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EXPERIMENTAL

**J. Dötsch**
**S. Demirakça**
**K. Zepf**
**J. Hänze**
**S. Parida**
**W. Rascher**

# Recovery from withdrawal of inhaled nitric oxide and kinetics of nitric oxide-induced inhibition of nitric oxide synthase activity in vitro

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J. Dötsch (✉) · K. Zepf · W. Rascher
Department of Pediatrics,
Friedrich-Alexander University
of Erlangen-Nürnberg, Loschgestrasse 15,
D-91054 Erlangen, Germany
e-mail: JoergWDoetsch@yahoo.com
Tel.: + 49-9131-8533131
Fax: + 49-9131-8536097

S. Demirakça
University Children's Hospital, Haus 28,
D-68135 Mannheim, Germany

J. Hänze
Department of Internal Medicine,
Justus-Liebig University of Giessen,
Friedrichstrasse 24, D-35392 Giessen,
Germany

S. Parida
Department of Microbiology,
Justus-Liebig University of Giessen,
Frankfurterstrasse 107, D-35392 Giessen,
Germany

**Abstract** *Objective*: To examine the kinetics of successful nitric oxide (NO) withdrawal in vivo and in vitro.
*Design and setting*: Prospective study in a university pediatric intensive care ward and research laboratory.
*Patients and materials*: Nineteen patients with acute respiratory distress syndrome (ARDS) or persistent pulmonary hypertension of the newborn (PPHN). Primary porcine pulmonary artery cells in vitro.
*Interventions*: NO inhalation and withdrawal in patients; exposure to NO donor sodium nitroprusside (SNP) and gaseous NO in vitro.
*Measurements and results*: In patients: a slight, but significant, increase of oxygenation index (OI) from 4.57 ± 0.24 $cmH_2O$/torr (mean ± SEM) to 4.90 ± 0.26 $cmH_2O$/torr after withdrawal of NO ($p < 0.001$). Recovery of OI (4.43 ± 0.23 $cmH_2O$/torr) 30 min after weaning, a significant drop after 4 h (3.72 ± 0.17 $cmH_2O$/torr; $p < 0.001$), values restored after 12 h.
In vitro: NO synthase (NOS) activity was significantly lower in SNP-incubated cells (20.0 ± 4.0 μM/min) than in control cells (37.6 ± 7.0 μM/min; $p < 0.05$). Thirty minutes after SNP withdrawal there was NOS activity of 35.8 ± 10.0 μM/min with a significant increase by 4 h ($p < 0.05$). No alteration of endothelial NOS (ENOS) mRNA expression by NO (Northern Blot).
*Conclusion*: In patients there is a slight, but significant, reversible increase of OI after successful weaning from NO. In vitro, NO leads to a reversible decrease of ENOS activity on a post mRNA level, resembling clinical observations.

**Key words** Intensive care · Critical care · Acute respiratory distress syndrome · Persistent pulmonary hypertension of the newborn · Feedback inhibition

## Introduction

Nitric oxide (NO) has become an important therapeutic agent in pediatric and adult intensive care units [1, 2]. Although NO is not officially approved as a drug in most countries yet, it promises to become an important clinical tool in the future if approved [3, 4]. Among its potential hazards are methemoglobinemia [5, 6], and cytotoxicity caused by oxidation or DNA cleavage after endonuclease activation [7]. Weaning, even from low doses of NO, may worsen the patient's oxygenation. We have recently demonstrated that the chances for successful weaning are high when an oxygenation index (OI) of 5 $cmH_2O$/torr is reached [1]. It is unknown, however, if and to what degree worsening of the patient's oxygenation must be expected even after successful weaning from NO. In addition, it is not clear how long after weaning the patient's oxygenation recovers.

Autoinhibition has been demonstrated previously for brain and macrophage NO synthases (NOS) [8, 9,

10]. This effect has been confirmed for systemic endothelial cells derived from bovine aorta [11]. These observations clearly indicate that NO inhibits NOS activity. Neuronal (n) NOS and endothelial (e) NOS are more sensitive than iNOS to the inhibitory action of NO. Not only exogenously added NO but also enzymatically generated NO inhibits the activity of nNOS and eNOS. The mechanism by which NO inhibits NOS appears to involve the heme iron prosthetic group of NOS. Moreover, the oxidation state of the heme iron is critical in determining the magnitude of inhibition of NOS by NO [12].

