Nitro blue tetrazolium inhibits but does not mimic hypoxic vasoconstriction in isolated rabbit lungs

NORBERT WEISSMANN, FRIEDRICH GRIMMINGER, ROBERT VOSWINCKEL, JÖRG CONZEN, AND WERNER SEEGER

Department of Internal Medicine, J. justus-Liebig-University Giessen, 35392 Giessen, Germany

Weissmann, Norbert, Friedrich Grimminger, Robert Voswinckel, Jörg Conzen, and Werner Seeger. Nitro blue tetrazolium inhibits but does not mimic hypoxic vasoconstriction in isolated rabbit lungs. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L721–L727, 1998.—It has been suggested that hypoxic pulmonary vasoconstriction (HPV) may mainly proceed via loss of normoxic vasodilation, forwarded by tonic O₂-dependent formation of nitric oxide and superoxide (23). Both agents may stimulate guanylate cyclase, the latter via conversion to hydrogen peroxide and formation of compound I with catalase. We probed this hypothesis in perfused rabbit lungs, employing the superoxide scavenger superoxide dismutase (SOD), 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), and nitro blue tetrazolium (NBT) and the catalase inhibitor aminotriazole (AT). NBT turned out to be a potent dose-dependent inhibitor of HPV in a concentration range of 200 nM to 1 µM, and superimposable dose-inhibition curves were obtained when lung synthesis of nitric oxide and vasodilatory prostanooids was preblocked by N ω-monomethyl-L-arginine (L-NMMA) and acetylsalicylic acid (ASA). The NBT effect was specific because no inhibition in the vasoconstrictor responses to the stable thromboxane analog U-46619 and angiotensin II was observed. In contrast, SOD and Tiron were ineffective. AT exerted non-specific inhibition of the hypoxia- and chemical vasoconstrictor-induced pressor responses. When applied under normoxic conditions, however, NBT alone or coapplied with L-NMMA or ASA, both for blockage of parallel vasodilatory pathways, did not mimic the hypoxia-induced vasoconstrictor response. In conclusion, the present study supports an important role for superoxide in the mechanisms of HPV. However, even simultaneous blockage of parallel vasodilatory pathways did not substantially affect the baseline pulmonary vascular tone; i.e., it does not mimic HPV. Hypoxia-induced downregulation of the toxic production of NO thus appears to contribute to HPV but may not fully explain this mechanism.

MATERIALS AND METHODS

Reagents. Tiron, SOD, NBT, AT, ferricytochrome c, and [Asn¹,Val⁵]angiotensin (ANG) II were purchased from Sigma (Munich, Germany). Acetylsalicylic acid (ASA; Aspisol) was obtained from Bayer (Leverkusen, Germany). N ω-monomethyl-L-arginine (L-NMMA) was from Calbiochem (Frank-
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Khartung, Germany, and U-46619 (stable thromboxane analog) was obtained from Paesel + Lore (Frankfurt, Germany). The perfusate was provided by Serag-Wiessner (Naila, Germany). All other biochemicals were purchased from Merck (Munich, Germany).

Lung isolation, perfusion, and ventilation. The model of isolated perfused rabbit lungs has been described previously (28, 29, 37). Briefly, pathogen-free rabbits of either sex (body weight 2.2–3.2 kg) were deeply anesthetized and anticoagulated with heparin (1,000 U/kg body weight). The lungs were excised while being perfused with Krebs-Henseleit buffer through cannulas in the pulmonary artery and left atrium. The buffer contained 125.0 mM NaCl, 4.3 mM KCl, and 1.1 mM HEPES to pH 7.4. The perfusate was provided by Serag-Wiessner (Naila, Germany). By this device, the partial pressure was set at 3.6–4.0 kPa (28, 29), and the perfusate was contained at a temperature of 37°C.

