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Early Determinants of H_2O_2-Induced Endothelial Dysfunction

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Abstract

Reactive oxygen species (ROS) can stimulate nitric oxide (NO•) production from the endothelium by transient activation of endothelial nitric oxide synthase (eNOS). With continued or repeated exposure, NO• production is reduced, however. We investigated the early determinants of this decrease in NO• production. Following an initial H_2O_2 exposure, endothelial cells responded by increasing NO• production measured electrochemically. NO• concentrations peaked by 10 min with a slow reduction over 30 min. The decrease in NO• at 30 min was associated with a 2.7 fold increase $O_2^{\bullet-}$ production ($p<0.05$) and a 14 fold reduction of the eNOS cofactor, tetrahydrobiopterin (BH_4, $p<0.05$). Used as a probe for endothelial dysfunction, the integrated NO• production over 30 min upon repeat H_2O_2 exposure was attenuated by 2.1 fold ($p=0.03$). Endothelial dysfunction could be prevented by BH_4 cofactor supplementation, by scavenging $O_2^{\bullet-}$ or peroxynitrite ($ONOO^-$), or by inhibiting the NADPH oxidase. Hydroxyl radical (•OH) scavenging did not have an effect. In summary, early H_2O_2-induced endothelial dysfunction was associated with a decreased BH_4 level and increased $O_2^{\bullet-}$ production. Dysfunction required $O_2^{\bullet-}$, $ONOO^-$, or a functional NADPH oxidase. Repeated activation of the NADPH oxidase by ROS may act as a feed forward system to promote endothelial dysfunction.

Keywords

Nitric Oxide [D01.339.387]; Hydrogen Peroxide [D01.248.497.158.685.750.424]; Endothelium [A10.272.491]; Nitric-Oxide Synthase [D08.811.682.135.772]; Electrochemistry [H01.181.529.307]

LIST OF ABBREVIATIONS

1. BAECs - bovine aortic endothelial cells; 2. BH_2 - 7, 8-dihydrobiopterin; 3. BH_4 - tetrahydrobiopterin; 4. CMH - 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine; 5. ESR - electron spin resonance; 6. eNOS - endothelial nitric oxide synthase; 7. L-NAME - N_ω-Nitro-L-arginine methyl ester; 8. MAECs - mouse aortic endothelial cells; 9. PBS - phosphate buffered saline; 10. PEG-SOD - polyethelene glycol conjugated superoxide dismutase; 11. ROS - reactive oxygen species; 12. SOD - superoxide dismutase

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Introduction

Reactive oxygen species (ROS) have been shown to contribute to cardiovascular disease, but some oxidative stress, such as that produced in moderate exercise, is associated with a decreased risk of cardiovascular disease(1–3). One possible explanation for this apparent paradox is that small amounts or episodic exposures to ROS increase the oxidative buffering capacity of the body, while a larger amounts or repeated exposures overpower these compensatory mechanisms. The determinants of when compensatory mechanisms fail are unclear, however.

H_2O_2, most commonly produced when $O_2^{\bullet-}$ is dismutated by superoxide dismutase (SOD), is the most stable and long lasting of the ROS(4). It is likely to play a critical role in maintaining the balance between $O_2^{\bullet-}$ and NO•, since it has been shown to act as a signaling molecule for both NADPH oxidases and endothelial nitric oxide synthase (eNOS). Presumably a compensatory response, exposure of endothelial cells to H_2O_2 causes upregulation of eNOS after several hours(5) and, on a shorter time scale (i.e., several minutes), activates eNOS through phosphorylation(6). On the other hand, continued exposure to H_2O_2 for an intermediate time period (30 min) results in reduced NO• bioavailibility(7). In addition, H_2O_2 activates the NADPH oxidases through phosphorylation of the NADPH oxidase subunit $p47^{phox}$ (4) to produce ROS, suggesting that continued or repeated ROS exposure may lead to reduced NO• by oxidation of BH_4, an essential eNOS cofactor, resulting in dysfunctional, uncoupled eNOS(8). In these experiments, we tested the determinants of reduced NO• production upon continued H_2O_2 exposures.

MATERIALS AND METHODS

Cell Culture

Bovine aortic endothelial cells (BAECs; Cell Systems, Kirkland, WA) were cultured on 0.05% gelatin using M199 media (Cellgro, Herndon, VA) containing 10% fetal bovine serum (FBS, Invitrogen-Gibco, Carlsbad, CA) and 1% of each of the following: penicillin/streptomycin (Cellgro), minimal essential media (MEM) vitamins (Cellgro), L-glutamine (Invitrogen-Gibco), and MEM Amino Acids (Invitrogen-Gibco). Experiments were performed at 37ºC on passage 4 or 5 cells that were 60–90% confluent. $p47^{phox}$-knockout and wild type mouse aortic endothelial cells (MAECs) were isolated as described previously(9) and cultured on 0.1% gelatin in Dulbecco's Modified Eagle Media (Invitrogen-Gibco) containing 1.25% MEM non-essential amino acids (Invitrogen-Gibco), 12.5% FBS (Atlanta Biologicals, Norcross, GA), 2.5 U/mL heparin (Baxter, Deerfield, IL), and 2% endothelial cell growth serum (isolated from bovine brain extract). Experiments were performed at 37ºC on cells from passages 6–10 at 60–90% confluence.

