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Role of Superoxide Anion on Basal and Stimulated Nitric Oxide Activity in Neonatal Piglet Pulmonary Vessels

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ABSTRACT

The superoxide anion ($O_2^{\cdot -}$) appears to be an important modulator of nitric oxide bioavailability. Enzymatic scavenging of $O_2^{\cdot -}$ is carried out by superoxide dismutase (SOD). The present study was designed to characterize the developmental changes on pulmonary vascular reactivity induced by *1*) exogenous Cu/Zn SOD, *2*) several putative SOD mimetics, and *3*) endogenous SOD inhibition. We also analyzed age-related changes on pulmonary SOD activity and vascular $O_2^{\cdot -}$ levels. SOD (1–300 U/mL) produced endothelium-dependent relaxation of U46619-contracted intrapulmonary arteries (fourth branch) and veins from 12- to 24-h-old and 2-wk-old piglets. SOD-induced relaxation was greater in pulmonary arteries and was abolished by the nitric oxide synthase inhibitor *N*ω-nitro-L-arginine methyl ester. SOD induced a greater pulmonary artery relaxation in the 2-wk-old than in the 12- to 24-h-old piglet. SOD (100 U/mL) did not modify acetylcholine-induced relaxation in pulmonary arteries. In contrast, endogenous SOD inhibition by diethyldithiocarbamate (3 mM) impaired acetylcholine-induced relaxation in pulmonary arteries from newborn but not from 2-wk-old piglets. Total SOD activity in lung tissue did not change with postnatal age. With the use of dihydroethidium, an oxidant-sensitive fluorescent probe, we did not find significant age- or vessel-related differences in $O_2^{\cdot -}$ presence. From the putative SOD mimetics tested, only the metal salts $MnCl_2$ and $CuSO_4$ reproduced the vascular effects of SOD. In summary, SOD produces endothelium-dependent pulmonary vascular relaxation by protecting nitric oxide from destruction by $O_2^{\cdot -}$. This effect was less marked in newborns than in 2-wk-old piglets. In contrast, pulmonary arteries from newborn piglets are more sensitive to the inhibition of endogenous SOD. **(*Pediatr Res* 54: 372–381, 2003)**

Abbreviations

ACh, acetylcholine
DETCA, diethyldithiocarbamate
DHE, dihydroethidium
L-NAME, *N*ω-nitro-L-arginine methyl ester
MnTMPyP, Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin
NBT, nitro blue tetrazolium
NO˙, nitric oxide
$ONOO^-$, peroxynitrite
PEG-SOD, polyethylene glycosylated SOD
PPHN, persistent pulmonary hypertension of the newborn
PTIYO, 4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide
SOD, superoxide dismutase
U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$

Successful adaptation of the newborn to postnatal conditions requires a dramatic transition of the pulmonary circulation from a high-resistance state *in utero* to a low-resistance state within minutes after birth. Some infants fail to achieve or sustain this normal decrease in pulmonary vascular resistance, which leads to severe respiratory distress and hypoxemia, referred to as PPHN (1)

NO˙, a free radical species produced by a wide variety of cell types, has gained recognition as a key mediator of diverse physiologic and pathologic processes, including the regulation of pulmonary vascular tone and the perinatal adaptation of the lung circulation (1). The free radical $O_2^{\cdot -}$ has been shown to interact with NO˙, preventing its vasodilator activity and producing the binary toxin $ONOO^-$ (2). The favorable kinetics of the reaction between NO˙ and $O_2^{\cdot -}$ intrinsically makes vascular $O_2^{\cdot -}$ levels an important determinant of NO˙ biologic activity (2). Enzymatic scavenging of $O_2^{\cdot -}$ is carried out by SOD (2).

The pulmonary vessels are continually exposed to free radicals and reactive oxygen species originating from inspired air

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or inflammatory cells, and pulmonary vascular tone is dramatically altered by reactive oxygen species such as $O_2^{\cdot -}$ (3, 4). At birth, the newborn encounters a much more oxygen-enriched world than the relatively hypoxic fetus. Investigators found late gestational chronology for the maturation of the protective pulmonary antioxidant enzymes in several species including humans (5–7). It has been suggested that $O_2^{\cdot -}$ accumulation combined with deficiencies in SOD activity may transiently compromise basal and agonist-induced $NO^{\cdot}$ activity in pulmonary vessels at birth (8). In a lamb model of PPHN, Steinhorn *et al.* (9) demonstrated that a single intratracheal dose of SOD reduced pulmonary vascular resistance and improved the pulmonary vasodilatory action of inhaled $NO^{\cdot}$. In contrast, Carpenter *et al.* (10) did not observe changes in pulmonary vascular resistance after i.v. infusion of SOD in 3- to 7-d-old lambs. Moreover, we have reported that exogenous SOD improved the response to exogenous $NO^{\cdot}$ in pulmonary arteries from 2-wk-old but not from newborn piglets (11). Therefore, our current knowledge about the influence of reactive oxygen species on pulmonary vascular reactivity in the perinatal period is rather limited.

Authentic SOD is known to produce endothelium-dependent relaxation by protecting basal $NO^{\cdot}$ from destruction by endogenously produced $O_2^{\cdot -}$ (12, 13). The aim of the present study was to determine the effects of SOD supplementation and inhibition on basal and stimulated $NO^{\cdot}$ activity in neonatal pulmonary vessels. We also examined the ability of several putative SOD mimetics to induce changes in pulmonary vascular contractility. The SOD mimetics examined were the simple metal salts $CuSO_4$ and $MnCl_2$, the spin trap agents tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), tempol (4-hydroxy 2,2,6,6-tetramethylpiridine-1-oxyl), and PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide), and the metal-based compound MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin). Some of these compounds have been shown to share the ability of SOD to promote endothelium-dependent relaxation (13).

METHODS

Tissue preparation. Experimental procedures followed Dutch laws for animal experimentation. Neonatal piglets aged 12–24 h ($n = 24$) and 2 wk ($n = 25$), obtained from a local farm, were killed by exsanguination after being anesthetized with sodium pentobarbitone (100 mg/kg). The lungs were rapidly immersed in cold (4°C) Krebs-Ringer bicarbonate buffer (composition in mM: NaCl, 118.5; KCl, 4.75; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; glucose, 5.5). Pulmonary arteries and veins (fourth branch, *in situ* external diameter 1–2 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm of length under a dissection microscope (11, 14). In some experiments, the endothelium of the vessels was removed by gently rubbing the intimal surface of the rings with a metal rod. For SOD activity assays, lung and liver tissue samples (50–100 mg) were flash-frozen in liquid nitrogen and stored at −80°C until studied.

Isometric force measurement. After dissection, two L-shaped stainless-steel wires were inserted into the arterial lumen, and the rings were introduced in Allhin organ chambers filled with Krebs solution at 37°C, gassed with 95% O_2-5% CO_2. One wire was attached to the chamber and the other to an isometric force-displacement transducer (model PRE 206-4, Cibertec, Madrid, Spain). The isometric force signal was amplified, converted from analog to digital form (PowerLab; AD-Instruments, Castle Hill, Australia), and recorded (Chart v3.4; AD-Instruments). The rings were initially stretched to a resting tension of 0.3 g (pulmonary arteries of 12- to 24 h-old animals) or 0.5 g (pulmonary arteries of 2-wk-old animals and pulmonary veins of both groups) and allowed to equilibrate for 60–90 min. During this period tissues were restretched and washed every 30 min with warm Krebs solution.

After equilibration, the rings were precontracted with the thromboxane A_2 mimetic U46619 (30 nM). In previous experiments, we demonstrated that this concentration produces approximately 60–70% of the maximal U46619-induced contraction in piglet pulmonary vessels (14). A number of the experimental procedures used, however, affected the level of tone induced by U46619 (*e.g.* L-NAME and DETCA enhanced and SOD reduced U46619-induced contraction). To compensate for these changes in sensitivity, the concentrations of U46619 were adjusted (10–100 nM) to ensure that the level of tone was 60–70% of the maximum seen in control rings. Independently of their effect on vascular tone, the different concentrations of U46619 could directly interfere with vascular relaxation. Subsequently, control studies were carried out by precontracting pulmonary arteries and veins of both groups of age with U46619 (10, 30, and 100 nM) to demonstrate that U46619 concentration did not affect ACh-induced relaxation (data not shown). All experiments were conducted in the presence of catalase (1000 U/mL) to prevent the accumulation of H_2O_2.

When the U46619-induced contractile response reached a stable tension, cumulative concentration-response curves were constructed for Cu/Zn SOD (0.1–300 U/mL) and the SOD mimetics. The SOD mimetics examined were $CuSO_4$ (100 nM–1 mM), $MnCl_2$ (100 nM–1 mM), tiron (100 nM–1 mM), tempol (100 nM–1 mM), PTIYO (100 nM–1 mM), and MnTMPyP (10 nM–10 μM). In some experiments, the vascular effects of SOD or the SOD mimetics were tested in the presence of the $NO^{\cdot}$ synthase inhibitor L-NAME (0.1 mM).

