Reoxygenation-induced Constriction in Murine Coronary Arteries

THE ROLE OF ENDOTHELIAL NADPH OXIDASE (gp91phox) AND INTRACELLULAR SUPEROXIDE*

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Previous work suggests that superoxide mediates hypoxia/reoxygenation (H/R)-induced constriction of isolated mouse coronary arteries (CA). To determine the source of superoxide overproduction during H/R we studied CA obtained from transgenic (Tg) mice overexpressing human CuZn-superoxide dismutase (SOD) and mice lacking gp91phox using an in vitro vascular ring bioassay. We found that under normoxic conditions CA isolated from wild type (wt) mice, CuZn-SOD Tg mice and gp91phox knock-out mice had similar contractile responses to U46619 and hypoxia and similar dilation responses to acetylcholine. In wt CA, 30 min of hypoxia (1% O2) followed by reoxygenation (16% O2) resulted in further coronary vasoconstriction (internal diameter from 105 ± 11 to 84.5 ± 17.9 μm), whereas this response was completely blocked in both CuZn-SOD Tg and gp91phox knock-out CA (104.3 ± 10.5 to 120.7 ± 14 μm and 143.3 ± 15.3 to 172.7 ± 12.5 μm, respectively, p < 0.01). Furthermore, we show that H/R enhances the generation of superoxide radicals in wt CA (25.8 ± 0.7 relative light units per second (RLUs)), whereas CuZn-SOD Tg CA (12.2 ± 0.8 RLU/s, p < 0.01) and gp91phox CA (12.5 ± 0.9 RLU/s, p < 0.01) showed reduced levels. These results demonstrate that H/R-induced vasoconstriction is mediated by intracellular superoxide overproduction via endothelial NADPH oxidase gp91phox. Therefore, increasing endogenous levels of CuZn-SOD in CA may provide a novel cardioprotective strategy for maintaining coronary perfusion under conditions of H/R.

In coronary arteries, hypoxia/reoxygenation (H/R)1 induces both contraction and endothelial dysfunction (1–6) and may be caused, at least in part, by an increased production of superoxide radicals (1, 7, 8). In the heart, superoxide radicals generated during ischemia/reperfusion (I/R) and/or H/R may cause direct injury to cardiac myocytes leading to impaired cardiac function, but they may also worsen conditions indirectly by aggravating coronary vasoconstrictor tone and therefore coronary perfusion. In some models, the exogenous addition of CuZn-superoxide dismutase (SOD) has been shown to reduce H/R-induced superoxide production in various tissues (9–11) and in the endothelium (9, 10, 12). Although it has been shown that superoxide plays a significant role in the pathogenesis of I/R and H/R injury, studies that involve the exogenous addition of CuZn-SOD have shown mixed results in preventing these injuries (13). For example, in a canine model of myocardial I/R, exogenous CuZn-SOD did not reduce infarct size nor did it improve regional myocardial contractile function (14, 15). On the other hand, in a dog and pig model of I/R, the addition of CuZn-SOD protected myocardial tissue from reperfusion injury (16, 17).

One explanation for why the exogenous administration of CuZn-SOD may fail to prevent injury could be the inability of CuZn-SOD to penetrate the cellular membrane and therefore reduce intracellular levels of superoxide anion species. This could be critical if the injury process is caused, at least in part, by an increased generation of intracellular superoxide anions.

Recent advances in gene transfer and knock-out techniques have provided genetic approaches to investigating the role that endogenous SODs play in I/R myocardial injury. Overexpression of each of the three human SODs, CuZn-SOD, Mn-SOD, and extracellular SOD, can prevent I/R myocardial injury by decreasing superoxide levels from different cellular compartments (intracellular, mitochondrial, or extracellular) (18–21). Overall, these studies indicate that superoxide overproduction in specific compartments probably plays a key role in I/R myocardial injury. However, there is very limited information available identifying the cellular compartment and biochemical pathway responsible for the coronary artery superoxide overproduction following H/R.

