an important role in this process. (Circulation. 2003;108:2934-2940.)

Key Words: angiotensin □ vagus nerve □ free radicals □ nitric oxide □ coronary disease

Many studies have examined nitric oxide (NO)–dependent vasodilation and demonstrated changes in the endothelial function under pathophysiological conditions.1–3 Acute pressure overload of the left ventricle (LV) and the coronary circulation could occur in various pathological as well as physiological states. Continuous arterial pressure recordings from normal subjects and hypertensive patients show variations of >50 mm Hg in mean systolic blood pressure (BP) occurring repeatedly throughout the day,4 and increases in BP to >200 mm Hg have been reported during static exercise.5 In addition, patients with malignant hypertension have acute increases in BP to >200 mm Hg. However, few studies have examined the effect of acute pressure overload on the endothelial function in coronary arteries. Recently, Higashi et al6 reported that patients with renovascular hypertension and activation of the systemic renin-angiotensin system (RAS) have impaired forearm endothelial function, which is improved by renal-artery angioplasty through a reduction in oxidative stress. Because this observation was performed during systemic hypertension, it is hard to separate the effects of acute pressure overload itself (ie, mechanical force) on endothelial function from the effects of various systemic neurohumoral factors. Therefore, we examined the effects of acute pressure overload in the absence of systemic hypertension on endothelial function in the present study.

Endothelial function is controlled by a balance between the production of NO and oxygen (O2) free radicals.7,8 In states in which NO production is not altered, its bioavailability may be reduced because of oxidative inactivation by excessive production of superoxide anion (O2−) in the vascular wall.9 Many studies have demonstrated that increased vascular production of O2− contributes to impaired endothelium-dependent vasodilation.8,10,11 Angiotensin II (Ang II) may be generated in the
various tissues in response to pressure overload. Ang II stimulation produces O$_2$• through the activation of NAD(P)H oxidase in the endothelial cell and vascular smooth muscle cell in vitro. We hypothesized that brief episodes of acute pressure overload lead to impairment of NO-dependent dilation, which is a result of the inactivation of NO by increased O$_2$•, and the activation of NAD(P)H oxidase by Ang II plays an important role in these processes.

von-Bezold and Hirt first reported that intravenous administration of veratrum alkaloids resulted in bradycardia and hypotension. The sensory receptors of the Bezold-Jarisch reflex are located in the LV of the heart, and the afferent and efferent arms of the Bezold-Jarisch reflex are vagal. Activation of ventricular receptors not only results in bradycardia and hypotension but also causes coronary vasodilation that is mediated by a cholinergic mechanism. Our recent studies have indicated that the coronary vasodilation induced by activation of the Bezold-Jarisch reflex is NO dependent, because nitro-L-arginine, an NO synthase (NOS) inhibitor, blocks this coronary vasodilation. Furthermore, vagally mediated coronary vasodilation is selectively impaired after pacing-induced heart failure and enhanced after short-term exercise training in conscious dogs in proportion to the altered release of NO from the vascular endothelium because of upregulation or downregulation of endothelial constitutive NOS (ecNOS).

The goals of our in vivo experiments were to determine (1) whether brief episodes of acute pressure overload lead to impairment of NO-dependent coronary microvascular dilation after activation of the Bezold-Jarisch reflex and (2) the potential mechanism responsible. Because NO plays an important role in modulating myocardial O$_2$ consumption (MV$\dot{O}_2$), which can be used as an in vitro bioassay for NO, we investigated the relationship between Ang II–induced O$_2$• production and the NO-dependent control of MV$\dot{O}_2$. The goals of in vitro experiments were to determine (1) the effects of Ang II on MV$\dot{O}_2$ regulation in LV tissues from normal dogs and (2) the role of increased O$_2$• production.

**Methods**

**Experiment 1**

**Surgical Preparation**

Dogs (n=20; weight, 21 to 30 kg) were anesthetized, intubated, and ventilated. A thoracotomy was performed, and catheters for the aorta and left atrium, LV pressure gauge (P 6.5, Konigsberg Instruments Inc), a Doppler flow transducer (Craig Hartley), and a pair of pacing electrodes were instrumented as we described previously. A hydraulic occluder was implanted around the supravalvular ascending aorta to constrict the aorta (AOC). The dogs were allowed 10 to 14 days to recover fully and were trained to lie quietly on the laboratory table. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the current National Institutes of Health and American Physiological Society Guidelines for the Use and Care of Laboratory Animals.

