The reciprocal relationship between heme oxygenase and nitric oxide synthase in the organs of lipopolysaccharide-treated rodents

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Abstract
The production of nitric oxide (NO) by inducible NO synthase (NOS) and carbon monoxide (CO) by inducible heme oxygenase (HO) contributes greatly to endotoxemia. Reciprocal relationships have been proposed between the NO/NOS and CO/HO systems. However, the interaction between these systems during endotoxemia is unclear, and it is unknown whether the interactive behavior differs among organs. Using endotoxic rats, we studied the effects of the inducible NOS (iNOS) inhibitor L-canavanine (CAN), and the HO inhibitor zinc protoporphyrin (ZPP) on gene expression and protein levels of iNOS, endothelial NOS (eNOS), inducible HO (HO-1), and constitutive HO (HO-2) in the brain, lung, heart, liver and kidney tissue. Intravenous injection of LPS significantly increased iNOS and HO-1 gene expression in all organs. The effects of LPS on eNOS gene expression differed among organs, with increased expression in the liver and kidney, and no change in the lung, brain and heart. ZPP administration down-regulated the LPS-induced increase in HO-1 expression and produced a further increase in iNOS expression in all organs. These data suggest that the CO/HO system modifies the NO/NOS system in endotoxic organs, and that there were only minor organ-specific behaviors in terms of the relationship between these systems in the organs examined.

Endotoxemia, which can lead to shock, is a detrimental consequence of severe Gram-negative bacterial infection. Endotoxic shock is initiated by the release of bacterial cell wall-derived lipopolysaccharide (LPS) and the subsequent production of cytokines and vasoactive mediators (13). Gaseous mediators, namely nitric oxide (NO) and carbon monoxide (CO), and the major endogenous sources of these mediators, nitric oxide synthase (NOS) and heme oxygenase (HO), serve a pivotal role in endotoxemia.

NO is an extensively studied molecule, and is known for its vasorelaxation and oxidative stressor properties during endotoxemia and septic shock. NO is produced by three distinct NOS enzymes. Two of these NOS isozymes are predominantly constitutively expressed in endothelial cells (eNOS) and in neuronal cells (nNOS), whereas the expression of the third isozyme (iNOS) is inducible in a variety of cells (e.g., macrophages, hepatocytes, vascular smooth muscle cells and cardiac myocytes) by numerous stimuli, including LPS (5, 13). iNOS is responsible for producing most of the NO that causes hypotension and oxidative stress during endotoxic shock, while eNOS is reported to have only a minor role in the pathophysiology of endotoxemia (9, 18).

CO has been perceived for many years to be a life-threatening gas; recently, however, CO has become recognized to have anti-inflammatory and vasorelaxative properties and is essential for life. HO is the rate-limiting enzyme in the catabolism of heme, a process that promotes the formation of equimolar amounts of the bile pigment biliverdin, free iron, and CO (1). Together with the anti-inflam-
matory and anti-oxidative functions of HO, the CO/HO system has recently been recognized as a vasodilator as well as a cytoprotector. HO has two distinct isozymes. HO-1 is highly inducible by heme and a vast array of non-heme substances, including LPS. By contrast, HO-2 is expressed in a constitutive fashion, and appears to function as a heme-binding molecule in normal cells (19). The similarities and diversities of the NO/NOS and CO/HO systems were well established in numerous studies (11, 14), but the interactions between these systems have not yet been elucidated. The interactive behavior of the two systems has been examined in mononuclear cells (7), vascular endothelial cells (4), and vascular smooth muscle cells (22), and accumulating evidence from these studies has shown that the NO/NOS system induces the CO/HO system, while the CO/HO system reciprocally regulates the NO/NOS system. The relationship between the two systems in whole organs, on the other hand, has not been well studied, and it is unknown whether the two systems interact in the same manner in all organs or whether the two systems interact in an organ-specific manner.

