Effects of NADH and NADPH on superoxide levels and cerebral vascular tone

SEAN P. DIDION AND FRANK M. FARACI
Departments of Internal Medicine and Pharmacology and Cardiovascular Center, University of Iowa College of Medicine, Iowa City, Iowa 52242

Received 3 July 2001; accepted in final form 4 October 2001

Didion, Sean P., and Frank M. Faraci. Effects of NADH and NADPH on superoxide levels and cerebral vascular tone. Am J Physiol Heart Circ Physiol 282: H688–H695, 2002. First published October 11, 2001; 10.1152/ajpheart.00576.2001.—Reactive oxygen species are important modulators of cerebral vascular tone. Recent evidence, mainly from the aorta, suggests that NAD(P)H oxidase is a major source of vascular superoxide. The goal of the present study was to examine the effects of NADH and NADPH that are commonly used to stimulate NAD(P)H oxidase activity, on superoxide levels and cerebral vascular tone. Basilar arteries and cerebral arterioles from normal rabbits were studied in vitro using isolated tissue baths and in vivo using a cranial window, respectively. In the basilar artery, NADH produced a biphasic response; low concentrations (0.1–10 μM NADH) produced marked relaxation, whereas higher concentrations (30–100 μM NADH) produced contraction. Responses to NADH were significantly (P < 0.05) inhibited in the presence of 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron; a scavenger of superoxide, 10 mM). In contrast, NADPH (10–100 μM) produced moderate contraction of the basilar artery, which was inhibited in the presence of Tiron. In vivo, NADH produced Tiron-sensitive dilatation of cerebral arterioles. NADH and NADPH dose dependently increased superoxide levels in the basilar artery, as detected by lucigenin (5 mM)-enhanced chemiluminescence, but increases in superoxide were significantly greater for NADPH than NADH. These increases in superoxide were markedly reduced in the presence of polyethylene glycol-superoxide dismutase (300 U/ml) or diphenylene iodonium [0.1 mM, an inhibitor of flavin-containing enzymes, including NAD(P)H oxidase] but were not affected by indo- methacin, N(G)-nitro-l-arginine, or allopurinol. These data suggest that NADH- and NADPH-induced changes in cerebral vascular tone are mediated by superoxide, produced by a flavin-containing enzyme, most likely NAD(P)H oxidase, but not xanthine oxidase or nitric oxide synthase.

basilar artery; cerebral arterioles; rabbit; reactive oxygen species

Reactive oxygen species appear to be key mediators of cellular signaling; however, mechanisms responsible for generation of superoxide in the vasculature are only beginning to be understood (20, 41, 50, 54). Although some insight into these mechanisms has been made in extracranial vessels (primarily the aorta), essentially nothing is known in this regard in the cerebral circulation. Potential enzymatic sources of superoxide in blood vessels include cyclooxygenase, nitric oxide synthases, lipoxygenase, and xanthine oxidase (4, 15, 41). More recently, a membrane-bound NAD(P)H oxidase, distinct from that of the neutrophil type, has been proposed as an important source of vascular superoxide (4, 16, 17). However, characterization of this vascular oxidase and its role in vascular function is only beginning to be defined. Currently, data support the contention that the oxidase is a multisubunit complex, consisting of p22(phox), p47(phox), p67(phox), gp91(phox), or Nox (a gp91(phox) homolog) and Rac (a low-molecular-weight G protein) (1, 4, 16, 17, 29, 50). It also appears that vascular NAD(P)H oxidase utilizes both NADH and NADPH as electron donors in the generation of superoxide, and, as such, NADH and NADPH have been commonly employed to increase NAD(P)H oxidase activity in intact vascular segments, intact vascular cells, and vascular cell homogenates (7, 36, 46, 47).

Previous studies by us and others (6, 23, 24, 30, 40, 43, 44, 51) have shown that reactive oxygen species alter cerebral vascular tone. However, it is not known whether NAD(P)H oxidase-generated superoxide has important effects on tone in cerebral vessels. As an initial effort to pursue this question, the goal of the present study was to determine whether NADH and/or NADPH affect cerebral vascular tone via NAD(P)H oxidase-mediated increases in superoxide.