**Recording Techniques**

Arterial pressure, LV pressure, and left circumflex coronary blood flow (CBF) were measured, heart rate (HR) was monitored, and mean arterial pressure (MAP) and mean CBF were derived as we described previously. Mean coronary resistance (CR) was calculated as the quotient of mean arterial pressure and CBF.

**Experimental Protocols**

**Effects of AOC on Activation of Bezold-Jarisch Reflex by Veratrine.**

In conscious dogs (n=20), veratrine at a dose of 5 μg/kg was administered as a bolus injection (1 mL) into the left atrium through the implanted catheter with HR constant (150 bpm). AOC increased LV systolic pressure (LVSPP) to 200 mm Hg for 30 minutes by inflating the hydraulic occluder. Veratrine was given again with the HR controlled at 3 minutes, 10 minutes, 30 minutes, 60 minutes, and 120 minutes after the release of AOC.

**Effect of Ascorbic Acid, Losartan, or Apocynin on Activation of Bezold-Jarisch Reflex by Veratrine After AOC.**

Five to 7 days later, ascorbic acid (n=5), losartan (n=5), or apocynin (n=4) was infused intravenously. Ascorbic acid was administered at an initial dose of 2000 mg, followed by a constant infusion at 25 mg/min at 90 minutes before AOC. Losartan was administered at a dose of 10 mg/kg over a period of 30 minutes and apocynin at a dose of 10 mg/kg over a period of 2 hours. Ascorbic acid was used as the antioxidant, losartan as Ang II type 1 (AT$_1$) receptor antagonist, and apocynin as an inhibitor of NAD(P)H oxidase activation. These doses were chosen on the basis of the previous data and the above-described protocols were repeated.

**NO Production From Coronary Microvessels**

Dogs were killed at 10 minutes after release of AOC (AOC dog), and coronary microvessels were isolated by the method of Zhang et al. NO production stimulated by acetylcholine (ACh) or bradykinin (BK) was measured in coronary microvessels. The formation of NO after activation of the Bezold-Jarisch reflex and (2) the role of increased O$_2$• production.

**Inhibition of MV$\dot{O}_2$ by BK and Exogenous NO**

BK stimulates kinin B$_2$-receptor on the endothelium to stimulate NO production. The preparation of LV cardiac muscle segments and the measurement of MV$\dot{O}_2$ were as described previously. Muscle segments were incubated with 10$^{-10}$ to 10$^{-5}$ mol/L Ang II 30 minutes before measurements of MV$\dot{O}_2$. In separate experiments, the effects of 10$^{-5}$ mol/L Ang II on MV$\dot{O}_2$ were also studied in the presence of 10$^{-2}$ mol/L ascorbic acid, 10$^{-3}$ mol/L 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), 10$^{-6}$ mol/L losartan, or 10$^{-3}$ mol/L apocynin.

**Chemicals**

Losartan was purchased from DuPont Pharmaceuticals. Other drugs were purchased from Sigma-Aldrich Co.

**Data Analysis**

All data are presented as mean±SEM. In vivo studies, the responses are the peak after administration of veratrine. The statis-
The changes in NO stimulation from ratio of F values indicated a significant difference, significance was determined with repeated-measures ANOVA. When the changes were considered significant at a value of $P<0.05$.

### Results

#### Effects of Veratrine on the Coronary Circulation and Hemodynamics

The heart was paced at 150 bpm to avoid the effects of veratrine-induced bradycardia on CBF. Figure 1 shows a typical response of CBF to veratrine. Veratrine caused significant increases in CBF and significant decreases in CR (to $1.74 \pm 0.11$ mm Hg $\cdot$ min$^{-1} \cdot$ mL$^{-1}$ from $2.67 \pm 0.15$ mm Hg $\cdot$ min$^{-1} \cdot$ mL$^{-1}$). The changes in systemic hemodynamics and CBF induced by veratrine are shown in the Table.

#### Effects of Veratrine After AOC

AOC increased LVSP to $211 \pm 4$ mm Hg. After release of AOC, the CBF and hemodynamics were not altered (Table). Figure 1 shows marked decreases in the vasodilation to veratrine in the same dog 10 minutes after release of AOC. Veratrine caused significantly smaller increases in CBF and decreases in CR 10 minutes after release of AOC (Figure 2, Table). These responses to veratrine were reduced for 2 to 24 hours. We refer to the reduced vasodilation to veratrine after AOC as “endothelial stunning.”

