Cytosolic NADPH may regulate differences in basal Nox oxidase-derived superoxide generation in bovine coronary and pulmonary arteries

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Our previous studies characterized NAD(P)H oxidases in bovine pulmonary arteries (BPA) and bovine coronary arteries (BCA) in the context of examining their potential role in regulation of basal superoxide generation. We characterized the NAD(P)H oxidases present in these vascular segments and how cytosolic NADPH may regulate differences in oxidase activity. BPA had 4.2-fold higher levels of NADPH than BCA. The activity and protein levels of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme generating cytosolic NADPH, were 1.5-fold higher in BPA than BCA. Thus BPA differ in that they have higher levels of G6PD activity, NADPH, and superoxide. Because both arteries have similar levels of oxidase expression and activity, elevated levels of cytosolic NADPH may contribute to increased superoxide in BPA.

Glucose-6-phosphate dehydrogenase: lactate; NAD(P)H oxidase; Nox2; Nox4; pentose phosphate pathway.

Materials

Many of the reagents used in the present study were obtained from sources previously described (6, 9, 16–20, 22, 23). Most vasoactive and mechanistic probes were purchased from Sigma Chemical (St. Louis, MO).

Measurement of NAD(P)H, NAD(P)+, and ATP in BCA and BPA. Isolated endothelium-removed left anterior descending coronary and secondary branch of pulmonary arterial rings were prepared from slaughterhouse-derived bovine hearts and lungs and used in the study (6, 9, 16–20, 22, 23). The rings were incubated in individually
thermostated (37°C) 10-ml baths (Metro Scientific) for 2 h in Krebs bicarbonate buffer (pH 7.4) containing the following (in mM): 118 NaCl, 4.7 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.2 KH₂PO₄, and 5.6 glucose, gassed with 21% O₂-5% CO₂ (balance N₂). After a 2-h equilibration period, the levels of NAD(P)H in BCA were determined by HPLC using adaptations of previously published methods (6, 7). Briefly, BCA and BPA were pretreated with and without different drugs and immediately frozen in liquid nitrogen. The frozen tissues were crushed and homogenized in an extraction medium consisting of 0.02 N NaOH, containing 0.5 mM cysteine at 0°C. The extracts were then heated at 60°C for 10 min and neutralized with 2 ml of 0.25 M glycylglycine buffer, pH 7.6. Acidic extracts were prepared by homogenizing the tissues in hot 0.1 N HCl followed by neutralization. The NAD(P)H was eluted on a reverse-phase HPLC column (4.6 × 250 mm; Bondapak C18, Shiseido) at room temperature using HP 1100 Series (Agilent Technologies) and buffer system consisting of 100 mM potassium phosphate (pH 6.0) (buffer A), and 100 mM potassium phosphate (pH 6.0) containing 5% methanol (buffer B). The column was eluted with 100% buffer A from 0 to 8.5 min, 80% buffer A plus 20% buffer B from 8.5 to 14.5 min, and 100% buffer B from 14.5 to 40 min. The flow rate was 1.0 ml/min, and the ultraviolet absorbance was monitored at 260 nm.

**Determination of glucose-6-phosphate dehydrogenase activity and glucose-6-phosphate levels in BCA and BPA.** Glucose-6-phosphate dehydrogenase (G6PD) activity was measured as previously described (27). Briefly, enzyme activity was determined in BPA and BCA homogenates using a plate-reader spectrophotometer (Bio-Tek PowerWave 200) by measuring the rate of increase of absorbance at 340 nm from the conversion of NADPH to NADP⁺ by G6PD. Substrate concentrations used were glucose-6-phosphate (G6P, 200 μM) and NADP⁺ (100 μM).

G6P levels were determined using the method described by Jungling et al. (11). This method was adopted after a slight modification. G6P level was determined in homogenate (50 μl) incubated in 400 μl K₂HPO₄ buffer (pH 7.6) containing NADP⁺ (27 mM), flavin mononucleotide (20 μM), G6PD (350 U/ml), and diithiothreitol (12.5 mM). After incubation for 30 min at 37°C, increase in fluorescence from the conversion of NADP⁺ to NADPH by G6PD was measured. The acceptable level of significance was Bonferroni correction was used when multiple comparisons were made. The acceptable level of significance was P < 0.05. The number of experimental determinations (n) in all cases is equal to the animals from which an arterial ring was employed for a treatment or a control group in all studies. Values are means ± SE.

