Potential Role of a Membrane-Bound NADH Oxidoreductase in Nitric Oxide Release and Arterial Relaxation to Nitroprusside

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Abstract—The site of metabolism in vascular smooth muscle responsible for the release of nitric oxide (NO) from nitroprusside is not well established. In this study we observed that a membrane-bound NADH oxidoreductase in the pulmonary artery activates nitroprusside to release NO, and we examined whether this process could potentially participate in relaxation to nitroprusside. Relaxation to nitroprusside in bovine calf pulmonary artery is inhibited by a scavenger of NO and by an antagonist of NO stimulation of guanylate cyclase. A flavoprotein probe that inhibits pulmonary artery NADH oxidoreductase (1 \( \mu \)mol/L diphenyliodonium) and electron acceptors for NADH oxidoreductase (0.3 mmol/L nitroblue tetrazolium and 0.1 mmol/L ferricyanide) inhibited pulmonary artery relaxation to nitroprusside, but not to nitroglycerin. Pulmonary arteries were observed to promote the release of NO from nitroprusside in vitro, and NO release was inhibited by the presence of nitroblue tetrazolium, ferricyanide, and diphenyliodonium. In homogenates of pulmonary arteries, NADH (0.1 mmol/L) increased the release of NO from nitroprusside by \( \approx 6 \)-fold, whereas NADPH, mitochondrial substrates, and other redox cofactors had minimal effects on NO release, and the action of NADH on nitroprusside was inhibited by nitroblue tetrazolium, ferricyanide, and diphenyliodonium. A membrane fraction enriched in NADH oxidoreductase activity showed a NADH-dependent release of NO from nitroprusside; nitroprusside caused NADH consumption, and it also inhibited the NADH-dependent reduction of nitroblue tetrazolium. Thus, a membrane-bound NADH oxidoreductase appears to contribute to the release of NO from nitroprusside, but not nitroglycerin, in calf pulmonary artery. (Circ Res. 1999;84:220-228.)

Key Words: electron transport ■ guanylate cyclase ■ nitric oxide ■ nitroprusside ■ redox

Nitroprusside is a clinically used vasodilator that appears to be metabolized across the peripheral vascular bed. Incubation of sodium nitroprusside (SNP) with isolated vascular tissue results in the generation of nitric oxide (NO), and it is thought that nitrosodiolator such as SNP and glycerol trinitrate (GTN) mediate their vasodilatory action through release of NO and activation of guanylate cyclase. Previous studies on the development of tolerance to nitrosodi- lators in isolated vascular tissue suggested that GTN and SNP appeared to differ in their pathways of metabolism to the active vasodilator species. However, very little is known regarding the actual metabolic mechanisms in vascular tissue that promote the release of NO from these nitrosodiolators.

It was first thought that SNP spontaneously released NO. Then it was suggested that chemical or enzymatic electron transfer reactions promoted NO release, because a variety of redox agents or systems appear to catalyze the release of its NO group. Multiple reducing agents, including NAD(P)H, thiols, ascorbate, reduced iron, hemoproteins, and cytochrome P-450 reductase, appear to enzymatically or chemically release NO from SNP in buffer systems. Early studies on the activation of guanylate cyclase by SNP typically included concentrations of thiols that probably contributed to NO release. In hepatocyte extracts, NAD(P)H-dependent cytochrome P-450 reductase and mitochondria have been reported to participate in the reductive metabolism of SNP. In vascular preparations, NADPH, NADH, and thiols have been reported to release NO from SNP, and the dominant site of metabolism appears to be membrane associated. In contrast, the metabolism of GTN to NO or its active metabolites has been suggested to involve systems including cytochrome P-450, glutathione transferase, and a plasma membrane–bound enzyme identified by Chung and Fung requiring a free thiol that appears to be active in the absence of NAD(P)H. Thus, evidence exists for multiple potential mechanisms of NO release from SNP, and the mechanism of NO generation from SNP appears to differ from that of GTN in vascular tissue. However, the primary metabolic pathway involved in the vasodilator actions of SNP remains to be defined.

In this study we examined the mechanism of NO release from SNP in bovine calf pulmonary artery (PA). This
preparation was selected, because it was initially observed during our characterization of a membrane-bound NADH oxidase that NADH oxidoreductase activity obtained in this PA preparation was able to efficiently catalyze the release NO from SNP. In the previous work on the membrane fraction containing SNP metabolizing activity, we characterized some of the electron transfer properties of NADH oxidoreductases present, including what appears to be a NADH oxidase system containing a b558-type cytochrome and a flavoprotein reductase of this cytochrome. In this study, we report the actions of probes previously developed in studies on the membrane-bound NADH oxidoreductases, including the electron acceptors nitroblue tetrazolium (NBT, 0.3 mmol/L) and potassium ferricyanide (FeCN, 0.1 mmol/L) and the flavoprotein inhibitor diphenyliodonium (DPI, 1 μmol/L), are examined for their actions on PA release of NO and relaxation to SNP. Because GTN is thought to produce vascular relaxation through a mechanism similar to that of SNP, GTN was used in vascular reactivity studies to provide evidence for the selective actions of probes used to study the metabolic activation of SNP.