Despite the known NO feedback inhibition on the endothelium, vascular endothelial cells have been found to perform differently depending on their origin [13, 14]. From a clinical point of view pulmonary arterial cells appear to be more appropriate than systemic cells. The present studies were designed to assess the time course of the patients' oxygenation after successful weaning from NO. Our second goal was to relate the clinical results to the kinetics of NOS inhibition in vitro and to examine NOS gene expression following NO exposure.

## Material and methods

### In vivo study

The study was approved by the ethics committee of the University of Giessen and informed consent was obtained from the patients' parents. For the present study only patients with successful weaning were included. The mean OI before successful withdrawal of NO was 4.6 ± 1.0 (range 2.2–6.8) $cmH_2O$. None of the patients received nitroglycerin, nitroprusside or other NO donors. Nineteen patients receiving NO for persistent pulmonary hypertension of the newborn (PPHN) or acute respiratory distress syndrome (ARDS) were enrolled (Table 1). Treatment with inhaled NO was initiated when an OI of 20 $cmH_2O$/torr ( = mean airway pressure × $FIO_2$ × 100/$PaO_2$) was exceeded under conventional therapy. Besides treatment of the underlying disease, conventional therapy included pressure-limited inverse ratio ventilation with PEEP and permissive hypercapnia [15, 16]. The patients studied in this survey had been examined under different aspects in a previous study [1]. NO was initially administered at 20 ppm for 1 h. After NO withdrawal for 15 min, a dose variation with 1, 5, 10, 20, 40 and 80 ppm NO was performed over 15 min for each step. Details of the protocol, the time course of NO inhalation, ventilator settings and monitoring of NO concentrations are described elsewhere [1]. The determination of the OI (mean airway pressure x $FIO_2$ x 100/$PaO_2$) was part of the protocol of a study of NO inhalation [1].

The OI was determined immediately before weaning from NO at 2 min, 30 min, 4 h and 12 h after weaning. Withdrawal was attempted as soon as a stable respiratory status (PEEP of 6 $cmH_2O$, I:E 1:2, $FIO_2$ of ≤ 0.8) and 5 ppm NO had been achieved. Withdrawals under less stable conditions generally were not successful. Most patients had failed withdrawal of NO one or more times before weaning was possible.

**Table 1** Characteristics of 19 patients with persistent pulmonary hypertension of the newborn or acute respiratory distress syndrome inhaling nitric oxide in the *in vivo* study. The underlying diagnosis was defined according to international criteria (1, 30, 31)

| | |
|---|---|
| Patients' ages (number) | 1 day (2), 2 days (6), 3 days (2), 1 month (2), 3 months (1), 4 months (1), 1 year (3), 4 years (1), 6 years (1) |
| Sex (male/female) | 10/9 |
| Inclusion criteria | oxygenation index > 20 $cmH_2O$/torr under conventional treatment |
| Exclusion criteria | treatment with nitric oxide donors, administration of drugs known to induce methemoglobinemia, inherited or acquired methemoglobinemia |
| Diagnosis | Meconium aspiration syndrome (n = 4), sepsis (n = 10), pneumonia (n = 3), congenital diaphragmatic hernia (n = 1), perinatal asphyxia (n = 1) |
| Multiorgan system failure | 6 (32 %) |

### Primary cell culture

Pulmonary artery endothelial cells prepared from porcine central pulmonary artery were cultured up to four passages in cell growth medium containing 10 % FCS and 2 % penicillin/streptomycin at 37 °C in a humidified atmosphere with 5 % $CO_2$.

#### *Characterization of porcine pulmonary artery cells*

Cells isolated from the porcine pulmonary artery were grown to confluency. Cells were characterized by immunostaining with the monoclonal antibodies to Von Willebrand factor (VWF)-(Factor VIII), CD31, CD34, CD44 (all four antibodies from Dianovova, Hamburg, Germany) and analyzed by flow cytometry for endothelial cell characterization. By flow cytometry, strongly positive staining was found in the cases of CD44 and VWF, while very little change was observed with CD31 and no staining was observed with CD3. The cell population was found to be pure as more than 99 % of cells were found to be positive for VWF. Details are given elsewhere [17].