Agonist-stimulated activity of $NO^{\cdot}$ was determined by assessing ACh-induced relaxation. When the effects of authentic exogenous Cu/Zn SOD (100 U/mL) on ACh-induced relaxation were assessed, SOD was given as a 20-min pretreatment before concentration-response curves to ACh were constructed. In these experiments, rings were constricted with a higher concentration of U46619 (100 nM) to compensate for the reduction in U46619-induced tone produced by SOD. Using a similar protocol, the effects of PEG-SOD (100 U/mL) on ACh-induced relaxation were also assessed.

In certain experiments, the effects of irreversible inhibition of endogenous Cu/Zn SOD with the copper chelator DETCA (15) were investigated on relaxations induced by ACh. In these experiments, vascular rings were incubated with DETCA (3

mM) for 60 min before being repeatedly washed out. The tissues were then contracted with U46619, and cumulative concentration-response curves to ACh were constructed. Some of these experiments were performed in the presence of SOD (100 U/mL) or PEG-SOD (100 U/mL). SOD and PEG-SOD were given as a 20-min pretreatment after DETCA incubation and were maintained during the concentration-response curve to ACh.

SOD activity measurement. Lung and liver tissue were homogenized in 1 mL of ice-cold buffer (145 mM NaH_2PO_4, pH 7.4), and the homogenate was centrifuged (2500 × *g*, 5 min). Total SOD activity in the supernatant was measured by monitoring the SOD-induced inhibition of NBT reduction to blue formazan by the $O_2^{\cdot -}$ generated in a xanthine/xanthine oxidase system (16). Briefly, 10 μL of supernatant was added to 940 μL of assay solution (consisting of 50 μM xanthine and 50 μM NBT in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 50 μL of a 1 U/mL xanthine oxidase solution and mixing well. The rate of increase in absorbance was continuously recorded with a spectrophotometer (Lambda 2; PerkinElmer Inc., Shelton, CT, U.S.A.) at 560 nm for 2 min. One unit of SOD was defined as the quantity of SOD required to produce 50% inhibition of the rate of reduction of NBT. The activity of SOD was standardized against milligrams of protein per milliliter. The amount of protein in the samples was determined using the bicinchoninic acid assay (using BSA as the standard).

$O_2^{\cdot -}$ detection. DHE, an oxidative fluorescent dye, was used to localize $O_2^{\cdot -}$ in vessel segments *in situ*. DHE can enter the cell and be oxidized by $O_2^{\cdot -}$ to yield ethidium, which binds to DNA to produce bright red fluorescence. The increase in ethidium-DNA fluorescence is suggestive of $O_2^{\cdot -}$ production within cells (17, 18).

Vascular rings were opened longitudinally, transferred to a 12-well plate, and incubated for 1 h in Krebs solution (at 37°C and gassed with 95% O_2-5% CO_2) in the presence of vehicle, DETCA (3 mM), or PEG-SOD (100 U/mL). Then, the vascular segments were repeatedly washed out and incubated at room temperature for 30 min in Krebs solution containing DHE (10 μM). After being washed out, segments were placed, endothelial face up, on a microscope slide and enclosed with Immumount (Shandon, Pittsburgh, PA, U.S.A.) and a coverslip. Preparations were examined on a Leica DM RXA fluorescence microscope. Using a 20× objective, three separated areas of each preparation (368 × 287 μm, 736 × 574 pixels) were photographed with a digital charge-coupled device camera. Images were saved for off-line analysis. Ethidium-stained cells were counted by a single observer (unaware of the experimental conditions) with the assistance of an automated image analysis software (SigmaScan Pro; Jandel Scientific, San Rafael, CA, U.S.A.).

Drugs. L-NAME, U46619, SOD (Cu/Zn superoxide dismutase from bovine erythrocytes), PEG-SOD (from bovine erythrocytes), catalase (from bovine liver), xanthine, xanthine oxidase, NBT, DETCA, tiron, tempol, PTIYO, $CuSO_4$, and $MnCl_2$ were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), ACh chloride from Janssen Chimica (Beersen, Belgium), MnTMPyP from Alexis Corporation Ltd (Nottingham, U.K.), and DHE (5 mM solution in DMSO) from Molecular Probes Europe (Leiden, The Netherlands). All the drugs were dissolved initially in distilled deionized water and further dilutions were made in Krebs-Ringer bicarbonate buffer.

Analysis of data. Results are expressed as mean ± SEM, and *n* reflects the number of animals. The contractile responses were expressed as absolute values (grams), and the relaxant responses as a percentage of the precontractile tone. The significance of differences between mean values was assessed by one-way ANOVA followed by Bonferroni *post hoc t* test (for parameters normally distributed) or by the Mann-Whitney *U* test (for parameters nonnormally distributed). Differences were considered significant at a $p < 0.05$.

RESULTS

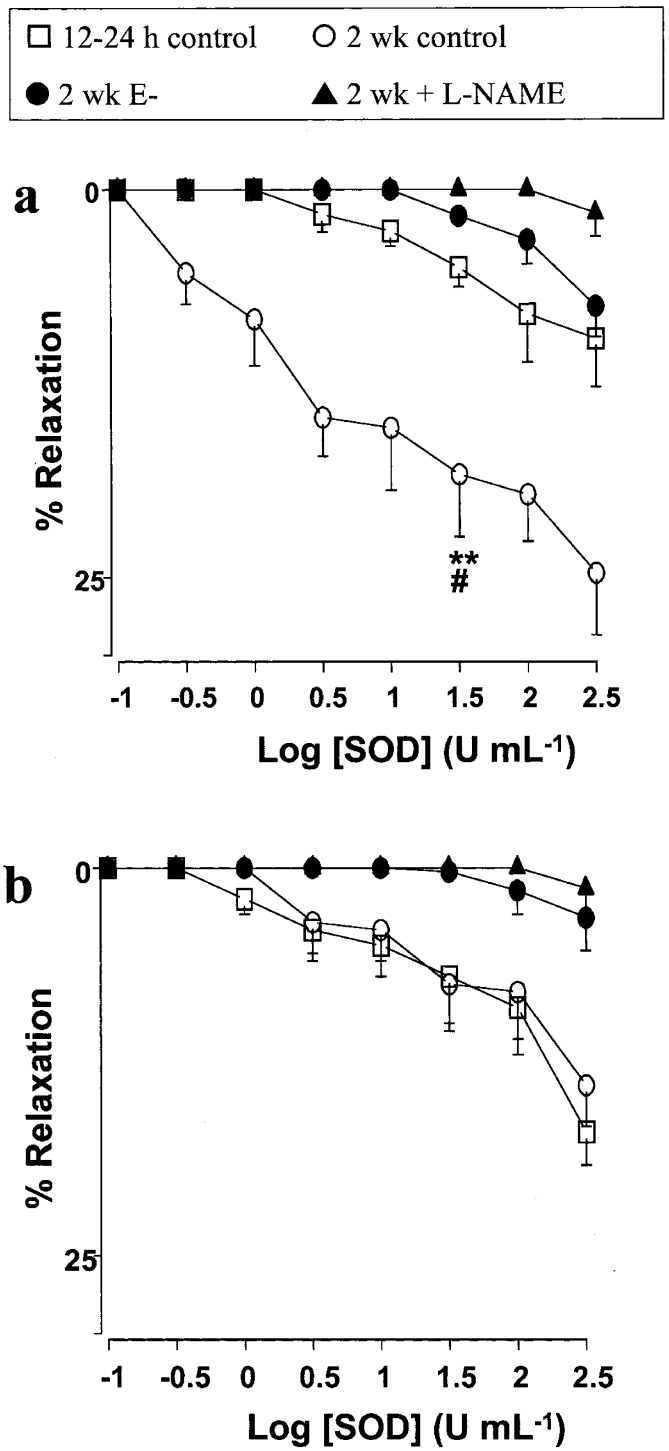
Effects of SOD on basal and ACh-stimulated activity of NO. During U46619-induced contraction in endothelium-containing rings, SOD produced a concentration-dependent relaxation in pulmonary arteries and pulmonary veins from 12- to 24-h-old and 2-wk-old piglets (Fig. 1). The amplitude of SOD-induced relaxation increased with postnatal age in pulmonary arteries but not in pulmonary veins (Fig. 1). In the 2-wk-old piglets, SOD-induced relaxation was significantly ($p < 0.05$) greater in pulmonary arteries (relaxation at the maximum SOD concentration tested was 24.7 ± 3.38%) than in pulmonary veins (17 ± 2.14%), whereas in the 12- to 24-h-old piglets SOD-induced relaxation was similar in pulmonary arteries and veins (Fig. 1).