Measurement of NBT effects on lung superoxide release. For evaluation of NBT effects on lung superoxide release, an on-line photometric assay of ferricytochrome c reduction was established as described for repetitive perfusate analysis by Bongard et al. (5). Briefly, 25 µM ferricytochrome c was added to the perfusate throughout, and absorption was continuously measured at the outflow of the lung at 550 nm. Experiments were performed in the absence and presence of NBT. Values are expressed as changes from a baseline concentration of reduced cytochrome (set at 100%) assessed 15 min after perfusate admixture of the dye.

Statistics. Data are means ± SE. Analysis of variance or a two-sided t-test was performed; statistical significance was assumed when P ranged ≤0.05.

RESULTS

Under baseline conditions, PAP values ranged between 3.6 and 7.6 mmHg in all experiments. A 3% hypoxic challenge (PAO2 ~ 23 mmHg) provoked a rapid increase in PAP, with maximum pressure elevations of 2.9 ± 0.2 mmHg (n = 32 lungs). U-46619 injections into the arterial line produced pressure elevations, with a maximum of 3.3 ± 0.3 mmHg (n = 8 lungs); those elicited by ANG II were 3.2 ± 0.2 mmHg (n = 8 lungs). The HPV maneuvers were readily reproducible within the same lung, with a slight increase in the strength of the pressor response within each sequence of maneuvers (Fig. 1). Reproducibility of the U-46619- and ANG II-provoked pressure elevations was also apparent (Fig. 2).

Admixture of the superoxide scavengers SOD (100–2,000 U/ml) and Tiron (10 µM to 10 mM) to the perfusate did not affect the strength of the HPV (Fig. 1). In addition, both agents did not change the baseline PAP and did not affect the relaxation induced by posthypoxic reoxygenation (Table 1). In contrast to SOD and Tiron, NBT caused a dose-dependent inhibition of HPV in a concentration range between 200 nM and 1 µM (Fig. 2). The HPV response was reduced by >70% in relation to the reference response. The efficacy of NBT was specific for the hypoxia-induced vasoconstrictor response because the U-46619- and ANG II-elicited pressure elevations were not significantly affected. NBT, however, did not change the baseline PAP and did not affect the relaxation induced by posthypoxic reoxygenation (Table 1).

Additional experiments were performed while the pulmonary NO generation was blocked by the admixture of 400 µM L-NAME to the perfusate throughout the experiments. In a previous study from our laboratory (30), this L-NAME concentration was shown to reduce the rabbit lung NO generation virtually completely. Under these conditions, the baseline PAP did not significantly change, but the strength of the refer-
ence hypoxic vasoconstrictor response was increased to 7.1 ± 0.6 mmHg (n = 8 lungs). This enhanced pressor response was again dose dependently inhibited by NBT in the same concentration range as employed in the absence of L-NMMA (Figs. 3 and 4). In contrast, vasoconstrictor responses elicited by U-46619 and ANG II in the presence of L-NMMA were not significantly suppressed by NBT. In further studies, the baseline generation of both NO and vasodilatory prostanoids was blocked by the presence of both L-NMMA (400 µM) and ASA (1 mM). Dose-dependent inhibition of HPV by NBT also occurred under these conditions (Fig. 4). In contrast to its influence on the hypoxia-elicited vasoconstrictor response, NBT did not affect the baseline PAP and thus did not turn out as a hypoxia mimic even when lung NO synthesis and vasodilator prostanoid generation were simultaneously blocked. These data support a role for superoxide and H2O2 in the mechanism of hypoxic vasoconstriction but question the concept that a drop in some tonic H2O2- and NO-related cGMP formation might be a sufficient trigger of the vasoconstrictor response.