NO• Measurement

NO• was measured using an NO•-specific carbon electrode. Nafion-coated carbon electrodes (100 μm exposed length x 30 μm diameter) were obtained commercially (World Precision Instruments, Sarasota, FL) and coated with o-phenylenediamine (o-PD) as described previously(6,10–12). NO• was measured amperometrically against an AgCl reference electrode, with the voltage of the electrode held constant above the oxidation potential of NO• (+900 mV). Electrodes were calibrated with known concentrations of NO• (obtained by bubbling NO• through N_2-degassed deionized H_2O to saturation). To insure the faradic current represented only NO•, current seen in the presence of the eNOS inhibitor, L-NAME (N_ω-Nitro-L-arginine methyl ester, 2 mM) was subtracted from the unprocessed current to give the L-NAME suppressible current used in all further analysis. In control experiments, the L-NAME

suppressible current represented 79% of the total faradic current. All electrodes used showed linear calibration curves with $R^2 > 0.95$.

Cell Treatments

Prior to experiments, cells were incubated in freshly made 60 μM BH_4 for 10 min to compensate for variable cell culture-induced BH_4 deficiencies(13–15). Experimental results in the absence of an initial BH_4 supplementation were qualitatively identical but with lower NO• production, suggesting that pretreatment was not likely to affect the interpretation of the results. NO• measurements were made in phosphate buffered saline (PBS; pH 7.4, 37°C), with the electrode positioned with a micromanipulator 5 μm above the monolayer. With BH_4 removed from the media to prevent any interaction with the electrode, cells were exposed to 50 μM H_2O_2, and the resulting increase in NO• production was measured for 30 min ("H_2O_2 exposure 1"). In order to probe the degree of endothelial dysfunction induced by this exposure, a second exposure was used. The same cells were then rinsed with PBS and again exposed to 50 μM H_2O_2 as the increase in NO• production was measured for 30 min ("H_2O_2 exposure 2"). The time between H_2O_2 exposures was limited to that necessary to change the media and was never more than 10 min.

To determine possible mediators of the reduced NO• production upon repeated H_2O_2 exposure, cells were pre-incubated for 30 min with apocynin (600 μM), $O_2^{\bullet-}$ scavenger polyethelene glycol conjugated superoxide dismutase (PEG-SOD) (450 U/mL), $ONOO^-$ scavenger uric acid (100 μM), or •OH scavenger mannitol (3 mM) prior to H_2O_2 exposure 1 or with 60 μM BH_4 for 10 min prior to H_2O_2 exposure 2. While there is no specific scavenger of $ONOO^-$, uric acid was chosen because it has minimal activity against $O_2^{\bullet-}$ and H_2O_2(16). To maximize the scavenger effects but prevent faradic contamination when measuring NO•, uric acid, mannitol, and apocynin treatments were extended into H_2O_2 exposure 1 but not into H_2O_2 exposure 2. All other treatments were terminated prior to H_2O_2 exposure 1 by washing the cells with PBS. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) unless otherwise noted.

Measurements of Biopterin Content

Measurements of biopterin content in BAECs were performed using HPLC analysis and a differential oxidation method as described previously(17). The amount of BH_4 was determined from the difference between total (i.e., BH_4, 7,8-dihydrobiopterin (BH_2), and biopterin) and alkaline-stable oxidized biopterin (BH_2 and biopterin). A Nucleosil C-18 column (4.6 x 250 mm, 5 μm) was used with 5% methanol/95% water as a solvent at a flow rate of 1.0 mL per min. The fluorescence detector was set at 350 nm for excitation and 450 nm for emission.

Measurements of Superoxide Production

All sample analyses were performed in pairs as previously described(18). The cell permeable spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (CMH; Alexis Corp., San Diego, CA) was used to examine $O_2^{\bullet-}$ production using electron spin resonance spectroscopy (ESR). Cells treated as delineated above were washed twice with PBS, scraped from the plates, resuspended in 1 mL of PBS, and collected by centrifugation. Freshly isolated cells were allowed to equilibrate in deferoxamine-chelated Krebs-HEPES solution containing CMH (0.5 mM), deferoxamine (50 μM), and DETC (5 μM) for 90 min at 37°C. After the incubation period, a mixture containing 60% Krebs-HEPES buffer, 30% cell suspension, 10% CMH stock (2.4 mg/mL), with or without 2% SOD was transferred to a 100 μL capillary tube. Superoxide production by BAECs was detected by following the low-field peak of the nitroxide ESR spectra using a time scan with the following ESR settings: microwave frequency 9.78 GHz, modulation amplitude 2 G, microwave power 10 dB, conversion time 1.3 s, time constant 5.2 s. Analyses of the slopes of the time scans were used to quantify the amount of $O_2^{\bullet-}$ produced by the cells and compared to spectra in the presence of SOD. Superoxide

production normalized for the total protein content was calculated as the difference in $O_2^{\bullet-}$ production in paired samples with and without SOD present.

Data Analysis

Current was obtained and analyzed using pClamp 8.0 (Axon Instruments, Union City, CA). Unprocessed currents were low-pass filtered using an 8-pole Bessel filter with 2 Hz cutoff frequency. Statistical analysis was performed using Microsoft Excel (Redmond, WA), Graph pad Prism (Graph pad Software, Inc., San Diego, CA), or SPSS (Chicago, IL). NO• production as a function of time in response to H_2O_2 was compared using two-way ANOVAs. Multiple means were compared using one-way ANOVAs with post hoc testing for multiple comparisons or t tests with corrections for multiple comparisons as appropriate. A $p<0.05$ was considered statistically significant.