In endothelium-denuded pulmonary vessels and in the presence of the $NO^{\cdot}$ synthase inhibitor L-NAME (0.1 mM), SOD-induced relaxation was significantly impaired. This was observed in vessels of 12- to 24-h-old (not shown) and 2-wk-old piglets (Fig. 1).

After contraction induced by U46619 (100 nM), treatment for 20 min with SOD (100 U/mL) failed to affect ACh-induced relaxation in pulmonary arteries from the two age groups studied (Fig. 2) and in pulmonary veins from 12- to 24-h-old piglets (Fig. 3). PEG-SOD (100 U/mL) also failed to affect ACh-induced relaxation in pulmonary arteries from both age groups (data not shown). In contrast, SOD significantly increased ACh-induced relaxation in pulmonary veins from 2-wk-old piglets (Fig. 3).

Effects of DETCA on ACh-induced relaxation. Treatment of endothelium-containing rings of piglet pulmonary vessels with DETCA (3 mM) for 60 min (followed by washout) to inactivate endogenous Cu/Zn SOD significantly enhanced U46619-induced tone (Fig. 4). DETCA treatment led to an impairment of ACh-induced relaxation in pulmonary arteries (Fig. 2) and pulmonary veins (Fig. 3) of newborn piglets. The blockade induced by DETCA was unaffected by treatment with exogenous SOD at 100 U/mL (Figs. 2 and 3). In contrast, PEG-SOD (100 U/mL) reversed DETCA-induced impairment of ACh-induced relaxation in neonatal pulmonary vessels (Figs. 2 and 3). DETCA did not affect ACh-induced relaxation in pulmonary vessels from 2-wk-old piglets (Figs. 2 and 3).

Effects of SOD mimetics on pulmonary vascular tone. $CuSO_4$ (Fig. 5) and $MnCl_2$ (Fig. 6) each relaxed endothelium



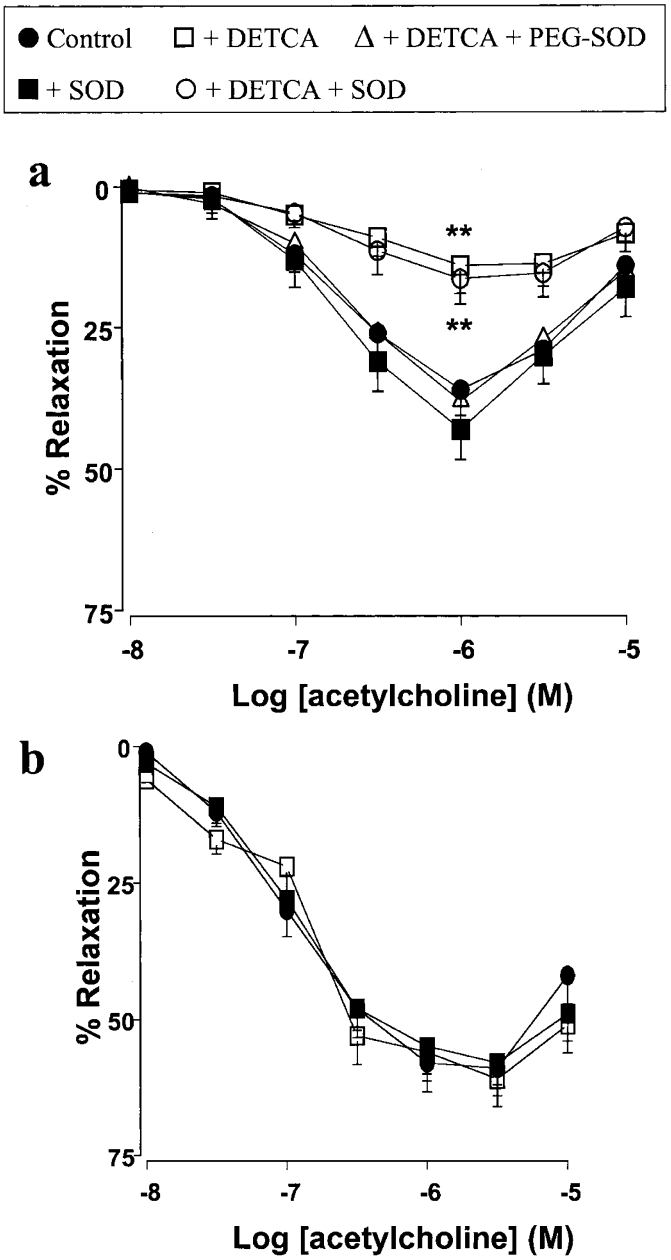


Figure 1. Concentration-dependent relaxant effects of Cu/Zn SOD in pulmonary arteries (*a*) and pulmonary veins (*b*) of 12- to 24-h-old ($n = 8$) and 2-wk-old ($n = 8$) piglets. The effects of pretreatment with the $NO^{\cdot}$ synthase inhibitor L-NAME (100 μM) and of endothelial removal (*E*-) are also shown (only in 2-wk-old piglets). Changes in tension induced by SOD are expressed as percent reversal of the contraction induced by U46619. Each point represents mean ± SEM. $^{**}p < 0.01$ 12- to 24-h-old *vs* 2-wk-old. $\#p < 0.05$ pulmonary artery *vs* pulmonary vein. Only levels of statistical significance for the highest difference are shown.

Figure 2. Effect of Cu/Zn SOD (100 U/mL) and SOD inhibition (by DETCA, 3 mM) on ACh-induced relaxation in endothelium-intact pulmonary arteries of 12- to 24-h-old (*a*, $n = 8$) and 2-wk-old (*b*, $n = 7$) piglets. Changes in tension induced by ACh are expressed as percent reversal of the contraction induced by U46619. Each point represents mean ± SEM. $^{**}p < 0.01$ *vs* control. Only levels of statistical significance for the highest difference are shown.

containing rings of pulmonary arteries and pulmonary veins, and this effect was abolished by the presence of L-NAME (Fig. 5; data of $MnCl_2$ -induced relaxation in the presence of L-NAME not shown). Pulmonary arteries from either 12- to 24-h-old or 2-wk-old animals were more sensitive ($p < 0.05$) to the relaxant effects of $CuSO_4$ compared with pulmonary veins. In contrast, no differences between arteries and veins were detected in $MnCl_2$-induced relaxations. Relaxations induced by $CuSO_4$ and $MnCl_2$ significantly increased with postnatal age in pulmonary arteries (Figs. 5 and 6).

Tiron, tempol, and PTIYO did not affect U46619-induced contractions in pulmonary arteries or pulmonary veins from the two age groups studied (data not shown).

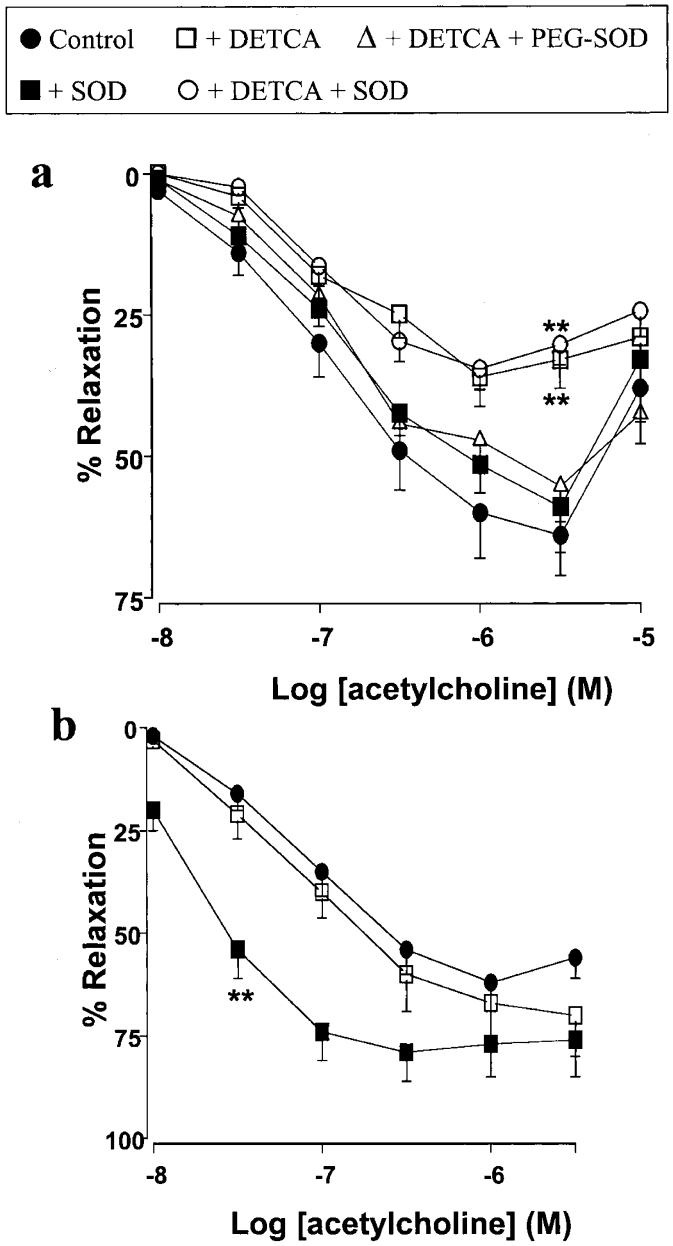


Figure 3. Effect of Cu/Zn SOD (100 U/mL) and SOD inhibition (by DETCA, 3 mM) on ACh-induced relaxation in endothelium-intact pulmonary veins of 12- to 24-h-old (*a, n* = 7) and 2-wk-old (*b, n* = 7) piglets. Changes in tension induced by ACh are expressed as percent reversal of the contraction induced by U46619. Each point represents mean ± SEM. $^{**}p < 0.01$ *vs* control. Only levels of statistical significance for the highest difference are shown.