Materials and Methods

Materials
Collagenase (type V), soybean trypsin inhibitor (type 1-S), elastase (type IV), MOPS, NADH, NADPH, glutathione (reduced form), glutathione (oxidized form, disodium salt), rotenone, malate (disodium salt), glutamate (monoammonium salt), bovine blood superoxide dismutase (CuZn-SOD), catalase from bovine liver (type C-40), sodium nitrite, sodium nitrate, N-1-naphthyl-ethylenediamine dihydrochloride, and sulfinilamide were purchased from Sigma Chemical Co. DPI chloride was purchased from Aldrich Chemical Co. Nitroglycerin solutions were prepared by dissolving 0.4-mg sublingual tablets (Parke-Davis) in distilled water. The scavenger of NO carboxy-PTIO or 1H-imidazol-1-yl-oxy, 2-(4-carboxyphenyl)-4,5,5-tetramethyl-3-oxide, potassium salt (PTIO), and the inhibitor of NO-elicited guanylate cyclase activation 1H-1,2,4-oxadiazolo[4,3-c]pyridine-3-carboxylic acid (ODQ) were obtained from Cayman Chemical Co. The NO donor S-nitroso-N-acetylpenicillamine (SNAP) was synthesized as previously described. Hb was reduced by reacting solutions containing 0.1 mmol/L Hb with a 10-fold excess of sodium dithionite, followed by dialysis to remove the dithionite decomposition products. Other chemicals were of analyzed reagent grade from Baker Chemical Co.

Measurement of Changes in Force in Bovine Calf PA

Isolated arterial rings of 4 mm in diameter and length with endothelium removed were prepared from the second and third branches of the main lobar PA of bovine calf lungs obtained immediately after slaughter, and the endothelium was removed by gentle rubbing, as previously described. Briefly, PA rings were mounted on stainless steel hooks attached to Grass force displacement transducers (type FT-03) for measurements of changes in isometric force on a Grass polygraph (model 7). Arteries were incubated for 2 hours at an optimal passive tension of 5 g in individually thermostated (37°C) 10-mL baths (Metro Scientific). These studies were conducted in Krebs-bicarbonate buffer, pH 7.4, and gassed with 21% O2-5% CO2 (balance N2). After 2 hours of equilibration, the vessels were depolarized with Krebs-bicarbonate containing KCI in place of NaCl. The arteries were then reequilibrated with Krebs buffer for 30 minutes before the experiments were conducted. The functional removal of endothelium was typically examined by confirmation that PAs precontracted with 10−7 mol/L serotonin did not show a relaxant response on exposure to 10−4 to 10−6 mol/L doses of acetylcholine. After a 30-minute equilibration, arterial rings were contracted to ~7 g with 20 to 30 mmol/L KCl. Once a stable contraction was obtained, cumulative increasing concentrations of SNP, GTN, or SNAP were added to each artery studied. In these studies, PA rings were preincubated for 15 minutes before contraction with KCl in the absence or presence of 10 μmol/L ODQ, 0.1 mmol/L PTIO, 1 μmol/L DPI, 0.1 mmol/L FeCN, or 0.3 mmol/L NBT, as indicated in Results, and the probes remained present during exposure to SNP or GTN. The doses of the probes used were generally the highest concentrations of these agents that appeared to avoid nonspecific effects (such as the depression of force) that were generally observed at higher doses. In some experiments, the response to SNP was studied under a severely hypoxic environment (Po2 ~8 to 10 torr) produced by gassing the tissue bath with 95% N2-5% CO2.

Preparation of the PA Homogenate and Membranes Enriched With NADH Oxidoreductase Activity
The PA homogenate was prepared as previously described. Membranes enriched with NADH oxidoreductase activity were prepared by centrifuging a filtered homogenate suspension at 5°C in a Sorvall SS-34 rotor at 2900g for 15 minutes. The supernatant obtained from the 29000g centrifugation was transferred to a Beckman ultracentrifuge equipped with a Ti50 rotor, and this fraction was centrifuged for 60 minutes at 100 000g. The sedimented pellet obtained (yield=0.60±0.05 mg protein/g PA) was previously shown to be enriched in plasma membrane and microsomal marker enzyme activities. This membrane pellet was washed with 2 mL of a sucrose-MOPS buffer solution that contained 250 mmol/L sucrose and 20 mmol/L MOPS (adjusted to pH 7.4 with KOH), and it was stored in 2 mL of the same buffer at 0°C to 5°C.

