Effects of hypoxia on relationships between cytosolic and mitochondrial NAD(P)H redox and superoxide generation in coronary arterial smooth muscle

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Gao Q. Wolin MS. Effects of hypoxia on relationships between cytosolic and mitochondrial NAD(P)H redox and superoxide generation in coronary arterial smooth muscle. Am J Physiol Heart Circ Physiol 295: H978–H989, 2008. First published June 20, 2008; doi:10.1152/ajpheart.00316.2008.—Since controversy exists on how hypoxia influences vascular reactive oxygen species (ROS) generation, and our previous work provided evidence that it relaxes endothelium-denuded bovine coronary arteries (BCA) in a ROS-independent manner by promoting cytosolic NADPH oxidation, we examined how hypoxia alters relationships between cytosolic and mitochondrial NAD(P)H redox and superoxide generation in BCA. Methods were developed to image and interpret the effects of hypoxia on NAD(P)H redox based on its autofluorescence in the cytosolic, mitochondrial, and nuclear regions of smooth muscle cells isolated from BCA. Aspects of anaerobic glycolysis and cytosolic NADH redox in BCA were assessed from measurements of lactate and pyruvate. Imaging changes in mitosox and dehydroethidium fluorescence were used to detect changes in mitochondrial and cytosolic-nuclear superoxide, respectively. Hypoxia appeared to increase mitochondrial and decrease cytosolic-nuclear superoxide under conditions associated with increased cytosolic NADH (lactate/pyruvate), mitochondrial NAD(P)H, and hyperpolarization of mitochondria detected by tetramethylrhodamine methyl-ester perchlorate fluorescence. Rotenone appeared to increase mitochondrial NAD(P)H and superoxide, suggesting hypoxia could increase superoxide generation by complex I. However, hypoxia decreased mitochondrial superoxide in the presence of contraction to 30 mM KCl, associated with decreased mitochondrial NAD(P)H. Thus, while hypoxia augments NAD(P)H redox associated with increased mitochondrial superoxide, contraction with KCl reverses these effects of hypoxia on mitochondrial superoxide, suggesting mitochondrial ROS increases do not mediate hypoxic relaxation in BCA. Since hypoxia lowers pyruvate, and pyruvate inhibits hypoxia-elicited relaxation and NADPH oxidation in BCA, mitochondrial control of pyruvate metabolism associated with cytosolic NADPH redox regulation could contribute to sensing hypoxia.

The redox status of NAD(P)H in cytosolic and mitochondrial subcellular regions is likely to have an important role in controlling both redox-regulated signaling mechanisms and the production of ROS in vascular smooth muscle. NADH and NADPH are the two major substrates for cytosolic ROS generation by Nox oxidases in vascular smooth muscle (12, 19), and mitochondrial NADH is thought to be utilized for ROS production by the electron transport chain (6, 40). Mitochondrial and cytosolic sources of superoxide, hydrogen peroxide, and the metabolism of these ROS seem to be important factors in regulating signaling mechanisms that control vascular function (25, 38, 40). In addition, promoting oxidation of cytosolic NADPH by inhibiting the pentose phosphate pathway appears to be able to control a vasodilator mechanism in BCA, which is not influenced by scavenging hydrogen peroxide (11, 13). Contraction of vascular smooth muscle can also have a metabolic influence on aspects of NAD(P)H redox (1–3, 13). For example, it has been reported from measurements of metabolite indicators of redox that 80 mM KCl decreased the mitochondrial ratio of NADH to NAD+ and increased the cytosolic NADH/NAD+ ratio in carotid artery smooth muscle (3). Thus interactions between cytosolic and mitochondrial NAD(P)H redox systems and ROS could potentially have important roles in vascular smooth muscle regulation.

Many aspects of NAD(P)H redox control and relationships between NAD(P)H redox and ROS signaling in vascular smooth muscle remain to be elucidated. Mitochondria and cytoplasm have a territory that includes the regulation of vascular smooth muscle tone and the production of ROS. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
different systems controlling the redox status of NADH and NADPH redox. Vascular smooth muscle is thought to have the shuttles that help maintain low levels of cytosolic NADH by promoting the formation of mitochondrial NADH (2), and the pentose phosphate pathway of glucose metabolism appears to have an important role in maintaining cytosolic NADP(H), predominantly in the form of NADPH (11). While there is only limited information available on mitochondrial NAD(P)H redox in vascular smooth muscle, studies in other tissues provided evidence that mitochondrial NADH rapidly changes in response to the balance of processes associated with regulation by intracellular calcium, the metabolic demands of energy metabolism, and availability of oxygen (8, 31, 39). Krebs’ cycle enzymes and NADH-dependent transhydrogenase activities are thought to maintain high levels of mitochondrial NADPH. Mitochondrial complex I and III inhibitors, rotenone and antimycin, respectively, can increase mitochondrial NADH (31), but the effect of increasing mitochondrial NADH on cytosolic and nuclear NAD(P)H redox has generally not been considered in previous studies. Rotenone can either increase or decrease mitochondrial superoxide generation, depending on if the primary site of superoxide generation in the mitochondrial electron transport chain is complex I (36) or complex III (7), respectively, whereas the action of antimycin on complex III seems to increase mitochondrial superoxide generation from both of these sites. Based on studies in different tissue types, hypoxia often shifts the redox status of cellular NAD(H) to a more reduced form (10, 22, 30, 31, 39, 42). Smooth muscle cells (SMCs) produce large amounts of lactate, even when oxygen is available (2, 28), and lactate production and release can be increased during hypoxia (33, 35). The ratio of lactate to pyruvate is traditionally viewed as an indicator of cytosolic NADH redox state, and this has been confirmed in vascular smooth muscle (1). In addition, exposure of coronary arteries to high physiological levels of lactate (10 mM) under normoxic conditions appears to increase superoxide generation through a mechanism that may involve increasing cytosolic NADH (23, 24). While it is possible to assess subcellular changes in NAD(P)H redox through the detection of its cellular autofluorescence in the near ultraviolet region (5, 8, 29, 31), the complexity of current approaches available for imaging these redox changes has hampered the progression of research in this field. Unique features of the control of NAD(P)H redox systems in subcellular organelles make components of these systems optimal indicators of how oxygen influences mitochondrial function and energy metabolism, which may provide insight into regulatory aspects of hypoxia.