#### *Incubation of porcine pulmonary artery cells*

Confluent pulmonary artery cells were washed twice with phosphate-buffered saline (PBS) before being used for the experiments. To examine the kinetics of NO inhibition the cells were treated with 100 μM of the NO donor sodium nitroprusside (SNP; Sigma, Munich, Germany) for 2 h. After being washed with PBS, the cells were either used for the determination of NOS activity immediately or incubated with cell culture medium for various time spans. To assess the effect of NO on NOS, mRNA expression cells were either incubated as monolayers with 100 μM of SNP for 24 h or incubated in suspension with 32 ppm of gaseous NO. For the latter experiment, the suspension was exposed to artificial air containing NO as described previously [3]. The cells were exposed to NO for 12 h. Due to the reduction of cell number in the suspension culture, the exposure was not prolonged.

*Measurement of NOS activity using the oxyhemoglobin assay*

Cells were extracted at 0 °C by homogenization in five volumes of a buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM DL dithiotheitol, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptine, 10 μg/ml soybean trypsin inhibitor and 2 μg/ml aprotinin, brought to pH 7.0 at 20 °C with HCl. The homogenates were then centrifuged at 12,000 × g for 20 min. The supernatants, stored on ice, were used within 3 h of preparation.

Nitric oxide synthase activity was assessed using the oxyhemoglobin assay [18, 19]. The incubation medium contained 1.6 μM oxyhemoglobin, 200 μM $CaCl_2$, 1 mM $MgCl_2$, 100 μM L-arginine, 100 μM NADPH, 40 mM potassium phosphate, pH 7.2 and up to 20 % (v/v) cell extract. The change in the difference of absorbency at 401 and 421 nM was monitored with a dual wavelength recording spectrophotometer (Ultrospec III, Pharmacia, Freiburg, Germany). The oxyhemoglobin assay was chosen because it is less susceptible to constraints imposed by competing reactions (as opposed to the citrulline assay) and oxyhemoglobin resembles the potential target of feedback inhibition of the NOS, that is the hemoglobin-like domain [17]. Therefore, although neither oxyhemoglobin nor citrulline assays are specific for eNOS, the oxyhemoglobin assay was applied.

*Endothelial NOS mRNA expression*

RNA extraction and Northern Blot were performed as described elsewhere [20]. All specific mRNA signals were normalized to $\beta$-actin expression. Specific cDNA probes for human endothelial NOS (ENOS) and human $\beta$-actin [21], both cloned in pGEM4, were used. The ENOS probe was synthesized by RT-PCR using the following primers:

5' GACGCTGCCTGGGCTCCCTGGTAT 3'
5' TTGGCTGGGTCCCCCCGCACAGAG 3'

After cloning of the PCR product the 510 bp long fragment was confirmed to be ENOS using an automated sequencer (Prism 310 Genetic Analyzer, Perkin Elmer, Weiterstadt, Germany).

Statistic evaluation

All results are given as mean ± SEM. After revealing significant differences in the OI using ANOVA for repeated measurements, comparisons between the initial OI and the subsequent values were carried out by paired *t*-test. The NOS activity and the ENOS mRNA expression over time were compared by ANOVA and, where appropriate, by unpaired *t*-test. In cases of multiple comparisons, *p* values were corrected according to Bonferoni. A *p* value of less than 0.05 was considered significant.

## Results

In vivo study

After successful withdrawal of NO there was a slight, but statistically significant, increase in the patients' mean OI from 4.57 ± 0.24 $cmH_2O$/torr (mean ± SEM) to 4.90 ± 0.26 $cmH_2O$/torr ($p < 0.001$). Thirty minutes after successful weaning the OI had regained values similar to those before withdrawal (4.43 ± 0.23 $cmH_2O$/torr). After 4 h the mean OI was significantly lower than before discontinuation of NO (3.72 ± 0.17; $p < 0.001$). After 12 h without NO the OI was similar to the initial values (4.38 ± 0.21 $cmH_2O$/torr; Fig. 1). The highest increase of the OI within the first 30 min after weaning was 0.7 $cmH_2O$/torr, the smallest increase 0.1 $cmH_2O$/torr.