METHODS

Experimental animals and in vitro preparations. New Zealand White rabbits of either sex (n = 44, 2.3–2.6 kg body wt) were euthanized with pentobarbital sodium (200 mg/kg) via the marginal ear and exsanguinated. The entire brain was removed and placed in cold Krebs buffer (pH 7.4) of the following ionic composition (in mmol/l): 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose. The basilar artery was dissected, and loose connective tissue was removed from the adventitial surface. The basilar artery was cut into a series of four to six rings and used for either vascular tone studies or measurement of superoxide levels (described in Measurement of superoxide).

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For studies of vascular tone, basilar artery rings were mounted on pairs of triangular hooks and suspended in individual organ chambers containing 20 ml of Krebs solution maintained at 37°C and bubbled continuously with 95% O2-5% CO2. Rings were connected to force transducers that measured isometric tension (contraction and relaxation). Resting tension was increased stepwise to reach a final resting tension of 0.5 g. This amount of tension was found to be optimal in preliminary experiments. We have used these methods previously (9, 10).

**Experimental protocol for in vitro studies.** Basilar artery rings were allowed to equilibrate for 45 min (during which time Krebs buffer was replaced with fresh buffer every 15 min) before addition of agonists. Rings were then contracted submaximally (70–80% of maximum) with histamine (0.1–1 μM) or KCl (30–40 mM). After a stable contraction plateau was reached, concentration-response curves were generated to NADH (0.1–100 μM) or NADPH (0.1–100 μM) in the presence or absence of 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron; 10 mM), a scavenger of superoxide. Because superoxide can readily be converted to hydrogen peroxide and because hydrogen peroxide can cause relaxation of some blood vessels (30, 52, 55), in separate experiments we also examined responses to NADH in the presence of catalase (150 U/ml). Also, because potassium channels have been shown to mediate relaxation in response to reactive oxygen species, we examined the response of basilar arteries to NADH in the presence of tetraethylammonium (TEA; 3 mM), an inhibitor of calcium-activated potassium channels (14, 38). At the end of each experiment, we obtained concentration-response curves to histamine to determine the maximal contractile responsiveness of each vessel.

**In vivo cranial window preparation.** In vivo experiments were performed on New Zealand White rabbits of either sex (n = 36, 2.5–3.2 kg body wt), which were anesthetized with pentobarbital sodium (40 mg/kg iv). Pentobarbital was supplemented regularly at 10 mg kg⁻¹ h⁻¹. The trachea was cannulated, and the animals were ventilated mechanically with air and supplemental oxygen. Arterial blood gases were cannulated, and the animals were ventilated mechanically either NADH (1 μM) or NADPH (10 μM). In separate experiments, after the 60-min recovery period, application of NADH was repeated after preincubation (30 min) and in the presence of either allopurinol (0.1 mM), indomethacin (10 μM), TEA (3 mM), or Tiron (10 mM).

**Measurement of superoxide.** Superoxide levels were measured by lucigenin-enhanced chemiluminescence as described previously (8, 33, 35, 37). This assay has been used extensively for measurement of superoxide levels in blood vessels (2, 8, 11, 18, 21, 33, 35, 37). More importantly, results obtained using this modification of the lucigenin assay (described below) are similar to those obtained using ferricytochrome c reduction, coelenterazine, or electron-spin resonance measurements of superoxide in intact vessels and in vascular homogenates (11, 18, 45).

Briefly, ring segments of basilar artery (obtained as described in Experimental animals and in vitro preparations) were placed in polypropylene tubes containing 5 μM lucigenin. Tubes were then read in a Femtomaster FB12 (Zytox) luminoimeter, which reported relative light units (RLU) emitted integrated over 30-s intervals for 5 min. We found that counts did not significantly increase with longer periods of measurements. Basal (control) levels of superoxide are reported as the value of tissue plus lucigenin-containing buffer minus background. Surface area of the vessel lumen was imaged with a videocamera and calculated using NIH Image software (version 1.61) to normalize superoxide levels.