In the present study, we developed a wide-field fluorescence microscopy imaging method to quantify relative changes in subcellular NAD(P)H redox by monitoring NAD(P)H autofluorescence from freshly isolated BCA SMCs, which retained contractile function and responses to hypoxia seen in intact arteries. Mitochondrial inhibitors and uncouplers, exogenously added lactate or pyruvate, were studied to document and develop an understanding of relationships observed to exist in the control of redox. This approach was used to evaluate how hypoxia was influencing the interactions of NAD(P)H redox between different cellular compartments. The levels of lactate and pyruvate in BCA and released into the incubation media were measured so that the effects of hypoxia on cytosolic NAD(H) redox (lactate-to-pyruvate ratios) and mitochondrial metabolic function could be evaluated. Fluorescent detectors mitosox and dihydroethidium (DHE) were used to detect relative changes in BCA superoxide derived from mitochondrial and extramitochondrial sources that are likely to be influenced by cytosolic NAD(P)H redox, respectively (17). These superoxide detectors were employed to study the effects of exposure to hypoxia in the presence or absence of a contractile agent (30 mM KCl), which does not seem to influence ROS (23, 24), to evaluate relationships between metabolic control of NAD(P)H redox and cellular superoxide generation under the conditions in which BCAs relax to hypoxia.

**MATERIALS AND METHODS**

**Measurement of force generation and loading of fluorescence superoxide indicators.** Isolated endothelium-removed artery rings were prepared from left anterior descending limb of BCAs of bovine calfs hearts obtained from a slaughterhouse immediately after slaughter. As previously described (13, 15, 24), arterial rings of ~3–4 mm in diameter and length were mounted on wire hooks attached to Grass force displacement transducers for measurement of changes in isometric force on a Grass Instruments polygraph. Arteries were incubated in Krebs buffer for 1 h and gassed with 21% O2, 5% CO2, and 74% N2 at an optimal passive tension of 5 g. Then the vessels were depolarized with Krebs buffer containing 130 mM KCl in place of NaCl. After washing with Krebs buffer, the vessels were reequilibrated with Krebs buffer for another 1 h before the experiments were conducted. The rings were contracted with 30 mM KCl under 21% O2, 5% CO2, and 74% N2 to obtain a control contractile response in the absence of any treatments. BCAs were exposed to different treatments 15 min after wash out of the 30 mM KCl, as indicated in the RESULTS. Fluorescent detectors mitosox (5 μM) or DHE (5 μM) were loaded into BCA rings at the beginning of the treatment to detect the superoxide that accumulated over 40 min. The rings were then fixed in 4% paraformaldehyde and embedded in OCT, and a 20-μm-thickness slice of cryosection was used. Images were obtained by a confocal microscope (Bio-Rad MRC 1024 ES) with ×40 oil immersion objective (Olympus, UApo) at 568-nm excitation and 605-nm emission for mitosox and DHE-labeled BCA slices. A green nuclear indicator, sytox (5 nM), which stained the nuclei, was imaged by the same confocal microscope at 488-nm excitation and 522-nm emission. Mean fluorescence intensity of labeled and nonlabeled BCA slices, measured using Bio-Rad laser sharp software, was used for quantification. The fluorescence intensity of nonlabeled BCA slices was used as background and subtracted from the fluorescence intensity of labeled BCA slices.

**BCA SMC isolation.** SMCs were freshly isolated from BCA according to Ge et al. (9), with some modifications. In brief, an endothelium-denuded BCA segment from a slaughterhouse-derived bovine calf heart was cut into small pieces and placed in an enzymatic digestion mixture containing 2 mg/ml collagenase type II, 1 mg/ml elastase, 1 mg/ml trypsin inhibitor, 1 mM dithiothreitol, and 1% bovine serum albumin in low-calcium (10 μM) Hank’s buffer (Media Tech). The tissue-enzyme mixture was incubated for 90 min in a shaking water bath at 37°C with constant 2% O2 and 5% CO2 gassing on top of the solution. By the end of the incubation, the supernatant was diluted 10 times in low-calcium Hank’s buffer, it was poured into a glass chamber, and viable elongated cells settled down by gravity. Cells were kept at 4°C, and calcium was gradually added back to avoid the “calcium paradox” (26). After cells settled down, the solution was changed to fresh Hank’s buffer. Experiments were conducted at room temperature, and the cells were used on the same day as they were prepared. Data examining changes in cell length on exposure to 30
bicarbonate buffer for 1 h at 37°C. After fresh Krebs buffer was were equilibrated in 21% O2, 5% CO2, and 74% N2 gassed Krebs measurement of lactate and pyruvate were adopted from the assay re-