#### NO Production From Coronary Microvessels

Nitrile production in response to ACh and BK by coronary microvessels from AOC dogs (ACh 145, BK 80 pmol $\cdot$ mg$^{-1} \cdot$ min$^{-1}$, n=2) was not decreased compared with normal dogs (ACh 83±13, BK 72±6 pmol $\cdot$ mg$^{-1} \cdot$ min$^{-1}$, n=7). The NO production to both ACh and BK was blocked by $N^\bullet$-nitro-L-arginine methyl ester (L-NAME).

#### Effects of Ascorbic Acid on the Responses to Veratrine After AOC

It is well known that O$_2^-$ inactivates NO.$^9$ To test whether the endothelial stunning is caused by O$_2^-$, ascorbic acid was given to scavenge O$_2^-$. AOC increased LVSP to $204 \pm 2$ mm Hg during ascorbic acid infusion. After AOC, the baseline systemic hemodynamics were not altered in the dogs with ascorbic acid, but CBF was increased (Table). Ascorbic acid preserved the response of CBF to veratrine 10 minutes after AOC (Figure 3).

#### Effects of Losartan on the Responses to Veratrine After AOC

Ang II could be generated in the arterial wall in response to increased vascular wall tension (AOC).$^{12,13}$ Ang II increases vascular oxidative stress.$^{16,27}$ AOC increased LVSP to $209 \pm 1$ mm Hg after infusion of losartan ($P=NS$ versus LVSP during AOC in control dogs). Losartan preserved the response of CBF to veratrine 10 minutes after AOC (Figure 3).

#### Effects of Apocynin on the Responses to Veratrine After AOC

Ang II stimulation produces O$_2^-$ through the activation of NAD(P)H oxidase in the endothelial$^{14}$ and vascular smooth muscle cells.$^{15,16}$ To test whether the endothelial stunning is associated with the activation of NAD(P)H oxidases, apocynin was given.$^{24}$ AOC increased LVSP to $204 \pm 7$ mm Hg after infusion of apocynin. Apocynin prevented the reduced responses of CBF and CR to veratrine 10 minutes after AOC (Figures 3 and 4). This effect of apocynin lasted for the entire duration of the experiments (Figure 4).

#### Effects of Ang II on MVO$_2$ in Tissue in Response to BK and SNAP

Cumulative doses of BK caused concentration-dependent decreases in MVO$_2$ in control tissue segments. BK-induced
reduction in MV\(_{\text{O}}\) was significantly attenuated in a concentration-dependent manner by preincubation with low concentration of Ang II (Figure 5A). The inhibitory effects of Ang II on MV\(_{\text{O}}\) in response to BK were completely inhibited with coincubation with losartan, ascorbic acid, tempol, or apocynin (Figure 5B). In contrast to BK, SNAP-induced reduction in MV\(_{\text{O}}\) was not affected by preincubation with Ang II (Figure 5C).

O\(_{2}^{-}\) Production by Ang II

Ang II resulted in significant increases in O\(_{2}^{-}\) production, which were inhibited by coincubation with BK, losartan, ascorbic acid, tempol, or apocynin (Figure 6). This inhibitory effect of O\(_{2}^{-}\) production by BK disappeared with coincubation with L-NAME (Figure 6).

Discussion

The most important finding of present study is that acute pressure overload by supravalvular AOC leads to impairment of NO-dependent coronary microvascular dilation. We refer to this phenomenon as endothelial “stunning” because of the transient nature of this response. Nitrite production from coronary microvessels was not decreased at the time of endothelial stunning. Most importantly, the endothelial stunning was prevented by preadministration of ascorbic acid, losartan, or apocynin. Therefore, the endothelial stunning we observed in vivo was associated with increased O\(_{2}^{-}\), which was mediated by increased Ang II--induced activation of NAD(P)H oxidase. Moreover, Ang II stimulation at pathophysiological concentration increased O\(_{2}^{-}\) through the activation of NAD(P)H oxidase and inhibited NO-dependent control of MV\(_{\text{O}}\) in cardiac tissues from normal dogs.