RESULTS

Previously, we have shown that exposure of endothelial cells to H_2O_2 results in a transient, immediate increase in eNOS-dependent NO• production(6,12). Suggesting endothelial dysfunction, this presumably compensatory increase in NO• production began to decrease over 30 min of continued exposure (figure 1). After H_2O_2 exposure 1, the reduction in NO• production was associated with an increase in $O_2^{\bullet-}$ production and a decrease in cellular BH_4 (figure 1, panels C and D). To quantify the degree of endothelial dysfunction induced, we used a second exposure to H_2O_2. After this second exposure, BAECs showed a reduction in peak and total NO• release when compared to the first exposure. Cells exposed to 50 μM H_2O_2 (H_2O_2 exposure 1) increased NO• production after 10 min to 356±94 nM (mean ± standard error mean, n=9). After 30 min, the NO• concentration decreased to 51.4% of the peak concentration. As an index of the total amount of NO• produced by cells, the integrated NO• signal over 30 min was 11261±2193 nM·min. After rinsing in PBS, the cells were again exposed to 50 μM H_2O_2 (H_2O_2 exposure 2). During H_2O_2 exposure 2, the peak NO• and the integrated NO• productions were reduced by 2.3- ($p<0.05$) and 2.0-fold ($p<0.05$) respectively when compared to that measured during H_2O_2 exposure 1.

The reduced NO• production was completely ameliorated by BH_4 supplementation prior to the second H_2O_2 exposure. Comparing either the time courses of NO• production or the integrated NO• values indicated that NO• production from cells supplemented with BH_4 just prior to H_2O_2 exposure 2 was not statistically different from the NO• production during H_2O_2 exposure 1 ($p=0.4$ and $p=0.7$, respectively). BH_4 supplementation concomitantly reduced $O_2^{\bullet-}$ production and raised cellular BH_4 levels to those seen before any H_2O_2 exposure (figure 1, panels C and D).

$O_2^{\bullet-}$ Scavenging and NADPH Oxidase Inhibition Prevented the Loss of NO• Production

Because NADPH oxidases are activated by H_2O_2 and produce $O_2^{\bullet-}$ (19), we tested if H_2O_2-induced $O_2^{\bullet-}$ might contribute to the endothelial dysfunction observed. Treating cells with the cell permeable $O_2^{\bullet-}$ scavenger PEG-SOD prevented the decreased NO• production seen during the second H_2O_2 exposure (figure 2). This NO• production after a second exposure to H_2O_2 in PEG-SOD-treated cells was statistically improved when compared to a second exposure response in the absence of treatment ($p<0.01$) and was similar to that seen with a second BH_4 supplementation treatment ($p = 0.2$). This result suggested that $O_2^{\bullet-}$ was causing the resultant dysfunction after repeat exposures to H_2O_2, possibly through BH_4 oxidation.

Suggesting that the NADPH oxidase was required for the $O_2^{\bullet-}$-mediated endothelial dysfunction seen, inhibiting NADPH oxidase assembly with apocynin (600 μM) prevented the dysfunction. A comparison of integrated NO• values indicated that the integrated NO•

produced by apocynin was statistically different from the H_2O_2 control ($p<0.05$) and that apocynin, BH_4, and PEG-SOD treatments resulted in indistinguishable salvage of NO• production as measured by a second H_2O_2 treatment ($p=0.34$ by one-way ANOVA).

To support results seen with chemical inhibition of the NADPH oxidase, we repeated the experiments in aortic endothelial cells isolated from $p47^{phox}$-knockout mice, which lack a critical subunit of the oxidase. Although there was a significant difference between the integrated H_2O_2 exposures 1 and 2 NO• productions in wild-type MAECs ($p<0.05$), NO• production was similar during both H_2O_2 exposures in $p47^{phox}$-knockout cells ($p=0.7$). Integrated NO• production decreased 2.1 fold during the second exposure, an identical decease to that seen in BAECs. In $p47^{phox}$-knockout cells, a second challenge with H_2O_2 caused a similar increase in NO• production (figure 3), eliminating the reduction seen in the wild-type cells.

ROS Scavengers Prevent the Loss of NO• Production with Repeated Exposures to H_2O_2

Production of $O_2^{\bullet-}$ also increases cellular levels of $ONOO^-$ and •OH, since $ONOO^-$ forms by reaction of $O_2^{\bullet-}$ with NO•, and •OH forms by degradation of protonated $ONOO^-$ or through the Haber-Weiss reaction(20,21). BH_4 is thought to be preferentially oxidized by these free radical species in comparison to $O_2^{\bullet-}$(22). Therefore, we sought to determine whether these species were playing a role in the reduced NO• production seen with H_2O_2 exposure. Treating cells with the $ONOO^-$ scavenger, uric acid, prevented the endothelial dysfunction seen during the second exposure (figure 4). NO• production after uric acid was statistically indistinguishable from the effect of BH_4 ($p=0.96$). Treating cells with the •OH scavenger, mannitol, did not have a significant effect ($p>0.05$), however.

DISCUSSION

Many disease states are associated with decreased NO• and increased ROS, yet exposure to moderate levels of oxidative stress induces increased NO• production and production capacity (5,6), presumably compensatory mechanisms. Therefore, it seems likely that some failure of these mechanisms is associated with the progression of disease. In this study, we explored early events leading to endothelial dysfunction during H_2O_2 exposure. We demonstrate that endothelial NO• production is increased acutely with exposure to H_2O_2 but that this production wanes with time and is reduced upon repeat exposure. This reduction in NO• is accompanied by decreased cellular BH_4 and increased $O_2^{\bullet-}$ production, thought to be hallmarks of eNOS uncoupling. Supporting this possibility, the endothelial dysfunction observed during a second H_2O_2 exposure could be ameliorated with BH_4 supplementation.