Detection of these changes. Simultaneous imaging of NAD(P)H and fluorescence, suggesting that the deconvolution methods used were cellular region-specific differences in the direction of changes in

The length of SMCs freshly isolated from BCAs was initially incubated in Krebs bicarbonate buffer for 30 min in separate experiments; TMRM (Molecular Probes), BCAs were equili-

Mitochondrial membrane potential (Ψm) was studied in endothelium-denuded BCA segments using fluorescence indicator tetramethylrhodamine methyl-

Molar ratio of TMRM red fluorescence to associated with increased TMRM red fluorescence. Mean fluorescence intensity of in-focus area with subtracted background area was used for quantification.

RESULTS show that some of the interventions employed caused sub-

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential (Ψm) was studied in endothelium-denuded BCA segments using fluorescence indicator tetramethylrhodamine methyl-

Measurement of lactate and pyruvate. Methods for the mea-

Statistical analysis. Values are shown as means ± SE. Statistical analyses were performed by Student’s t-test or by ANOVA followed by Tukey-Kramer multiple-comparison test for comparisons between two groups or multiple groups, respectively. The number of arteries from different animals or cells studied from at least three different animals is reported as n, and P < 0.05 was considered statistically significant.

RESULTS

Comparison of the influence of 30 mM KCl and hypoxia on contraction of arteries and SMCs isolated from BCAs. This study is designed to compare different types of experiments on the control of redox processes based on the feasibility of conducting experiments in SMCs that are either present in intact arteries or isolated by enzymatic digestion of BCA. Thus the functional responses of these two preparations were compared with document that the isolated cells retain contractile function and responses to hypoxia that are seen in functional arteries. The length of SMCs freshly isolated from BCAs typically ranged from 60 to 100 μm, and an ~32% shortening of SMCs was observed 10 min after 30 mM KCl treatment (See Fig. 1). Treatments examined in this study, such as lactate, pyruvate, mitochondrial probes, and hypoxia, did not cause a cell length change or an effect on force under baseline conditions (Fig. 1 or data not shown). Whereas, as shown in Fig. 1, SMCs showed less shortening to 30 mM KCl under hypoxia. As reported in previous studies (13, 23, 24), exposure of BCAs precontracted with 30 mM KCl to hypoxia caused a 39 ± 3%
relaxation \((n = 23)\) of the force generated by 30 mM KCl. Thus SMC and arterial ring preparations derived from BCAs show a similar decreased contractile response to 30 mM KCl when exposed to hypoxia.

**Imaging monitoring of subcellular NAD(P)H autofluorescence in SMCs freshly isolated from BCAs.** Representative images of live BCA SMCs are shown in Fig. 2. Under bright field, SMCs are elongated, relaxed, and loosely attached to the bottom of a glass chamber. The image representing NAD(P)H autofluorescence in Fig. 2, *top left*, is a projected wide-field fluorescence deconvolution microscopy image from \(\sim 20\) Z-stack images. There are bright areas in the perinuclear regions and throughout the cell that are similar to the mitochondrial-specific dye mitotracker labeling pattern shown in these cells in Fig. 2, *top left*. The similar distribution patterns of NAD(P)H and mitotracker are consistent with previous observations \(\text{(8, 29, 31)}\) that the majority of cell/tissue regions of high levels of NAD(P)H fluorescence are mitochondria. As mitotracker appeared to cause changes in NAD(P)H autofluorescence, the images shown in Fig. 2, *top left*, were obtained from different cells. Since studies by others have provided evidence that there appears to be a good colocalization of mitochondrial NAD(P)H and oxidized flavoprotein fluorescence \(\text{(29)}\), we examined if this occurred in BCA SMCs. As shown in Fig. 2, *top right*, the areas of high levels of NAD(P)H autofluorescence were very well colocalized with the fluorescence observed in spectral regions associated with mitochondrial flavoprotein fluorescence. This image was obtained using a Zeiss ApoTome grid projection method of removing out-of-focus light. The ApoTome imaging tended to cause time-dependent changes in NAD(P)H autofluorescence, and we were not successful with obtaining Z-stack images of flavoprotein fluorescence with intensities suitable for deconvolution. Thus we focused on examining deconvoluted images for analysis of the effects of interventions on subcellular NAD(P)H autofluorescence because it did not show time-dependent changes as a result of the imaging process (see Fig. 2, *bottom*). In the resting state, the cytosolic NAD(P)H autofluorescence is \(\sim 33 \pm 3\%\) of the mitochondria fluorescence intensity. Nuclear NAD(P)H autofluorescence is slightly higher than cytoplasm and is \(\sim 41.5 \pm 4\%\) of mitochondrial autofluorescence (Fig. 2, *bottom*).