Measurement of NO Head-Space Gas Production by Intact and Homogenized PA Fractions

The release of NO from SNP was quantified by an adaptation of previously described methods. Major lobar PAs with endothelium removed were cleaned, cut to small strips, and incubated for 30 minutes with bicarbonate-buffered Krebs solution. Tissue (~300 mg) or arterial homogenate fractions (0.5 to 1.5 mg of protein) were then placed in Fernbach flasks (total volume, 6 mL; VWR Scientific) filled with 2 mL of 10 mmol/L HEPES-NaOH–buffered Krebs solution (pH 7.4), and then the flasks were covered with rubber-sleeve septa. Flasks were incubated at 37°C, and the contents of the flasks were then deoxygenated with argon gas for 15 to 20 minutes. After deoxygenation, drugs in deoxygenated solutions were injected with the use of gas-tight syringes, and then the flasks were incubated for the time period used to measure NO accumulation, as indicated in Results. Some preliminary experiments were also conducted in the absence of deoxygenation. One head-space gas sample (0.5 mL) was taken from each flask with a gas-tight syringe. Measurements of the NO content of the aliquots of head-space gas were then conducted by injection into a model 207B Redox chemiluminescence detector (Sievers Research, Inc.). This instrument quantifies the chemiluminescence that originates from injected NO in the sample, when it is reacted with ozone. This chemiluminescence method for NO was calibrated using calibration gases obtained from MG Industries, and the intensity of the emitted light was proportional to the amount of NO in the sample. NO standard samples were injected into the NO analyzer from gas-tight syringes that were flushed with argon before use and between the samples. The peak area (voltage) values of the individual NO standards were plotted against known NO concentrations. The slope and the y intercept of the line were determined by linear regression analysis. All incubations with SNP showed a small (presumably photoschemic) generation of NO in the absence of PA or isolated membranes, and the inhibitory probes used for probing

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NADH oxidoreductase did not significantly alter the detected amount of NO derived spontaneously from SNP. This background level of NO was subtracted from all data reported in Results. Although the quantity of NO released from PAs by 0.1 mmol/L SNP was observed to be similar under an atmosphere of 21% O2 compared with an argon atmosphere (n=6), detailed studies on the release of NO by this head-space gas method were conducted under argon atmosphere, because previous studies with this method used severe hypoxia and because preliminary studies indicated that an atmosphere of 21% O2 caused a marked impairment (>90% inhibition, n=3) of our ability to detect NAD(P)H-elicited release of NO from SNP in the homogenate preparation. Because the presence of 0.3 μmol/L CuZn-SOD (n=3) slightly enhanced the detection of NO under 21% O2, but not under argon, from the homogenate preparation, it is likely that the redox control of superoxide metabolism was altered in the homogenate preparation.

Measurement of SNP-Derived NO From PA Membranes by Hb Oxidation

NO was measured in isolated membranes with a spectrophotometric assay that determines the amount of NO-elicited methemoglobin formation from oxyhemoglobin (oxyHb), which is catalyzed by NO.21 This method was chosen to permit studies with isolated PA membranes to be conducted under similar conditions. Briefly, the changes in absorbances of Hb at 577 and 592 nm were measured, and an ε377–592 of 11.2 (mmol/L)−1 cm−1 was used to calculate the concentration of the NO formed. The assay mixture contained (final concentration): 5 μmol/L oxyHb, 0.3 μmol/L CuZn-SOD (to inhibit the removal of NO caused by NADH oxidase-derived superoxide), 0.1 μmol/L catalase (to prevent the oxidation of oxyHb by hydrogen peroxide), 1 mmol/L glutathione, 0.1 mmol/L SNP, and ~0.05 to 0.08 mg of membrane-fraction protein in air-equilibrated sucrose/MOPS buffer. The assay was initiated at 37°C by addition of 0.1 mmol/L (final concentration) NADH.

Measurement of SNP-Derived Nitrile (NO2−) From PA Membranes

The nitrite assay was performed as an additional method of measurement of NO release from SNP. Briefly, the assay mixture in air-equilibrated sucrose/MOPS buffer contained the following: 0.05 to 0.09 mg of membrane-fraction protein, 0.1 mmol/L SNP, and 1 mmol/L glutathione. The reaction was initiated at 37°C by addition of 0.1 mmol/L NADH in a final volume of 1.0 mL. The reaction was terminated by the addition of 1 mL of 1% sulfanilamide and 100 μL of 0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride. The mixture was then vortexed and incubated for 10 minutes at room temperature, and measurements of the absorbance at 540 nm were used to quantify the amount of nitrite present. Quantification was based on a comparison with the absorbances of known amounts of nitrite, under the conditions used to measure nitrite production by the membrane fractions. Under these conditions, the amounts of NADH used had no effect on the detection of nitrite.

