Organ Dysfunction among Piglets Treated with Inhaled Nitric Oxide and Intravenous Hydrocortisone during Prolonged Endotoxin Infusion

Sofie Paues Göranson, Waldemar Gozdzik, Piotr Harbut, Stanislaw Ryniak, Stanislaw Zielinski, Caroline Gillis Haggerstrand, Andrzej Kubler, Goran Hedenstierna, Claes Frostell, Johanna Albert

Abstract

Objective: It has previously been shown that a combination of inhaled nitric oxide (iNO) and intravenous (IV) steroid attenuates endotoxin-induced organ damage in a 6-hour porcine endotoxemia model. We aimed to further explore these effects in a 30-hour model with attention to clinically important variables.

Design: Randomized controlled trial.

Setting: University animal laboratory.

Subjects: Domestic piglets (n = 30).

Interventions: Animals were randomized into 5 groups (n = 6 each): 1) Controls, 2) LPS-only (endotoxin/lipopolysaccharide (LPS) infusion), 3) LPS + iNO, 4) LPS + IV steroid, 5) LPS + iNO + IV steroid.

Measurements and Main Results: Exposure to LPS temporarily increased pulmonary artery mean pressure and impeded renal function with elevated serum creatinine and acidosis compared to a control group over the 30-hour study period. Double treatment with both iNO and IV steroid tended to blunt the deterioration in renal function, although the only significant effect was on Base Excess (p = 0.045). None of the LPS + iNO + IV steroid treated animals died during the study period, whereas one animal died in each of the other LPS-infused groups.

Conclusions: This study suggests that combined early therapy with iNO and IV steroid is associated with partial protection of kidney function after 30 hours of experimental LPS infusion.

Introduction

Despite modern intensive care the hospital mortality in sepsis with multi-organ failure varies between 30–75%, with the highest mortality in patients with septic shock [1–4]. Inhaled nitric oxide (iNO) improves arterial oxygenation and attenuates pulmonary hypertension by selective relaxation of vascular smooth muscle cells in ventilated lung regions [5,6]. Moreover, it has been increasingly accepted that iNO also has systemic effects [7,8].

Neither the mechanisms, nor the extent of iNO’s extrapulmonary effects, are sufficiently known. Nitric oxide (NO) in tissue has anti-inflammatory effects and inhibits the expression of cytokines, adhesion molecules, interleukins, and other inflammatory mediators [9–15]. NO and corticosteroid administration have both been shown to modulate the inflammatory process by, among other actions, inhibiting the activation of NF-kB, which regulates the expression of many inflammatory and immune genes [12,14,16].
Anesthetic doses were increased during instrumentation and medetomidine (5.3–8.2 mg/kg/h) was used. The trachea was intubated and the piglet was ventilated in a pressure-controlled mode using a Servo 900C ventilator (Siemens Medical Systems, Valencia, CA). Adequate anesthesia or sedation was used throughout the period of the experiment. Muscle relaxants were not used. Animals surviving to the 30-hour endpoint were euthanized with a lethal dose of IV barbiturate. Animals surviving the protocol period. The control group was used solely to enable comparison with the LPS group.

The study was designed to include six piglets in each group and an experimental length of 30 hours, followed by sacrifice of animals surviving the protocol period. The control group was used solely to enable comparison with the LPS group.

Administration of iNO

Inhaled nitric oxide (iNO) (Pulmonox-Messer Griesheim Singapore) was delivered at 30 ppm by a ventilator (Pulmonox Mini [Messer Griesheim, Gumpoldskirchen, Austria]) to the inspiratory limb of the ventilator as previously described by us [8].

Monitoring and hemodynamics

The animals were monitored continuously with a three-lead electrocardiogram (ECG), recordings of heart rate (HR, beats per min), mean systemic, pulmonary arterial, and central venous pressures, (MAP, MPAP and CVP, mmHg), pulse oximetry (SpO2, %), fraction of inspired oxygen (FiO2), and end-tidal carbon dioxide concentration (EtCO2, kPa) (General Electric health care AS/3 Instrumentarium, OY Helsinki, Finland). Pulmonary

Materials and Methods

Ethics statement

The Animal Research Ethics Committee of the Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wroclaw, Poland approved the study (#7/03). Death was not used as a primary endpoint. This allowed us to humanely euthanize an animal that was unresponsive to treatment of severe circulatory collapse. Adequate anesthesia or sedation was used throughout the period of the experiment. Muscle relaxants were not used. Animals surviving to the 30-hour endpoint were euthanized with a lethal dose of IV barbiturate.