**Effects of mitochondrial probes, lactate, and pyruvate on subcellular NAD(P)H autofluorescence.** The effects of mitochondrial electron transport chain inhibitors and an uncoupler were examined to further document the origins NAD(P)H autofluorescence signals and to characterize interactions between the subcellular NAD(P)H systems in BCA SMCs. Rotenone \(\text{(10 \(\mu\)M)}, a\ complex\ I\ inhibitor, increased NAD(P)H autofluorescence intensity by 73\% in the mitochondrial region compared with control mitochondria autofluorescence intensity. In the presence of rotenone, the autofluorescence intensity in cytosolic and nuclear regions increased by 136 and 102\%, respectively (Fig. 3). Antimycin A \(\text{(10 \(\mu\)M)}, a\ mitochondrial\ complex\ III\ inhibitor, increased autofluorescence intensity by 160\% in the mitochondrial region compared with control mitochondria autofluorescence intensity. Cytosolic and nuclear autofluorescence intensity increased by 139 and 295\%, respectively (Fig. 3). FCCP \(\text{(4 \(\mu\)M)}, a\ mitochondrial\ uncoupler, decreased mitochondrial autofluorescence intensity by 41\%, but it did not cause a detectable change in cytosolic or nuclear autofluorescence intensity (Fig. 3). These results demonstrate...
that the direction of mitochondrial autofluorescence changes in response to the electron transport inhibitors and an uncoupler is consistent with the detection of their anticipated effects on inhibiting and maximizing mitochondrial NADH consumption, respectively. While the direction of changes in NAD(P)H autofluorescence in the cytosolic and nuclear regions is similar to each other, the data with FCCP suggest that these changes can be independent of those occurring in mitochondria.

The ratio of lactate to pyruvate can influence cytosolic NADH/NAD\(^+\) redox potential as a result of a rapid equilibration through the lactate dehydrogenase reaction involving lactate + NAD -> pyruvate + NADH. Exposure of BCA SMCs to 10 mM lactate increased mitochondrial, cytosolic, and nuclear NAD(P)H autofluorescence significantly 1 min after treatment, and this increase remained elevated 10 min later (See Fig. 3). Pyruvate (10 mM) only transiently increased mitochondrial NAD(P)H levels, because mitochondrial autofluorescence was elevated 1 min after treatment, but it returned to the control level 10 min later. In contrast, cytosolic and nuclear NAD(P)H fluorescence levels were significantly lower than control in BCA SMCs exposed to pyruvate (Fig. 3).

Cytosolic NAD(P)H autofluorescence changes 10 min after treatment with lactate and pyruvate appeared to be consistent with their anticipated influence on cytosolic NAD(H) redox. In addition, the data suggest that there appears to be a rapid equilibration of cytosolic and nuclear NADH redox systems.

Effects of hypoxia and 30 mM K\(\text{Cl}\) on subcellular NAD(P)H autofluorescence. Based on the consistency of changes in autofluorescence with anticipated changes in subcellular NAD(P)H, we examined the effects of hypoxia, 30 mM K\(\text{Cl}\), and 30 mM K\(\text{Cl}\) plus hypoxia on the changes of subcellular NAD(P)H autofluorescence. Representative images and summary data of subcellular imaging shown in Fig. 4 provide evidence that hypoxia increases mitochondrial NAD(P)H autofluorescence by 42%. Cytosolic and nuclear NAD(P)H autofluorescence increased by 70 and 50%, respectively. A combination of cellular contraction and a decrease in the intensity of mitochondrial autofluorescence in the presence of 30 mM K\(\text{Cl}\) made it difficult to analyze changes in cytosolic autofluorescence. Thus only nuclear NAD(P)H autofluorescence was examined, and it did not appear to be significantly altered by hypoxia (Fig. 4). Since some cells exposed to 30 mM K\(\text{Cl}\) plus hypoxia appeared to change in shape in a manner associated with membrane blebbing, these cells were not exposed to an initial imaging, and the fluorescence from imaging untreated cells from the same blood vessel segment was used as the pretreatment controls. The data in Fig. 4 show that mitochondrial NAD(P)H autofluorescence did not change in the presence of 30 mM KCl plus hypoxia, suggesting that the presence of 30 mM KCl appears to prevent the increases in autofluorescence caused by hypoxia that were seen in mitochondria and other cellular regions. In addition, the combination of 30 mM KCl plus hypoxia appeared to prevent the decrease in mitochondrial autofluorescence caused by 30 mM KCl. Nuclear NAD(P)H autofluorescence appeared to increase in the presence of 30 mM KCl plus hypoxia in a manner that was similar to the effects of hypoxia in the absence of 30 mM KCl.

