Importance of endothelial nitric oxide synthase for the hypothermic protection of lungs against ischemia-reperfusion injury

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Objectives: The hypothesis that the protective effects of mild hypothermia against the pulmonary ischemia-reperfusion injury are mediated by endothelial nitric oxide synthase was tested.

Methods: Endothelial nitric oxide synthase knock-out and wild-type mice were sham operated or underwent a 1-hour occlusion of the left pulmonary hilum, followed by 5 hours of reperfusion. Temperature in the left pleural cavity during ischemia was maintained at either 36°C (normothermia) or 32°C (hypothermia). Inflammatory response (myeloperoxidase activity), endothelial barrier function (extravasation of Evans blue–labeled albumin), and endothelial nitric oxide synthase expression and phosphorylation were determined at the end of reperfusion.

Results: After normothermic ischemia both strains had a similar mortality (wild-type, 22.9%; knock-out, 15.4%), which was completely abolished by hypothermia. Endothelial barrier function was disturbed after normothermic ischemia in both wild-type and knock-out mice. Mild hypothermia significantly reduced pulmonary Evans blue extravasation in wild-type mice, but not in knock-out mice. Myeloperoxidase activity increased after normothermic ischemia to the same degree in both strains. This response was significantly attenuated by hypothermia in wild-type mice, but not in knock-out mice. In wild-type mice, endothelial nitric oxide synthase expression and phosphorylation were higher after hypothermic ischemia than after normothermic ischemia. No effect of ischemia on expression of inducible nitric oxide synthase was found in wild-type or knock-out mice.

Conclusion: Hypothermic protection against pulmonary ischemia-reperfusion injury is dependent on endothelial nitric oxide synthase and is associated with increased expression and phosphorylation of endothelial nitric oxide synthase.

Pulmonary dysfunction after cardiopulmonary bypass or lung transplantation is a frequently observed phenomenon that is associated with lung ischemia-reperfusion (I/R) injury. Among the main detrimental consequences of I/R are endothelial dysfunction and the inflammatory response. Release of nitric oxide (NO) may significantly modulate I/R injury. Numerous studies suggested that NO can exert both protective and deleterious effects depending on the amount and the source of NO production. At low concentrations, NO released by endothelial NO synthase (eNOS) is protective by preserving endothelial function and inhibiting the inflammatory response. However, higher concentrations of NO produced by inducible NO synthase (iNOS) can exacerbate reperfusion injury through formation of the toxic free radical peroxynitrite. During I/R, down-regulation of eNOS activity and up-regulation of iNOS has been described in several studies, and such alterations of the NO system are associated with endothelial dysfunction and proinflammatory reactions.
Abbreviations and Acronyms

EB = Evans blue
eNOS = endothelial nitric oxide synthase
iNOS = inducible nitric oxide synthase
nNOS = neuronal nitric oxide synthase
I/R = ischemia/reperfusion
KO = knock-out
MPO = myeloperoxidase
NO = nitric oxide
SEM = standard error of the mean
WT = wild-type

It has recently been reported that hypothermia can exert its protective effect against I/R injury by modulation of the NO system. Particularly, studies on brain7,8 and mesenteric9 tissue demonstrated the inhibition of iNOS/neuronal NOS (nNOS) expression and NO production by hypothermia during ischemia, leading to attenuation of the posts ischemic inflammatory response.3 Whether hypothermia produces similar effects on eNOS remains unclear. Using eNOS knock-out (KO) mice, we have recently shown that eNOS is crucial in protection of lungs against I/R injury by attenuation of leukocyte infiltration and adhesion molecule expression.10 In the present study, applying the same model, we tested whether eNOS plays a role in hypothermia-induced protection against I/R injury of lungs.

Material and Methods

Surgical Procedure

All animal experiments were carried out in accordance with the guidelines of the local regulatory agencies and confirmed with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Six- to eight-week-old female wild-type (WT, n = 68) mice (C57BL/6NCrl; Charles River, Sulzfeld, Germany) and eNOS knock-out (KO, n= 50) mice (B6.129P2-Nos3; Charles River) were randomly divided into four groups: group I: WT, normothermic lung ischemia (n = 35); group II: WT, hypothermic lung ischemia (n = 25); group III: KO, normothermic lung ischemia (n = 19); group IV: KO, hypothermic lung ischemia (n = 26). Five further animals of each species were sham-operated and 3 WT mice were performed without lung ischemia.