H_2O_2 activates not only eNOS but also the $O_2^{\bullet-}$-producing NADPH oxidase(4). Furthermore, increased NADPH oxidase activity is associated with hypertension and progression of atherosclerosis, suggesting that this enzyme may be part of the pathogenic cascade that leads to uncompensated oxidative stress in these diseases(6). Therefore, we tested whether early H_2O_2-induced endothelial dysfunction could be mediated by the NADPH soxidase and its product, $O_2^{\bullet-}$. Either genetic inhibition of the NADPH oxidase in $p47^{phox}$-knockout cells or inhibition of the oxidase by apocynin resulted in a recovery of $H_2O_2^-$induced NO• production to the level seen with BH_4 supplementation, suggesting that the NADPH oxidase was an upstream mediator of the endothelial dysfunction seen.

Activation of the NADPH oxidase results in increased $O_2^{\bullet-}$. Scavenging $O_2^{\bullet-}$ had effects on NO• production that were indistinguishable from apocynin or genetic inhibition of the oxidase. This implies that this ROS may be a downstream effector of NADPH oxidase activation. The trend toward increased NO• in PEG-SOD treated cells as compared to the cells with inhibited NADPH oxidase could be explained if additional protection from oxidative degradation of

NO• was provided by scavenging oxygen radicals from other sources such as xanthine oxidase, cyclooxygenase, cytochrome P450, and mitochondria. Nevertheless, the majority of PEG-SOD effect could be attributed to dismutation of NADPH oxidase-produced $O_2^{\bullet -}$.

Although $O_2^{\bullet -}$ can oxidize BH_4, $ONOO^-$ and •OH are known to be more potent BH_4 oxidizers (22–24). Because $ONOO^-$ is formed when $O_2^{\bullet -}$ and NO• react, scavenging of $O_2^{\bullet -}$ would indirectly prevent $ONOO^-$ formation. $ONOO^-$ scavenging with uric acid resulted in NO• production statistically similar to that if cells were supplemented a second time with BH_4. Scavenging of •OH with mannitol did not result in recovery of NO• production, suggesting it is not important in the overall effect. Uric acid is not absolutely specific for $ONOO^-$, however, but was chosen because of its minimal effects on $O_2^{\bullet -}$ and H_2O_2(16). The results with other ONOO agents such as Manganese (III) tetrakis (1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP) would have been even more difficult to interpret in this case because of their effects on other ROS. Therefore, the most parsimonious interpretation of the scavenger data is that much of the endothelial dysfunction is mediated by the $O_2^{\bullet -}$ downstream product, $ONOO^-$, but the involvement of other ROS cannot be rigorously excluded.

The simplest explanation for the NO• production profile seen upon H_2O_2 exposure involves activation of both eNOS and the NADPH oxidase. NADPH oxidase-derived $O_2^{\bullet -}$ reacts with NO• to form $ONOO^-$. $ONOO^-$ reacts with BH_4 to create uncoupled eNOS, resulting in a reduced NO• production in response to subsequent H_2O_2 exposures (figure 5). This hypothesis is supported by the similarity of BH_4 supplementation with the effects of PEG-SOD, apocynin, and urate, suggesting that uncoupling of eNOS by BH_4 oxidation is sufficient to explain the observed reduction in NO• produced with repeat H_2O_2 exposures. Alternatively, BH_4 could be acting as a ROS scavenger. This possibility is less likely because BH_4 has been shown to be an inefficient $O_2^{\bullet -}$ scavenger(25). Nevertheless, the possibility of $ONOO^-$ scavenging by BH_4 cannot be eliminated.(15) In either case, the data support the fact that continued H_2O_2 exposure generates endothelial dysfunction and that this effect requires ROS production and NADPH oxidase activation.

BH_4 oxidation in response to increased ROS may be part of the explanation for the failure of oxidative stress compensatory mechanisms during the time period studied. Moreover, this may explain the ability of BH_4 to lower blood pressure in hypertensive mice,(26) if BH_4 supplementation reduces uncoupled eNOS production and improves NO• bioavailability. Nevertheless, the effects of persistent oxidative stress are likely to be more complicated. Any effect of ROS to generate uncoupled eNOS may be exacerbated by dihydrofolate reductase downregulation(27), an enzyme that catalyzes reduction of oxidized dihydropterin to BH_4, and counterbalanced by H_2O_2-induced BH_4 production by GTP cyclohydrolase I (24).

In conclusion, although H_2O_2 caused an initial increase in NO• production, NO• levels declined over time with additional exposures to H_2O_2. The decline could be prevented by BH_4 supplementation, scavengers of ROS, and NADPH oxidase inhibition. The similarity of these effects suggests that endothelial dysfunction with repeat H_2O_2 exposures could be explained by oxidation of BH_4 by $ONOO^-$ produced when H_2O_2 activates both eNOS and NADPH oxidase. These findings may have implications for the progression of disease states associated with oxidative stress.