**RESULTS**

**Superoxide levels detected by lucigenin chemiluminescence in BCA and BPA.** Detection of O₂⁻ levels using lucigenin (5 μM) chemiluminescence revealed that BPA rings generated ~60–80% higher basal O₂⁻ level compared with BCA (Fig. 1). This was further confirmed by a treatment of BPA with O₂⁻ scavengers polyethylene glycol (PEG)-SOD (137 U/ml) and tiron (10 mM), which inhibited O₂⁻ by 60% and 82%, respectively. Treatment of BCA with similar concentrations of PEG-SOD and tiron suppressed O₂⁻ levels by 80% and 93%, respectively. Because of the markedly greater amount of vascular smooth muscle cells compared with other cells present in the adventitia of the endothelium-removed conduit-sized arteries studied, the levels of O₂⁻ detected by lucigenin are interpreted as representing primarily the contribution of this cell type.

**Determination of the influence of Nox oxidase activation and pentose phosphate pathway NADPH on O₂⁻ in BCA and BPA.** To determine whether Nox activation by binding its p47^phox subunit was influencing the source of O₂⁻ that may contribute to the higher levels observed in BPA, we tested the effect of apocynin (10 μM), an NAD(P)H oxidase inhibitor, on the O₂⁻ levels from BPA and BCA. Our data in Fig. 2 indicated that apocynin suppressed the O₂⁻ levels by 51% and 37% in BPA and BCA, respectively. In contrast, stimulation of NAD(P)H oxidase activity by activating protein kinase C with phorbol-12,13-dibutyrate to promote p47^phox phosphorylation and binding to Nox2 (12) significantly increased O₂⁻ levels in both BCA and BPA. The increase in lucigenin chemiluminescence was more than 15-fold in BPA and 5-fold in BCA, and this resulted in BPA showing 5-fold greater levels of O₂⁻ than BCA.
Furthermore, to investigate whether PPP-derived NADPH influences NAD(P)H oxidase activity in BCA and BPA, the effect of 6-aminonicotinamide (6-AN) (1 mM), a probe previously used by us to inhibit this pathway (6), was studied for its influence on O$_2^•$ levels in these arterial segments (Fig. 2). A dose of 1 mM 6-AN was selected for use based on observations that it lowered NADPH by 50–70% in BCA and rat lungs (6, 8). 6-AN inhibited basal O$_2^•$ levels by 75% in BPA, and it decreased O$_2^•$ levels by only 40% in BCA.

Evaluation of the NADH and NADPH dependence of O$_2^•$ generation in BPA and BCA homogenates and isolated microsomes. To compare the influence of NADPH and NADH on O$_2^•$ generation in BPA and BCA, the concentration dependence of O$_2^•$ generation by these pyridine nucleotides was evaluated in BPA and BCA homogenates (see Fig. 3). In the BPA homogenate, NADPH (0.1–1,000 μM) and NADH (0.1–1,000 μM) increased O$_2^•$ generation in a concentration-dependent
manner. However, at higher concentrations NADH (100 and 1,000 µM) promoted significantly more O$_2^-$ generation than NADPH. Nevertheless, at more physiologically relevant lower concentrations (e.g., 1–10 µM), NADPH generated significantly higher O$_2^-$ (see Fig. 3, inset) than NADH. Although in BCA both NADPH and NADH elevated O$_2^-$ levels in a concentration-dependent manner, there was no difference in the generation of O$_2^-$ at each concentration of NADH and NADPH examined (Fig. 3). In the microsomal membrane fraction of BPA, although a distinct NADPH-dependent production of O$_2^-$ was observed at the 100 µM concentration examined, 100 µM NADH resulted in a markedly greater level of O$_2^-$ generation. A similar pattern of NADH and NADPH-dependent O$_2^-$ production was observed in the microsomal membrane fraction obtained from BCA.