As previously described (37), the current perfused lung system employs a membrane oxygenator in the perfusion circuit to keep the PO2 and PCO2 values in the

DISCUSSION

The present study employed buffer-perfused rabbit lungs to investigate the role of superoxide and H2O2 formation in the mechanisms of HPV in an intact organ model. NBT, an agent that traps superoxide in a manner that prevents H2O2 formation (21, 22), dose dependently inhibited HPV, whereas SOD and Tiron (agents permitting H2O2 appearance; Refs. 21, 22) were ineffective. The NBT effect was specific for the hypoxia-induced vasoconstrictor response and may not be explained by interference with lung NO synthesis. However, under normoxic conditions, NBT did not affect baseline PAP and thus did not turn out as a hypoxia mimic even when lung NO synthesis and vasodilator prostanoid generation were simultaneously blocked. These data support a role for superoxide and H2O2 in the mechanism of hypoxic vasoconstriction but question the concept that a drop in some tonic H2O2- and NO-related cGMP formation might be a sufficient trigger of the vasoconstrictor response.
Table 1. Effects of SOD, Tiron, NBT, and AT on baseline pulmonary arterial pressure and relaxation induced by posthypoxic reoxygenation

<table>
<thead>
<tr>
<th>Challenge 3</th>
<th>Challenge 4</th>
<th>Challenge 5</th>
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<td><strong>A</strong></td>
<td><strong>B</strong></td>
<td><strong>A</strong></td>
<td><strong>B</strong></td>
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<tr>
<td>Control HPV</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.1</td>
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<td>L-NMMA, control HPV</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>0.1 ± 0.1</td>
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<tr>
<td>L-NMMA, ASA, control HPV</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
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<td>HPV, SOD</td>
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<td>HPV, Tiron</td>
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<td>600 nM NBT</td>
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<td>L-NMMA, HPV, NBT</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>L-NMMA, ASA, HPV, NBT</td>
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<td>0.1 ± 0.0</td>
<td>0.0 ± 0.1</td>
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<td>L-NMMA, ASA, HPV, AT</td>
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<td>0.0 ± 0.1</td>
<td>−0.5 ± 0.1</td>
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Values are means ± SE in mmHg of changes in pulmonary arterial pressure within 5 min after addition of either agent to perfusate (A) and differences in pulmonary arterial pressure as assessed directly before and 10 min after a hypoxic challenge (B); n = 4 lungs/group. SOD, superoxide dismutase; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid; NBT, nitro blue tetrazolium; AT, aminotriazole; HPV, hypoxic pulmonary vasoconstriction; L-NMMA, N⁰-monomethyl-L-arginine; ASA, acetylsalicylic acid. Positive values, rise in pulmonary arterial pressure; negative values, decrease in pulmonary arterial pressure. In experiments with L-NMMA (400 µM) or L-NMMA (400 µM) and ASA (1 mM), these substances were present in perfusate throughout experimental protocol.

In a very low concentration range of 200 nM to 1 µM, the superoxide scavenger NBT was found to cause strong dose-dependent inhibition of the hypoxia-induced pressor response, whereas the PAP increase provoked by the stable thromboxane analog U-46619 and by ANG II was not affected at all. Impressively, superimposable dose-inhibition curves were again obtained when lung NO synthesis (L-NMMA) or both the generation of NO and the vasodilatory prostanooids (L-NMMA and ASA) were blocked throughout the experiments; i.e., the NBT effect may not be explained by some interference with these important vasoregulatory systems. The efficacy of NBT thus supports a preceding study (22) in isolated calf pulmonary arteries, in which NBT inhibited hypoxic constriction together with superoxide scavenging, however, in a much higher dose range (300 µM). Other NBT effects in addition to scavenging of superoxide are unlikely to contribute to the inhibition of HPV. These might include 1) inhibition of NADPH diaphorase generating NO. Such effects can be excluded because NBT turned out to be fully active in experiments with prior blockade of the NO system; 2) generation of superoxide by reoxidation of the NBT radical (4). This is highly improbable because the lung tissue and tubing system increasingly changed their color to blue, indicating that NBT was continuously reduced to the insoluble blue formazan; 3) generation of molecular O₂ due to the addition of NBT (4) that might reduce the strength of hypoxia. However, even entire conversion of the highest NBT dose presently used would generate O₂ quantities that are more than four orders of magnitude lower than the quantity of O₂ per
se transported to the lung during a 10-min period of hypoxia. Thus such a mechanism may not be responsible for the inhibition of HPV by NBT; and 4) reduction of NBT by other than superoxide-generating chemical and enzymatic systems (4, 33). As shown by the data