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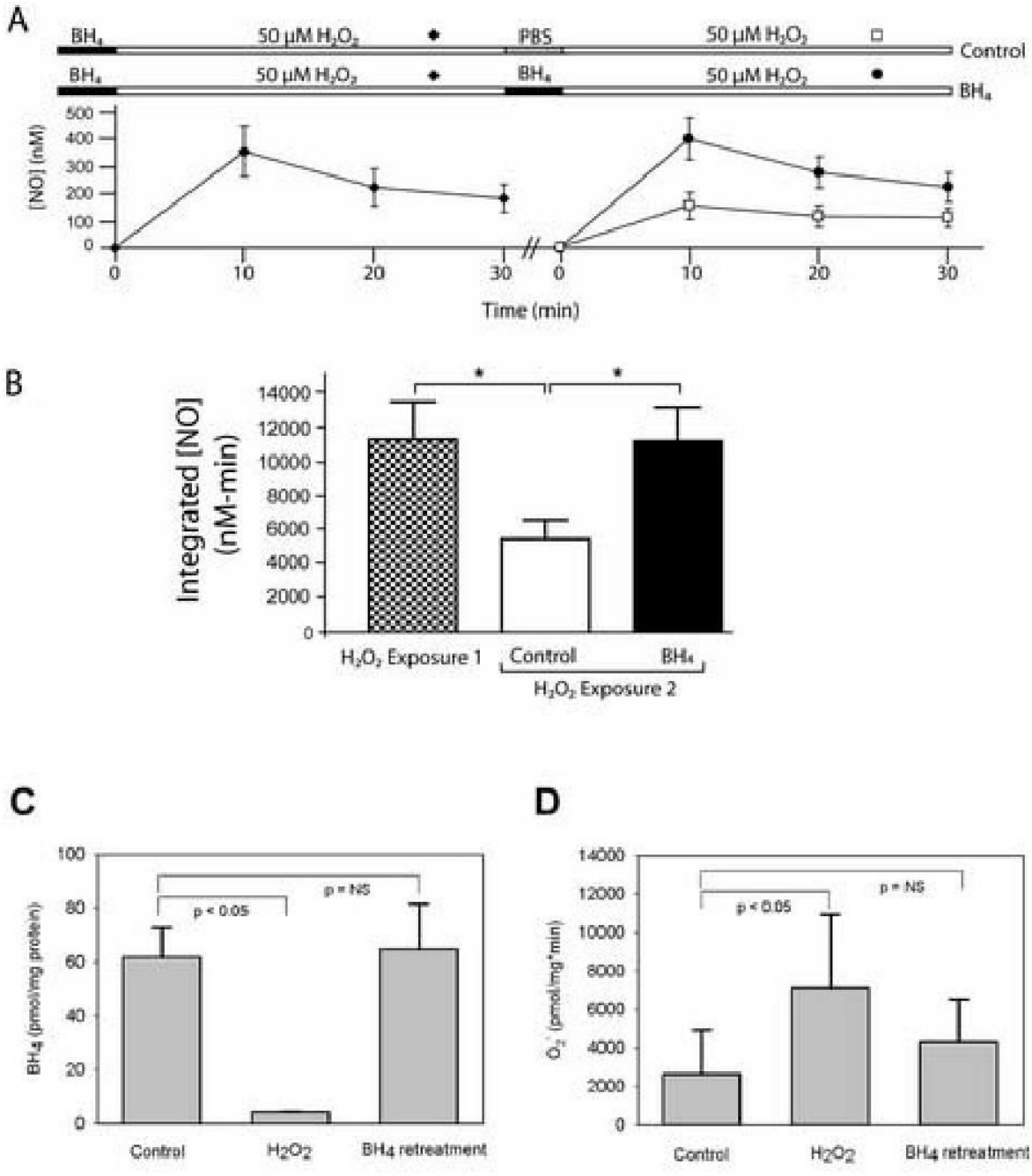

Figure 1.

H_2O_2-induced endothelial dysfunction. Panel A: Following addition of H_2O_2 to BAECs, NO• production increases initial but then decreases over time (n=9). Demonstrating endothelial dysfunction, a second addition of H_2O_2 reveals a statistically smaller increase in NO• production (n=9; $p<0.05$). BH_4 supplementation prior to rechallenging cells with H_2O_2 ameliorated the reduction in H_2O_2-induced NO• production (n=4). Panel B: BH_4 supplementation prevents a reduction in NO• production between the first and second H_2O_2 exposures (p=0.7). Panel C: The reduction in NO• production after an initial 30 min H_2O_2 exposure was accompanied by a decrease in cell BH_4 levels ($p<0.05$) that was completely corrected by BH_4 supplementation. Panel D: The reduction in NO• production after an initial

30 min H_2O_2 exposure was accompanied also by an increase in $O_2^{\bullet -}$ production ($p<0.05$) that was corrected by BH_4 supplementation.