The aim of the present study was to determine the cellular location and source of superoxide production in murine coronary arteries that affect vasoconstrictor responsiveness under normoxia and hypoxia/reoxygenation conditions. Additionally, we wanted to determine whether these genetic manipulations would protect against H/R-induced coronary artery (CA) superoxide overproduction and vasoconstriction.

EXPERIMENTAL PROCEDURES

Preparation of Animals—The Animal Care and Use Committees at Johns Hopkins Medical Center and at Duke University Medical Center approved the experimental protocols used in this study. Adult male (8–20 weeks old) heterozygous transgenic mouse strains overexpressing the human CuZn-SOD gene (B6SJL-TgN 2Gur, stock number 002297 (23)), wild type C57BL/6 (stock number 000664) control mice, and hemizygous male NADPH gp91phox KO mice (∼Y, C57BL/6 background, stock number 002365 (23)) were obtained from Jackson Laboratory (Bar Harbor, ME).

CuZn-SOD mRNA levels were measured by reverse transcriptase-PCR analysis. Coronary artery and left ventricle tissue was isolated, and total RNA was purified using the RNAqueous Micro kit (Ambion, Austin, TX). This kit included the treatment of total RNA with DNase...
I to remove trace amounts of contaminating genomic DNA. The synthesis of single-stranded DNA from RNA was performed using the SuperScript First-Strand Synthesis system for reverse transcriptase-PCR (Invitrogen), according to the protocol provided by manufacturer. The forward (5′-AGG TAC CAG TGC AGG-3′) and reverse (5′-GTC TCC TGA GAG TGA CAT CAC A-3′) primers were designed to amplify both human and mouse CuZn-SOD. PCR reactions were performed using the SmartStart Taq polymerase (Stratagene, La Jolla, CA) at 95 °C for 5 min followed by 32 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The amplified DNA (162 bp) was electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. Murine glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified using primers from TaqMan Rodent GAPDH control reagents kit (Applied Biosystems, Foster City, CA). The relative ethidium bromide fluorescence was quantified using NIH Image software (version 1.29).

**Coronary Artery Ring Biosassay System**—Mouse CA rings were isolated and mounted, and isotonic shortening contraction was measured as described previously (6). Briefly, the isolated mouse left main CA (~70–90 μm in diameter, 1-mm length) was placed into a microvascular chamber, cannulated at both ends with glass micropipettes, and pressurized. In some vessels, endothelial cells were removed by gently rubbing the intraluminal surface with a steel wire. The vascular intraluminal pressure (Ptm) was measured with a pressure transducer positioned at the level of the vessel lumen. In the microvascular chamber vessels were superfused constantly with recirculating Krebs-Ringer bicarbonate solution containing (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25.0 NaHCO3, and 11.1 glucose. The perfused Krebs-Ringer bicarbonate solution was gassed with 16% O2, 5% CO2, balance N2 (pH 7.35–7.45) and maintained at 37 °C. A custom-built Plexiglas cover was placed over the chamber to control oxygen tension over the superfusate. An oxygen electrode (Microelectrode, Inc., Bedford, NH) was passed through a port in the cover into the superfusate and positioned near the vessel to provide continuous measurement of oxygen tension. The vascular intraluminal diameter (ID) was measured continuously by a video dimension analyzer (Living Systems Instrumentation, Burlington, VT). The oxygen tension, vascular ID, and Ptm were recorded. Initially the isolated CA was allowed to equilibrate in the microvascular chamber for 30 min at a Ptm of 10 mm Hg. Ptm was increased to 60 mm Hg in 10-mm Hg steps at 5–7-min intervals and held constant thereafter. Measurement of ID at Ptm = 60 mm Hg (ID60) began 5 min after Ptm was increased to 60 mm Hg (time 0) and continued throughout the experiment.

The generation of superoxide anions in isolated murine CA was measured using a lucigenin (bis-N-methyacridinium nitrate)-enhanced chemiluminescence technique (6, 24). The total volume of lucigenin (5 μL) buffer solution was 1 mL. After base-line chemiluminescence stabilized for 5 min, the coronary artery was placed in the chemilumino-meter and photon emission was recorded continuously. The chemiluminescence signal was recorded as relative light units per second (RLUs).