Acute hypertension (190 to 200 mm Hg in mean arterial BP) during intravenous norepinephrine administration leads to the appearance of O\(_{2}^{-}\) in the cerebral extracellular space and abolition of endothelium-dependent vascular relaxation of cerebral arterioles to ACh in anesthetized cat.28 These effects were prevented by SOD. Patients with renovascular hypertension and activation of the RAS have impaired endothelium-mediated forearm vasodilation, which is improved by renal-artery angioplasty and reduced oxidant stress.6 Furthermore, infusion of ascorbic acid improved the endothelial function in renovascular hypertension before angioplasty.6
However, because these studies were performed in patients with renin-dependent hypertension, it is hard to separate the effects of mechanical force on endothelial function from the effects of various neurohumoral factors. Lamping and Dole showed that acute elevations of coronary perfusion pressure for 15 minutes increased the constrictor response of large epicardial coronary arteries to serotonin. With this in mind, we examined the effects of acute pressure overload on coronary microvascular endothelial function in conscious dogs. Acute pressure overload increased LVSP to 200 mm Hg in our study but did not increase systemic pressure and HR. This suggests that the RAS and sympathetic nervous system were not systematically activated in our model. Ghaleh et al showed that mean arterial pressure and HR were not increased and plasma renin was not activated even in conscious dogs with supravalvular aortic banding for 10 months.

Our results showed that reflex cholinergic coronary vasodilation after activation of the Bezold-Jarisch reflex was selectively depressed in conscious dogs after release of AOC.

The abnormalities in vagally mediated coronary vasodilation after release of AOC could be present at the cardiac receptor level, in the afferent nerves, in the central nervous system, or in the efferent nerves. This is most likely not the case, however, because reflex hypotension was still preserved. The coronary vasodilation induced by activation of the Bezold-Jarisch reflex is NO dependent, because nitro-L-arginine, an NOS inhibitor, blocks such coronary vasodilation. Furthermore, vagally mediated coronary vasodilation is selectively impaired after pacing-induced heart failure or enhanced after short-term exercise training in conscious dogs. The changes in reflex cholinergic NO-dependent coronary vasodilation were proportional to the altered release of NO from the vascular endothelium because of altered gene expression of eNOS. We examined NO production from coronary microvessels in response to ACh, but there were no changes. This suggests that the endothelial stunning is not a result of the effects of altered muscarinic receptor function. Furthermore, we also used another agonist, BK, whose effect is not mediated by muscarinic receptors to stimulate the release of NO from the endothelium. Again, there were no changes in NO production. Therefore, the endothelial stunning we observed is not because of altered NO production or the altered function of receptors mediating release of NO.

\[ \text{O}_2^- \text{ production by vascular tissues and its interaction with NO} \]

The effects of ascorbic acid on the endothelial stunning. Ascorbic acid prevented the endothelial stunning, perhaps directly scavenging \( \text{O}_2^- \) and thereby decreasing NO inactivation and inactivation of target enzymes. Although ascorbic acid is an effective scavenger of \( \text{O}_2^- \) with a bimolecular rate constant for the reaction of \( 2.7 \times 10^9 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1} \) and \( \text{O}_2^- \) reacts with NO, reducing NO bioactivity, producing the oxidant peroxynitrite. Therefore, we examined the effects of ascorbic acid on the endothelial stunning. Ascorbic acid prevented the endothelial stunning, perhaps directly scavenging \( \text{O}_2^- \) and thereby decreasing NO inactivation and inactivation of target enzymes. Although ascorbic acid is an effective scavenger of \( \text{O}_2^- \), with a bimolecular rate constant for the reaction of \( 2.7 \times 10^9 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1} \), and NO, reducing NO bioactivity, producing the oxidant peroxynitrite.
interaction when present in millimolar concentrations. Ascorbic acid was infused intravenously at an initial dose of 2000 mg followed by a constant infusion at 25 mg/min in the present study. It is estimated that a plasma concentration of $10^{-7}$ mol/L, ascorbic acid is achieved in vivo by our method. Furthermore, $\dot{O}_2^-$ generated from intracellular sources is scavenged primarily within cells, because $\dot{O}_2^-$ is poorly transported across membranes. The intracellular concentration of ascorbic acid is increased in concentration- and time-dependent manners as its extracellular concentration is increased. Because ascorbic acid was infused for 90 minutes before AOC in the present study, a sufficient intracellular concentration might be achieved to scavenge $\dot{O}_2^-$ within cell.