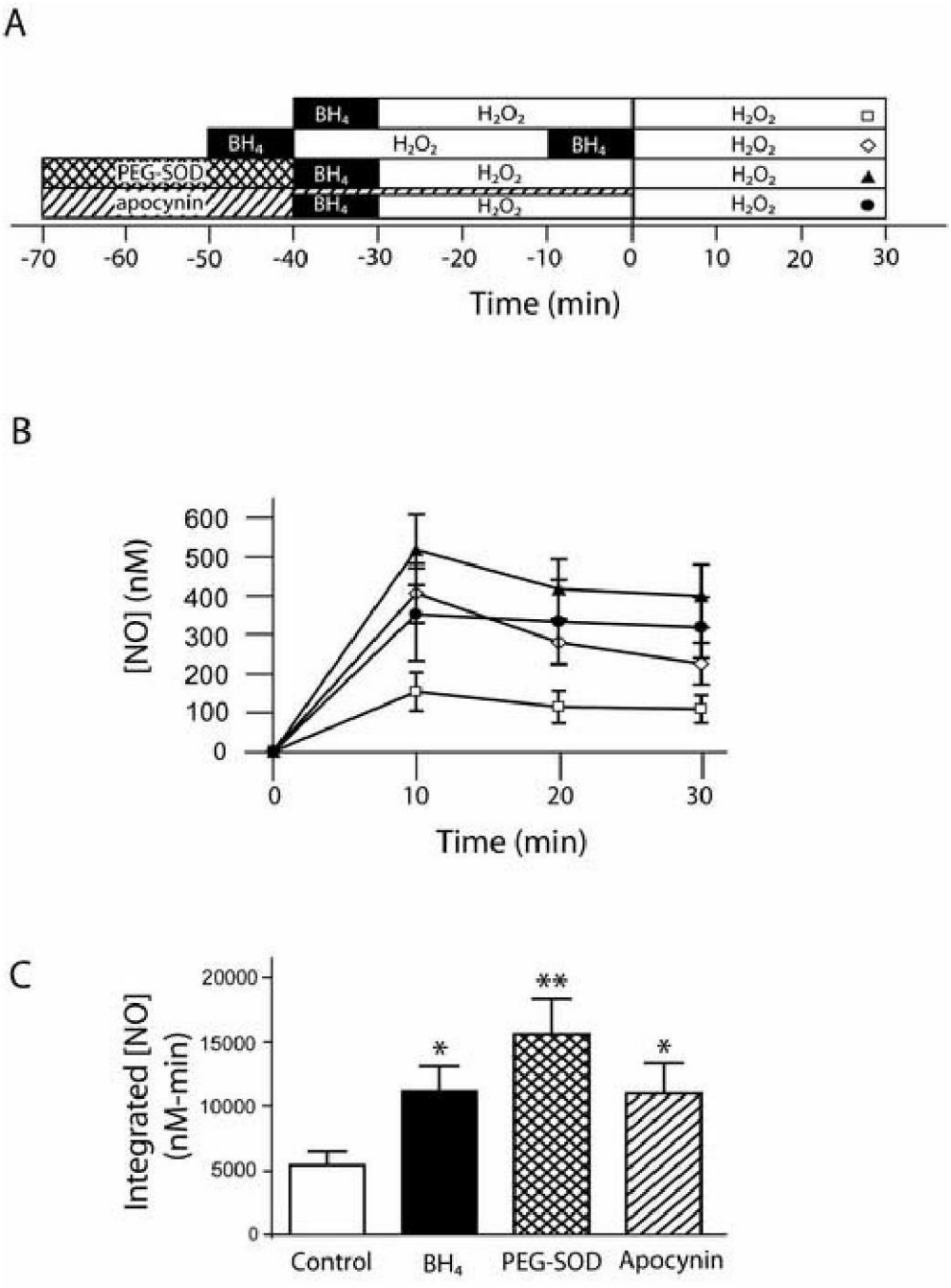

Figure 2.

Superoxide scavenging and NADPH oxidase inhibition prevent the endothelial dysfunction. Panel A: Time courses of treatments. Panel B: Comparison of NO• production over time during the second H_2O_2 exposure with control (n=9, open squares), BH_4 (n=4, open diamonds), PEG-SOD (n=4, filled triangles), and apocynin (n=4, filled circles) treatments. Panel C: Integrated NO• production over 30 min. BH_4, PEG-SOD, and apocynin treatments were statistically different from control (* = $p<0.05$, ** = $p<0.01$) but not from each other (p=0.3).