In this study, we examined the interactions between the two systems in whole organs by measuring the expression of mRNA and protein for iNOS, eNOS, HO-1, and HO-2 in LPS-treated rat brain, lung, heart, liver, and kidney. We also administered L-canavanine (CAN), a selective iNOS inhibitor and zinc protoporphyrin (ZPP), an HO inhibitor, to determine the reciprocal relationship between the two systems.

MATERIALS AND METHODS

Animal treatment and organ preparation. Male Wiistar rats aged 8–10 weeks, weighing 230–270 g, were purchased from Saitama Experimental Animal Supply (Saitama, Japan). The experimental protocol was approved by the Animal Experimental Ethical Review Committee of Nippon Medical School. Rats were anesthetized by an intravenous injection of 50 mg/kg pentobarbital followed by continuous infusion of 25 mg/kg/h. To ensure the airway remained open, a polyethylene catheter was intubated into the trachea, and spontaneous ventilation of atmospheric air was maintained throughout the study. To measurement of blood pressure, a polyethylene catheter was inserted into the left femoral artery. Animals were assigned into six groups (n = 8 per group): the lipopolysaccharide (LPS) group, in which 10 mg/kg LPS was administered instead of LPS; the LPS+CAN group, in which 10 mg/kg LPS was initially injected followed by a continuous infusion of 20 mg/kg/h CAN; the LPS+ZPP group, in which an initial dose of 10 mg/kg LPS was followed by hourly injections of 0.1 μmol/kg/h ZPP; the CAN group, in which initial saline injection was followed by a continuous infusion of 20 mg/kg/h CAN; and the ZPP group, in which the initial saline injection was followed by hourly injections of 0.1 μmol/kg/h ZPP. Blood pressure and pulse rate were recorded at three time-points: immediately prior to the initial injection of LPS or saline, and 3 and 6 h after the injection. Animals were decapitated after 6 h and the brains, lungs, hearts, livers, and kidneys were extracted. All samples were stored at −80°C before determination of mRNA expression and protein levels of HO-1, HO-2, iNOS, and eNOS.

Real-time reverse transcriptase-polymerase chain reaction (Real-time PCR). Total RNA was extracted from each organ using the chaotropic Trizol method followed by isogen-chloroform extraction and isopropanol precipitation (3). The mRNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan, Tokyo, Japan) and PCR Express (Thermo Fisher Scientific, Waltham, MA, USA). We used TaqMan® Gene Expression Assays (Applied Biosystems Japan) for real-time PCR primers and TaqMan fluorogenic probes. The accession numbers of the primers and probes are presented in Table 1. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as an endogenous control.
and primer set (Pre-Developed TaqMan® Assay Reagents; Rat GAPD Endogenous control Probe, Primer Code number 4352338E, NCBI RefSeq accession number NM_017008.3) (Applied Biosystems Japan) was purchased and included alongside the unknown samples as an endogenous control. The relative amounts of all mRNAs were measured using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems Japan) and calculated by the comparative CT method (Applied Biosystems, Foster City, CA, USA).

**Immunoblotting.** Protein expression was measured for the groups that showed a significant difference in mRNA expression. Protein was extracted from homogenized organ samples showing average mRNA expression in each group. Samples were lysed with T-PER® (PIERCE, Rockford, IL) lysis buffer and each lysate was then diluted to 1 mg/mL with Laemmli sample buffer (Bio-Rad Japan, Tokyo, Japan) containing β-mercaptoethanol and boiled at 95°C for 10 min. The lysates (5 μg) were fractionated on Tris-HCl 12.5% Ready-Gels® (Bio-Rad Japan) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with ChemiBLOCKER™ blocking solution (Millipore, Billerica, MA) and then incubated with primary antibodies as follows: rabbit polyclonal anti-HO-1 (1:1,500; Millipore), rabbit polyclonal anti-iNOS (1:1,000; Abcam, Cambridge, MA), rabbit polyclonal anti-eNOS (1:1,000; Abcam), and rabbit polyclonal anti-GAPDH (1:2,500; Abcam). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000–1:10,000; Millipore) secondary antibody and processed with ECL Plus™ Western blotting detection reagents (GE Healthcare KK, Tokyo, Japan) for enhanced chemiluminescence.