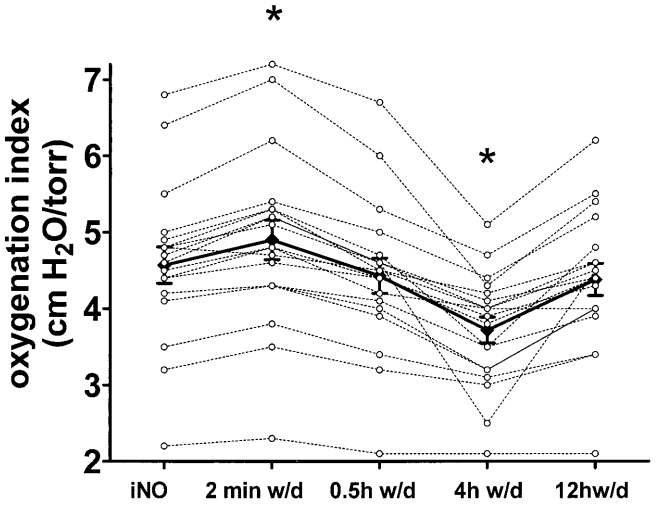


**Fig. 1** Course of oxygenation indices before and after successful withdrawal of inhaled nitric oxide (NO) in 19 patients who received NO for the treatment of persistent pulmonary hypertension of the newborn or ARDS. *Open circles* and *dotted lines* show the individual values, *closed squares* and *thick line* represent mean ± SEM. Significant differences between the initial value during NO inhalation and the subsequent values are marked by asterisks ($p < 0.001$) (*iNO* before discontinuation of inhaled nitric oxide, *w/d* time after withdrawal of nitric oxide)

In vitro

Nitric oxide activity in pulmonary artery endothelial cell was significantly reduced to one-half after 2 h of incubation with 100 μM of the NO donor SNP (20.0 ± 4.0 μM/min vs 37.6 ± 7.0 μM/min in the controls; $p < 0.05$). Thirty minutes after withdrawal from SNP, NO activity was similar to that in the controls (35.8 ± 10.0 μM/min). Four hours after NO-donor discontinuation NOS activity reached twice the control value (72.4 ± 14.8 μM/min; $p < 0.05$) and declined gradually thereafter (59.2 ± 20.2 μM/min; Fig. 2).

There was no alteration of eNOS mRNA concentration after the incubation of suspended endothelial cells with gaseous NO. Similarly, the pretreatment of monolayer cells with the NO donor SNP did not change the mRNA expression of endothelial NOS (Fig. 3 A, B).

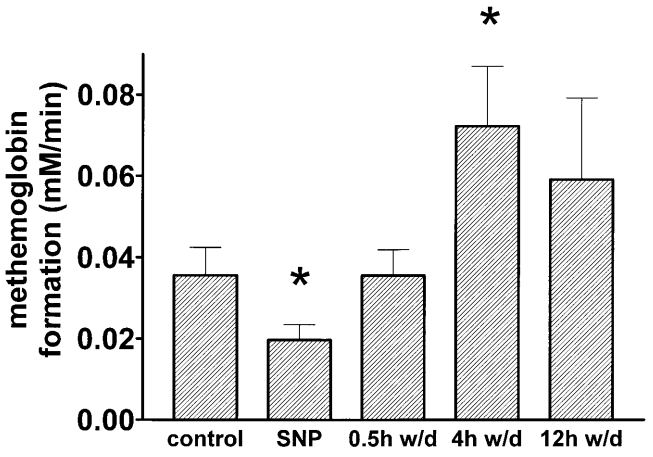


**Fig. 2** Nitric oxide synthase activity assessed by the oxyhemoglobin assay as a function of time after withdrawal of the NO donor sodium nitroprusside (SNP). Values are expressed as mean ± SEM. Significant differences between the initial value before SNP incubation and the subsequent values are marked by asterisks ($p < 0.05$) (*SNP* after 2 h of incubation with SNP, *w/d* time after withdrawal of SNP)