Anesthesia and instrumentation

The experiments were conducted at the Institute of the Experimental Surgery and Biotechnology Research, Wroclaw University of Medicine. We studied 30 domestic piglets, 2 months old, and with a median body weight of 21 kg (range 15–24 kg). We have previously described this model in detail [8,19]. The piglets were randomized into five groups and observed for an experimental length of 30 hours, followed by sacrifice of animals surviving the protocol period. The control group was used solely to enable comparison with the LPS group.

1. Control: No LPS infusion, iNO, or IV steroid.
2. LPS-only: Continuous IV infusion of LPS for the whole study period (30 hours).
3. LPS + iNO: iNO at 30 ppm started after 3 hours of LPS infusion and continued until the end of the experiment.
4. LPS + IV steroid: IV hydrocortisone 75 mg started after 3 hours of LPS infusion and repeated every 8 hours thereafter.
5. LPS + iNO + IV steroid: Both continuous iNO at 30 ppm and IV hydrocortisone 75 mg every 8 hours starting 3 hours after the LPS infusion and continued until the end of the experiment.

The piglets were randomized into five groups and observed for 30 hours as follows:

1. Control: No LPS infusion, iNO, or IV steroid.
2. LPS-only: Continuous IV infusion of LPS for the whole study period (30 hours).
3. LPS + iNO: iNO at 30 ppm started after 3 hours of LPS infusion and continued until the end of the experiment.
4. LPS + IV steroid: IV hydrocortisone 75 mg started after 3 hours of LPS infusion and repeated every 8 hours thereafter.
5. LPS + iNO + IV steroid: Both continuous iNO at 30 ppm and IV hydrocortisone 75 mg every 8 hours starting 3 hours after the LPS infusion and continued until the end of the experiment.

The protocol was designed to evaluate the effects of various interventions on prolonged LPS-induced organ dysfunction. A sepsis-like condition was established by continuous IV infusion of endotoxin [LPS polysaccharide (LPS) from Escherichia coli (L2630-25MG), SIGMA, Gothenburg, Sweden, Chemical lot 110K41 10, mixed in sterile water = 2 mg·kg⁻¹] as a bolus dose. An initial dose of 5 μg·kg⁻¹·h⁻¹ was administered for two hours after baseline measurements and then lowered to 1 μg·kg⁻¹·h⁻¹ for the remaining 28 hours of the study period. A flowchart describing the various experimental procedures is given in Figure 1. Fluid and vasopressor support were administered per protocol (see below) to all groups.

The piglets were randomized into five groups and observed for 30 hours as follows:

1. Control: No LPS infusion, iNO, or IV steroid.
2. LPS-only: Continuous IV infusion of LPS for the whole study period (30 hours).
3. LPS + iNO: iNO at 30 ppm started after 3 hours of LPS infusion and continued until the end of the experiment.
4. LPS + IV steroid: IV hydrocortisone 75 mg started after 3 hours of LPS infusion and repeated every 8 hours thereafter.
5. LPS + iNO + IV steroid: Both continuous iNO at 30 ppm and IV hydrocortisone 75 mg every 8 hours starting 3 hours after the LPS infusion and continued until the end of the experiment.

The study was designed to include six piglets in each group and an experimental length of 30 hours, followed by sacrifice of animals surviving the protocol period. The control group was used solely to enable comparison with the LPS group.
capillary wedge pressure (PCWP, mmHg) and thermodilution cardiac output (CO, L×min⁻¹) were measured every 4 hours. Urinary output was measured and the bladder emptied at 12, 24, and 30 hours. Body temperature was monitored by the PAC thermistor in order to keep the animals normothermic (37–38°C) using heating blankets or external cooling if needed. The animals were turned side to side every four hours.

Hypotension, defined as MAP less than 60 mmHg for longer than 3 minutes, was treated initially with a 300 mL bolus of lactated Ringers solution or infusion of up to 750 mL hydroxyethyl starch. If MAP remained below 60 mmHg an infusion of norepinephrine (NE) (40 μg×ml⁻¹) was started. We aimed at maintaining CVP between 6 and 8 mmHg. The summed doses of NE given at 10 time-points during the study (1, 2, 3, 4, 8, 12, 16, 20, 24 and 28 hours) were reported as “total NE” for each animal.