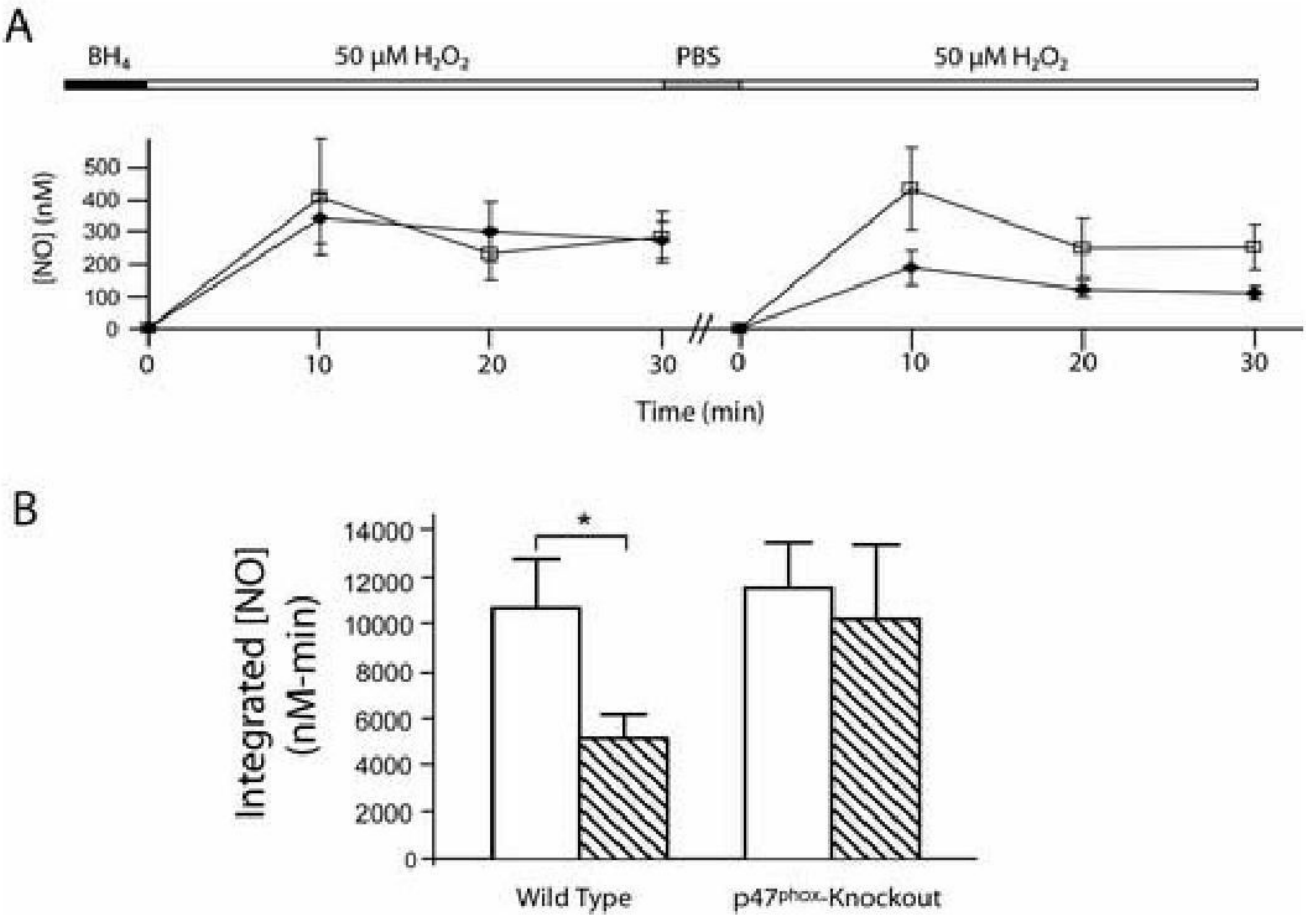

Figure 3.

Endothelial dysfunction with H_2O_2 exposure is prevented in $p47^{phox}$-knockout MAECs. Panel A: NO• production during subsequent H_2O_2 exposures was statistically different in wild-type cells (filled diamonds) but not in $p47^{phox}$-knockout cells (open squares). Panel B: Integrated NO• production over 30 min in wild type and $p47^{phox}$-knockout MAECs (H_2O_2 exposure 1, open bar, H_2O_2 exposure 2, diagonal stripes). Integrated NO• production was reduced with subsequent H_2O_2 exposures in wild type (* = $p<0.05$) but not in knockout cells ($p=0.7$).

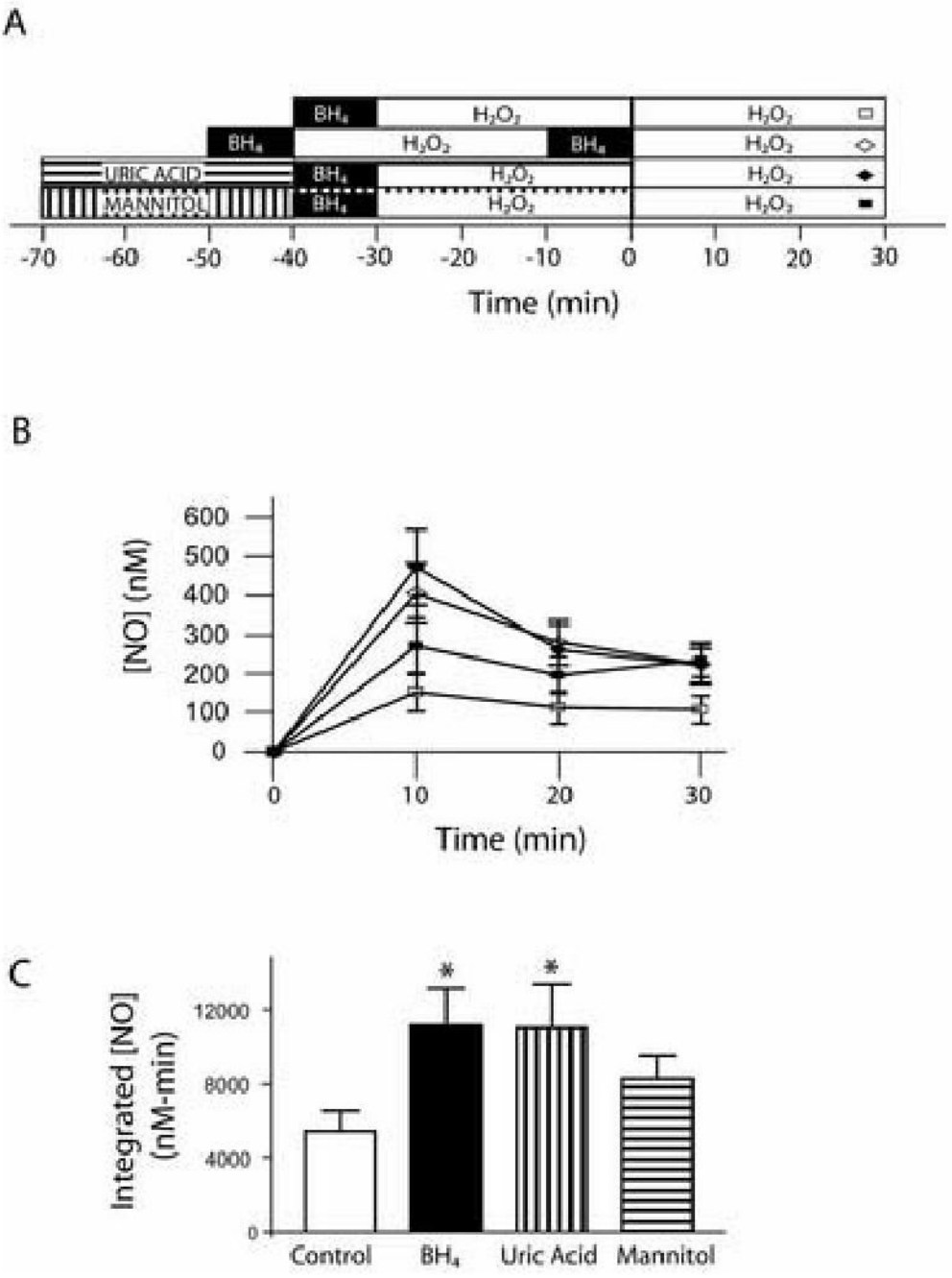

Figure 4.