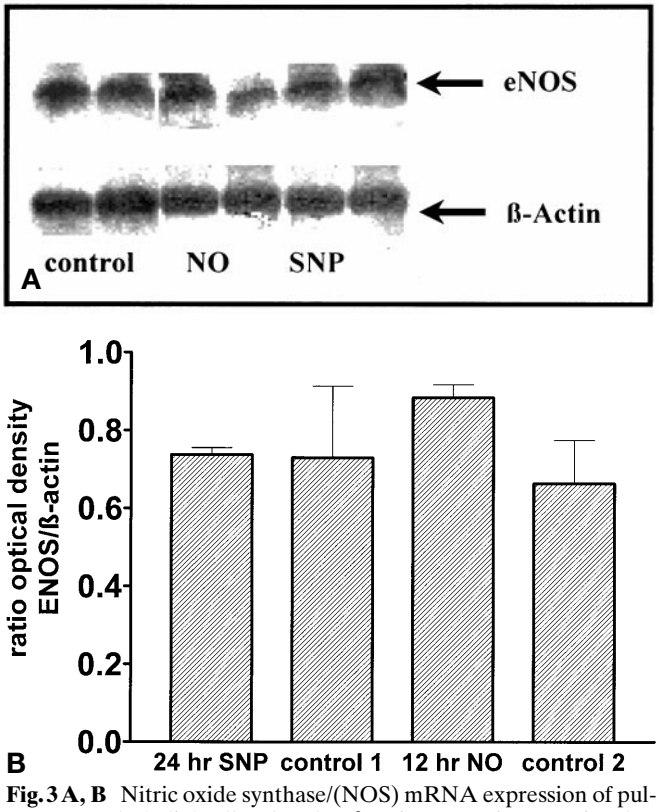


**Fig. 3 A, B** Nitric oxide synthase/(NOS) mRNA expression of pulmonary endothelial cells related to $\beta$-actin expression after incubation with sodium nitroprusside (SNP) or gaseous nitric oxide (NO) as compared to adequate controls. **A** Original Northern Blot. The first two lanes represent control cells that were incubated as monolayers for 24 h, lanes 3 and 4 show results from incubation with gaseous NO and lanes 5 and 6 represent eNOS and $\beta$-actin mRNA after incubation with SNP. **B** Optical density ratios obtained from four experiments. Values are expressed as mean ± SEM. There were no significant differences between treated cells and their respective controls (*SNP* sodium nitroprusside incubation *NO* nitric oxide incubation – with respective controls))

## Discussion

The present study indicates a slight, but significant, worsening of the patients' OI immediately after weaning from inhaled NO. The OI recovers to initial values 30 min after withdrawal. After premature withdrawal of inhaled NO in pediatric patients, an increase of OI or pulmonary artery resistance is observed, necessitating the reapplication of NO [1]. Premature weaning, that is unsuccessful discontinuation of inhaled NO, has to be distinguished from the temporary inhibition of the ENOS by exogenously applied NO. Since the latter effect is not very pronounced and self-limiting after approximately 30 min, sustained impairment of oxygenation after half an hour may indicate further need for exogenous, inhaled NO. We therefore suggest that the reapplication of inhaled NO be assessed half an hour after withdrawal if the patient's situation does not necessitate immediate intervention. A second guideline may be the increase in the OI: in our study the maximal augmentation in patients successfully weaned was 0.7 $cmH_2O$/torr, whereas in patients failing NO withdrawal the minimal increase of the OI was 1.4 torr/$cmH_2O$ [1].

These kinetics of recovery from NO weaning resemble our in vitro experiments showing the reversibility of NOS activity suppression by SNP in pulmonary artery cells after half an hour. This process not only was reversible but even leads to a transiently increased NOS activity. As should be expected from the relatively fast changes in NOS activity, the inhibition of this activity was not caused by suppression of mRNA concentration. Autoinhibition of NOS has been demonstrated previously [8, 9, 10, 11, 12]. We now extend these findings to pulmonary artery endothelial cells. In our experiments we used SNP as the NO donor although purer ones exist. However, since not the feedback inhibition of NOS as such, but the kinetics of this inhibition were the objective of the present study, we tried to use an NO donor with as short a half-life as possible. The half-life of SNP is 3.9 ± 0.2 min, SNAP, for instance, has a half-life of 37 ± 4 h [22].