from the ferricytochrome c admixture to the perfusion fluid, the presently used low NBT concentration caused significant quenching of extracellular cytochrome c-reducing capacity. This finding may be assumed to indicate superoxide scavenging by NBT. It has been shown in detail that besides superoxide, only ascorbate release substantially contributes to the cytochrome c-reducing capacity of the rabbit lung vasculature (5). Moreover, there is multiple evidence from biological systems that NBT scavenges superoxide (21, 22).

The fact that the profiles of NBT (assumed to scavenge superoxide) and diphenyleneiodonium (reducing superoxide generation) in rabbit lung HPV are virtually identical strongly favors the assumption that some reduction in the superoxide levels by NBT underlies the HPV inhibitory effect of this agent (22). In contrast to the assumption of Mohazzab-H. and Wolin (22), there is evidence that NBT scavenges extracellular superoxide (12, 33). Recently, Marshall et al. (19) showed that extracellular superoxide is increased during hypoxia in small pulmonary arterial smooth muscle cells, the predominant site of HPV.

In contrast to NBT, the superoxide scavengers SOD and Tiron added to the perfusate did not affect the hypoxia-induced vasoconstriction, even when applied in very high concentrations (2,000 U/ml of SOD; 10 mM Tiron). Three major explanations may underlie these findings.

1) SOD and Tiron might not have sufficient access to the sites of O_2 sensing (assumed to be the precapillary smooth muscle cells; Ref. 19) and superoxide generation and efficacy. This explanation may well hold true
for SOD because it may not be able to pass the endothelial barrier in sufficient quantities due to its molecular weight of \( \sim 31,200 \). Additionally, steric hindrances and/or electrostatic effects could also be responsible for the ineffectiveness of SOD (13). Moreover, intracellular regions are hardly reached by SOD, which is known to demand vehicle systems such as polyethylene glycol or liposomes for entrance into the intracellular space in substantial quantities (6, 32). In contrast, the low-molecular-weight superoxide scavenger Tiron may enter the cell and has repeatedly been shown to trap even intracellular \( \mathrm{O}_2 \) radicals (18, 20), and such a high concentration as the currently employed 10 mM must thus be assumed to be distributed into all cellular and extracellular compartments related to hypoxia sensing and subsequent signal transduction. Overall, distribution of substantial concentrations of buffer-admixed Tiron into all compartments relevant for superoxide generation and efficacy may be assumed from the basic pharmacological features of this agent, even if the present study may not provide a direct proof for this assumption.

2) Tiron and SOD may not affect superoxide and \( \mathrm{H}_2\mathrm{O}_2 \) concentrations because of the high rate constant (efficacy) of native SOD or spontaneous dismutation of superoxide.

3) Both SOD and Tiron are scavengers of superoxide that result in the formation of \( \mathrm{H}_2\mathrm{O}_2 \) from this \( \mathrm{O}_2 \) radical (9, 21). This is in contrast to the effect of NBT, known to trap superoxide without the formation of \( \mathrm{H}_2\mathrm{O}_2 \) (10). SOD and Tiron on one hand and NBT on the other, although all being scavengers of extracellular superoxide, must thus be assumed to have different effects on the downstream appearance of \( \mathrm{H}_2\mathrm{O}_2 \).

Notwithstanding the fact that the reasons for the discrepancy between the efficacy of NBT and the ineffectiveness of SOD and Tiron are not fully resolved, the present study supports the notion that changes in the basic pharmacological features of this agent, even if the present study may not provide a direct proof for this assumption.

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vasoconstrictor event occurring in HPV remain to be further elucidated.

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Address for reprint requests: W. Seeger, Dept. of Internal Medicine, J ustus-Liebig-Univ. Giessen, Klinikstrasse 36, 35392 Giessen, Germany.

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