$ONOO^-$ but not •OH scavengers prevent endothelial dysfunction. Panel A: Time course of treatments with $ONOO^-$ scavenger uric acid (100 μM), •OH scavenger mannitol (3 mM), and H_2O_2 (50μM). Panel B: The time courses of NO• production during H_2O_2 exposure 2 for control (open squares, n=9), BH_4 (open diamonds, n=4), uric acid (closed diamonds, n=4), and mannitol (closed squares, n=6) are shown. Panel C: Integrated NO• production over 30 min during H_2O_2 exposure 2 indicates that BH_4, uric acid, and mannitol improve NO• bioavailability when compared to control cells. Uric acid treatment resulted in a statistically significant improvement from control (* = $p<0.05$) that was indistinguishable from that of BH_4 ($p=0.65$). Mannitol did not improve statistically NO• bioavailability ($p>0.05$).

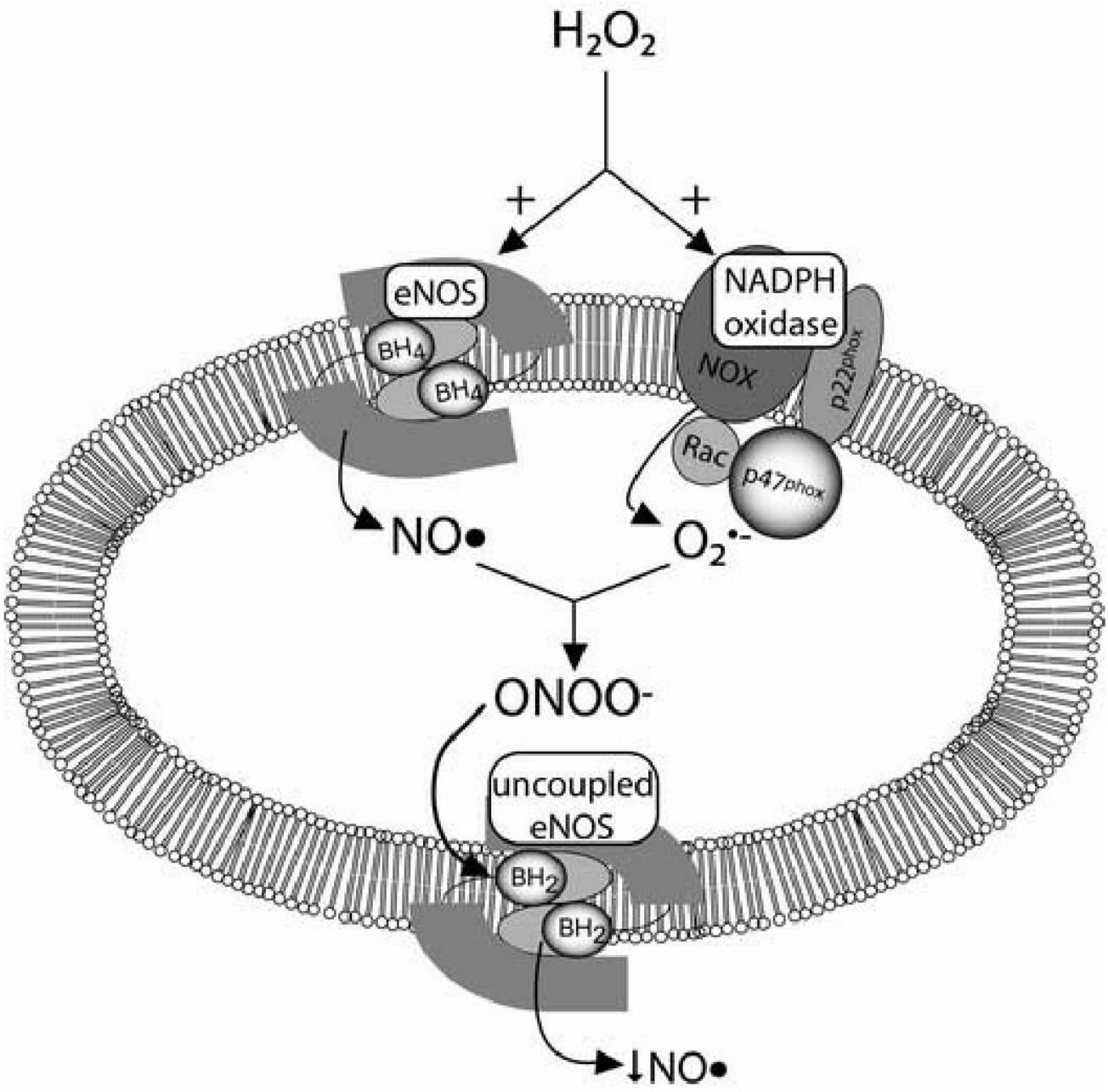

Figure 5.

A unified hypothesis explaining the NO• production decrease in response to H_2O_2 exposure. Simultaneous activation of eNOS and the NADPH oxidase results in $ONOO^-$. This species and possibly other ROS oxidize BH_4 to BH_2, causing uncoupling of eNOS. This reduces NO• measured on subsequent applications of H_2O_2.