**Chemical Reagents**—Acetylcholine chloride, bovine CuZn-SOD, lucigenin, and papaverine were obtained from Sigma. U46619 was purchased from Cayman Chemical Co. (Ann Arbor, MI). Stock solutions of drugs were prepared fresh each day in deionized water and stored at 4 °C during the experiment. All drug concentrations are expressed as final molar concentration (mol/liter) in the chamber superfusate.

**Data Analysis**—Vascular responses to H/R and pharmacological stimulation were expressed as changes of intraluminal diameter (μm). The measurements of superoxide anions (chemiluminescence signal) were expressed as RLUs. Data were expressed as mean ± S.E. for n number of animals. Unpaired Student’s t test or multivariate analysis of variance was used for statistical analysis as appropriate.

**RESULTS**

**Determination of CuZn-SOD mRNA Levels in Murine Heart and Coronary Artery**—mRNA levels of CuZn-SOD were measured in both CA and heart tissue isolated from wild type and CuZn-SOD Tg mice. The samples were obtained as follows. Coronary arteries were isolated from three mice, pooled, and total RNA was purified as described under “Experimental Procedures.” The same was done with murine hearts. The two groups of mice analyzed were wild type and CuZn-SOD Tg. A, total RNA was reverse transcribed into single-stranded DNA with (+RT) or without (−RT) reverse transcriptase. A 162-bp CuZn-SOD amplification product was generated as described under “Experimental Procedures.” DNA bands were visualized by ethidium bromide. B, the DNA band intensity was quantified using NIH Image software and normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

**Coronary Artery Responses to Hypoxic Reoxygenation**—After the isolated murine CA equilibrated for 20 min at a Ptm of 60 mm Hg, 1% hypoxia was introduced for 30 min, and changes in intraluminal diameter of the vessel were recorded. Universally 30 min of hypoxia resulted in a similar pattern of contraction in vessels isolated from wt mice, CuZn-SOD Tg mice, and gp91phox KO mice (Fig. 2). Removal of the endothelium from the CA of both CuZn-SOD Tg and wt mice (6) had no effect on hypoxia-induced vasoconstriction.

Following hypoxia, reoxygenation with 16% oxygen for 30 min resulted in further vasoconstriction in wt + EC intact coronary arteries (ID = 105 ± 10.9 to 84.5 ± 17.9 μm, n = 6) (Fig. 2). However, CA isolated from CuZn-SOD Tg mice, with endothelium both intact (ID = 104.3 ± 10.5 to 120.7 ± 14 μm, n = 6, p < 0.01) and denuded (ID = 87.7 ± 11.4 to 122.3 ± 15.4 μm, n = 6, p < 0.01), showed a time-dependent vessel relaxation response to reoxygenation (Fig. 2). In addition, this time-dependent vasorelaxation during reoxygenation was also observed in CA isolated from gp91phox KO mice (ID = 143.3 ± 15.3 to 172.7 ± 12.5 μm, n = 7, p < 0.01) (Fig. 3).

**FIG. 1.** CuZn-SOD mRNA levels were measured in coronary artery and heart tissue obtained from wild type and CuZn-SOD Tg mice. The samples were obtained as follows. Coronary arteries were isolated from three mice, pooled, and total RNA was purified as described under “Experimental Procedures.” The same was done with murine hearts. The two groups of mice analyzed were wild type and CuZn-SOD Tg. A, total RNA was reverse transcribed into single-stranded DNA with (+RT) or without (−RT) reverse transcriptase. A 162-bp CuZn-SOD amplification product was generated as described under “Experimental Procedures.” DNA bands were visualized by ethidium bromide. B, the DNA band intensity was quantified using NIH Image software and normalized to glyceraldehyde-3-phosphate dehydrogenase expression.
Coronary arteries were isolated and pressurized to 60 mm Hg and ID_{60} at determined base line all under normoxic conditions. Endothelium was left either intact or denuded, as indicated. Serial vessel responsiveness to the thromboxane A\textsubscript{2} analog U46619 (0.01 \textmu M) was determined followed by that of acetylcholine (ACh) (1 \textmu M) and then by papaverine (30 \textmu M). The intraluminal diameter (\mu m) was measured. Values shown are the mean ± S.E. n = number of animals.