Nitric oxide has been shown to interact with a number of vasoactive mechanisms besides its own synthesis. The neuropeptide Y1 receptor as well as the endothelin$_A$ receptor function are suppressed by NO [23, 24]. Similarly, catecholamine and endothelin-1 [25, 26] concentrations are reduced by NO while the secretion of the vasoactive peptide neuropeptide Y is increased [27]. By activation of the cyclooxygenase, NO also increases

the synthesis of prostaglandins [28]. Inactivation of proteins takes place via an alteration of thiol groups [29].

The exact link between our in vivo and in vitro data is still subject to speculation. In this context, animal studies studying NOS tissue activity might be beneficial. Also the determination of eNOS protein levels would be warranted to rule out a reduction of the eNOS protein concentration by NO. If NOS inhibition is the cause of the worsening of the OI, the attenuated in vivo effect might be explained by other vasoactive mechanisms partially compensating for the changes in NOS activity. In addition, the low NO doses applied during weaning may allow for a certain recovery of the endogenous NOS activity before withdrawal itself.

In summary, the slight initial increase of the OI after NO withdrawal is reversible within 30 min if the timing for weaning within the disease process has been appropriate. NO suppresses the ENOS of pulmonary artery cells in a spontaneously reversible way even resulting in transiently increased enzyme activity. ENOS mRNA expression is not influenced by NO.