Superoxide production was stimulated with cumulative additions of NADH (1–100 μM) or NADPH (1–100 μM). Superoxide levels were also determined in the presence (30-min preincubation) or absence (30-min preincubation with vehicle) of either diphenylehydantoin [DPI; a selective inhibitor of flavin-containing enzymes including NAD(P)H oxidase, 0.1 mM] (39), polyethylene glycol (PEG)-superoxide dismutase (SOD) (300 U/ml), indomethacin (10 μM), Nω-nitro-L-arginine (l-NNA; 10 μM), or allopurinol (10 μM). In some experiments, Tiron (a nonenzymatic cell-permeable superoxide scavenger, 10 μM) was added at the end of the protocol to quench the superoxide signal.

**Drugs.** Allopurinol, DPI, indomethacin, l-NNA, lucigenin, NADH, NADPH, TEA, Tiron, PEG-SOD, and papaverine were obtained from Sigma (St. Louis, MO) and were all dissolved in saline with the exceptions of DPI, which was dissolved in DMSO (final concentration < 0.01%), and indomethacin, which was dissolved in Na2CO3 (0.1 M). Histamine hydrochloride was obtained from RBI (Natick, MA) and dissolved in saline. All other reagents were of standard laboratory grade.

**Statistical analysis.** All values are reported as means ± SE. Responses are expressed as the percent relaxation from maximum. Single comparisons were made using Student's paired or unpaired t-test. Multiple comparisons were made using ANOVA followed by Fisher's exact test. A probability value of <0.05 was considered significant.

**RESULTS**

**Effect of NADH and NADPH on basilar artery tone.** NADH altered the tone of basilar arteries precontracted with histamine (Figs. 1 and 2). However, this response was biphasic; low concentrations of NADH (0.1–10 μM) produced marked relaxation, whereas higher concentrations (>10 μM) of NADH produced contraction of the basilar artery. For example, 10 μM

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\[ \text{H689EFFECTS OF NADH AND NADPH ON CEREBRAL VESSELS} \]
NADH produced 65 ± 6% relaxation, whereas 0.1 mM NADH produced only 50 ± 8% relaxation of the basilar artery.

NADPH (10–100 μM) produced modest contraction of basilar arteries precontracted with histamine, whereas lower concentrations (≤3 μM) had no effect on vascular tone (Figs. 1 and 2). For example, 100 μM NADPH produced 16 ± 10% contraction of the basilar artery. Addition of vehicle had no significant effect (P > 0.05) on basilar arteries precontracted with histamine.

Role of superoxide and membrane potential on NADH- and NADPH-induced changes on basilar artery tone. NADH-induced relaxation of basilar artery was inhibited markedly by Tiron (Fig. 3), suggesting that superoxide is the mediator of NADH-induced vasorelaxation. In contrast, incubation of basilar arteries with catalase (150 U/ml) had no effect (P > 0.05) on NADH-induced relaxation (n = 4; data not shown), suggesting that relaxation in response to NADH is not mediated by hydrogen peroxide. We (43) have shown previously that 100 U/ml catalase is effective in inhibiting relaxation of cerebral vessels to hydrogen peroxide. In addition, NADPH-induced contraction was abolished in the presence of Tiron (data not shown), suggesting that contraction of the basilar artery in response to NADPH is also mediated by superoxide.

KCl (30–40 mM), which depolarizes vessels, was used to contract rings to a similar degree as that produced by histamine. In arteries precontracted with KCl, NADH failed to produce relaxation compared with arteries precontracted with histamine (control) (Fig. 4). These results suggest that relaxation of the basilar artery in response to NADH is dependent on changes in membrane potential (i.e., hyperpolarization). Furthermore, in the presence of TEA (3 mM), an inhibitor of calcium-activated potassium channels, relaxation of the basilar artery in response to NADH was markedly reduced (P < 0.05; Table 1), suggesting that calcium-activated potassium channels mediate a major portion of the response of the basilar artery to NADH.

Fig. 1. Original recordings demonstrating the effect of NADH and NADPH on basilar artery tone. Arteries were precontracted with histamine before generation of dose-response curves to cumulative additions of NADH (top) or NADPH (bottom).

Fig. 2. Effect of NADH (n = 20) and NADPH (n = 11) on tone of basilar arteries precontracted with histamine. The response to NADH and NADPH is expressed as percent relaxation compared with histamine-induced precontraction. Values are means ± SE. *P < 0.05 vs. baseline.