In order to compensate for fluid loss and maintain the blood glucose level a mixture of 2.5% dextrose in saline 0.9% (Glu/NaCl 1:1) (Braun, Melsungen, Germany) was given at a basal infusion rate of 100 mL×h⁻¹. High or low blood glucose levels were not treated. Glycopyrrolate 0.2 mg×1500 mL⁻¹ was added to the infusion in order to counter bradycardia. In addition, all animals received cefuroxime (GlaxoSmithKline, Solna, Sweden) 500 mg IV every 8 hours to counter accidental bacterial contamination during instrumentation.

Laboratory specimens
Blood samples were drawn at baseline (after instrumentation and 1-hour recovery period), at regular intervals for monitoring, and after 30 hours of endotoxin exposure. Measurements included white blood cell count (WBC), interleukin 1 (IL-1), tumour necrosis factor-α (TNFα), creatinine, and urea. Point-of-care analysis of blood gases (iSTAT, Abbot, East Windsor, USA) included pH, PaCO₂, PaO₂, and base excess (BE). Blood drawn for cytokine levels was immediately centrifuged (Hettich Zentrifugen Universal 16R, Hettich GmbH&H, Tuttingen, Germany) for ten minutes at 4000 g×min⁻¹. The supernatants were stored at −70°C until analyzed. WBC, creatinine, and urea were analyzed within one hour.

Interventions
The protocol allowed for certain pre-specified actions to mimic clinical treatment. These included fluid boluses and IV norepinephrine if MAP remained below 60 mm Hg for more than 3 minutes, increased inspiratory pressure and FIO₂ to maintain normocarbia and avoid hypoxemia, and endotracheal suction for secretion clearance. Metabolic acidosis was not treated.

Statistics
Differences within groups between baseline and 30 hours were tested using the exact Wilcoxon signed rank test. Treatment effect, measured as the difference from baseline to 30 hours, was compared between the three treatment groups (iNO, IV steroid and iNO + IV steroid) and the LPS-only group using the exact Wilcoxon-Mann-Whitney test. Given the complexity of the study with experiments lasting for 35 hours or more (including setup and termination) we chose to limit group size to 6 animals to make the study feasible. For animals that died prior to the full study period we used the last antemortem data for analysis, provided that the animal had lived at least 20 hours. This was the case in 2 of the 3 deaths. Multiplicity adjustments were done using the Bonferroni-Holm procedure for the between-groups comparisons. A much larger number of animals would have been required for comparisons and multiplicity adjustments within groups, and was not the main focus of the study. Two-sided tests were used. A p-value of <0.05 was taken to indicate a statistically significant difference.

Results
General findings
Before LPS infusion there were no baseline differences between study groups in respiratory or circulatory variables or renal function, although the double treatment group tended to have higher PaO₂/FIO₂ than the LPS-only group (Tables 1–3). No significant effects were seen over time or between groups on HR, CVP, or PCWP (data not shown). The total amount of anesthetic and fluids given during the study period was not significantly different between all groups receiving LPS. No animals died in the control group or the LPS + iNO + IV steroid (“double-treatment”) group, and there was 1 death each in the remaining three LPS-exposed groups. One animal died of respiratory failure (pulmonary edema) at 6 hours, one animal died of sudden arrhythmia late in the study period. A third animal was euthanized per protocol (close to the end) after having developed severe and unresponsive hypotension.

LPS group
LPS exposure over 30 hours was associated with increased peak inspiratory pressure compared to baseline (p = 0.05) and compared...
### Table 1. Respiratory variables.