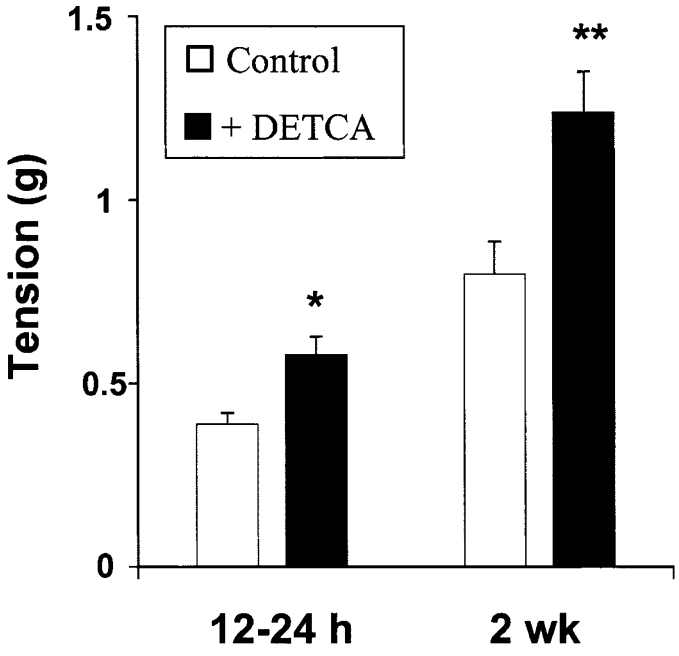


Figure 4. Effect of Cu/Zn SOD inhibition (by DETCA, 3 mM) on U46619 (30 nM)-induced tone in endothelium-intact pulmonary arteries of 12- to 24-h-old (n = 8) and 2-wk-old (n = 8) piglets. Each bar represents mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* control.

MnTMPyP produced an enhancement of U46619-induced tone in pulmonary arteries and pulmonary veins (Fig. 7). This effect of MnTMPyP was more marked in pulmonary arteries than in pulmonary veins and decreased with postnatal age in both types of vessels (Fig. 7). Pretreatment with L-NAME or SOD almost abolished the enhancement of U46619-induced tone produced by MnTMPyP (Fig. 7).

SOD activity. Total lung and liver SOD activities were not significantly different when measured in tissues from 12- to 24-h-old and 2-wk-old piglets (Fig. 8).

Detection of $O_2^{\cdot -}$ generation. After 30 min of incubation with the oxidative dye DHE, numerous fluorescent ethidium-stained cells were identified in segments of pulmonary arteries and veins (Fig. 9). The density of ethidium-stained cells increased after DETCA treatment and decreased after PEG-SOD treatment, confirming specificity for $O_2^{\cdot -}$ (Fig. 9). No differences in ethidium-stained cell density were observed between the two age groups or between pulmonary arteries and veins (Fig. 9).

DISCUSSION

Adequate $O_2^{\cdot -}$ scavenging appears to be imperative for normal $NO^{\cdot}$-mediated vasomotor function. The present study was designed to determine the effect of SOD activity augmentation and inhibition on $NO^{\cdot}$-mediated relaxations in neonatal pulmonary vessels. We demonstrated that exogenous Cu/Zn SOD induced endothelium-dependent relaxation in pulmonary arteries and pulmonary veins from newborn and 2-wk-old piglets. SOD-induced relaxation increased with postnatal age in pulmonary arteries and was impaired by the presence of L-NAME, a $NO^{\cdot}$ synthase inhibitor. This suggests that SOD induced its relaxant effects by protecting basal $NO^{\cdot}$ from the destructive action of endogenously produced $O_2^{\cdot -}$. We also demonstrated that inhibition of endogenous Cu/Zn SOD by DETCA produced an impairment of ACh-induced endothelium-dependent relaxation in neonatal pulmonary vessels. This effect of DETCA on ACh-induced relaxation was not affected by exogenous Cu/Zn SOD supplementation, but was reversed by PEG-SOD. Finally, we have tested several putative SOD mimetics and observed that the ability of Cu/Zn SOD to promote endothelium-dependent relaxation is shared by $CuSO_4$ and $MnCl_2$, but not by tiron, tempol, or PTIYO. The low molecular weight manganese-porphyrin-based SOD mimetic, MnTMPyP, produced an augmentation of vascular tone that

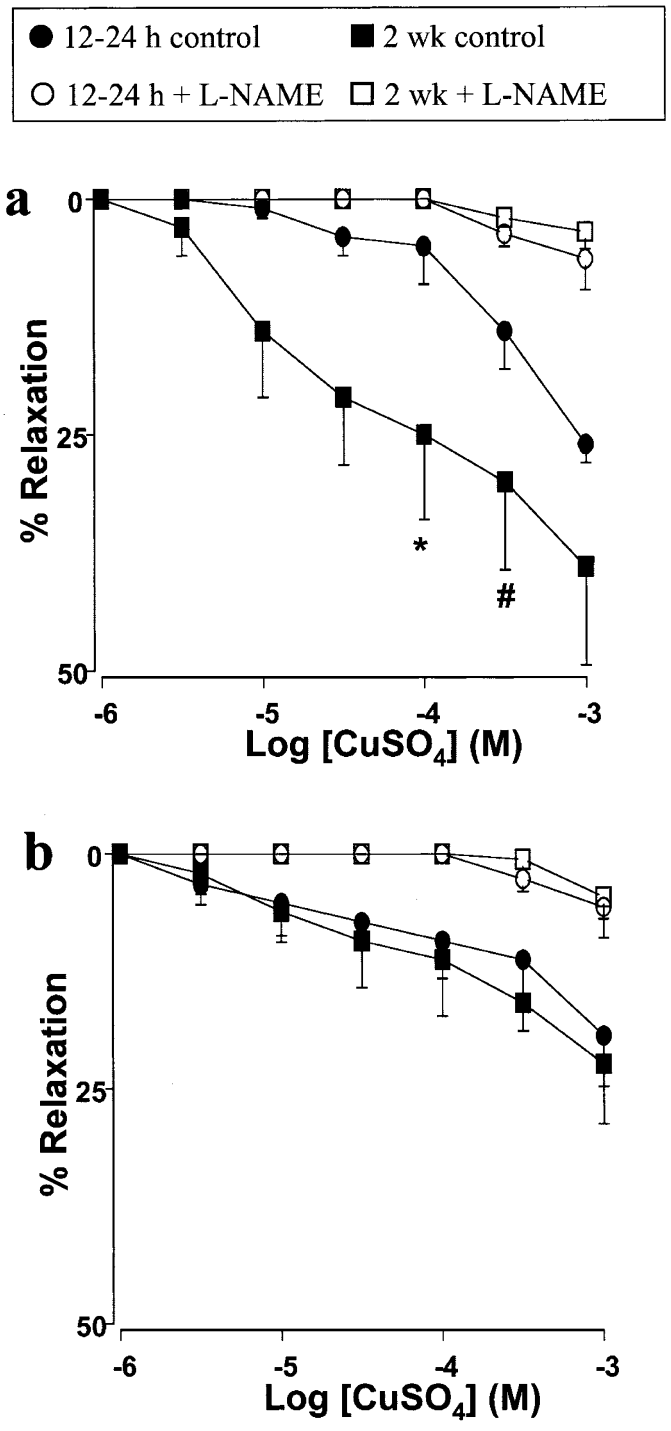


Figure 5. Concentration-dependent relaxant effects of $CuSO_4$ in endothelium-containing pulmonary arteries (*a*) and pulmonary veins (*b*) of 12- to 24-h-old ($n = 7$) and 2-wk-old ($n = 7$) piglets. The effects of pretreatment with the NO· synthase inhibitor L-NAME (100 μM) are also shown. Changes in tension induced by $CuSO_4$ are expressed as percent reversal of the contraction induced by U46619. Each point represents mean ± SEM. *$p < 0.05$ 12- to 24-h-old *vs* 2-wk-old. #$p < 0.05$ pulmonary artery *vs* pulmonary vein. Only levels of statistical significance for the highest difference are shown.