Wung et al. using human umbilical vein endothelial cells, showed that cyclic strain induced $\dot{O}_2^-$. In this study, when the endothelial cells were exposed to cyclic strain for 30 minutes, intracellular $\dot{O}_2^-$ was increased. Furthermore, Hishikawa and Luscher found that pulsatile stretch for 1 hour increased $\dot{O}_2^-$ production from human aortic endothelial cells, and this was reduced by diphospheneiodionium chloride, an NADPH oxidase inhibitor. These in vitro studies using cultured endothelial cells support our data. We have not measured $\dot{O}_2^-$ directly at the time of endothelial stunning, but these data strongly suggest that the endothelial stunning induced by acute pressure overload is a result of NO inactivation subsequent to mechanical stretch-induced $\dot{O}_2^-$ production from the coronary microvessel endothelial cells.

Components of the RAS have been demonstrated in various tissues in the heart. Increased gene transcript levels for renin, angiotensinogen, angiotensin receptors, and ACE have been identified in an experimental model of pressure overload. The results of the present study suggest that an acute pressure overload for LV and coronary circulation occurs during stress and malignant hypertension. The results of the present study suggest that an acute transient increase in pressure might lead to coronary endothelial stunning. Furthermore, repeated episodes of acute transient increases in pressure might lead to the irreversible endothelial dysfunction. The relationship between Ang II-induced $\dot{O}_2^-$ production and the NO-dependent control of MVO$_2$ remains unknown. In the present study, we showed that pathophysiological concentrations of Ang II inhibited the NO-dependent control of MVO$_2$. These effects of Ang II might be one of the mechanisms responsible for the development of cardiac dysfunction.

In summary, the present results demonstrate that (1) the coronary vasodilation after activation of the Bezold-Jarisch reflex is depressed in conscious dogs after a brief episode of acute pressure overload; (2) the endothelial stunning we observed is associated with increased $\dot{O}_2^-$, mediated by increased Ang II–induced activation of NAD(P)H oxidase; and (3) pathophysiological concentrations of Ang II increase $\dot{O}_2^-$ in the heart, which inhibits NO-dependent control of myocardial oxygen consumption.

We examined the same phenomenon from the opposite point of view by measuring $\dot{O}_2^-$ production in LV tissue samples with lucigenin chemiluminescence. These results suggest that NO scavenges Ang II–induced $\dot{O}_2^-$. The concentrations ($10^{-10}$ to $10^{-8}$ mol/L) of Ang II we used in the present study are the same as those used in previous reports in cultured cells. We do not know exactly whether the concentrations of Ang II we used in vitro experiments are attained in the coronary microcirculation after AOC in our in vivo model, but the estimated pathological plasma concentration of Ang II is $10^{-10}$ mol/L, and the interstitial level of Ang II may be more than 100 times that of plasma.

The vascular NAD(P)H oxidase is composed of both cytosolic and membrane-bound components. Combinations of the cytosolic p47phox, p67phox, and rac-1 associate, translate to, and activate the membrane-bound complex composed of the p22phox and gp91phox subunits. Cytosolic subunit binding is thought to enhance the conversion of molecular oxygen to $\dot{O}_2^-$. Apocynin has shown to prevent the binding of the cytosolic subunits of the NAD(P)H oxidase to the membrane-bound p22phox/Nox or gp91phox subunits, preventing oxidase activation and subsequent production of $\dot{O}_2^-$ in coronary artery. The amount of apocynin we used in this in vivo experiment could prevent $\dot{O}_2^-$ production by activation of NAD(P)H oxidase.

Acute transient pressure overload for LV and coronary circulation occurs during stress and malignant hypertension. The results of the present study suggest that an acute transient increase in pressure might lead to coronary endothelial stunning. Furthermore, repeated episodes of acute transient increases in pressure might lead to the irreversible endothelial dysfunction. The relationship between Ang II–induced $\dot{O}_2^-$ production and the NO-dependent control of MVO$_2$ remains unknown. In the present study, we showed that pathophysiological concentrations of Ang II inhibited the NO-dependent control of MVO$_2$. These effects of Ang II might be one of the mechanisms responsible for the development of cardiac dysfunction.

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References


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