Fig. 3. Effect of Tiron (10 mM, n = 10) on NADH-induced relaxation of the basilar artery. Values are means ± SE. *P < 0.05 vs. control.

Fig. 4. NADH-induced relaxation of basilar arteries precontracted with histamine (control, n = 4) or KCl (30–40 mM, n = 4) before generation of dose-response curves to cumulative addition of NADH. Values are means ± SE. *P < 0.05 vs. control.
Effect of NADH and NADPH on cerebral arterioles in vivo. Baseline diameter of cerebral arterioles averaged 94 ± 4 μm. NADH and NADPH produced concentration-dependent dilatation of cerebral arterioles, but the response to NADH was greater than that achieved with NADPH (Fig. 5). Dilatation of cerebral arterioles was not different (P > 0.05) in time-control experiments with repeated application of either NADH or NADPH (data not shown). Dilatation of cerebral arterioles in response to NADH was significantly (P < 0.05) inhibited in the presence of Tiron (Fig. 6), suggesting that dilatation of cerebral arterioles to NADH is mediated, in large part, by superoxide. Dilatation of cerebral arterioles in response to NADH was also significantly (P < 0.05) reduced in the presence of TEA (3 mM; Table 1), suggesting that calcium-activated potassium channels mediate the responses of cerebral arterioles to NADH. In contrast, allopurinol (0.1 mM) and indomethacin (10 μM) had no effect on dilator responses to NADH (data not shown), suggesting that neither the activity of xanthine oxidase nor cyclooxygenase are important sources of superoxide in cerebral arterioles in response to NADH. Because responses to NADPH were much smaller, we did not examine effects of Tiron on NADPH-induced vasodilatation.

Effect of NADH and NADPH on superoxide levels. Superoxide levels were relatively low in the basilar artery under normal conditions (1.8 ± 0.2 RLU·min⁻¹·mm⁻²) (Fig. 7). NADH (1–100 μM) produced concentration-dependent increases in superoxide in the basilar artery (P < 0.05; Fig. 7). Addition of NADPH (1–100 μM) to the basilar artery also increased superoxide over control levels (Fig. 7). However, superoxide levels in response to NADPH were much greater than that elicited with NADH. In control experiments, addition of either vehicle, NADH, or NADPH to lucigenin-containing buffer did not result in increases in the

Table 1. Effect of 3 mM TEA on response of the basilar artery and cerebral arterioles to NADH

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<th>NADH, μM</th>
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<tr>
<td></td>
<td>0.1</td>
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<tr>
<td>Basilar Artery</td>
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<td>Relaxation, %</td>
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<tr>
<td>Control (n = 12)</td>
<td>10 ± 5</td>
<td>38 ± 10</td>
<td>76 ± 9</td>
<td>55 ± 13</td>
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<td>TEA (n = 3)</td>
<td>5 ± 5*</td>
<td>10 ± 10*</td>
<td>9 ± 16*</td>
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<td>Cerebral Arterioles</td>
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<td>Diameter, %Change</td>
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<tr>
<td>Control (n = 12)</td>
<td>6 ± 1</td>
<td>20 ± 2</td>
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<td>TEA (n = 6)</td>
<td>2 ± 2*</td>
<td>12 ± 2*</td>
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Values are means ± SE; n = no. of vessels. TEA, triethylammonium. *P < 0.05 vs. control.

Fig. 5. Response of cerebral arterioles to NADH (n = 12) and NADPH (n = 7). Values are means ± SE. *P < 0.05 vs. NADH.

Fig. 6. Response of cerebral arterioles to repeated application of NADH (1 and 10 μM) under control conditions and in the presence Tiron (10 mM, n = 8). Values are means ± SE. *P < 0.05 vs. control.

Fig. 7. Effect of NADH (A; n = 14) and NADPH (B; n = 13) on basilar artery superoxide levels as measured using lucigenin-enhanced chemiluminescence. RLU, relative light units. Values are means ± SE. *P < 0.05 vs. baseline.