Effects of hypoxia or rotenone on BCA lactate and pyruvate levels and release in the presence or absence of 30 mM K\(\text{Cl}\). Lactate and pyruvate tissue levels and release into the extracellular media from BCA were measured under normoxic or hypoxic conditions in the presence or absence of 30 mM K\(\text{Cl}\) to detect evidence for augmentation of anaerobic glycolysis (lactate release) and elevated cytosolic NADH redox, with tissue lactate-to-pyruvate ratios being considered as a more precise indicator of the cytosolic NADH-to-NAD ratio (18). Figure 5 contains the measured amounts of lactate and pyruvate present in BCA, and the quantities of these metabolites that were released from these arteries during a 20-min incubation under different conditions were examined. Hypoxia and 30 mM K\(\text{Cl}\) in the absence and presence of hypoxia significantly increased tissue and media lactate levels, with 30 mM K\(\text{Cl}\) plus hypoxia having the greatest effect among these conditions (Fig. 5). The media and tissue levels of pyruvate decreased significantly during hypoxia when examined in either the absence or presence of 30 mM K\(\text{Cl}\). In contrast to the observed changes in lactate, 30 mM K\(\text{Cl}\) did not appear to influence the release or tissue levels of pyruvate, either in the absence or presence of hypoxia (Fig. 5). Figure 5 also shows the ratio of tissue and media lactate to pyruvate under the conditions examined. Hypoxia significantly increased lactate-to-pyruvate ratios, both...
in BCA and in the media released from BCA under all conditions examined. The presence of 30 mM KCl increased tissue lactate-to-pyruvate ratios in the absence and presence of hypoxia. Whereas 30 mM KCl appeared to increase the ratio of lactate/pyruvate released from BCA, these changes were not statistically significant. Thus hypoxia appears to be increasing cytosolic NADH and anaerobic glycolysis.

Since rotenone had similar effects as hypoxia on the changes in subcellular NAD(P)H autofluorescence in SMC isolated from BCA, we examined the effect of rotenone on incubating media and tissue levels of lactate and pyruvate in BCA. Rotenone increased incubating media and tissue levels of lactate significantly (Fig. 6). Rotenone also decreased incubating media levels of pyruvate significantly, but not tissue levels.

Fig. 4. Representative images (A) and summary data (B) showing the subcellular NAD(P)H response to hypoxia (n = 7). C: summary data showing the subcellular NAD(P)H response to 30K (n = 8). D: summary data showing subcellular NAD(P)H response to 30K + hypoxia. (n = 21–24). Values are means ± SE.
of pyruvate (Fig. 6). Thus the effect of rotenone on tissue levels of pyruvate is different from that of hypoxia. The ratios of lactate to pyruvate from incubating media and extracts of BCA (tissue) are both significantly increased by rotenone (Fig. 6).

Effect of hypoxia on BCA smooth muscle mitochondrial and cytosolic-nuclear superoxide generation in the presence or absence of force induced by 30 mM KCl. Loading of the DHE and mitosox detectors of superoxide did not have an effect on BCA force generation induced by 30 mM KCl (data not shown). Representative confocal images from 20-μm-thick cryosections and a statistical analysis of the effects of treatments data are shown in Fig. 7 for DHE and mitosox-treated BCA. The data in Fig. 7 show that hypoxia, but not 30 mM KCl alone, significantly increased mitochondrial superoxide generation detected by mitosox labeling (red). Nuclei are stained with sytox (green), and a colocalization of these two fluorescent probes is shown in Fig. 7 to highlight the perinuclear staining of mitochondria by mitosox. In the presence of force generation elicited by 30 mM KCl, hypoxia significantly decreased mitochondrial superoxide generation relative to normoxia. Since the fluorescence of oxidation products of DHE are markedly enhanced by binding DNA, the fluorescence of cells treated with this probe appear to be concentrated in nuclei of the SMCs present in BCA. The changes in fluorescence detected by the oxidation of DHE were markedly different from those seen with mitosox, in that 30 mM KCl, hypoxia, and 30 mM KCl plus hypoxia all decreased fluorescence intensity significantly, with 30 mM KCl plus hypoxia having the greatest effect compared with the normoxia control. Thus,

Fig. 6. Release into the incubating media (n = 9; A) and tissue levels (n = 5; B) of lactate (left), pyruvate (middle), and lactate-to-pyruvate ratios (right) in the absence or presence of rotenone. Values are means ± SE.

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Fig. 7. Representative images (top) and summary data (means ± SE; bottom) showing mitosox-detected mitochondrial superoxide and dihydroethidium (DHE) detection of extramitochondrial (cytosolic) superoxide in response to hypoxia (H) in the presence or absence of 30K contraction on BCA (n = 4). N, normoxia.
whereas hypoxia appears to increase mitochondrial superoxide, this effect of hypoxia is reversed to a decrease in superoxide when BCAs are contracted with 30 mM KCl. In contrast, the presumably cytosolic-nuclear sources of superoxide generation detected by DHE appear to decrease under conditions of hypoxia and/or 30 mM KCl.

Effect of rotenone on BCA smooth muscle mitochondrial and cytosolic-nuclear superoxide generation. The effect of rotenone on superoxide generation in BCA detected by the fluorescence dyes mitosox and DHE was examined to probe for the effects of inhibition of electron transport on sites of smooth muscle ROS generation and to enable comparisons with the effects of hypoxia. Rotenone significantly increased mitochondrial superoxide generation detected by mitosox to 161% of the fluorescence detected on untreated control BCA (see Fig. 8A), whereas the data in Fig. 8A show that rotenone did not have any effect on the sources of superoxide generation detected by DHE fluorescence. Rotenone also increased total cellular superoxide generation as detected by lucigenin-enhanced chemiluminescence to 193% of the level observed in untreated control BCA (see Fig. 8B). Treatment of BCA with 10 μM antimycin increased lucigenin chemiluminescence by 184%. Thus the potential for superoxide production by complex III + complex I appears to be similar to the amounts produced by complex I. Inhibiting electron transport with rotenone did not appear to have an effect on the nonmitochondrial sources of superoxide detected by DHE. Since hypoxia and rotenone have similar effects on changes in mitochondrial NAD(P)H redox and superoxide, we examined whether rotenone could inhibit the contraction of BCA to 30 mM KCl in a manner similar to hypoxia. Rotenone (10 μM) treatment for 30 min did not have an effect on the subsequent force generation induced by 30 mM KCl in BCA (Fig. 8C).