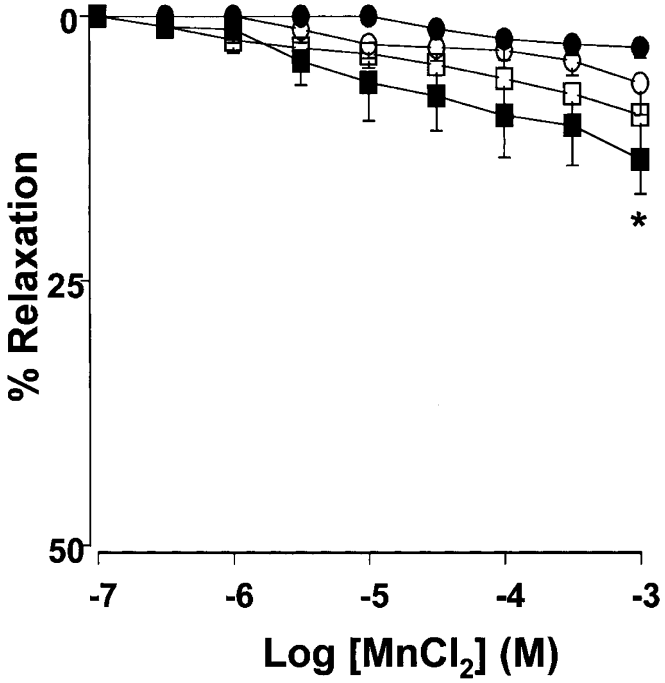


Figure 6. Concentration-dependent relaxant effects of $MnCl_2$ in endothelium-containing pulmonary arteries (*solid symbols*) and pulmonary veins (*open symbols*) of 12- to 24-h-old (*circles,* $n = 7$) and 2-wk-old piglets (*squares,* $n = 7$). Changes in tension induced by $MnCl_2$ are expressed as percent reversal of the contraction induced by U46619. Each point represents mean ± SEM. *$p < 0.05$ 12- to 24-h-old *vs* 2-wk-old. Only levels of statistical significance for the highest difference are shown.

was abolished by NO· synthase inhibition, suggesting that its actions are more consistent with destruction rather than potentiation of basal NO· activity.

SOD augmentation and basal NO· activity. The age-related increase in SOD-induced relaxation of pulmonary arteries that we describe in the present work may reflect developmental changes in basal NO· bioactivity or, alternatively, may be the consequence of a reduced ability of SOD to reduce $O_2^{\cdot -}$ levels in the neonatal vessels. Basal NO· bioactivity, as demonstrated by the contractile effects of NO· synthase inhibition, increased during the first days of postnatal life in piglets (11, 19) and rabbits (8). In addition, agonist-stimulated NO· activity also increased during the first days of life in piglet [(11, 20), and present work], sheep (21), and rabbit pulmonary arteries (8). In contrast, levels of lung endothelial NO· synthase protein and enzymatic activity were similar in 5-min-old and 3-d-old piglets (22). The endothelium-independent response of piglet pulmonary arteries to exogenous NO· also increased with postnatal age (11), suggesting that the age-dependent increase in response to endogenous NO· is related not only to increased NO· synthesis but also to an increased action or decreased destruction of NO·. Morecroft and MacLean (8) found, as opposed to our results, that SOD decreased phenylephrine-induced contraction in pulmonary arteries of 0- to 12-h-old but not those of 4-d-old or adult rabbits. Moreover, ACh-induced relaxation was observed in pulmonary arteries from 0- to 12-h-old rabbits only in the presence of SOD, suggesting that the lack of response to ACh in the neonatal vessels was because of an increased accumulation of $O_2^{\cdot -}$ (8). In the present study, we used DHE to detect changes in pulmonary vascular $O_2^{\cdot -}$ presence *in situ*. Our findings indicate that $O_2^{\cdot -}$ levels were similar in pulmonary vessels from 12- to 24-h-old and 2-wk-old piglets. In addition, we have not detected differ-

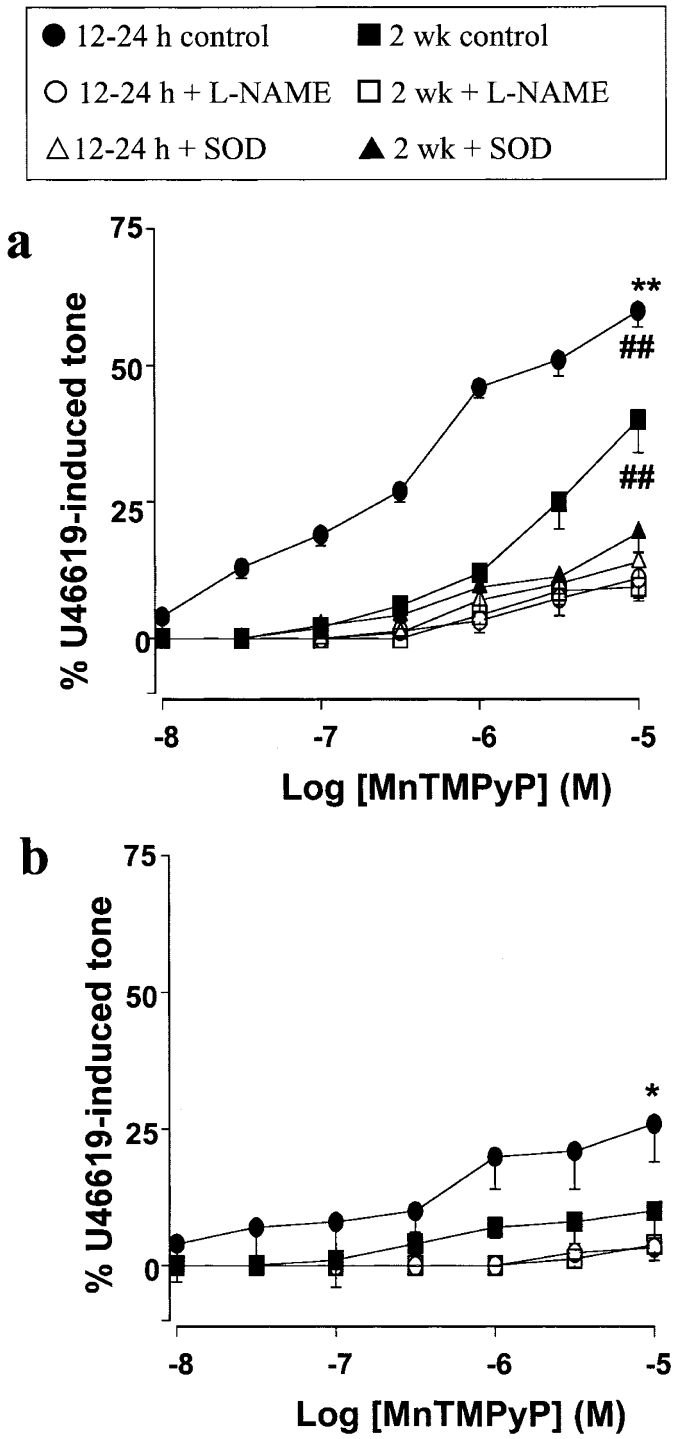


Figure 7. Concentration-dependent contractile effects of MnTMPyP in endothelium-containing pulmonary arteries (*a*) and pulmonary veins (*b*) of 12- to 24-h-old ($n = 7$) and 2-wk-old piglets ($n = 7$). The effects of pretreatment with the NO· synthase inhibitor L-NAME (100 μM) are also shown. Changes in tension induced by MnTMPyP are expressed as percent increase of the contraction induced by U46619. Each point represents mean ± SEM. *$p <$ 0.05, **$p < 0.01$ 12- to 24-h-old *vs* 2-wk-old. ##$p < 0.01$ pulmonary artery *vs* pulmonary vein.