| Group                          | Baseline          | 30 h               | p<sup>1</sup> | p<sup>2</sup> | p<sup>3</sup> (P adj) |
|-------------------------------|-------------------|--------------------|---------------|--------------|-----------------------|
| **Peak Inspiratory Pressure (cmH₂O)** |                   |                    |               |              |                       |
| Control                       | 16; 13–19         | 19; 18–22          | 0.06          | Ref.         |                       |
| LPS-only                      | 15; 15–24         | 28; 23–35          | 0.05          | 0.005        |                       |
| LPS + iNO                     | 17; 16–19         | 24; 22–40          | 0.03          | 1.0 (1.0)    |                       |
| LPS + IV steroid              | 18; 13–26         | 27; 17–40          | 0.09          | 0.86 (1.0)   |                       |
| LPS + iNO + IV steroid        | 17; 15–20         | 20; 14–37          | 0.16          | 0.64 (1.0)   |                       |
| **PaO₂/FIO₂ (kPa)**           |                   |                    |               |              |                       |
| Control                       | 48; 36–77         | 45; 35–98          | 0.68          | Ref.         |                       |
| LPS-only                      | 52; 45–57         | 48; 13–57          | 0.36          | 0.78         |                       |
| LPS + iNO                     | 64; 51–92         | 45; 28–79          | 0.16          | 1.00 (1.0)   |                       |
| LPS + IV steroid              | 54; 43–112        | 44; 19–76          | 0.03          | 0.43 (1.0)   |                       |
| LPS + iNO + IV steroid        | 66; 59–98         | 56; 44–90          | 0.22          | 0.93 (1.0)   |                       |
| **PaCO₂ (kPa)**               |                   |                    |               |              |                       |
| Control                       | 5.79; 4.45–7.01   | 4.48; 3.37–6.49    | 0.07          | Ref.         |                       |
| LPS-only                      | 5.67; 4.70–6.93   | 5.53; 4.60–5.82    | 0.44          | 0.41         |                       |
| LPS + iNO                     | 5.73; 3.72–6.88   | 4.70; 4.17–6.55    | 0.44          | 0.66 (1.0)   |                       |
| LPS + IV steroid              | 5.22; 4.48–7.39   | 5.28; 4.28–9.30    | 1.00          | 0.54 (1.0)   |                       |
| LPS + iNO + IV steroid        | 5.17; 4.75–6.67   | 5.09; 4.67–7.43    | 0.84          | 0.57 (1.0)   |                       |

**Statistics:** Values are shown as median and range.
<sup>1</sup>Within group from baseline to 30 hours.
<sup>2</sup>Comparison of distribution of differences between LPS-only and control.
<sup>3</sup>Comparison of distribution of differences against LPS-only (Bonferroni-Holm adjusted p-values within parenthesis).
doi:10.1371/journal.pone.0096594.t001

### Table 2. Circulatory variables.

| Group                          | Baseline          | 30 h               | p<sup>1</sup> | p<sup>2</sup> | p<sup>3</sup> (P adj) |
|-------------------------------|-------------------|--------------------|---------------|--------------|-----------------------|
| **Cardiac output (L/min)**    |                   |                    |               |              |                       |
| Control                       | 2.4; 2.2–4.4      | 2.1; 1.2–6         | 0.06          | Ref.         |                       |
| LPS-only                      | 2.0; 1.3–2.3      | 1.4; 1.1–3.7       | 0.81          | 0.24         |                       |
| LPS + iNO                     | 2.5; 1.9–4.4      | 3.5; 1.4–4.5       | 0.62          | 0.73 (0.92)  |                       |
| LPS + IV steroid              | 2.4; 1.9–4.4      | 3.5; 1.4–4.5       | 0.12          | 0.46 (0.92)  |                       |
| LPS + iNO + IV steroid        | 2.7; 2.1–2.8      | 1.9; 1.3–2.8       | 0.09          | 0.19 (0.58)  |                       |
| **MAP (mmHg)**                |                   |                    |               |              |                       |
| Control                       | 101; 92–124       | 84; 63–96          | 0.15          | Ref.         |                       |
| LPS-only                      | 93; 79–151        | 110; 87–138        | 0.81          | 0.64         |                       |
| LPS + iNO                     | 91; 52–103        | 82; 63–138         | 1.00          | 0.69 (1.0)   |                       |
| LPS + IV steroid              | 95; 75–103        | 82; 63–138         | 1.00          | 1.00 (1.0)   |                       |
| LPS + iNO + IV steroid        | 92; 63–124        | 100; 72–129        | 0.78          | 0.70 (1.0)   |                       |
| **MPAP (mmHg)**               |                   |                    |               |              |                       |
| Control                       | 15; 12–19         | 17; 12–18          | 0.85          | Ref.         |                       |
| LPS-only                      | 15; 11–21         | 25; 17–34          | 0.12          | 0.01         |                       |
| LPS + iNO                     | 12; 7–19          | 15; 12–37          | 0.06          | 0.56 (0.56)  |                       |
| LPS + IV steroid              | 15; 13–23         | 32; 15–41          | 0.06          | 0.08 (0.24)  |                       |
| LPS + iNO + IV steroid        | 13; 11–21         | 14; 8–38           | 0.50          | 0.26 (0.51)  |                       |
| **Total NE adm. (µg/kg)**     |                   |                    |               |              |                       |
| Control                       | 0.0               | 0–0                |               |              |                       |
| LPS-only                      | 162; 9–1169       | 0.001              |              |              |                       |
| LPS + iNO                     | 64; 6–1071        | 0.63 (0.63)        |              |              |                       |
| LPS + IV steroid              | 37; 0–72          | 0.20 (0.40)        |              |              |                       |
| LPS + iNO + IV steroid        | 7; 0–45           | 0.04 (0.11)        |              |              |                       |