Measurement of SNP-Elicited NADH Oxidation by PA Membranes

NADH oxidation by and 0.05 to 0.10 mg/mL of membrane fraction protein was measured in air-equilibrated sucrose/MOPS buffer in the absence and presence of 0.1 mmol/L SNP by following the change in NADH absorption at 340 nm.22 The extinction coefficient of 6.23 (mmol/L)−1 cm−1 was used to calculate the rate of NADH oxidation.

Measurement of the Effect of SNP on NADH-Elicited NBT Reduction by PA Membranes

NBT reduction by and 0.05 to 0.10 mg/mL of membrane fraction protein was measured in air-equilibrated sucrose/MOPS buffer in the absence and presence of 0.1 mmol/L SNP by modification of a method previously described.23 The change in absorbance of 62.5 μmol/L NBT at 360 nm was used to calculate the rate of NBT reduction based on an extinction coefficient of 15 (mmol/L)−1 cm−1.

Figure 1. Effects of a scavenger of NO (PTIO) and an inhibitor of NO stimulation of guanylate cyclase (ODQ) on relaxation of PA to SNP (A) and GTN (B). PAs with endothelium removed were incubated in the absence (Control) and presence of 0.1 mmol/L PTIO or 10 μmol/L ODQ for 15 minutes before the vessels were contracted with 20 to 30 mmol/L KCl and the arteries exposed to increasing cumulative concentrations of SNP (n=12 to 22) or GTN (n=18 to 27).

Statistical Analysis

Data were analyzed by Student’s t test or by 1-way ANOVA using a post hoc Duncan’s test for the determination of statistical significance between groups. A P<0.05 was used to determine statistical significance. Enzyme-kinetic parameters were determined using the computer program EnzymeKinetics (Trinity Software). The number of experimental determinations (n) in all cases is equal to the number of animals from which an arterial segment, homogenate, or isolated membrane fraction was used as a control or treatment group. Data in the figures are depicted as mean±SE.

Results

Effects of a Scavenger of NO (PTIO) and an Inhibitor of NO Stimulation of Guanylate Cyclase (ODQ) on Relaxation of PA to SNP and GTN

The effects of a scavenger of NO, PTIO,23 on the relaxation of PA to SNP (and GTN) were used to confirm the role of NO in vasodilator responses. As shown in Figure 1, PTIO inhibited the concentration-dependent relaxation of PA to SNP, but not to GTN. The effects of an inhibitor of NO-elicited stimulation of soluble guanylate cyclase (ODQ24) were used to confirm the importance of this process in the mechanism of PA relaxation to SNP and GTN. As shown in Figure 1, ODQ essentially eliminated the relaxant responses to both SNP and GTN.
Effects of Electron Acceptors (NBT and FeCN) and a Flavoprotein Inhibitor (DPI) on Relaxation of PA to SNP and GTN