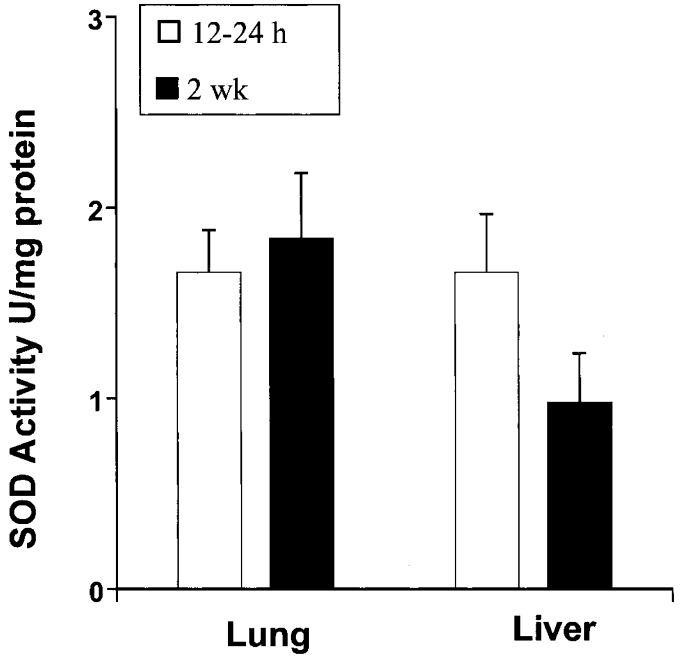


Figure 8. Total SOD activity levels in 12- to 24-h-old ($n = 10$) and 2-wk-old ($n = 10$) piglet lung and liver. Each bar represents mean ± SEM.

ences in $O_2^{\cdot-}$ presence between pulmonary arteries and pulmonary veins. Although the density of ethidium-stained cells may be unable to provide an adequate estimation of actual $O_2^{\cdot-}$ levels, when compared with direct measurements of ethidium fluorescence (18), relative increases and decreases of ethidium-stained cells were assessed after respective treatment with DETCA and PEG-SOD. This indicates that the method that we used is relatively sensitive to identify differences in vascular $O_2^{\cdot-}$ presence. Another limitation of the conventional fluorescent microscopy, which we used to analyze ethidium staining, was the inability to detect the specific population of cells responsible for $O_2^{\cdot-}$ production. Further investigations, using fluorescent confocal microscopy, are warranted to analyze differential vascular $O_2^{\cdot-}$ presence in cells from the endothelial, medial smooth muscle, and adventitial layers of neonatal pulmonary vessels.

Developmental regulation of SOD appears to be species specific (7, 23). Therefore, differences in the pattern of development might explain the age-related differences in the vascular actions of SOD observed between piglet (present work) and rabbit (8) pulmonary arteries. SOD activity increased in rabbit lungs between birth and 1 mo of postnatal life (23). In contrast, we have not observed age-related changes in piglet pulmonary SOD activity. Moreover, Perez-Vizcaino *et al.* (24) demonstrated that the expression of Cu/Zn SOD by Western blot analysis decreased in piglet lungs between birth and 2 wk of postnatal life. Interestingly, it has been reported that extracellular SOD is intracellular in preterm and term rabbit lungs and that secretion of SOD into the extracellular compartment increases with age, beginning in the first week of postnatal life (23). How these developmental changes in the distribution of extracellular SOD occur in the porcine lung remains to be investigated.

SOD augmentation and Ach-stimulated NO· activity. In the present study, SOD resulted in endothelium-dependent relaxation but failed to affect ACh-induced relaxation in pulmonary arteries of 12- to 24-h-old and 2-wk-old piglets and in pulmo-

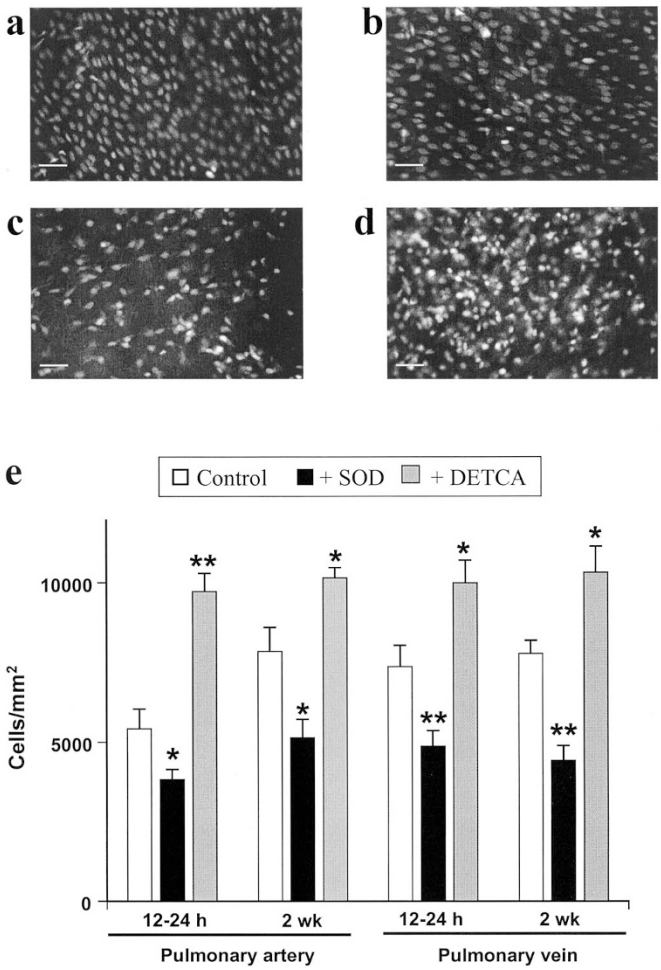


Figure 9. *In situ* detection of $O_2^{\cdot-}$ presence in piglet pulmonary arteries and veins. Representative black and white fluorescent photomicrographs of neonatal (12- to 24-h-old) piglet pulmonary artery (*a, c, d*) and vein (*b*) segments labeled with the oxidative dye DHE (red fluorescence when oxidized to ethidium by $O_2^{\cdot-}$) are shown. Vascular segments were incubated with vehicle (*a, b*), 100 U/mL PEG-SOD (*c*), or 3 mM DETCA (*d*), for 1 h before assay with DHE. Scale bar = 20 μm. *e,* summarized data of ethidium-stained cell density in pulmonary arteries and veins from 12- to 24-h-old (n = 6) and 2-wk-old piglets (n = 8). Each bar represents mean ± SEM. *$p < 0.05$, **$p < 0.01$ *vs* control.

nary veins of 12- to 24-h-old piglets. Accordingly, other investigators demonstrated that SOD did not affect endothelium-dependent relaxation induced by ACh or the calcium ionophore A-23187 in small pulmonary arteries from pigs (4) or juvenile lambs (25). In contrast, SOD enhanced the relaxant effects of ACh in bovine intrapulmonary arteries (26) and in conduit pulmonary arteries of 0- to 12-h-old but not of adult rabbits (8). A differential sensitivity of basal and ACh-stimulated $NO^{\cdot}$ relaxation to inhibition by endogenous $O_2^{\cdot-}$ has been documented in rat aorta by Mian and Martin (12). They suggest that Cu/Zn SOD activity lowers levels of $O_2^{\cdot-}$ to such an extent that low levels of $NO^{\cdot}$ produced under basal conditions can be destroyed but high levels produced by ACh cannot (12).

SOD inhibition and $NO^{\cdot}$ activity. Treatment with high concentrations of DETCA inactivates both intracellular and extracellular isoforms of Cu/Zn SOD and leads, in a number of blood vessels, to increased levels of $O_2^{\cdot-}$ together with severe impairment of endothelium-dependent relaxation (15, 27, 28). We showed that DETCA significantly increases $O_2^{\cdot-}$ levels in pulmonary arteries and veins from neonatal and 2-wk-old piglets, and we observed that treatment of neonatal pulmonary vessels with DETCA impaired ACh-induced relaxation. However, DETCA did not affect ACh-induced relaxation in pulmonary vessels from 2-wk-old piglets. The effect of DETCA in neonatal vessels was not reversed by SOD so may be mainly a result of inhibition of SOD intracellularly where exogenous SOD cannot penetrate (12). In fact, we observed that PEG-SOD reversed DETCA-induced impairment of ACh-induced relaxation. It has been demonstrated that PEG conjugation to SOD enhanced enzyme uptake by endothelial cells and provided greater resistance to oxidant stress than the native enzyme (29).

DETCA pretreatment did not affect the relaxation induced by the $NO^{\cdot}$ donors sodium nitroprusside and *S*-nitroso-*N*-acetyl-penicillamine (30), or by the endothelial $NO^{\cdot}$ synthase stimulator ACh (present study) in pulmonary arteries from 2-wk-old piglets. Accordingly, DETCA pretreatment did not significantly affect vasorelaxation induced by ACh or *S*-nitroso-*N*-acetyl penicillamine in rat aorta (31). A higher antioxidant action of basal $NO^{\cdot}$ to regulate $O_2^{\cdot-}$ levels after the inhibition of tissue SOD might explain the lack of effect of DETCA on ACh-induced relaxation in the older piglets. However, we have found a similar DETCA-induced increase of ethidium-stained cells in vessels from 12- to 24-h-old and 2-wk-old piglets.