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## References


1. Demirakca S, Dötsch J, Knothe C, Magsaam J, Reiter HL, Bauer J, Kuehl PG (1996) Inhaled nitric oxide in neonatal and pediatric acute respiratory distress syndrome: dose response, prolonged inhalation and weaning. Crit Care Med 24: 1913–1919
2. Rossaint R, Falke KJ, Lopez F, Slam K, Pison U, Zapol WM (1993) Inhaled nitric oxide for the adult respiratory distress syndrome N Engl J Med 328: 399–405
3. Hart CM (1999) Nitric oxide in adult lung disease. Chest 115: 1407–1417
4. Abman SH, Kinsella JP (1998) Inhaled nitric oxide therapy for pulmonary disease in pediatrics. Curr Opin Pediatr 10: 236–242
5. Dötsch J, Demirakça S, Hamm R, Knothe C, Bauer J, Kühl PG, Rascher W (1997) Extracorporeal circulation increases nitric oxide induced methemoglobinemia in vivo and in vitro. Crit Care Med 25: 1153–1156
6. Dötsch J, Demirakça S, Hahn D, Katz N, Kühl PG, Rascher W (1999) Accuracy of methemoglobin measurements: comparison of six different commercial devices and one manual method. Crit Care Med 27 (6): 1191–1194
7. Brune B, Messmer UK, Sandau K (1995) The role of nitric oxide in cell injury. Toxicol Lett 82–83: 233–237
8. Rogers NE, Ignarro LJ (1992) Constitutive nitric oxide synthase from cerebellum is reversibly inhibited by nitric oxide formed from L-arginine. Biochem Biophys Res Commun 30; 189: 242–249
9. Rengasamy A, Johns RA (1993) Regulation of nitric oxide synthase by nitric oxide. Mol Pharmacol 44: 124–128
10. Assreuy J, Cunha FQ, Liew FY, Moncada S (1993) Feedback inhibition of nitric oxide synthase activity by nitric oxide. Br J Pharmacol 108: 833–837
11. Buga G, Griscavage J, Rogers N, Ignarro L (1993) Negative feedback regulation of endothelial cell function by nitric oxide. Circ Res 73: 808–812
12. Griscavage JM, Hobbs AJ, Ignarro LJ (1995) Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds. Adv Pharmacol 34: 215–234
13. Johnson ME, Sill JC, Uhl CB, Halsey TJ, Gores GJ (1996) Effect of volatile anesthetics on hydrogen peroxide-induced injury in aortic and pulmonary arterial endothelial cells. Anesthesiology 84: 103–116
14. Dahm P, Thome J, Zoucas E, Martensson L, Myhre E, Blomquist S (1997) Differential effects of nitric oxide synthase modulation on porcine systemic and pulmonary circulation in vivo. Crit Care Med 25: 280–285
15. Royall JA, Levin DL (1988) Adult respiratory distress syndrome in pediatric patients. II. Management. J Pediatr 112: 335–347
16. Rappaport SH, Shpiner R, Yoshihara G, Wright J, Chang P, Abraham E (1994) Randomized prospective trial of pressure-limited versus volume-controlled ventilation in severe respiratory failure. Crit Care Med 22: 22–32
17. Parida SK, Domann E, Rohde M, Muller S, Darji A, Hain T, Wehland J, Chakraborty T (1998) Internalin B is essential for adhesion and mediates the invasion of Listeria monocytogenes into human endothelial cells. Mol Microbiol 28: 81–93
18. Knowles RG, Palacios M, Palmer RM, Moncada S (1990) Kinetic characteristics of nitric oxide synthase from rat brain. Biochem J 269: 207–210
19. Feelisch M, Kubitzek D, Werringloer J (1996) The oxyhemoglobin assay. In: Feelisch M, Stamler JS (eds) Methods in nitric oxide research, Wiley, New York, pp 455–478
20. Hänze J, Kummer W, Haass M, Lang RE (1991) Neuropeptide Y mRNA regulation in rat sympathetic ganglia: effect of reserpine. Neurosci Lett 124: 119–123
21. Moos M, Gallwitz D (1983) Structure of two human beta-actin-related processed genes one of which is located next to a simple repetitive sequence. EMBO J 3: 757–761
22. Ferrero R, Rodriguez-Pascual F, Miras-Portugal MT, Torres M (1999) Comparative effects of several nitric oxide donors on intracellular cyclic GMP levels in bovine chromaffin cells: correlation with nitric oxide production. Br J Pharmacol 127: 779–787
23. Dötsch J, Hänze J, Beste O, Behrendt J, Weber W-M, Dittrich K, Rascher W (1997) Suppression of the neuropeptide Y-1 receptor function in SK-N-MC cells by nitric oxide. Am J Physiol 273: C618–621
24. Goligorsky M, Tsukahara H, Magazine H, Andersen TT, Asrar BM, Wadie FB (1994) Termination of endothelin signaling: role of nitric oxide. J Cell 158: 485–494
25. Hinson JP, Kapas S, Cameron LA (1996) Differential effects of endogenous and exogenous nitric oxide on release of endothelin-1 from the intact perfused rat adrenal gland in situ. FEBS Lett 379: 7–10
26. Macarthur H, Mattammal MB, Westfall TC (1995) A new perspective on the inhibitory role of nitric oxide in sympathetic neurotransmission. Biochem Biophys Res Commun 216: 686–689
27. Dötsch J, Hänze J, Dittrich K, Demirakça S, Haberberger R, Rascher W (1997) Stimulation of neuropeptide Y release in rat pheochromocytoma cells by nitric oxide. Eur J Pharmacol 331: 313–317

28. Watkins DN, Garlepp MJ, Thompson PJ (1997) Regulation of the inducible cyclo-oxygenase pathway in human cultured airway epithelial (A549) cells by nitric oxide. Br J Pharmacol 121: 1482–1488
29. Ishii T, Sunami O, Nakajima H, Nishio H, Takeuchi T, Hata F (1999) Critical role of sulfenic acid formation of thiols in the inactivation of glyceraldehyde-3-phosphate dehydrogenase by nitric oxide. Biochem Pharmacol 58: 133–143
30. Wilkinson JD, Pollack MM, Glass NL, Kanter RK, Katz RW, Steinhart CM (1987) Mortality associated with multiple organ system failure and sepsis in pediatric intensive care unit. J Pediatr 111: 324–328
31. Hayden WR (1994) Sepsis terminology in pediatrics (letter). J Pediatr 124: 657–658