Effect of rotenone and hypoxia on Fm in BCA. The Fm was detected using a low concentration of the fluorescence probe TMRM (100 nM), to avoid the quenching effects seen at higher concentrations of this probe (32). Representative images and summary data shown in Fig. 9 provide evidence that rotenone (10 μM) and hypoxia treatments were both associated with an increase in TMRM fluorescence or a hyperpolarization of mitochondria membrane potential in BCA (Fig. 9). In contrast, exposure to the mitochondrial uncoupler 4 μM FCCP caused a marked decrease in TMRM fluorescence, consistent with its expected depolarizing effect on Fm. Thus increased BCA mitochondrial superoxide generation in response to rotenone and hypoxia occurs under conditions in which mitochondrial hyperpolarization is observed.

DISCUSSION

Data in the present study provide evidence that hypoxia can increase BCA mitochondrial superoxide in a manner that appears to result from increased mitochondrial NAD(P)H. However, when BCA are contracted with 30 mM KCl and undergo relaxation to hypoxia, superoxide appears to be decreased in mitochondrial and extramitochondrial cellular regions. Hypoxia promoted a decrease in pyruvate, which may be a key factor in metabolic control mechanisms regulating cytosolic NADPH redox and its role in expression of the relaxation to hypoxia. These observations may provide insight into physiological mechanisms that control NAD(P)H redox and ROS changes potentially involved in vascular regulation by hypoxia through relationships that are shown in the model in Fig. 10.

Hypoxia and the control of NAD(P)H redox systems. The data in the present study demonstrate that subcellular NAD(P)H redox changes can be studied in BCA SMCs through monitoring and analyzing NAD(P)H autofluorescence using wide-field fluorescence microscopy. Different cellular compartments, including mitochondria, cytosolic, and nuclear regions of SMCs, have different levels of NAD(P)H redox that can be monitored under biologically relevant conditions. The different interventions examined provide evidence for the detection of both confirmatory and novel aspects of interactions controlling subcellular NAD(P)H redox between different cellular compartments. Interestingly, hypoxia increased NAD(P)H redox in mitochondrial, cytosolic, and nuclear regions to a similar extent. Since it appears hypoxia increased NAD(P)H levels to a degree that was less than antimycin in all compartments examined, hypoxia may have only been partially inhibiting mitochondrial respiration under the conditions used for monitoring autofluorescence that are associated with decreased contraction to 30 mM KCl. NADH redox is thought to be the predominant source of the autofluorescence in the spectral regions examined that is seen in mitochondria, whereas NADPH is thought to be the major contributor to cytosolic fluorescence due to the presence of very low levels of cytosolic NADH in most tissues that have

![Fig. 8. Effect of rotenone on BCA superoxide generation detected by mitosox and DHE (n = 4; A), lucigenin-enhanced chemiluminescence (n = 8; B), and force generated in the presence of 30K (n = 12; C). Values are means ± SE.](http://ajpheart.physiology.org/)}
been studied (8, 29). Observations in the present study with mitochondrial inhibitors and with lactate and pyruvate generally support interpretations made in other systems on the contribution of NADH to the high levels of mitochondrial fluorescence that was detected. However, the observed lowering of cytosolic NAD(P)H fluorescence by pyruvate under aerobic conditions associated with pyruvate increasing cytosolic NADPH in BCA (13) suggests that NADH was a significant contributor to the cytosolic fluorescence that is seen under basal conditions. This interpretation is consistent with previous evidence that pyruvate oxidizes cytosolic NADH in vascular smooth muscle (1). Vascular smooth muscle is thought to have the malate-aspartate and glycerol-phosphate shuttles to metabolically transfer cytosolic-generated NADH into mitochondria and lactate dehydrogenase to consume cytosolic NADH (2). Limitations in the efficiency of mitochondrial shuttling of cytosolic NADH (2) and compartmentalization of glycolysis (28) have been proposed to explain why vascular smooth muscle releases high levels of lactate. These systems appear to be functioning under basal conditions in BCA in a manner that maintains relatively high levels of cytosolic NADH that are associated with lactate release under aerobic conditions, and this cytosolic redox control system could be an important factor in the response to hypoxia.