**Optical densitometry for protein band quantitation.** Optical densitometry for protein band quantitation was performed on scanned films using Image J (National Institutes of Health, USA). The background density was subtracted from the density of each band and the densitometric reading of the protein of interest was normalized to GAPDH readings, which served as loading controls. The value of each sample was calculated as: (target protein/GAPDH protein) and then divided by the value of the control group for normalization.

**Statistical analysis.** Non-repeated-measures analysis of variance (ANOVA) and Bonferroni’s multiple-comparison tests were performed to determine differences in gene expression between the CON and LPS groups. The same test was also performed to distinguish differences in mean arterial pressure (MAP) and pulse rate between the groups. Repeat-measures ANOVA and Bonferroni’s multiple-comparison tests were performed to determine differences in MAP and pulse rate over time. P < 0.05 was considered to indicate a significant difference. All statistical analyses were carried out using Dr. SPSS® (SPSS Inc. Chicago, IL). Data are expressed as means ± standard deviation (SD).

**RESULTS**

Figs. 1, 2 and 3 show the relative mRNA expression of HO-1, iNOS, and eNOS, respectively. The levels of HO-1 and iNOS mRNA increased in all five organs in the LPS group compared with the CON group (Figs. 1 and 2). LPS-induced iNOS expression decreased in the lung, heart, liver, and kidney in the LPS+CAN group while showing no significant change in the brain, although there was a trend towards a decrease. The LPS+ZPP group showed a decrease in LPS-induced HO-1 mRNA expression and a further increase in iNOS mRNA gene expression in all five organs. Except for the brain, there was no organ-specific expression for either inducible isoforms. eNOS mRNA levels increased in the liver and kidney of animals in the LPS group, but remained constant in the brain, lung and heart, compared with animals in the CON group (Fig. 3).

Figs. 4, 5 and 6 show the protein bands of the immunoblotting analysis and the normalized optical densitometric values of HO-1, iNOS and eNOS, respectively. The changes in protein expression in each sample were similar to the mRNA expression observed in the real-time PCR analysis. HO-2 did not change in mRNA expression and protein level in all organs examined in any group of the study (data not shown).

Fig. 7 and Fig. 8 show the time-course of changes in the MAP and the pulse rate. The MAP is one of the most practical clinical measures for sepsis which is known to decrease in sepsis which is known to increase in endotoxemia, mainly due to overproduction of NO, various cytokines, and CO (2, 13). The pulse rate is also known as a measure for sepsis which is known to increase in endotoxemia (8). The decrease in MAP along with the increase in pulse rate was measured to confirm the systemic response to LPS and other agents administered. The MAP decreased in the LPS group compared with
Fig. 1  Effects of L-canavanine (CAN) and zinc protoporphyrin (ZPP) on the expression of HO-1 mRNA in the brain, lung, heart, liver and kidney tissue after administration of LPS or saline. The HO-1 expression was increased in the LPS group and showed no difference between the LPS+CAN group and the LPS group. n=8 per group. **, P < 0.01 vs CON group. ##, P < 0.01 vs LPS group. Abbreviations are shown in the legend for Fig. 1.