We have previously reported\textsuperscript{15} that NADH oxidoreductase transfers electrons from NADH to NBT and FeCN causing a previously quantified, spectrally detectable reduction of these electron acceptors. Because both NBT and FeCN accept electrons from NADH oxidoreductase activity present in the membranes of PA,\textsuperscript{15} we used the electron-accepting properties of these probes as a potential method of investigating the role of this electron transport system in the mechanism of SNP-mediated relaxation. As shown in Figure 2, 0.3 mmol/L NBT markedly attenuated PA relaxation to SNP without altering relaxation of GTN. The data in Figure 3 indicate that 0.1 mmol/L FeCN caused a marked inhibition of relaxation to SNP without altering relaxation to GTN. To further confirm that NBT and FeCN do not scavenge NO or alter the mechanism through which it mediates relaxation, experiments (n=7 or 8) were conducted examining the effects of FeCN and NBT on the relaxant response to the NO donor SNAP. The relaxation of 69.4±8.8% to 1 μmol/L of SNAP was 81.4±3.9% in the presence of 0.3 mmol/L NBT and was 64.1±3.7% in the presence of 0.1 mmol/L FeCN. Because these probes do not alter the relaxant response to SNAP, NBT and FeCN do not appear to scavenge NO or alter the mechanism through which it mediates relaxation under the conditions used in the present study. We have previously shown\textsuperscript{16} that NADH oxidoreductase activity in the homogenate of PA can be inhibited by a flavoprotein-selective inhibitor, 1 μmol/L DPI. As shown in Figure 4, DPI caused a modest inhibition of PA relaxation to SNP, whereas GTN-mediated relaxation was not affected. Because most of the studies on the release of NO from SNP were conducted under an atmosphere of severe hypoxia, the effects of an atmosphere of 95% N\textsubscript{2}-5% CO\textsubscript{2} (P\textsubscript{O\textsubscript{2}} 5-8 to 10 torr) on relaxation to this agent in the absence and presence of NBT, FeCN, and DPI were examined. Severe hypoxia caused an increase in the sensitivity to relaxation by SNP, but it did not appear to alter the effects of the probes. For example, 0.1 μmol/L SNP caused a relaxation of 93±6% under N\textsubscript{2} compared with 63±7% relaxation under 21% O\textsubscript{2} atmosphere (n=8, P<0.05). The response to this dose of SNP was inhibited 56% (41±10% relaxation, n=8) under N\textsubscript{2} compared with 87% inhibition under 21% O\textsubscript{2} atmosphere by NBT, 62% (36±9% relaxation, n=8) under N\textsubscript{2} compared with 89% inhibition under 21% O\textsubscript{2} atmosphere by FeCN, and 64% (33±12% relaxation, n=8) under N\textsubscript{2} compared with 37% inhibition under 21% O\textsubscript{2} atmosphere by DPI.

Effects of NBT, FeCN, and DPI on the Generation of NO From SNP by PA

In this part of the study, we examined the effect of NBT, FeCN, and DPI on NO release from SNP in PA segments with endothelium removed. As shown by the data in Figure 5A, the release of NO from SNP by PA was attenuated by a flavoprotein inhibitor of NADH oxidoreductase (DPI) and...
was markedly inhibited by the electron acceptors of NADH oxidoreductase (NBT and FeCN). In confirmation of observations previously made in isolated rabbit aorta, GTN (0.1 mmol/L) did not elicit a detectable release of NO under the conditions used for the SNP studies in the presence of either an air or an argon atmosphere (n = 4, not shown).

Effects of Cellular Redox Cofactors on the Generation of NO From SNP in PA Homogenates

Previous studies suggest that NADPH, NADH, glutathione, and mitochondrial electron transport should be considered as sources of electrons that could potentially cause the reductive release of NO from SNP. The data in the Table indicate that NADH (0.1 mmol/L) was the most active electron donor in promoting the release of NO from SNP (0.1 mmol/L). NADPH (0.1 mmol/L) and glutathione (1 mmol/L) also caused a detectable release of NO, whereas substrates for mitochondrial transport (5 mmol/L glutamate + maleate) and oxidized glutathione (1 mmol/L) did not release NO. Because rotenone did not significantly modify the NADH-dependent release of NO from SNP, mitochondrial NADH dehydrogenase of the electron transport chain did not appear to be involved. Under conditions similar to those in studies with SNP, GTN (0.1 mmol/L) did not produce a detectable release of NO in the absence or presence of 0.1 mmol/L NADH (n = 4, not shown).

Effects of NBT, FeCN, and DPI on the NADH-Dependent Generation of NO From SNP by PA Homogenates

The data in Figure 5 indicate that NBT, FeCN, and DPI all showed a pattern of inhibition of NADH-dependent release of NO from SNP that was similar to the effects of these probes on NO release from SNP in PA shown in Figure 5A. While

Effects of Redox Cofactors on the Release of NO From SNP by PA Homogenates

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>NO Production (nmol/min per mg PA)</th>
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<tbody>
<tr>
<td>Control (0.1 mmol/L SNP)</td>
<td>0.58 ± 0.10</td>
</tr>
<tr>
<td>NADH (0.1 mmol/L)</td>
<td>4.25 ± 0.63*</td>
</tr>
<tr>
<td>NADH + rotenone (50 µmol/L)</td>
<td>3.67 ± 1.08*</td>
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<tr>
<td>NADPH (0.1 mmol/L)</td>
<td>1.32 ± 0.23*</td>
</tr>
<tr>
<td>NADPH + NBT (0.3 mmol/L)</td>
<td>1.18 ± 0.20*</td>
</tr>
<tr>
<td>NADPH + FeCN (0.1 mmol/L)</td>
<td>0.54 ± 0.08†</td>
</tr>
<tr>
<td>NADPH + DPI (1 µmol/L)</td>
<td>1.32 ± 0.21*</td>
</tr>
<tr>
<td>Glu (5 mmol/L) + Mal (5 mmol/L)</td>
<td>0.52 ± 0.09</td>
</tr>
<tr>
<td>GSH (1 mmol/L)</td>
<td>1.08 ± 0.33*</td>
</tr>
<tr>
<td>GSSG (1 mmol/L)</td>
<td>0.88 ± 0.20</td>
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</table>

Glu indicates glutamate; Mal, maleate; GSH, glutathione (reduced form); and GSSG, glutathione (oxidized form).