Inhibition of SOD with DETCA unmasked a constitutive inhibition of $NO^{\cdot}$ by $O_2^{\cdot-}$ in vascular tissue, as suggested by the increase in U46619-induced contraction observed in DETCA-treated pulmonary arteries. The source of $O_2^{\cdot-}$ in 2-wk-old piglet pulmonary arteries has been studied by Lopez-Lopez *et al.* (30) by using inhibitors of the main enzymatic $O_2^{\cdot-}$-generating systems, *i.e.* membrane NAD(P)H oxidase, $NO^{\cdot}$ synthase, cyclooxygenase, lipoxygenase, cytochrome P-450 oxidase, xanthine oxidase, and the mitochondrial electron transport chain. They demonstrated that adventitial NAD(P)H oxidase was the main source of $O_2^{\cdot-}$ in these vessels (30). NAD(P)H-mediated $O_2^{\cdot-}$ production was localized to adventitial fibroblasts in rabbit aorta (32). Enhanced capacity of growth of fetal and neonatal bovine pulmonary artery adventitial fibroblasts has been demonstrated (33), and more connective tissue was present in piglet pulmonary arteries at 14 d than at birth (34). Connective tissue deposition was also augmented with postnatal age in human pulmonary arteries (35). Whether these facts result in differences in vascular $O_2^{\cdot-}$ production remains to be investigated.

SOD and pulmonary veins. Pulmonary veins are the major site of action of a number of vasoactive factors in different animal species and at different ages (11, 36, 37). In newborn piglets we observed that the responses to exogenous and endogenous $NO^{\cdot}$ were greater in pulmonary veins than in pulmonary arteries (11). However, in the present work we observed that the vascular effects of SOD and the SOD mimetics were consistently smaller in pulmonary veins than in pulmonary arteries. In contrast, the pulmonary veins of 2-wk-

old piglets were the only vessel that demonstrated SOD-induced increase of ACh-induced relaxation. Accordingly, SOD increased the response to ACh in 4-wk-old ovine (25) and adult bovine (26) pulmonary veins. Pulmonary veins are exposed to higher oxygen concentrations than pulmonary arteries, but it seems that this fact does not imply a higher activity of antioxidant defenses. SOD activity (but also $O_2^{\cdot -}$ generation and $NO^{\cdot}$ formation) was found to be lower in pulmonary vein than in pulmonary artery segments from adult humans (38). Moreover, pulmonary vein endothelial cells are more susceptible than pulmonary artery ones to free radical–induced damage, suggesting a reduced development of antioxidant defenses (39). However, we have not found significant differences in $O_2^{\cdot -}$ levels between pulmonary arteries and pulmonary veins from newborn and 2-wk-old piglets.

Vascular effects of SOD mimetics. Multicenter, randomized trials have shown that inhaled $NO^{\cdot}$ improves oxygenation and decreases the need for extracorporeal membrane oxygenation in infants with PPHN (40). By scavenging $O_2^{\cdot -}$, SOD may increase the bioavailability of inhaled $NO^{\cdot}$ while simultaneously reducing toxic $ONOO^-$ formation. Steinhorn *et al.* (9) demonstrated that a single intratracheal dose of recombinant human Cu/Zn SOD enhanced the pulmonary vascular effects of inhaled $NO^{\cdot}$. However, the main limitations for the therapeutic use of Cu/Zn SOD are its large size, which limits cell permeability, short circulating half-life, and expense. An increasing number of low-molecular weight SOD mimetics have been proposed to overcome some of these limitations (41). In the present study, the ability of Cu/Zn SOD to promote endothelium-dependent relaxation of piglet pulmonary vessels was shared by the simple metal salts $CuSO_4$ and $MnCl_2$. MacKenzie *et al.* (13) described similar effects of $CuSO_4$ and $MnCl_2$ in rat aorta, but the sensitivity of that preparation to the metal salt–induced relaxation was significantly higher than that presently observed in piglet pulmonary vessels. The free metal ions released from $CuSO_4$ and $MnCl_2$ at the high concentrations that are needed to induce vascular effects limit their potential use as SOD mimetics (13).

The spin trap agents tiron, tempol, and PTIYO are effective *in vitro* scavengers of $O_2^{\cdot -}$, but less potent than SOD or the metal-based SOD mimetics (13, 42–44). In the present study we showed that none of these compounds shared the ability of Cu/Zn SOD to promote endothelium-dependent relaxation of piglet pulmonary vessels. Again, similar results have been reported in rat aorta (13). Spin trap compounds react with and destroy $NO^{\cdot}$ (45). This fact limits the use of these agents as SOD mimetics (13)

Manganese-based metalloporphyrin complexes have been shown to possess distinct antioxidant properties. These include scavenging $O_2^{\cdot -}$, H_2O_2, $ONOO^-$, and lipid peroxyl radicals (41). However, it has been demonstrated that the low molecular weight manganese-porphyrin-based compound, MnTMPyP, can be either a net scavenger or generator of $O_2^{\cdot -}$ depending on the prevailing redox environment (46). We found that MnTMPyP failed to produce relaxation but produced augmentation of U46619-induced tone. This contractile effect of MnTMPyP was abolished by the presence of the $NO^{\cdot}$ synthase inhibitor L-NAME and was significantly reduced by the presence of SOD. A similar L-NAME- and SOD-prevented contractile effect of MnTMPyP has been described in the aorta of adult rats (13). This suggests that, at least in the experimental conditions in which isolated vessels are studied, MnTMPyP acts more as an $O_2^{\cdot -}$ generator than as an $O_2^{\cdot -}$ scavenger (13).

Limitations of the study. Important segmental differences in vascular contractility are present in the pulmonary circulation. Therefore, information obtained in large or small conduit pulmonary arteries should be cautiously extrapolated to resistance vessels and to *in vivo* situations. In addition, the Krebs buffer used for *in vitro* studies of vascular contractility provides prime conditions for contamination artifacts with $O_2^{\cdot -}$ (2). It contains high concentrations of glucose that forms $O_2^{\cdot -}$ by autoxidation, is contaminated with trace iron and copper, is bubbled with 95% oxygen, and is incubated under the UV radiation of fluorescent lights (2). In fact, the relaxant effects of SOD in pulmonary arteries of fetal lambs were blunted by lowering the tissue bath oxygen tension from 94 to 20% (47). In contrast, the relaxant response of piglet pulmonary arteries to authentic $NO^{\cdot}$ did not change when the organ chambers were bubbled with 95%, 21%, or 0% oxygen (11), suggesting that formation of $O_2^{\cdot -}$ was not the result of such high oxygen concentrations. Finally, *in vivo* constant removal of $NO^{\cdot}$ by Hb will allow SOD to successfully reduce $ONOO^-$ formation. However, *in vitro* addition of SOD does not necessarily slow the production of $ONOO^-$, unless vast amounts are added, because little else other than $O_2^{\cdot -}$ in the system can remove $NO^{\cdot}$ (2).

CONCLUSIONS

In summary, we assessed the effects of SOD supplementation and inhibition on pulmonary vascular reactivity and found that the ability of SOD to produce pulmonary vascular relaxation by protecting basal $NO^{\cdot}$ from destruction by $O_2^{\cdot -}$ was lower in neonatal than in 2-wk-old piglets. In contrast, inhibition of endogenous SOD affected ACh-induced $NO^{\cdot}$ activity only in the pulmonary vessels of newborn piglets. Further investigations into the early postnatal changes in antioxidant defenses and sources of $O_2^{\cdot -}$ are warranted to improve our understanding of the functional maturation of the pulmonary circulation during this critical period of lung development.