| Genotype          | Endothelium | Base line | U46619 | ACh   | Papaverine | n  |
|-------------------|-------------|-----------|--------|-------|------------|----|
| Wild type         | Intact      | 157.4 ± 9.2 | 0.01 \mu m | 71.0 ± 6.7 | 118.0 ± 8.2 | 12.7 ± 6.1 | 12 |
| Wild type         | Denuded     | 116.3 ± 11.9 | 1 \mu m   | 70.6 ± 9.5 | 75.8 ± 8.9   | 156.3 ± 28.0 | 7  |
| Wild type\textsuperscript{a} | Intact      | 165.8 ± 15.5 | 30 mm    | 54.2 ± 5.2 | 99.9 ± 7.3 | 112.4 ± 7.6 | 5  |
| CuZn SOD Tg       | Intact      | 161.6 ± 11.8 |         | 78.7 ± 2.4 | 120.4 ± 7.3 | 122.5 ± 10.9 | 6  |
| CuZn SOD Tg       | Denuded     | 121.0 ± 3.5\textsuperscript{b} |        | 64.2 ± 4.4 | 64.2 ± 0.7\textsuperscript{b} | 144.0 ± 15.9 | 6  |
| gp91\textsuperscript{phox} KO | Intact      | 176.2 ± 13.2 |         | 89.9 ± 8.7 | 149.2 ± 14.3 |        |     |

\textsuperscript{a} p < 0.05 versus wild type + EC intact.
\textsuperscript{b} Intact wild type was treated with 150 units/ml SOD1.
\textsuperscript{c} p < 0.05 versus SOD1 Tg + EC intact.

DISCUSSION

In the coronary circulation, hypoxia-induced responses have been controversial and include both vasorelaxation and/or vasoconstriction. In some models, hypoxia is shown to cause coronary vasorelaxation, likely mediated by ATP-sensitive potassium channels (5, 25), prostaglandin (26, 27), and nitric oxide (28). This hypoxic coronary vasodilatation also is reported to be regulated by endothelium-dependent and/or -independent mechanisms (5, 25, 29). On the other hand, hypoxia is shown to cause coronary vasoconstriction. In these studies, endothelium-derived contracting factors are thought to play a major role in hypoxic coronary vasoconstriction (2, 30–32). Although these discrepancies in hypoxia-induced responses may be attributable to species, arterial size or locus, or method of measurement, our studies indicate that hypoxia causes CA vasoconstriction that is independent of the endothelium and or superoxide.

Following hypoxia, reoxygenation caused progressive vaso-
constriction in CA isolated from wt mice (Fig. 2). This reoxygenation-induced coronary vasoconstriction was prevented and reversed in vessels isolated from human CuZn-SOD Tg mice (Fig. 2) and gp91phox KO mice (Fig. 3). Furthermore, denuding the endothelium from the Tg mice appears to cause further relaxation. These findings, along with our previous findings where we have shown that denuding the endothelium from wt coronary arteries also prevents reoxygenation-induced constriction (6), led us to hypothesize that reoxygenation-induced vasoconstriction is mediated, at least in part, by the endothelial overproduction of superoxide anions via an NADPH oxidase gp91phox-dependent subunit protein. To further support the role of superoxide in this process, we measured superoxide levels using a lucigenin-enhanced chemiluminescence technique and found that CA isolated from wt mice showed significantly higher levels of chemiluminescence than that from CuZn-SOD Tg mice, from denuded wt CA (6), and from gp91phox KO CA (Fig. 4). This further supports the role of the endothelium in generating superoxide anion species (via NADPH oxidase containing gp91phox subunit protein), which in turn leads to enhanced smooth muscle constriction (Fig. 5).