*P < 0.05 vs control; †Effect of the inhibitory probe (P < 0.05; n = 13).
FeCN inhibited the modest release of NO from SNP catalyzed by NADPH-dependent oxidoreductase activity. NBT and DPI did not significantly alter the actions of NADPH (see Table). Preliminary experiments conducted during the development of this study indicated that the release of NO from a 20-fold lower dose of SNP (5 \( \mu \text{mol/L} \)) observed during a prolonged incubation of PA homogenates+0.1 mmol/L NADH (15-minute incubation, \( n = \text{11 or 12} \)) or PAs with endothelium removed (60-minute incubation, \( n = \text{11 to 31} \)) were also significantly inhibited by NBT and DPI (not shown). Experiments were conducted in the presence of 0.1 mmol/L NADH to estimate the \( K_m \) for SNP and the \( K_i \) for NBT and FeCN. On the basis of the amounts of NADH-elicited release of NO from SNP over the 25 \( \mu \text{mol/L} \) to 1 mmol/L concentration range and the effects of 30 and 100 \( \mu \text{mol/L} \) doses of NBT and 10 and 30 \( \mu \text{mol/L} \) doses of FeCN, the apparent \( K_m \) for SNP was 185 \( \mu \text{mol/L} \), the apparent \( K_i \) for NBT was 100 \( \mu \text{mol/L} \), and the apparent \( K_i \) for FeCN was 10 \( \mu \text{mol/L} \).

**Properties of SNP Metabolism by Isolated PA Membranes Enriched in NADH Oxidoreductase Activity**

Under the conditions used for head-space NO release measurements on the homogenate, isolated PA membranes showed an increase (\( P<0.05 \)) in the release of NO from 0.1 mmol/L SNP of 24±5 and 4±1 nmol/min per mg of protein (\( n = 3 \)) in the presence of 0.1 mmol/L NADH and NADPH, respectively. The supernatant fraction in the presence of NADH and NADPH showed increases that did not reach statistical significance of 4±1 and 1±1 nmol/min per mg (\( n = 3 \)), respectively. Because these preliminary studies indicated that the NADH-dependent release of NO from 0.1 mmol/L SNP was enriched ~6-fold (\( P<0.05 \)) in the 100 000g membrane fraction compared with the 100 000g supernatant fraction, further studies were conducted on the membrane fraction. In these studies, additional approaches were used to examine the metabolism of SNP under an air-equilibrated atmosphere. The effect of SNP on the rate of NADH oxidation by the membrane fraction NADH oxidoreductase was examined. As is shown in Figure 6A, 0.1 mmol/L SNP accelerated NADH oxidation by membrane-bound NADH oxidoreductase by ~50 nmol/min per mg of protein (\( P<0.001 \)). We previously have shown\(^\text{15} \) that membrane NADH oxidoreductase activity is able to reduce many electron acceptors, including NBT. In the presence of 0.1 mmol/L SNP, NBT reduction decreased by ~92% (see Figure 6B). Under conditions similar to the measurements of NADH consumption and NBT reduction, the production of NO from SNP was measured indirectly through quantification of nitrite in the presence of the isolated membrane fraction and NADH. As shown in Figure 7A, in the presence of NADH, nitrite formation was significantly increased by 51 nmol/10 minutes per mg of PA protein (\( P<0.05 \)). The production of NO was also examined directly through oxyHb oxidation in the presence of 0.1 mmol/L SNP. As shown in Figure 7B, in the presence of the membrane fraction and NADH, NO production was significantly increased by 34 nmol/10 minutes per mg of protein.

**Discussion**

The data in this study suggest that a membrane-bound NADH oxidoreductase system has properties consistent with its functioning in the reductive release of vascular relaxant levels of NO from SNP in PA. In addition to its being the most active NO-releasing redox system examined in the PA homogenate, electron acceptors (NBT and FeCN) and an inhibitor (DPI) of membrane NADH oxidoreductase, which attenuated the release of NO in the homogenate, also attenuated SNP-dependent NO release and relaxation of PAs with endothelium removed. Figure 8 contains a model for how the probes used in this study potentially interact with the hypothesized role for a membrane-bound NADH oxidoreductase in the mechanism of NO release and pulmonary arterial relaxation to SNP.