Fig. 2  Effects of L-canavanine (CAN) and zinc protoporphyrin (ZPP) on the expression of iNOS mRNA in the brain, lung, heart, liver and kidney tissue after administration of LPS or saline. The iNOS expression was increased in the LPS group and showed further increase in the LPS+ZPP group. n=8 per group. *, P < 0.05 vs CON group. **, P < 0.01 vs LPS group. Abbreviations are shown in the legend for Fig. 1.
Gaseous mediators in solid organs

The CON group at 3 and 6 h after administration of LPS. The LPS+CAN group and the LPS+ZPP group also showed decreases in MAP compared with the CON group, but these decreases were comparatively smaller than that in the LPS group at 6 h. The pulse rate increased in the LPS group at 6 h after administration of LPS, while that in the CON group decreased at 3 and 6 h. The LPS+CAN group and the LPS+ZPP group showed no changes in pulse rate throughout the study, resulting in a significant difference compared with the CON group at 3 and 6 h, and compared with the LPS group at 6 h after administration of LPS.

DISCUSSION

iNOS expression was suppressed by CO/HO activity

Previous experimental data suggest that NO is a potent inducer of HO-1 gene expression as a result of peroxynitrite formation by reacting with superoxide anion and/or glutathione depletion by NO (4). On the other hand, the CO/HO system has been suggested to modulate NO production (21). In our study, ZPP (an HO inhibitor) down-regulated the LPS-induced increase in HO-1 mRNA expression, and elicited a further increase in LPS-induced iNOS expression in all of the organs examined. This behavior of iNOS and HO-1 mRNA expression has been reported in several cells and tissues, including macrophages and the left ventricle tissue (14, 20), but the present study was the first to examine such behaviors in five solid organs at the same time-point of endotoxemia. Although there are some minor differences between the organs, we identified no organ-specific behavior in terms of mRNA and protein expression. The mechanisms responsible for the increase in iNOS mRNA are assumed to be: the abrogation of the anti-inflammatory effect of CO which increases levels of the inflammatory mediator iNOS, and the decrease of the degradation of heme, a cofactor of NOS, owing to inhibition of HO. Recent studies have suggested that direct inhibition of CO might be a dominating factor in the regulation of iNOS by the CO/HO system (21). These mechanisms have been extensively investigated using cell lines, and the results of our study suggest that these mechanisms may occur in vivo and may occur simultaneously in endotoxic organs.

HO-1 expression was not induced by NO/NOS activity

Administration of the selective iNOS inhibitor CAN did not significantly affect HO-1 mRNA levels in any of the organs tested. Previous reports have
Fig. 4  Effects of L-canavanine (CAN) and zinc protoporphyrin (ZPP) on the protein expression of HO-1 in the brain, lung, heart, liver and kidney tissue after administration of LPS or saline. Western blots are shown with optical densitometric analysis. CON, control group; LPS, lipopolysaccharide group; LPS+CAN, lipopolysaccharide with L-canavanine group; LPS+ZPP, lipopolysaccharide with zinc protoporphyrin group; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OD, optical densitometry; Ctrl, control.

Fig. 5  Effects of L-canavanine (CAN) and zinc protoporphyrin (ZPP) on the protein expression of iNOS in the brain, lung, heart, liver and kidney tissue after administration of LPS or saline. Western blots are shown with optical densitometric analysis. Abbreviations are shown in the legend for Fig. 4.