Endothelial Barri er Function

Capillary permeability was evaluated by measuring extravasation of Evans blue (EB) dye in the lung tissue. EB binds to serum albumin and has been extensively used as a tracer for transcapillary flux of macromolecules. EB was slowly injected into the left internal jugular vein (30 mg/kg, dissolved in 200 µL saline). Thirty minutes later the chest was opened and the lungs were perfused with 5 to 10 mL of phosphate buffer via a pulmonary arterial catheter to remove excess intravascular dye. Lungs were excised, homogenized, and incubated in 100% formamide (1 mL per 100 mg lung) at 37°C for 24 hours to extract EB dye. The concentration of EB dye extracted in formamide was analyzed by spectrophotometry at wavelengths of 620 and 740 nm. The correction of optical densities (E) for contaminating heme pigments was calculated as described previously11: E620 (corrected) = E620 – (1.426 × E740 + 0.03), and was expressed in arbitrary units.

Western Blotting

Frozen lung tissue was weighed and homogenized in ice-cold lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, 250 mmol/L sucrose, 20 mmol/L 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, 20 mmol/L phenylmethylsulfonyl fluoride, pH 7.4). After centrifugation at 16,000g for 15 minutes at 4°C, the supernatant was used for Western blotting. Fifty micrograms of protein per lane were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% gels. Equal loading of lines was confirmed by Coomassie blue staining. Gels were transferred to a nitrocellulose membrane (Hybond-ECL; GE Healthcare, Piscataway, NJ). The membranes were then blocked and incubated with the following primary antibodies overnight at 4°C: anti-eNOS (Calbiochem, EMD Biosciences, San Diego, Calif), anti-iNOS (BD Transduction Laboratories, Lexington, Ky), and anti-phosphorylated eNOS, specific to phosphorylated Ser-1177, (Calbiochem). Horseradish peroxidase–conjugated secondary antibody (goat anti-mouse immunoglobulin, Bio-Rad Microscience, Hercules, Calif) was used at 1:6,000 dilution. After being washed with TBS-Tween, proteins were detected with the use of opti-4CN Substrate (Bio-Rad). Optical densities of protein bands were normalized in each case to the density of β-actin band from the same sample and expressed in relative units.
Myeloperoxidase (MPO) Activity Assay
Neutrophil infiltration in the lungs was assessed by measuring the activity of myeloperoxidase (MPO), an enzyme specific for granulocyte lysosomes. This assay has been found to be a more sensitive method for the detection of entrapped neutrophils than quantitative histology. We modified the method first described by Laight and associates. In brief, lungs were washed with phosphate buffer and homogenized by ultrasonicator for 10 seconds in 1 mL of hexadecyl-trimethyl-ammoniumbromide (Sigma) buffer, followed by 3 freeze-and-thaw cycles. The tissue homogenate was incubated at 60°C for 2 hours to exclude nonthermore- stable peroxidases and centrifuged at 12,000 g for 10 minutes. Forty microliters of the supernatant was then allowed to react with 400 μL of 3,3-5,5 tetramethylbenzidine (Sigma). Changes in extinction at a wavelength of 655 nm were measured by photometer over 3 minutes at 30-second intervals. MPO activity was expressed in units per gram of lung tissue. One unit of MPO activity was defined as an increase of optical density by 1.0 per minute.

Statistical Analysis
Data are presented as mean ± SEM. Statistical analysis between groups was performed by 1-way analysis of variance followed by the Bonferroni post hoc test.

Results
Mortality
During 5 hours of reperfusion, mortality after normothermic (36°C) ischemia was 22.9% in WT mice (n = 8/35) and 15.4% in KO mice (n = 3/19), which was not significantly different between the groups (the Fisher exact test). Mild hypothermia (32°C) during ischemia completely abolished the postischemic mortality in both WT and KO mice (0% each). Similarly, no mortality was observed in sham-operated animals.

Endothelial Barrier Function
In sham-operated animals EB tissue content did not differ significantly between WT and KO mice. One hour of normothermic ischemia followed by 5 hours of reperfusion dramatically increased EB content in injured lungs in both mouse types to the same degree (Figure 1). Mild intrasenchymal hypothermia demonstrated a marked protection of endothelial function in WT mice: EB content (arbitrary units) in injured lungs was 0.179 ± 0.024 (n = 6) after normothermic ischemia versus 0.083 ± 0.019 (n = 5; P = .01) after hypothermic ischemia. In contrast, hypothermia failed to preserve endothelial function in KO mice: similar EB content in lungs after normothermic and hypothermic ischemia was observed.