Because CuZn-SOD has been found to be widely distributed in the cell cytosol and nucleus, is the primary antioxidant enzyme regulating intracellular superoxide levels (33), and is unlikely to be translocated across either the endothelium or smooth muscle membrane, we speculate that reoxygenation-induced vasoconstriction is mediated by an endothelium-derived intracellular superoxide source. In a mouse model of myocardial ischemia/reperfusion injury, Chen et al. (20) have reported that overexpression of Mn-SOD preserves myocardial function, suggesting that mitochondria are probably the intracellular source for enhanced superoxide production. In endothelium-denuded calf coronary arteries, Mohazzab et al. (34) observed that NADPH oxidase contributes to superoxide generation under reoxygenation conditions indicating that vascular smooth muscle could be another source of superoxide production. However, our results from NADPH oxidase gp91phox KO mice clearly demonstrated that endothelial NADPH oxidase gp91phox subunit protein plays a major role in H/R-induced superoxide overproduction.

In our earlier studies we demonstrated that the exogenous addition of CuZn-SOD prevented H/R-induced murine coronary vasoconstriction and superoxide overproduction (6). Because CuZn-SOD (32-kDa protein) would unlikely be rapidly translocated across either the endothelium or smooth muscle membrane (35), it is likely that extracellular superoxide plays a major role in this process. However, H/R-induced superoxide overproduction was significantly reduced in CA isolated from human CuZn-SOD Tg mice, suggesting that H/R-induced endothelial superoxide is initially generated from an intracellular space. Taken together, it is logical to conclude that under H/R conditions endothelial superoxide is overproduced in the intracellular space and then released into the extracellular space where its overall effects are to enhance coronary vasoconstriction. However, the mechanism by which superoxide diffuses across the plasma membrane into the extracellular space is not clear. One possibility is via a bicarbonate-dependent anion exchange protein (AE2) found in rat pulmonary artery. Recent studies demonstrate that AE2 releases intracellular superoxide into the extracellular spaces but that this process is dependent on extracellular bicarbonate (36, 37). Inhibition of AE2 significantly attenuates endothelial superoxide release under normoxic conditions suggesting that AE2 could be one of the superoxide transporters (36, 37).

NADPH oxidases are a major source of superoxide production in vascular tissues (38–40). Upon stimulation the cytosolic subunits p47phox, p67phox, and Rac1/2 are translocated to the plasma membrane where they interact with cytochrome b558 (gp91phox and p22phox) resulting in rapid activation of the NADPH oxidase complex (41, 42). Phosphorylation of p47phox by activated protein kinase C and/or protein kinase A has been shown to enhance NADPH oxidase activity (41–43). Unlike neutrophil NADPH oxidase activity, endothelial-associated NADPH oxidase activity is constitutively active at low levels even in the absence of stimulating conditions (38, 42, 43). The subunit protein gp91phox is an integral component of the native mouse endothelial NADPH oxidase complex (44). For example, in aorta isolated from gp91phox KO mice, angiotensin II-induced increases in superoxide levels are markedly reduced (44). On the other hand, knocking out p47phox activity, present in the NADPH oxidase complex of neutrophils, did not lead to protection against myocardial injury and dysfunction following ischemia/reperfusion injury (45).

In summary, the present study demonstrates that H/R-induced murine coronary vasoconstriction is mediated by an intracellular overproduction of superoxide in endothelium via an endothelium-associated NADPH oxidase complex (41). Phosphorylation of p47phox, p67phox, and Rac1/2 are translocated to the plasma membrane where they interact with cytochrome b558 (gp91phox and p22phox) resulting in rapid activation of the NADPH oxidase complex (41, 42). Phosphorylation of p47phox by activated protein kinase C and/or protein kinase A has been shown to enhance NADPH oxidase activity (41–43). Unlike neutrophil NADPH oxidase activity, endothelial-associated NADPH oxidase activity is constitutively active at low levels even in the absence of stimulating conditions (38, 42, 43). The subunit protein gp91phox is an integral component of the native mouse endothelial NADPH oxidase complex (44). For example, in aorta isolated from gp91phox KO mice, angiotensin II-induced increases in superoxide levels are markedly reduced (44). On the other hand, knocking out p47phox activity, present in the NADPH oxidase complex of neutrophils, did not lead to protection against myocardial injury and dysfunction following ischemia/reperfusion injury (45).

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