**Statistics:** Values are shown as median and range.
<sup>1</sup>Within group from baseline to 30 hours.
<sup>2</sup>Comparison of distribution of differences between LPS-only and control.
<sup>3</sup>Comparison of distribution of differences against LPS-only (Bonferroni-Holm adjusted p-values within parenthesis).
doi:10.1371/journal.pone.0096594.t002
Discussion

In this study we found no significant effect of combination therapy with inhaled nitric oxide and intravenous steroid in an experimental porcine model of extended challenge with endotoxin, although a tendency to a protection of kidney function was seen.

Based on our previous experience with prolonged experimental studies in a porcine model [8,19], this protocol involved 30 hours of endotoxin exposure using high-dose E. coli lipopolysaccharide (LPS) infusion, which resulted in multiple organ dysfunction after 12–24 hours. This study was intended to mimic the clinical setting, with an initial endotoxin insult and then resuscitative interventions initiated in real time over a prolonged study period.

Early in the experiment (4–6 hours) transient intense pulmonary hypertension was evident in all animals exposed to LPS. We did not focus on these early events as they have been described in detail in several earlier studies [18,20,21]. A majority of animals developed signs of impaired organ function and deaths occurred in all groups exposed to LPS except in the double-treated group.

When initially going over our data, it became clear to us that our interventions to some extent blunted the monitored pathophysiological response. Our protocol allowed for various inter-ventions, e.g. against hypotension, hypovolemia, hypoxemia and hypercarbia which we believe made most animals survive the study period of 30 hours. On the other hand, these interventions reduced the possibility to detect statistically significant differences in pathophysiology between the study groups.

The animals treated with the combination of iNO + IV steroid tended to be more stable and less sick during the protocol period of 30 hours of LPS-infusion, as compared to LPS-only animals. Several animals required vasopressor support in addition to increased crystalloid administration in order to maintain MAP > 60 mmHg. Although not statistically significant, this seemed to