Inflammatory Response
Tissue MPO activity was used as a quantitative marker of inflammatory response to I/R-induced injury. It was slightly but significantly higher in sham-operated KO animals than in WT mice: MPO activity (units per gram tissue) was 4.0 ± 0.68 (n = 5) in KO mice versus 1.8 ± 0.32 (n = 10) in WT mice (P = .001). This finding is in line with previously described increased baseline rolling and endothelial adherence of leukocytes in eNOS KO mice. After normothermic ischemia, MPO activity was significantly elevated in both WT and KO mice in comparison with sham-operated animals (Figure 2). The inflammatory response was significantly attenuated by hypothermia during the ischemic period in WT mice: 10.0 ± 1.1 (n = 5) after normothermic ischemia versus 6.84 ± 0.56 (n = 6) after hypothermic ischemia (P = .016). However, no significant effect of hypothermia on MPO activity was observed in KO mice.
NOS Isoform Expression

Protein expression of eNOS and iNOS after pulmonary I/R was analyzed by Western immunoblotting. In WT mice, normothermic ischemia followed by reperfusion did not significantly change eNOS protein expression (Figure 3), whereas reperfusion after hypothermic ischemia significantly increased expression of eNOS by approximately 2-fold ($P < 0.01$). The further analysis of the phosphorylation status of eNOS at Ser-1177, which is critical for eNOS activation, revealed a significant increase of phosphorylated eNOS in WT mice after both normothermic and hypothermic ischemia in comparison with sham-operated animals. The increase of eNOS phosphorylation was, however, significantly higher ($P = 0.033$) after hypothermic ischemia than after normothermic ischemia.

Because iNOS may also participate in postischemic tissue injury,$^2$ the expression of this NOS isoform was also analyzed. Baseline expression of iNOS was detectable in sham-operated animals. However, no effects of I/R in WT and KO mice on iNOS protein expression were observed (Figure 4). To exclude the false-negative results, animals were treated with 10 mg/kg lipopolysaccharide (LPS) from *Escherichia coli*, which resulted in a significant increase of iNOS expression 24 hours after treatment.

Discussion

The aim of this study was to test the importance of eNOS in intraschemic hypothermia-induced protection against I/R lung injury. Using a transgenic eNOS KO mouse model, we found that the hypothermia-induced preservation of endothelial barrier function and attenuation of the postischemic inflammatory response were eNOS dependent. This protective effect of hypothermia was associated with significant
up-regulation of the posts ischemic expression and phosphorylation of eNOS in the lungs.

Injury of the endothelium is a common consequence of an ischemic insult. During reperfusion, release of oxygen radicals and proinflammatory cytokines can additionally jeopardize endothelial function. In a previous study, we observed that the inflammatory response reached its peak 5 hours after ischemia. Similarly, in the present study, along with an increase of the MPO activity, the content of EB dye in lungs increased significantly after 1 hour of normothermic ischemia and 5 hours of reperfusion in both WT and KO mice, indicating a pronounced disturbance of endothelial integrity. Therefore, applying endothelial barrier function and inflammatory response as end points of assessment of the posts ischemic pulmonary injury, we analyzed the role of eNOS in the protective effect of hypothermia. For this purpose a mild hypothermia (32°C) was chosen to minimize the side effects of hypothermic exposure. Such mild hypothermia seems to play a substantial role in hypothermia-induced protection of lungs. Indeed, two lines of evidence demonstrate the importance of eNOS in the present study. First, hypothermia-induced protection against I/R injury was completely abolished in eNOS KO mice. Second, hypothermia was associated with posts ischemic up-regulation of eNOS.

There is increasing evidence indicating that eNOS plays an essential role in posts ischemic organ protection. Particularly, eNOS activity has been shown to be important for preservation of endothelial function and attenuation of posts ischemic inflammation in different tissues. Regarding lung tissue, only a few studies emphasized the function of eNOS in I/R injury. Our previous study suggested that up-regulation of eNOS attenuates endothelial cell-leukocyte adhesion via suppression of vascular cell adhesion molecule expression. A significant up-regulation of eNOS was, however, observed only 24 hours after reperfusion. In the present study, with the temperature being lowered during ischemia, up-regulation of eNOS expression occurred as early as 5 hours after ischemia. Thus, the acceleration of eNOS expression might be one of the intrinsic mechanisms of hypothermia-induced protection against I/R injury. In accordance with this finding, Liu and coworkers also demonstrated a marked increase of eNOS expression in rat lungs after hypothermic ischemia within 2 hours of reperfusion. How hypothermia may affect the eNOS-expression timing is still unclear. The eNOS expression is a function of the balance between the protein synthesis and degradation. The regulation of these processes is complex, and the precise analysis of the mechanisms involved in hypothermia-induced eNOS up-regulation was beyond the scope of the present study.

Conclusion

In summary, applying the murine eNOS KO model, we have provided evidence that protection against pulmonary I/R injury by mild intraschemic hypothermia is eNOS dependent and is accompanied by up-regulation of eNOS expression and phosphorylation. Further analysis of this eNOS-dependent mechanism of hypothermic protection could
provide new strategies for better pulmonary preservation and prevention of I/R injury.

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References