Identification mRNA for Nox2 and Nox4 by QRT-PCR in BCA and BPA. It has been reported that three of the isoforms of NAD(P)H oxidases, namely Nox1, Nox2, and Nox4, are expressed in the cultured vascular smooth muscle cells and/or intact arteries (5, 10, 26), but a comparison of the expression of these oxidases between a pulmonary and systemic artery has not been previously reported. Hence, QRT-PCR for Nox1, Nox2, and Nox-4 was performed to determine the source of O$_2^-$ in BPA and BCA. Although the message for Nox1 could not be detected by using several different primers designed from human and animal sequences, mRNA for both Nox2 and Nox4 were observed to be present in BPA and BCA in levels that appeared to be similar to each other (see Fig. 4).

Identification and expression of Nox2 and Nox4 protein by Western blot analysis. The levels of expression of Nox2 and Nox4 in BPA were compared with the levels observed in BCA by Western blot analysis (see Fig. 4). SDS gel electrophoresis was performed with BPA and BCA samples to detect Nox1, Nox2, and Nox4. Our results suggest that several bands were detected with the Nox2 antibody used, with one of the bands at ~65 kDa, which was similar to that reported in rat and mouse models (1, 13, 14). In addition, we have detected additional bands at ~120 and 50 kDa, which remain to be identified. Western blot analysis for Nox4 protein showed two bands around 80 and 65 kDa, and this is consistent with previous reports detecting bands of similar molecular mass (3, 10). An additional band at ~70 kDa that remains to be identified was
also detected in BCA. After each blot was analyzed for Nox protein expression, each blot was stripped and reprobed for the detection of α-actin protein levels to confirm uniform loading (not shown). The densitometry analysis demonstrated in Fig. 4 indicates that BPA and BCA had similar levels of both Nox2 and Nox4 based on an analysis of the protein bands we could identify (65 and 80 kDa) as originating from these Nox oxidases. Nox1 could not be detected under similar conditions.

Fig. 4. Estimation of the expression of Nox2 and Nox4 by QRT-PCR and Western blot analysis. The mRNA for NAD(P)H oxidases Nox2 and Nox4 was quantitated \( (n = 6) \) in the BPA and BCA by QRT-PCR and normalized by α-actin \( \text{top} \). Representative data of Western blot analysis \( (n = 12) \) for both the oxidases in BPA \( \text{lanes 1–4} \) and BCA \( \text{lanes 5–8} \) \( \text{middle} \), and summary of densitometry of Nox2 \( \text{bottom left; 65 kDa} \) and Nox4 \( \text{bottom right; 80 kDa} \) protein in BPA and BCA are shown together with a HL-60 whole cell lysate (Santa Cruz; SC-2209), which was used as a positive control in Western blot studies.
Measurement of NADP(H), NAD(H), and ATP levels in BCA and BPA. Pyridine nucleotide levels in BPA and BCA were determined by HPLC. As shown in Fig. 5, we found fourfold more NADPH levels in BPA compared with BCA. A similar difference was found in NADP\(^+\) levels. The NADP\(^+\)-to-NADPH ratio in BPA was 1.3 and in BCA was 0.6. Although we could not distinguish between cytosolic or mitochondrial NAD(H), total NADH levels estimated in BCA homogenate were higher than BPA. In contrast, NAD\(^+\) levels were approximately fourfold higher in BPA. Additionally, the NAD\(^+\)-to-NADH ratio in BPA was 16.0 and in BCA was 1.8. ATP levels were about twofold higher in BCA, even though there were no differences in O\(_2\) consumption levels between BCA and BPA (Fig. 5, E and F).

Determination of G6PD activity and expression and G6P levels in BCA and BPA. To investigate the underlying cause for higher NADPH levels in BPA, we estimated the activity of G6PD, which is a rate-limiting enzyme in PPP, and G6P levels in coronary and pulmonary artery. Estimation of G6P levels indicated that BPA has 59% lower G6P content (Fig. 6).
results indicated that BPA has 1.6-fold higher G6PD activity than estimated in BCA (Fig. 6). Furthermore, the PPP inhibitor 6-AN (1 mM) attenuated G6PD activity in homogenates of BPA and BCA by 55% and 52%, respectively. Western blot analyses were also consistent with G6PD levels being elevated in BPA probably as a result of a higher expression of G6PD protein (Fig. 7).