AJP-Heart Circ Physiol • VOL 282 • FEBRUARY 2002 • www.ajpheart.org
lucigenin signal over background (P > 0.05; data not shown).

NADH- and NADPH-induced increases in superoxide levels were markedly (>90%) inhibited (P < 0.05) in the presence of PEG-SOD (300 U/ml; data not shown). Incubation of vessels with either L-NNA (10 μM), indomethacin (10 μM), or allopurinol (10 μM) had no significant (P > 0.05) effect on NADH-induced increases in superoxide levels (Fig. 8). However, NADH-induced increases in superoxide were significantly inhibited in the presence of DPI (Fig. 8), suggesting that a flavin-containing enzyme, most likely NAD(P)H oxidase but not xanthine oxidase or nitric oxide synthase, is a significant source of superoxide in cerebral arteries in response to NADH.

**DISCUSSION**

There are several major findings of the present study. First, application of NADH or NADPH to cerebral vessels (both in vitro and in vivo) produces changes in vascular tone. Second, both contraction and relaxation of cerebral vessels in response to NADH and NADPH appear to be mediated through increases in superoxide production. Third, although it may seem paradoxical that superoxide could produce both relaxation and contraction of cerebral vessels, these contrasting effects may be related to either the quantity of superoxide produced in response to each substrate or differences in compartmentalization of superoxide generation and/or diffusion. Fourth, increases in superoxide production in the basilar artery in response to NADH most likely involve activity of NAD(P)H oxidase and does not appear to involve cyclooxygenase, nitric oxide synthase or xanthine oxidase. Finally, relaxation of cerebral vessels in response to NADH appears to be mediated through activation of potassium channels.

**Effects of NADH and NADPH on vascular tone and superoxide levels.** The present study is the first, to our knowledge, to examine the effects of NADH and NADPH on cerebral vascular tone. We found that NADH induced a biphasic response in the basilar artery, where low concentrations of NADH produced relaxation, and high concentrations of NADH produced contraction. Similarly, NADH produced dilatation of cerebral arterioles in vivo. Both relaxation of the basilar artery and dilatation of cerebral arterioles to NADH were greatly reduced in the presence of Tiron, suggesting superoxide was the mediator of the response. In previous studies including our own (22, 30, 43, 52, 55), the dismutation product of superoxide (i.e., hydrogen peroxide) has been shown to cause relaxation of cerebral blood vessels. Our data suggest that hydrogen peroxide is not involved in responses to NADH because NADH-induced changes in basilar artery tone were unaffected by catalase. These results suggest a major role for superoxide, but not hydrogen peroxide, in vasorelaxation in response to NADH.

In addition to the effects of NADH on vascular tone, we found that NADPH induced modest contraction of the basilar artery (which was inhibited in the presence of Tiron) and very modest dilatation (as compared with NADH) in cerebral arterioles in vivo. Taken together, these data suggest that in the cerebral circulation NADH produces primarily relaxation of the basilar artery and dilatation of cerebral arterioles. On the other hand, the effect of NADPH on cerebral vascular tone appears to be minimal compared with that of NADH. It also appears that the contractile response to NADPH is mediated by superoxide because Tiron was efficacious in inhibiting this response. Because formation of reactive oxygen species is highly interdependent and because we did not perform experiments with NADPH in the presence of catalase, we cannot completely rule out a role for hydrogen peroxide in producing contraction. However, Tiron in addition to scavenging superoxide is known to promote hydrogen peroxide formation (36). Thus a role for hydrogen peroxide in the response to NADPH in the basilar artery seems unlikely because the contractile response to NADPH was not enhanced in the presence of Tiron.

To our knowledge, only one previous study (46) has examined the effects of NADH and NADPH on vascular tone. This previous study examined the response to a single concentration of NADPH (1.0 mM), which produced ~20% contraction of the mouse aorta. In contrast, the same concentration of NADH had no effect on tone in this previous study. The present finding that NADPH-induced contraction of the basilar artery is consistent with previous work in the aorta (46). In addition, the present study examined dose-response relationships of NADH and NADPH on vascular tone and on superoxide levels. The present study further extends results obtained in the aorta to cerebral blood vessels (the basilar artery and cerebral arterioles). Our study also presents the first data, to our knowledge, regarding the effects of NADH and NADPH on vascular responses in vivo.