| Table 3. Kidney function. |
|---------------------------|
| **Group** | **Baseline** | **30 h** | **P^1** | **P^2** | **P^3 (P adj)** |
| **pH** | | | | | |
| Control | 7.43; 7.42–7.54 | 7.46; 7.38–7.55 | 1.00 | Ref. |
| LPS-only | 7.46; 7.45–7.52 | 7.30; 7.25–7.32 | 0.06 | 0.004 |
| LPS + iNO | 7.46; 7.39–7.54 | 7.34; 7.21–7.42 | 0.03 | 0.33 (0.61) |
| LPS + IV steroid | 7.47; 7.33–7.52 | 7.33; 7.17–7.40 | 0.03 | 0.31 (0.61) |
| LPS + iNO + IV steroid | 7.47; 7.40–7.50 | 7.41; 7.27–7.54 | 0.56 | 0.18 (0.54) |
| **BE (mEq/L)** | | | | | |
| Control | +6.0; +1.0–+8.0 | +1.5; −9.0–+7.0 | 0.06 | Ref. |
| LPS-only | +6.0; +4.0–+8.0 | −8.0; −8.0–−5.0 | 0.06 | 0.004 |
| LPS + iNO | +6.5; +2.0–+9.0 | −6.5; −11.0–+4.0 | 0.03 | 0.83 (0.83) |
| LPS + IV steroid | +4.0; +3.0–+7.0 | −6.0; −9.0–+1.0 | 0.03 | 0.017 (0.045) |
| LPS + iNO + IV steroid | +4.0; +0.0–+6.0 | −0.5; −6.0–+8.0 | 0.31 | 0.015 (0.045) |
| **Urea (mmol/L)** | | | | | |
| Control | 4.67; 2.17–5.33 | 2.34; 1.67–3.00 | 0.03 | Ref. |
| LPS-only | 4.34; 3.50–6.67 | 11.00; 2.33–17.67 | 0.31 | 0.02 |
| LPS + iNO | 3.83; 3.50–6.33 | 8.92; 5.83–16.17 | 0.03 | 0.93 (1.0) |
| LPS + IV steroid | 3.67; 1.50–6.33 | 9.67; 4.83–27.17 | 0.03 | 0.93 (1.0) |
| LPS + iNO + IV steroid | 3.75; 2.33–7.60 | 8.17; 3.33–13.67 | 0.22 | 0.79 (1.0) |
| **Urine output 12–30 hours, (mL)** | | | | | |
| Control | 1040; 200–1500 | Ref. |
| LPS-only | 580; 400–1160 | 0.58 |
| LPS + iNO | 1058; 0–1790 | 0.66 (1.0) |
| LPS + IV steroid | 716; 150–2095 | 0.47 (1.0) |
| LPS + iNO + IV steroid | 1025; 400–2500 | 0.26 (0.79) |

**Statistics:** Values are shown as median and range.

1Within group from baseline to 30 hours (urine output: no baseline value, volume collected over time, from 12 to 30 hours).

2Comparison of distribution of differences versus LPS-only (Bonferroni-Holm adjusted p-values within parenthesis).

3Comparison of distribution of differences versus LPS-only (Bonferroni-Holm adjusted p-values within parenthesis).

doi:10.1371/journal.pone.0096594.t003
occur less frequently in the LPS + IV steroid and LPS + iNO + IV steroid groups. In general, we seldom encountered severe respiratory problems in the treatment groups, with only few animals requiring FIO2$\geq$0.50. We noted an increased peak inspiratory pressure (PIP) in several LPS-exposed animals, again with a tendency of less rise in the double treatment (Table 1). A rise in airway pressure could be a sign of an inflammatory response in lung tissue and is a hallmark of acute lung injury [22]. Acute kidney injury (AKI), signified by polyuria or oliguria plus elevated creatinine, urea, and decreasing Base Excess, was noted in many animals exposed to LPS. Clinical studies have shown that the presence of AKI as ‘risk’ increases hospital mortality from 8.4% to 20.9%, and when ‘injury’ is present, to 45.6% [23,24].

Figure 2. Changes in serum creatinine levels over time, unit = \(\mu\text{mol/L}\). LPS = lipopolysaccharide, IV steroid = intravenous hydrocortisone, iNO = inhaled nitric oxide. Values are shown as median and range. Data have been collected at 0, 12, 24 and 30 hours but spread along the time axis to be more distinguishable. A significant difference was seen between control and LPS alone (\(^*: p = 0.02\)). No significances between treatment groups (IV steroid, iNO, IV steroid + iNO) and LPS alone were noted.

doi:10.1371/journal.pone.0096594.g002

Table 4. Inflammatory mediators.