**DISCUSSION**

O$_2^*$ generation in both BPA and BCA appear to originate from Nox-type NAD(P)H oxidases based on data from previous studies (16–20, 22) in these arteries and the results of the present study. Whereas our earlier studies detected a much greater level of NADH than NADPH-dependent O$_2^*$ generation employing larger lucigenin concentrations, when examined at lower levels of lucigenin that are thought to be free of redox cycling artifacts, NADPH oxidase activity was nearly as prominent as NADH oxidase activity in artery homogenates, especially at concentrations of NAD(P)H that are thought to be physiologically relevant. Cytosolic NAD(H) is thought to be highly oxidized as a result of the lactate dehydrogenase reaction and function of mitochondrial shuttles for NADH removal (24). Thus it is likely that basal levels of cytosolic NADH are likely to be in the concentration range of 1 μM (28), and most of the NADH that was measured probably originates from mitochondrial sources. The function of the PPP appears to control the redox status of NADP(H) in the bovine arteries examined (6), and cytosolic NADPH levels are likely to be in the range of 10–20 μM based on the measurements made in the present study. This estimate of cytosolic NADPH concentrations is based on comparisons with the tissue levels of other well-characterized metabolites such as ATP and glutathione and based on the assumption that mitochondria are only a relatively minor contributor to the levels that were measured. Thus based on the NADH and NADPH concentration dependence of O$_2^*$ generation in BPA and BCA homogenates observed in the present study and estimates of the cytosolic levels of these metabolites that are likely to be present in these arteries, cytosolic NADPH may be a more important source of O$_2^*$ generation than NADH under the basal conditions examined in the present study. The effects of PPP inhibition with 6-AN on the levels of detected O$_2^*$ support these interpretations because lowering cytosolic NADPH levels by inhibiting the PPP decreased O$_2^*$ detection. Our previous studies suggest that attempting to further lower cytosolic NADH through the lactate dehydrogenase reaction with pyruvate did not alter basal O$_2^*$; however, lactate raised the levels of O$_2^*$ detected in the arteries studied (9, 17, 18, 23), suggesting that basal O$_2^*$ generation is regulated by increases in cytosolic NADH levels.

The Nox oxidases present in BPA and BCA appear to be Nox2 and Nox4. Nox1 could not be detected in BCA and BPA. Nox2 is the phagocytic cell gp91phox-type oxidase. Whereas the gp91phox system in phagocytes has an apparent 91-kDa molecular mass due to glycosylation, the antibody used in the present study, which is selective for unique amino acid sequences on this subunit, detected proteins with molecular masses in the range of 50–65 and 120 kDa. It is likely that the protein subunits observed with the Nox2 antibody originate...
Based on the QRT-PCR, Western blot analyses, and the properties of the Nox oxidase subunits expressed in BPA and BCA. The antibody used or if they originate from novel properties of the molecular mass bands observed are artifacts of the properties of the antibody employed in the present study. In other studies, antibodies for Nox2 have detected 65-, 75-, and 110-kDa proteins (1, 13, 14). The Nox4 antibody detected proteins with molecular masses in the region of 65 and 80 kDa, which are in the range of previously reported Nox4 subunits (3, 10). The source of the additional band at 50 kDa, and the new band at ~70 kDa observed only in BCA are not known at this time. Further investigation is needed to determine whether the additional molecular mass bands observed are artifacts of the properties of the antibody used or if they originate from novel properties of the Nox oxidase subunits expressed in BPA and BCA. Based on the QRT-PCR, Western blot analyses, and the properties of O$_2$ generation by oxidase activities present at physiological levels of NAD(P)H, there appear to be similar levels of Nox oxidase activity in BPA and BCA. Whereas little is known about the individual contributions of Nox2 and Nox4 containing oxidase systems to the basal levels of O$_2$ that were detected, it is likely that the actions of apocynin originate from a selective inhibition of Nox2, because Nox4 does not appear to be regulated by binding of its p47$^{phox}$ subunit (12).