Actions of the interventions used in NAD(P)H autofluorescence imaging experiments reported in the present study provide evidence for the detection of potential NAD(P)H redox interactions between mitochondrial, cytosolic, and nuclear regions, which are potentially important for understanding responses to hypoxia. Mitochondrial complex I and III inhibitors, rotenone and antimycin, respectively, increased mitochondrial NAD(P)H and superoxide production from complex I of the electron transport chain, whereas contracting BCA with 30K prevents hypoxia from increasing mitochondrial NAD(P)H and a decrease in mitochondrial superoxide is observed. Superoxide production in the cytosolic-nuclear region is lowered by hypoxia potentially as a result of decreased activity of cytosolic NAD(P)H-dependent Nox oxidases. Hypoxia increases BCA lactate and decreases pyruvate, indicating it increases the ratio of lactate/pyruvate, which is an indicator of increased cytosolic NADH/NAD redox. Our previous work (Ref. 13) documents evidence that hypoxia promotes BCA relaxation by increasing the ratio of NADP/NADPH associated with a lowering glucose-6-phosphate (G-6-P). This hypoxia-elicited impaired maintenance of G-6-P and cytosolic NADPH by the pentose phosphate pathway (PPP) is prevented by added pyruvate. Since hypoxia was observed to lower pyruvate, it may regulate mitochondrial-dependent metabolic pathways (described in the DISCUSSION), which control cytosolic NADPH redox through processes that include a feedback inhibition of glycolysis in a manner that maintains G-6-P levels and NADPH generation by the PPP. Note that the up and down arrows highlight documented and hypothesized effects of hypoxia observed in BCA.
combination of effects, including an increase in mitochondrial NAD(P)H impairing the shuttling of cytosolic NADH into mitochondria, and from inhibition of mitochondrial electron transport promoting anaerobic energy metabolism associated with increased glycolysis-derived cytosolic NADH. The mitochondrial uncoupler FCCP decreased mitochondrial, but not cytosolic and nuclear, NAD(P)H autofluorescence, suggesting that it diminished the influence of mitochondrial metabolism on the control of cytosolic NAD(P)H redox under the conditions examined. For example, the uncoupling of mitochondria could be hypothesized to stimulate glycolysis to produce more cytosolic NADH and also simultaneously enhance the efficiency of cytosolic NADH removal by mitochondrial shuttles under the conditions examined. Since nuclei are thought to be relatively permeable to NAD(P)H (41), it appears that the data in the present study are providing evidence for equilibration of NAD(P)H redox systems potentially occurring between the cytosolic and nuclear regions of BCAs. Hypoxia may increase and 30 mM KCl may decrease mitochondrial NADH by altering rates of its biosynthesis and consumption by the electron transport chain in a manner that resembles effects of mitochondrial probes. These changes in mitochondrial function could influence cytosolic redox and signaling mechanisms through metabolic interactions, which remain to be defined.

**Hypoxia and the modulation of intracellular ROS.** Data in the present study provide evidence that may help explain factors that contribute to the debate on why hypoxia may either increase or decrease superoxide generation in vascular smooth muscle. In this study, we appear to have observed differences in the direction of cytosolic-nuclear and mitochondrial superoxide generation in response to hypoxia (examined in the presence or absence of 30 mM KCl), based on changes in fluorescence from DHE and mitosox, respectively. Hypoxia decreased DHE fluorescence in a manner that was slightly increased in the presence of 30 mM KCl, suggesting that hypoxia was decreasing superoxide generation in the cytosolic-nuclear cellular regions. While hypoxia increased mitosox fluorescence consistent with it increasing superoxide, hypoxia appeared to cause a decrease in mitochondrial superoxide in BCA contracted with 30 mM KCl. The decreases in superoxide in cytosolic-nuclear and mitochondrial regions by hypoxia observed in the presence of 30 mM KCl are consistent with previous observations simultaneously measuring force generation and superoxide production. Hypoxia decreased superoxide production detected by lucigenin-enhanced chemiluminescence under conditions in which it relaxed BCA contracted with 30 mM KCl (24). Thus decreases in mitochondrial NADH associated with force generation may be a key factor in preventing the detection of hypoxia-elicited increases in vascular smooth muscle mitochondrial superoxide generation.

The increases in mitochondrial superoxide elicited by hypoxia and by rotenone (under aerobic conditions) were associated with increased mitochondrial autofluorescence, which is likely to be derived primarily from elevated levels of mitochondrial NADH, an electron donor used for the generation of superoxide by the mitochondrial electron transport chain. It has been known since the early work of Chance and colleagues (6) that mitochondria exposed to conditions of low-ADP and high-ATP concentrations maintain high levels of mitochondrial NADH and electron density in the proximal part of the electron transport chain associated with increased superoxide or peroxide formation from this mitochondrial site. Studies on multiple tissue preparations suggest that high ATP and low ADP levels are thought to be present in most tissues under basal physiological conditions. Imaging data in the present study suggest that hypoxia and rotenone increase mitochondrial NADH, and this could potentially be a factor in increasing electron density in sites such as complex I that autoxidize generating superoxide. The similar increases in superoxide elicited by antimycin and rotenone suggest that complex III may not be a prominent source of superoxide compared with complex I when electron transport is inhibited. Contractions with 30 mM KCl appeared to decrease mitochondrial NAD(P)H fluorescence and the detection of mitochondrial superoxide, suggesting that it may be lowering superoxide production by decreasing electron density in the proximal part of the electron transport chain. Our laboratory’s previous work provided evidence that ATP levels were not decreased by hypoxia in arteries exposed to 30 mM KCl (13). Thus a metabolic stress promoted by the increase in calcium and cellular work (e.g., increased ADP generation) activated by this contractile agent may have increased mitochondrial NADH consumption by the electron transport chain to a greater extent than stimulation of mitochondrial NADH production. Our observations of hypoxia and rotenone increasing superoxide in BCA under basal conditions are similar to a previous study in rat renal artery SMCs (21), which provided evidence that hypoxia and rotenone also increased mitochondrial ROS generation. However, superoxide production in renal arteries was associated with a more depolarized ƒ, whereas our study found hypoxia and rotenone increasing superoxide generation under conditions in which mitochondrial hyperpolarization was observed. While there is evidence supporting a partial depolarization mitochondria as being a stimulus for increased superoxide production, an interaction between increased mitochondrial NAD(P)H and hyperpolarization has also been documented to promote mitochondrial ROS generation (34). Overall, the data in the present study suggest that increased mitochondrial NADH may have a more dominant effect on the generation of mitochondrial superoxide than the limited availability of oxygen for superoxide generation under the hypoxic conditions examined.