Previous studies (3, 7, 33, 36, 37, 47) have demonstrated increased superoxide generation in arterial ring preparations, cultured vascular cells, and vascular membrane fractions of extracranial blood vessels in...
response to exogenous NADH and NADPH. Thus both NADH and NADPH have been commonly used to stimulate NAD(P)H oxidase activity. In the present study, superoxide levels in the basilar artery were relatively low under basal conditions. Cumulative addition of NADH or NADPH resulted in significant increases in vascular superoxide levels. The increase in superoxide in the basilar artery in response to NADPH was ~10-fold greater than that achieved with NADH.

An interesting observation can be made when one compares vascular function and superoxide levels. That is, low concentrations of NADH produced low superoxide levels and marked relaxation of cerebral vessels. In addition, levels of superoxide produced by higher concentrations of NADH are similar to levels produced by 10 μM NADPH. These observations support the novel concept that superoxide-mediated relaxation or contraction may, in part, correlate with the amount of superoxide produced in response to NADH or NADPH stimulation. A finding consistent with this possibility has been described previously in cerebral arterioles using a superoxide-generating system (acetaldehyde + xanthine oxidase), which produced dilatation at low substrate concentrations and a biphasic response (constriction followed by dilatation) at higher substrate concentrations (40). Alternatively, superoxide generation induced by NADH and NADPH may be restricted to different cellular compartments, suggesting that compartmentalization or differences in diffusion might occur resulting in different physiological effects. This idea is supported by a study (46) of the mouse aorta in which NADH (0.5 mM) and NADPH (0.5 mM) produced similar levels of superoxide, but only NADPH had an effect on tone.

Depending on the model, both relaxation and contraction in response to superoxide (or other reactive oxygen species) have been described in cerebral vessels (22, 23, 30, 40, 43, 45, 47). Reactive oxygen species have been shown to produce relaxation of cerebral blood vessels via activation of potassium channels (25–27, 34). Consistent with this idea, we found that the majority of the relaxation of the basilar artery and dilatation of cerebral arterioles to NADH could be inhibited by TEA. This finding suggests that activation of calcium-activated potassium channels play an important role in the response of cerebral vessels to NADH. In contrast, contraction of cerebral vessels to reactive oxygen species (namely, superoxide produced in response to NADPH) might reflect scavenging of basal nitric oxide, inhibition of activity of potassium channels, or activation of other undefined vasoconstrictor pathways.

**Role for a vascular NAD(P)H oxidase.** Recently, a membrane-bound NAD(P)H oxidase, distinct from the neutrophil type, has been proposed as an important source of vascular superoxide (4, 16, 17). These studies have been confined mainly to the aorta and vascular cells in culture. In the present study, it appears that a vascular NAD(P)H oxidase may be involved in generation of superoxide in response to NADH and NADPH for two reasons. First, in the present study, increases in superoxide levels in the basilar artery in response to NADH were significantly reduced in the presence of DPI. In previous studies (3, 36, 46, 47), DPI, a potent inhibitor of flavin-containing enzymes [e.g., NAD(P)H oxidase], has been demonstrated to selectively inhibit NADH- and NADPH-induced increases in superoxide. Second, superoxide production in response to NADH was not affected by indomethacin, L-NNA, or allopurinol, suggesting that neither cyclooxygenase, nitric oxide synthase, nor xanthine oxidase, the latter two of which are also flavin-containing enzymes, do not significantly contribute to the observed increases in superoxide in response to NADH.

On the basis of previous studies (primarily in the rabbit aorta), we speculate that smooth muscle and adventitia would be major sources of NAD(P)H-derived superoxide in cerebral vessels studied in the present study. In vivo, it is possible that changes in tone of cerebral arterioles might be related to NADH- and NADPH-induced generation of superoxide from parenchymal tissue because other cells also contain NAD(P)H oxidase (42, 48). However, our in vitro data indicate that cerebral vessels are capable of generating superoxide that is functionally important in response to NADH independent of parenchyma, suggesting that NAD(P)H oxidase is expressed within the cerebral vascular wall.