The function of the PPP in controlling cytosolic NADPH levels may be an important factor controlling the observed elevation of basal O$_2$ levels in BPA compared with BCA. Although BCA contained higher levels of NADH than BPA, it is likely that this reflects a property of basal mitochondrial function such as the maintenance of higher ATP levels in BCA and not a mechanism directly controlling O$_2$ levels, because most of the NADH detected is likely to be present in mitochondria. In addition, basal oxygen consumption rates were similar in BCA and BPA, and this suggests that the differences in NADH and ATP levels observed in these arteries are metabolic adaptations that are not likely to originate from factors that control mitochondrial respiration or basal levels of energy consumption. Because BPA were observed to have higher levels of G6PD expression and activity, it is likely that the known function of this enzyme as the rate-limiting step of the PPP is a primary factor in the observed higher levels of NADPH in these arteries compared with BCA. Based on the potential importance of cytosolic NADPH levels controlling basal O$_2$ production, the increased activity of the PPP in BPA may be a primary factor in the generating the increased levels of O$_2$ observed in these arteries compared with BCA.

Observations made in this study suggest that the levels of both cytosolic NADPH and NADPH are important factors influencing the Nox oxidase activity present in BPA and BCA. In previous studies, we have shown that modulating cytosolic NADH levels through the lactate dehydrogenase reaction influences responses to hypoxia-reoxygenation (23) and NO (9) potentially mediated through cGMP by alterations in the levels of hydrogen peroxide and superoxide, respectively, suggesting that basal levels of cytosolic NADH have an important role in controlling the signaling mechanisms linked to these responses. In contrast, lowering basal levels of NADPH by inhibiting the PPP decreased the levels of superoxide in both BCA and BPA, and this appears to be associated with the activation of a coordination of processes that lower intracellular calcium in a manner that appears to be independent of Nox oxidase-derived oxidant species (6). Cytosolic NADPH-dependent systems seem designed to work in a manner that coordinate the control of both Nox activity generating reactive oxygen species (ROS) and the impact of ROS on thiol redox signaling through the influence of NADPH availability on the activities of Nox oxidases and NADPH-dependent thiol-reducing systems, including glutathione reductase and thioredoxin reductase. The higher levels of G6PD and NADPH in pulmonary arteries should also increase their resistance to other sources of oxidative stress compared with coronary arteries. Fundamental differences exist between the response of BPA and BCA to hypoxia, with BPA contracting to hypoxia through processes that appear to originate from a lowering of basal peroxide levels (21), and BCA relaxing through a mechanism associated with lowering basal levels of NADPH (7, 8). The observed differences in control of cytosolic NADPH by the PPP between BCA and BPA may be a key factor in generating the differences in responses to hypoxia that are observed with BPA maintaining a higher basal level of Nox-derived peroxide as a result of the higher levels of NADPH present in these arteries and with hypoxia lowering NADPH in BCA as a result of the less effective ability of the PPP to compete with glycolysis potentially originating from the lower levels of G6P that are present in these vessels during hypoxia. Because mice lacking Nox2 (gp91$^{phox}$) show a normal response to hypoxia in a model also thought to involve a hypoxia-mediated decrease in peroxide (1), the Nox4 observed to be present in BPA may be the oxygen sensor involved in the pulmonary response to hypoxia.

**Fig. 7. Comparisons of the expression of G6PD in BPA and BCA.** A differential expression of G6PD protein was seen in the pulmonary and coronary arteries (top). Cumulative data of G6PD Western blot analysis in BPA (n = 9) and BCA (n = 9) demonstrate a significantly higher G6PD protein in BPA.

### Figure 7

- **G6PD Expression in BPA and BCA**
  - **BPA-G6PD**
  - **BCA-G6PD**
  - **Spot Density (% Arbitrary Units)**
  - **BPA vs. BCA**
  - **P<0.05 vs. BPA**

- **Observations**
  - Differential expression of G6PD in BPA and BCA.
  - Increased activity of the PPP in BPA.
  - Lowering basal levels of NADPH by inhibiting the PPP decreased the levels of superoxide in both BCA and BPA.

### References

1. Previous studies showing that...
2. Hypoxia-reoxygenation responses...
3. Glutathione reductase and thioredoxin...
4. Lowering basal levels of NADPH...
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REFERENCES