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REFERENCES

1. Abman SH, Stevens T 1996 Perinatal pulmonary vasoregulation: implications for the pathophysiology and treatment of neonatal pulmonary hypertension. In: Haddad G, Lister G (eds) Tissue Oxygen Deprivation: Developmental, Molecular and Integrative Function. Marcel Dekker, New York, pp 367–432
2. Beckman JS, Koppenol WH 1996 Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am J Physiol 271:C1424–C1437
3. Sanderud J, Norstein J, Saugstad OD 1991 Reactive oxygen metabolites produce pulmonary vasoconstriction in young pigs. Pediatr Res 29:543–547
4. Liu Q, Wiener CM, Flavahan NA 1998 Superoxide and endothelium-dependent constriction to flow in porcine small pulmonary arteries. Br J Pharmacol 124:331–336
5. Walther FJ, Wade AB, Warburton D, Forman HJ 1991 Ontogeny of antioxidant enzymes in the fetal lamb lung. Exp Lung Res 17:39–45
6. Frank L 1998 Development of the antioxidant defenses in fetal life. Semin Neonatol 3:173–182

7. Asikainen TM, Raivio KO, Saksela M, Kinnula VL 1988 Expression and developmental profile of antioxidant enzymes in human lung and liver. Am J Respir Cell Mol Biol 19:942–949
8. Morecroft I, MacLean MR 1998 Developmental changes in endothelium-dependent vasodilation and the influence of superoxide anions in perinatal rabbit pulmonary arteries. Br J Pharmacol 125:1585–1593
9. Steinhorn RH, Albert G, Swartz DD, Russell JA, Levine CR, Davis JM 2001 Recombinant human superoxide dismutase enhances the effect of inhaled nitric oxide in persistent pulmonary hypertension. Am J Respir Crit Care Med 164:834–839
10. Carpenter D, Larkin H, Chang A, Morris E, O'Neill J, Curtis J 2001 Superoxide dismutase and catalase do not affect the pulmonary hypertensive response to group B streptococcus in the lamb. Pediatr Res 49:181–188
11. Villamor E, Perez-Vizcaino F, Cogolludo AL, Conde-Oviedo J, Zaragoza-Arnaez F, Lopez-Lopez JG, Tamargo J 2000 Relaxant effects of carbon monoxide compared with nitric oxide in pulmonary and systemic vessels of newborn piglets. Pediatr Res 48:546–553
12. Mian KB, Martin W 1995 Differential sensitivity of basal and acetylcholine-stimulated activity of nitric oxide to destruction by superoxide anion in rat aorta. Br J Pharmacol 115:993–1000
13. MacKenzie A, Filippini S, Martin W 1999 Effects of superoxide dismutase mimetics on the activity of nitric oxide in rat aorta. Br J Pharmacol 127:1159–1164
14. Villamor E, Perez Vizcaino F, Tamargo J, Moro M 1996 Effects of group B streptococcus on the responses to U46619, endothelin-1, and noradrenaline in isolated pulmonary and mesenteric arteries of piglets. Pediatr Res 40:827–833
15. MacKenzie A, Martin W 1998 Loss of endothelium-derived nitric oxide in rabbit aorta by oxidant stress: restoration by superoxide dismutase mimetics. Br J Pharmacol 124:719–728
16. Beauchamp C, Fridovich I 1971 Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287
17. Li N, Yi FX, Spurrier JL, Bobrowitz CA, Zou AP 2002 Production of superoxide through NADH oxidase in thick ascending limb of Henle's loop in rat kidney. Am J Physiol Renal Physiol 282:F1111–F1119
18. Stepp DW, Ou J, Ackerman AW, Welak S, Klick D, Pritchard Jr KA 2002 Native LDL, and minimally oxidized LDL differentially regulate superoxide anion in vascular endothelium *in situ.* Am J Physiol Heart Circ Physiol 283:H750–H759
19. Levy M, Tulloh RM, Komai H, Stuart-Smith K, Haworth SG 1995 Maturation of the contractile response and its endothelial modulation in newborn porcine intrapulmonary arteries. Pediatr Res 38:25–29
20. Liu SF, Hislop AA, Haworth SG, Barnes PJ 1992 Developmental changes in endothelium-dependent pulmonary vasodilatation in pigs. Br J Pharmacol 106:324–330
21. Steinhorn RH, Morin FC, Gugino SF, Giese EC, Russell JA 1993 Developmental differences in endothelium-dependent responses in isolated ovine pulmonary arteries and veins. Am J Physiol 264:H2162–H167
22. Arrigoni FI, Hislop AA, Pollock JS, Haworth SG, Mitchell JA 2002 Birth upregulates nitric oxide synthase activity in the porcine lung. Life Sci 70:1609–1620
23. Nozik-Grayck E, Dieterle CS, Piantadosi CA, Enghild JJ, Oury TD 2000 Secretion of extracellular superoxide dismutase in neonatal lungs. Am J Physiol Lung Cell Mol Physiol 279:L977–L984
24. Perez-Vizcaino F, Lopez-Lopez JG, Santiago R, Cogolludo A, Zaragoza-Arnaez F, Moreno L, Alonso MJ, Salaices M, Tamargo J 2002 Postnatal maturation in nitric oxide-induced pulmonary artery relaxation involving cyclooxygenase-1 activity. Am J Physiol Lung Cell Mol Physiol 283:L839–L848
25. Steinhorn RH, Russell JA, Lakshminrusimha S, Gugino SF, Black SM, Fineman JR 2001 Altered endothelium-dependent relaxations in lambs with high pulmonary blood flow and pulmonary hypertension. Am J Physiol Heart Circ Physiol 280:H311–H317
26. Ignarro LJ, Byrns RE, Buga GM, Wood KS 1987 Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ Res 61:866–879
27. Cherry PD, Omar HA, Farrell KA, Stuart JS, Wolin MS 1990 Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. Am J Physiol 259:H1056–H1062
28. Mugge A, Elwell JH, Peterson TE, Harrison DG 1991 Release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity. Am J Physiol 260:C219–C225
29. Beckman JS, Minor Jr RL, White CW, Repine JE, Rosen GM, Freeman BA 1988 Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. J Biol Chem 15:6884–6892
30. Lopez-Lopez JG, Perez-Vizcaino F, Cogolludo AL, Ibarra M, Zaragoza-Arnaez F, Tamargo J 2001 Nitric oxide- and nitric oxide donors-induced relaxation and its modulation by oxidative stress in piglet pulmonary arteries. Br J Pharmacol 133:615–624
31. Laight DW, Kaw AV, Carrier MJ, Anggard EE 1998 Interaction between superoxide anion and nitric oxide in the regulation of vascular endothelial function. Br J Pharmacol 124:238–244
32. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT 1997 Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc Natl Acad Sci USA 94:14483–14488
33. Das M, Stenmark KR, Dempsey EC 1995 Enhanced growth of fetal and neonatal pulmonary artery adventitial fibroblasts is dependent on protein kinase C. Am J Physiol 269:L660–L667
34. Kelly DA, Hislop AA, Hall SM, Haworth SG 2002 Correlation of pulmonary arterial smooth muscle structure and reactivity during adaptation to extrauterine life. J Vasc Res 39:30–40
35. Allen K, Haworth SG 1988 Human postnatal pulmonary arterial remodeling: ultrastructural studies of smooth muscle cell and connective tissue maturation. Lab Invest 59:702–709
36. Raj JU, Hillyard R, Kaapa P, Gropper M, Anderson J 1990 Pulmonary arterial and venous constriction during hypoxia in 3- to 5-wk-old and adult ferrets. J Appl Physiol 69:2183–2189
37. Gao Y, Tolsa JF, Botello M, Raj JU 1998 Developmental change in isoproterenol-mediated relaxation of pulmonary veins of fetal and newborn lambs. J Appl Physiol 84:1535–1539
38. Schmalfuss CM, Chen LY, Bott JN, Staples ED, Mehta JL 1999 Superoxide anion generation, superoxide dismutase activity, and nitric oxide release in human internal mammary artery and saphenous vein segments. J Cardiovasc Pharmacol Ther 4:249–257
39. Grishko V, Solomon M, Wilson GL, LeDoux SP, Gillespie MN 2001 Oxygen radical-induced mitochondrial DNA damage and repair in pulmonary vascular endothelial cell phenotypes. Am J Physiol Lung Cell Mol Physiol 280:L1300-L1308
40. Finer NN, Barrington KJ 2002 Nitric oxide for respiratory failure in infants born at or near term (Cochrane Review). In: The Cochrane Library, Issue 2, Oxford: Update Software
41. Cuzzocrea S, Riley DP, Caputi AP, Salvemini D 2001 Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev 53:135–159
42. Mok JS, Paisley K, Martin W 1998 Inhibition of nitrergic neurotransmission in the bovine retractor penis muscle by an oxidant stress: effects of superoxide dismutase mimetics. Br J Pharmacol 124:111–118
43. Mitchell JB, Samuni A, Krishna MC, DeGraff WG, Ahn MS, Samuni U, Russo A 1990 Biologically active metal-independent superoxide dismutase mimics. Biochemistry 29:2802–2807
44. Krishna CM, Liebmann JE, Kaufman D, DeGraff W, Hahn SM, McMurry T, Mitchell JB, Russo A 1992 The catecholic metal sequestering agent 1,2-dihydroxybenzene-3,5-disulfonate confers protection against oxidative cell damage. Arch Biochem Biophys 294:98–106
45. Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, Maeda H 1993 Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO through a radical reaction. Biochemistry 32:827–832
46. Gardner PR, Nguyen DD, White CW 1996 Superoxide scavenging by Mn(II/III) tetrakis (1-methyl-4-pyridyl) porphyrin in mammalian cells. Arch Biochem Biophys 325:20–28
47. Steinhorn RH, Lakshminrusimha S, Gugino SF, Russell JA 2000 Superoxide dismutase relaxes pulmonary arteries from fetal sheep by a mechanism independent of NO-guanylate cyclase signaling. Nitric Oxide 4:225–226(abstr)