Our laboratory’s previous work (24) suggests that hypoxia can function to lower superoxide derived from Nox oxidases under conditions examined in the present study to be associated with the detection of increased BCA cytosolic-nuclear NAD(P)H autofluorescence and lactate-to-pyruvate ratios and decreased DHE fluorescence, suggesting that hypoxia was increasing the overall level of cytosolic NAD(P)H under conditions in which it decreased superoxide production. The primary electron donors for major superoxide generating systems associated with the cytosolic region of vascular smooth muscle of BCA are thought to be NADH and NADPH functioning as substrates for the Nox oxidases that are present in this vascular segment, with NADH and NADPH showing similar concentration-dependent effects on the generation of superoxide (12, 40). While hypoxia has been shown to lower cytosolic NADPH in BCA (13), measurements of lactate-to-pyruvate ratios indicate that hypoxia is increasing cytosolic NADH, suggesting that the increase in NADH is the major factor in the increase in cytosolic-nuclear autofluorescence that is observed. Since DHE fluorescence did not increase under conditions in which cytosolic-nuclear fluorescence increased by 102–136% in the presence of rotenone.
it is likely that the overall increase in NAD(P)H observed does not have a prominent effect on superoxide generation in this cellular region. Previous measurements of the dose-dependent effects of NAD(P)H on superoxide production in homogenates of BCA suggest that a doubling of NAD(P)H concentrations would have only a minimal effect on the rate of superoxide production that is observed (12). Since hypoxia increased cytosolic-nuclear autofluorescence to a lesser extent than rotenone, and it caused a decrease in DHE fluorescence, it appears that oxygen availability as a substrate for superoxide generation by Nox oxidases is likely to be more of an important factor in controlling the production of ROS in the cytosolic-nuclear region than the changes in NAD(P)H redox that are observed under the hypoxic conditions examined. Thus it is possible that the extreme diversity of published observations on hypoxia increasing or decreasing ROS in vascular smooth preparations may originate from small differences in experimental conditions markedly affecting the control of mitochondrial NAD(P)H redox, which appears to have a major influence on the control of superoxide generation under hypoxic conditions.

Pyruvate-dependent metabolic control of cytosolic NADPH redox may regulate the relaxation to hypoxia. While ROS do not appear to be primary mediators of the relaxation of BCA to hypoxia, some of the observations made in this study may provide insight into the potential origins of a pyruvate-inhibited metabolic stress produced by hypoxia that we hypothesized to be part of an oxygen-sensing mechanism involving cytosolic NADPH oxidation that appears to promote BCA relaxation under hypoxic conditions (13). The data from measurements of lactate and pyruvate in the present study provide evidence that hypoxia causes a shift to anaerobic glycolysis (increased lactate release) and increases cytosolic NADH/NAD, in a manner that seems to result in decreased pyruvate availability, under conditions in which the relaxation to hypoxia is observed. While rotenone appears to mimic many aspects of the effects of hypoxia that were examined in this study on NAD(P)H redox and superoxide generation, it does not elicit the decreased tissue levels of pyruvate or force caused by hypoxia. Thus a lowering of pyruvate by hypoxia could be a key factor in promoting the decrease in force that is observed. Under conditions in which relaxation is observed, hypoxia appears to be causing only a partial impairment of mitochondrial respiration in a manner that promotes a lowering of pyruvate without decreasing ATP levels in BCA. Pyruvate availability is potentially a key factor in a mitochondrial controlled feedback mechanism of stabilizing cytosolic NADPH by releasing citrate in amounts that elicit a feedback inhibition of phosphofructokinase and increased glucose-6-phosphate levels (20). Citrate may also promote cytosolic NADPH generation by providing substrates for isocitrate dehydrogenase and malic enzyme. Thus we hypothesize that the effects of hypoxia on pyruvate-dependent regulation of cytosolic NADPH redox by mitochondria are potentially an additional component of a previously described (13) oxygen-sensing mechanism promoting cytosolic NADPH oxidation and the observed relaxation of BCA to hypoxia. Evidence for pyruvate attenuating metabolic increases in blood flow is consistent with the potential physiological importance of this mechanism, because data in the present study show that pyruvate decreased cytosolic NAD(P)H fluorescence under conditions in which it appears (13) to increase cytosolic NADPH. Since NAD(P)H redox appears to control mechanisms that regulate gene expression (41), the hypoxia-elicited changes redox examined in the present study may also have important roles in the adaptation of smooth muscle to hypoxic conditions associated with the expression of vascular remodeling and pathophysiology.

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