The exact spacial orientation of the assembled vascular NAD(P)H oxidase, in terms of substrate binding and superoxide release (i.e., intracellular vs. extracellular), is not yet known. Data regarding the oxidase at the molecular level has yet to resolve this issue. Furthermore, the component (gp91phox) that appears most integral to oxidase function, because it contains both the flavin- and NADH/NADPH-binding sites, exists as various isoforms (i.e., the Nox family of gp91phox homologs), and its expression appears to vary among tissues (5, 29). Until the structure of vascular NAD(P)H oxidase is fully defined, we cannot conclude whether translocation of exogenous NADH and NADPH to the cytoplasm is necessary for oxidase activity. However, the finding in the present study that exogenous NADH/NADPH increase superoxide through an enzymatic mechanism (DPI-inhibitable) suggests that they reach the oxidase.

**Consideration of methods.** In the present study, superoxide was measured using lucigenin-enhanced chemiluminescence, a method previously shown to be a sensitive assay for detection of superoxide in vascular tissue (3, 31, 33, 35–37, 47). However, there are certain potential limitations associated with this assay. First, because all current methods of superoxide detection (lucigenin- and coelentrazine-enhanced chemiluminescence, electron-spin resonance, cytochrome c reductase, etc.) rely on a detector compounds, they are indirect methods of superoxide measurement. Second, lucigenin may undergo redox cycling and, in the presence of oxygen, can result in generation of superoxide when high concentrations of lucigenin (250 μM) are used (32, 49). Previous studies (33, 37, 45, 46) have shown that this problem can be circumvented with the
use of low (<25 μM) lucigenin concentrations. With this approach, the results obtained with lucigenin in blood vessels are similar to those obtained with coelenterazine-enhanced chemiluminescence, cytochrome c assay, electron-spin resonance, and hydroethidine (19, 46, 47). In accord with these previous studies, we used a 5 μM concentration of lucigenin, which produced a signal that was markedly reduced by PEG-SOD or Tiron, scavengers of superoxide. Perhaps more importantly, our conclusions do not rely exclusively on data obtained with lucigenin because the results obtained related to vascular function support the conclusions that NADH- and NADPH-induced changes in vascular tone are mediated by superoxide.

Implications in regard to vascular dysfunction. An important metabolic consequence associated with vascular dysfunction during hypoxia and hyperglycemia involves an increase in the cellular NADH-to-NAD<sup>+</sup> ratio (an index of the cellular reduction state) (28, 53). An increase in the cellular NADH-to-NAD<sup>+</sup> ratio would likely produce alterations in activity of enzyme systems dependent on NADH. In the case of the present study, an increase in the NADH-to-NAD<sup>+</sup> ratio (i.e., application of exogenous NADH) would presumably increase levels of superoxide via increased NAD(P)H oxidase activity. Alterations in the cellular NADH-to-NAD<sup>+</sup> ratio might also have important implications in pathophysiological conditions, such as atherosclerosis and hypertension, which are not only associated with impaired vascular function but also increased levels of superoxide and NAD(P)H oxidase activity (4, 16, 50). Whether NAD(P)H concentrations increase during such conditions is not clear; however, the present study suggests the cellular reduction state may have important implications on vascular tone.

In summary, this is the first study to examine the effects of NADH and NADPH on superoxide levels and tone of cerebral blood vessels. We found that NADH produced marked relaxation, whereas NADPH produced primarily contraction of basilar artery. Similarly, NADH produced greater dilatation of cerebral arterioles than NADPH. These responses appear to be superoxide mediated, because superoxide scavengers were efficacious in abolishing both NADH- and NADPH-induced increases in superoxide and changes in vascular tone. Our experimental data strongly suggest that NAD(P)H oxidase activity is a vascular source of superoxide in the cerebral circulation.

The authors acknowledge the excellent technical assistance of Keith R. Breese and Dale A. Kinzenbaw. We thank Drs. Donald D. Heistad and Chris A. Hathaway for critical evaluation of this manuscript.

This study was supported by National Institutes of Health Grants NS-24621, HL-38901, and HL-62984. S. P. Didion was a recipient of Individual National Service Research Award HL-10237.

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