During the course of examining the electron-transfer properties of NADH oxidoreductase activities present in membranes obtained from PA, it was observed that the membrane fraction studied contained a very high level of NADH-dependent reductase activity for electron acceptors, including FeCN and NBT.\(^\text{15} \) Because of structural similarities between FeCN (potassium ferricyanide or K\(_3\)Fe(CN)\(_6\)) and SNP (sodium nitroferricyanide, Na\(_2\)Fe(CN)\(_6\)NO), we suspected that SNP could be an electron acceptor for membrane-bound oxidoreductases, and we initially examined the effect of SNP on NADH oxidation and on NADH-elicited nitrite and NO formation by isolated PA membranes. In the presence of 0.1 mmol/L SNP, there was a marked increase in NADH oxidation, suggesting that SNP was an electron acceptor for...
membrane-bound NADH oxidoreductases. In addition, similar increases in NO and its aerobic decomposition product nitrite were observed in the presence of NADH. Although the increase in the consumption of NADH in the presence of SNP was greater than the amount of NO and nitrite production, this may be partially explained by a combination of factors, including the release of NO from SNP involving a multistep process, which is discussed below, and a potential inhibitory influence of aerobic conditions on the production and/or detection of NO from SNP by isolated tissue fractions. Since NBT is another electron acceptor that is reduced by NADH oxidoreductases present in PA membranes, we examined the effect of SNP on NADH-mediated NBT reduction. The reduction of NBT by NADH was also markedly inhibited in the presence of 0.1 mmol/L SNP. This observation suggests that SNP and NBT compete with each other for reduction, suggesting the potential involvement of a specific NADH oxidoreductase. While the data are consistent with the involvement of a specific NADH oxidoreductase in the release of NO from SNP, the normal function of the enzyme with this activity and its relationship with previously characterized sources of NADH-dependent superoxide production by these membranes remain to be defined. Because of the presence of this oxidase activity in the microsomes and difficulties detecting NADH-dependent release of NO from SNP by head-space gas methods under aerobic conditions, it is possible that the production of superoxide and its interaction with NO had an influence on some of the experiments conducted with microsomes under aerobic conditions. Overall, these data suggest that a NADH oxidoreductase activity present in PA membranes catalyzes a NADH-dependent reduction of SNP, and this is associated with the production of NO.

Previous studies have observed that SNP can undergo 1 electron reduction by the NAD(P)H-dependent oxidoreductase activities of microsomal cytochrome P-450 reductase or mitochondria derived from rat hepatocytes to form metal-nitroxyl radicals. Once SNP is reduced, it appears that NO, cyanide, and H₂O₂ are produced as byproducts of the further metabolism of SNP. Studies on the subcellular fractions of vascular tissue have detected during prolonged incubations with SNP that a NADPH-regenerating system promotes NO release from mitochondrial and membrane-enriched fractions, and that NADH also released NO. Our previous work suggests that isolated PA membranes contain relatively low levels of NADPH oxidoreductase activities, and preliminary experiments with isolated PA membranes determined that NADH (0.1 mmol/L) caused a 6-fold greater release of NO from SNP than NADPH (0.1 mmol/L). This was similar to observations made in the homogenate of PA, in which NADPH released only 20% of the amount of NO generated by a similar level of NADH. The differences between the observations made in the present study and previous work in vascular tissue may originate from the brief duration (1 minute) of the homogenate incubations used in the present study and the apparent absence of use of a NADH-regenerating system during the prolonged incubations used in the previous studies. The attenuation of NADH-elicited NO release by electron acceptors (FeCN and NBT) of membrane-bound NADH oxidoreductase activity and by the flavoprotein inhibitor (DPI) are consistent with an enzyme, with NADH oxidoreductase activity being the key NADH-dependent system present in the homogenate that metabolizes SNP to...
release NO. As shown by the data in Figure 5, NBT, FeCN, and DPI also caused inhibition of NO release from SNP by PAs, with endothelium removed in a manner similar to the effects of these probes on NADH-dependent NO generation from SNP in the PA homogenate. The inhibitory action of DPI also indicates that a flavoprotein participates in the release of NO from SNP by PA. Because NADPH-elicited NO release from SNP in the PA homogenate was inhibited by FeCN, but not significantly altered by NBT and DPI, NADPH oxidoreductases may not be a primary source of the observed generation of NO from SNP in PAs with endothelium removed. Mitochondrial electron transport did not seem to be a detectable source of NO release from SNP, since mitochondrial substrates (glutamate + maleate) did not cause NO release and since the NADH-dependent production of NO was not significantly altered by rotenone, an inhibitor of NADH dehydrogenase of the mitochondrial electron transport chain. Thus, PA membranes contain a NADH oxidoreductase activity that seems to have a more important role in the metabolic release of NO from SNP in intact and homogenized PA than the other redox systems examined.