| Group                  | Baseline         | 30 h             | \(P^1\) | \(P^2\) | \(P^3\) (P adj) |
|------------------------|------------------|------------------|---------|---------|----------------|
| **WBC**                |                  |                  |         |         |                |
| Control                | 11.9; 9.0–15.3   | 11.9; 8.5–14.7   | 0.44    | Ref.    |                |
| LPS-only               | 7.5; 4.5–13.3    | 18.3; 7.6–21.8   | 0.06    | 0.25    |                |
| LPS + iNO              | 8.8; 4.0–20.1    | 34.5; 3.9–43.9   | 0.50    | 1.00    | (1.0)          |
| LPS + IV steroid       | 7.4; 3.7–16.5    | 21.4; 8.5–65.5   | 0.06    | 0.15    | (0.45)         |
| LPS + iNO + IV steroid | 11.8; 5.8–18.8   | 17.5; 8.4–40.1   | 0.16    | 0.97    | (1.0)          |
| **IL-1**               |                  |                  |         |         |                |
| Control                | 17.5; 9.6–84     | 18.4; 4–48       | 1.00    | Ref.    |                |
| LPS-only               | 30.7; 4–68       | 117; 9.6–186     | 0.06    | 0.06    |                |
| LPS + iNO              | 26.2; 8–72       | 75; 42–116       | 0.18    | 0.41    | (1.0)          |
| LPS + IV steroid       | 44.1; 6–72       | 75; 41.8–116     | 0.25    | 0.90    | (1.0)          |
| LPS + iNO + IV steroid | 18.9; 2.70       | 50; 14–130       | 0.12    | 0.84    | (1.0)          |
| **TNF-\alpha**         |                  |                  |         |         |                |
| Control                | 88; 53–130       | 97; 66–117       | 0.81    | Ref.    |                |
| LPS-only               | 77; 63–110       | 59; 154–1077     | 0.06    | 0.02    |                |
| LPS + iNO              | 81; 62–130       | 52; 106–1243     | 0.12    | 0.73    | (1.0)          |
| LPS + IV steroid       | 72; 62–99        | 42; 277–679      | 0.12    | 0.91    | (1.0)          |
| LPS + iNO + IV steroid | 77; 54–203       | 243; 153–443     | 0.06    | 0.55    | (1.0)          |

Statistics: Values are expressed as median and range.

WBC = White blood cell count (x10^9/L); IL-1 = Interleukin 1 (pg/ml); TNF-\alpha = Tumor Necrosis Factor-\alpha (pg/ml); LPS = Lipopolysaccharide; iNO = Inhaled nitric oxide.

1 within group from baseline to 30 hours.

2 Comparison of distribution of differences between LPS-only and control.

3 Comparison of distribution of differences against LPS-only (Bonferroni-Holm adjusted p-values within parenthesis).

doi:10.1371/journal.pone.0096594.t004
Recent data from Hallström et al suggest interspecies differences in the response to iNO during experimental endotoxemia [25,26]. However the much smaller endotoxin dose used in these studies on healthy human volunteers compared to our experimental animals should also be kept in mind. In addition, the administration of endotoxin is not identical to a septic state, in which also live bacteria and possibly release of toxins other than LPS complicate the biochemistry and pathophysiology. It can also be discussed which array of cytokines and markers to follow, during the exposure to endotoxin. In a recent review by Pierrakos and Vincent [27], a total of 178 biomarkers were discussed and referenced. It was concluded that no one special marker has yet been proven in experimental and clinical studies to be of greater relevance and specificity than the others.

Furthermore, the present piglet study and other work to date have not discerned which doses of iNO and IV hydrocortisone are optimal for clinical effect, nor have they defined whether a preemptive treatment strategy would yield a stronger effect. In a recent review, Rivers et al [28] argue for start of immunotherapeutic intervention early, before the cytokine storm has blown over. Finally, we do not yet know the exact molecular mechanism for the modifying effect, if any, of a combined treatment with iNO and IV steroids, during an LPS infusion. Modified expression of the glucocorticoid receptor remains a possibility [18]. An interesting additional idea would be a reduced expression of Toll-like receptors, in similarity with what was preliminarily reported earlier this year from a study focusing on Toll-like receptor 4 (TLR 4) during ischemia-reperfusion injury in a piglet model [29].

Conclusions

We have studied sedated and mechanically ventilated piglets during a prolonged (30 hour) LPS infusion examining clinically meaningful endpoints while intervening with iNO and IV steroid separately and in combination. Although a tendency of organ protection was noted by the combination of iNO and IV steroid, in particular of the kidney, no clearly significant differences were seen. A more focused study in a larger material may reveal significant differences.

Acknowledgments

The authors would like to acknowledge Dr. John M. Litell from the Department of Pulmonary and Critical Care Medicine at Mayo Clinic for his assistance in preparing the manuscript.

Author Contributions

Conceived and designed the experiments: JA WG CF AK SPG. Performed the experiments: PH SZ SR SPG GH. Analyzed the data: SPG JA WG CF GH. Contributed reagents/materials/analysis tools: WG AK JA CF GH SZ. Wrote the paper: JA SPG GH CF WG. Lab organisation: WG JA AK. Provided comments: PH SR SZ CH AK.

References