Fig. 6  Effects of L-canavanine (CAN) and zinc protoporphyrin (ZPP) on the protein expression of eNOS in the liver and kidney tissue after administration of LPS or saline. Western blots are shown with optical densitometric analysis. Abbreviations are shown in the legend for Fig. 4.
shown inconsistent results regarding the regulation of HO-1 expression when iNOS inhibitors are administered in similar conditions. Similar to our results, Sakamoto et al. (15) and Yet et al. (22) reported no change in HO-1 expression after the administration of iNOS inhibitors, whereas HO-1 expression was significantly decreased after the administration of iNOS inhibitors in other studies (4, 7). The former authors speculated that HO-1 showed no change because there are other pathways independent of the NO pathway that can induce HO-1, even when iNOS inhibitors are administered, or that overstimulation of both NO/NOS and CO/HO pathways by administrating a lethal dose of LPS may disequilibrated the physiological balance and disturb the functional relationship. Our study, in which 10 mg/kg LPS was administered intravenously, may follow these hypotheses, because this dose was confirmed to be lethal in preliminary studies. As such, the dose of LPS used in the present study probably overstimulated and disequilibrated the NO/NOS and CO/HO interaction. Furthermore, the effect of CAN on HO-1 expression might have been masked by overstimulation of mediators independent of NO, such as inflammatory cytokines and other oxidative stressors. Our study protocol, using in vivo whole organs to determine the relationships between gaseous molecule systems, is less commonly used compared with other protocols because studies to date have mainly focused on monocytic cells (7), endothelial cells (4), and vascular smooth muscle cells (22). Therefore, there is also the possibility that the induction of HO-1 gene expression by the NO/NOS system, as examined in various cell lines, does not occur in vivo. Further studies are necessary to investigate whether this relationship is functional in vivo and, consequently, in human sepsis. If this relationship is functional, it will be important to investigate the level of stimulation (the concentration of LPS and/or the severity of sepsis) required to verify the NO/NOS induced HO-1 expression.

**The organ-specific changes in eNOS expression during endotoxemia**

The baseline eNOS mRNA gene expression is known to be moderate, but essentially efficient to life. Its expression is increased by stimuli such as shear stress, hydrogen peroxide and lysophosphatidylcholine, and decreased by LPS and tumor necrosis factor-α (16), but responses differ among organs and cells, and also according to the time-point of endotoxemia (6, 18). Endotoxemia typically increases eNOS expression in the early phase (begins within 60 min) and gradually decreases its expression in the late phase, as the pivotal role shifts to iNOS (17). In the late phase, LPS stimulation decreases the expression of eNOS in the lung, aorta, and heart and increases expression in the liver (10). Our study showed increased expression in the liver and kidney, while there was no change in the brain, lung and heart. Taken together with the previous reports, we assume that the up-regulation seen in the two organs might represent the organ-specific behavior that is reported to occur in the late phase, or because the time-point of endotoxemia was still in the early phase in those two organs. The unchanged expression of eNOS in the brain, lung and heart could be because these organs were in the transient phase between up-regulation and down-regulation. The results of our study were, at least in part, consistent...
Cardiac depression in endotoxemia is known to occur in the early phase (14), which is at least partly due to eNOS overexpression. Both NO and CO can relax vascular smooth muscle cells and can alter cardiac function via cGMP production, but the restoration of MAP by ZPP is not complete due to inhibition of CO. Because CO itself is only about 1/80 as effective as NO at producing cGMP (11), other factors are likely to be involved, such as the arginine vasopressin pathway (12). This suggests the existence of an endogenous substance similar to 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1), a synthetic compound that activates guanylate cyclase as effectively as NO when combined with CO (11).

In conclusion, our study demonstrates that high concentrations of LPS promote a dramatic increase in iNOS and HO-1 mRNA and protein expression, and that the CO/HO system modifies the NO/NOS system in the rat brain, lung, heart, liver and kidney tissue. We could not detect any functional relationship between these systems after the administration of CAN in this protocol. We also showed that there is no organ-specific relationship between the inducible types of both enzymes. The only organ-specific finding in our study was the LPS-induced up-regulation of eNOS mRNA and protein levels in the liver and kidney.

Recently, CO and HO inducers have been proposed as clinically feasible anti-inflammatory agents. In terms of the clinical use of the CO/HO system, our results suggest that systemic use of agents that modify the CO/HO system—such as hemoglobin and CO inhalation—may affect the NO/NOS system in conditions such as human sepsis, and that the organs examined in this study may react in the same fashion. CO inhalation is currently being evaluated in several clinical trials; however, we believe that not only the anti-inflammatory and anti-oxidative potential of this system, but also its relationship with NO/NOS system should be carefully observed.

REFERENCES