The data in this study are consistent with a role for NADH oxidoreductase–elicited release of NO in the mechanism of PA relaxation to SNP, but not to GTN. Since a trapping agent for NO (PTIO) and an inhibitor of guanylate cyclase stimulation in the mechanism of PA relaxation to SNP. In the presence of the electron acceptors for NADH oxidoreductase, NBT and FeCN, SNP-mediated relaxation was inhibited in what appears to be a competitive manner. This is consistent with a potential competition between NBT or FeCN with SNP for NADH oxidoreductase–derived electrons that are presumably required for the release of NO from SNP. On the other hand, NBT and FeCN did not significantly alter relaxation to GTN or the NO donor SNAP, which is consistent with these electron acceptors having a selective effect on the release of NO from SNP by a system such as the membrane-bound NADH oxidoreductase. DPI was also able to inhibit SNP-mediated, but not GTN-mediated, relaxation in PA. While the inhibition of SNP-mediated relaxation of PA by DPI also appeared to be of a competitive type, it is likely that this originates from both DPI restraining the rate of electron transfer at each dose of SNP and SNP concentrations being below the level that maximizes its rate of metabolism, since the $K_m$ for NO appears to be $\approx 185$ $\mu$mol/L. The absence of inhibition of relaxation to GTN by the DPI treatment used is also consistent with DPI having a selective effect on the release of NO from SNP by a system such as the detected membrane-bound NADH oxidoreductase and the absence of a role for this electron transport system in the metabolic activation of GTN.

The actual pathway through which GTN is metabolized by vascular tissue to promote vascular relaxation is not well established, but it is generally thought that the formation of NO and the activation of guanylate cyclase have key roles in this process. The potent inhibitory effect of ODQ on relaxation to GTN observed in the present study further supports previous work consistent with the importance of guanylate cyclase stimulation in the mechanism of PA relaxation to GTN. The absence of an inhibitory effect of a trapping agent for NO (PTIO) on PA relaxation to GTN is more difficult to interpret, since the metabolic generation of NO from GTN might occur in the proximity of guanylate cyclase and PTIO is thought to have only limited access to this intracellular site. The data obtained in this study are not consistent with NADH oxidoreductase having a role in the relaxant action of GTN, since the probes for this system (NBT, FeCN, and DPI) did not alter PA relaxation to GTN and isolated PA membranes did not show a detectable release of NO from GTN in the presence of NADH. The absence of an inhibitory effect of DPI should not be interpreted as eliminating a role for all flavoproteins in the metabolic activation of GTN, since other flavoproteins could potentially be less susceptible to the inhibitory effects of DPI. Thus, the data in the present study do not provide new information on the metabolic activation of GTN in vascular tissue other than that the NADH oxidoreductase that releases NO from SNP does not appear to play a key role in relaxation to GTN.

In summary, a NADH oxidoreductase activity previously characterized in a membrane fraction obtained from calf PA may play an important role in the metabolism and release of NO from SNP, but not from GTN. On the basis of the inhibitory actions of some of the probes used, the NADH oxidoreductase involved in the release of NO from SNP appears to be a flavoprotein that contains sites that transfer electrons to FeCN and NBT. Since FeCN is not thought to be readily transported across cell membranes, the membrane-bound NADH oxidoreductase activity examined in the present study may function to transfer electrons needed for the release of NO from intracellular cytosolic NADH to SNP on the extracellular surface. There is substantial evidence that many cell types have a transmembrane electron transport system, and the reduction of FeCN is typically used to assay this system. While it is possible that other enzymes, such as the inflammatory cell membrane-bound NADPH oxidoreductase, could cause NO release by transferring electrons to SNP, previous work examining the cell surface enzymatic activity that reduced FeCN (see References 27 and 28) seems to have only observed a primary role for NADH oxidoreductase in this response. The selective inhibitory effect of the trapping agent for NO (PTIO) on relaxation to SNP compared with GTN could be interpreted as being consistent with an extracellular generation of NO from SNP, but not from GTN. An observation that is consistent with the importance of an extracellular release of NO from SNP in vivo is the reported selective attenuation of the vasodilator response to SNP (compared with other NO-releasing agents) in the cerebral circulation by infusion of cross-linked Hb. Thus, the data in this study are consistent with the hypothesis that NO is released from SNP on the extracellular surface of cells in the vasculature by a transmembrane NADH oxidoreductase electron transport system.

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Nitric Oxide Production by Nitroprusside

References


Potential Role of a Membrane-Bound NADH Oxidoreductase in Nitric Oxide Release and Arterial Relaxation to Nitroprusside

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