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Forum Review

Alteration in Heme Oxygenase-1 and Nitric Oxide Synthase-2 Gene Expression During Endotoxemia in Cyclooxygenase-2-Deficient Mice

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ABSTRACT

Sepsis is a systemic inflammatory response to a blood-borne infection that is associated with an extremely high rate of morbidity and mortality. The present article reviews our recent studies involving the role of cyclooxygenase (COX)-2 in host responses to bacterial endotoxemia and its role in the regulation of nitric oxide synthase (NOS)2 and heme oxygenase (HO)-1. COX-2-deficient (−/−) mice display a blunted and delayed induction of the cytokine-inducible genes NOS2 and HO-1 after administration of *Escherichia coli* lipopolysaccharide (LPS or endotoxin). Translocation and activation of transcription factors important for signaling events during an inflammatory response, such as nuclear factor-κB and activating protein-1, are also reduced. In addition, COX-2$^{-/-}$ mice have reduced leukocyte infiltration into critical organs (kidneys and lungs) after LPS administration. Interestingly, the absence of COX-2 does not alter the LPS induction of several proinflammatory cytokines in tissue macrophages, but induction of the antiinflammatory cytokine interleukin-10 is exaggerated. After LPS administration, 50% of wild-type (+/+) mice die; however, COX-2$^{-/-}$ mice display a dramatic improvement in survival during endotoxemia. Taken together, our findings suggest that COX-2$^{-/-}$ mice are resistant to many of the detrimental consequences of endotoxemia. *Antioxid. Redox Signal.* 6, 850–857.

INTRODUCTION

SEPSIS is a systemic inflammatory response to a severe infection (6, 36). In a subset of patients, the release of bacterial cell wall-derived lipopolysaccharide (LPS or endotoxin) and other bacterial toxins initiates a cascade of inflammatory events. This response to infection is mediated, in part, by leukocyte activation and the release of cytokines that can result in end-organ damage. The production of proinflammatory mediators (such as inflammatory cytokines, chemokines, and eicosanoids) activates cellular defense mechanisms to fight the infection (6, 41). A secondary antiinflammatory response ensues in an attempt to prevent inflammation-induced tissue injury (6). If left unchecked, the proinflammatory response will result in a massive systemic reaction leading to multiple organ failure and death. However, if the compensatory antiinflammatory response is able to counterbalance the initial proinflammatory response, homeostasis can be achieved and permanent organ dysfunction avoided (6).

Eicosanoids are lipid mediators derived from arachidonic acid metabolism that play critical roles in host responses to infection. The cyclooxygenase (COX) enzymes convert arachidonic acid to prostanoids (17). Three different isoforms of COX [COX-1, COX-2, and the recently reported COX-3, which is a variant of COX-1 (7)] have been identified. In most tissues, COX-1 is constitutively expressed, whereas COX-2 is inducible by inflammatory mediators, such as LPS (11, 17). The importance of prostanoids in endotoxemia and sepsis was originally suggested many years ago by Northover

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and Subramanian (33). In this study, dogs were treated with aspirin and found to be resistant to LPS-induced hypotension. This study was followed by several others using nonsteroidal antiinflammatory drugs to nonselectively inhibit COX enzymes, which resulted in beneficial effects during endotoxemia and sepsis in different animal models (for review, see 13). More recently, the development of COX-1- and COX-2-selective inhibitors has allowed the investigation of isoform-specific pathways. Whereas nonspecific inhibition of COX isoforms improves survival in animal models of endotoxemia (14–16), selective inhibition of COX-2 by NS-398 in a recent study did not show late protection during endotoxemia (LPS) and bacterial peritonitis-induced sepsis (cecal ligation and puncture) (43). These data led to the speculation that COX-2 may not have been the primary COX isoform responsible for prostanoid-mediated pathophysiologic consequences in endotoxemia and sepsis (13). To address directly the role of COX-2 in host responses to bacterial endotoxemia, studies have been performed to assess the impact of *Escherichia coli* LPS on inflammatory cytokine-inducible gene expression [heme oxygenase (HO)-1 and nitric oxide synthase (NOS)2], acute inflammatory responses, and survival rates in COX-2$^{-/-}$ mice (10, 21, 30).

ROLE OF HO-1 AND NOS2 IN ENDOTOXEMIA

HO-1 is a cytoprotective enzyme that is induced by stimuli associated with oxidative stress (20). HO-1 catalyzes the degradation of heme (an oxidant) to generate biliverdin, carbon monoxide (CO), and iron (25, 26). Biliverdin is subsequently converted to bilirubin, a potent endogenous antioxidant (44). CO shares many similarities with nitric oxide (NO), such as its ability to increase cyclic GMP levels and promote vasodilation, thereby modulating tissue perfusion (8, 31). Moreover, studies report that CO has antiinflammatory properties in part by signaling through the mitogen-activated protein kinases pathway (35). Iron is subsequently sequestered by ferritin, which in itself has antioxidant properties. We have previously demonstrated that interleukin (IL)-1β and LPS markedly induce HO-1 expression in cultured vascular smooth muscle cells and several organs of endotoxemic rats, respectively (37, 49), suggesting that HO-1 may be involved in the pathogenesis of endotoxic shock. These data are supported by human studies showing an elevation in carboxyhemoglobin levels in septic trauma patients (29). We have also demonstrated that Zn-protoporphyrin IX, an inhibitor of HO activity, abrogates endotoxin-induced hypotension in rats (49). These results implied that the marked induction of HO-1 during endotoxemia contributed to the decrease in systemic blood pressure. Conversely, investigators have also shown that the administration of LPS to rats receiving high doses of HO inhibitors (34) or mice lacking HO-1 (42) leads to increased mortality. Using high doses of LPS (25 mg/kg), investigators showed that the increased mortality in HO-1 null mice is associated with hepatic necrosis *in vivo* (42).

To understand better the role of HO-1 in the pathophysiology of endotoxemia, we evaluated LPS-induced hypotension and end-organ dysfunction in HO-1-deficient mice (48). The goal of this study was to define the role of HO-1 in LPS-induced hypotension, and to determine whether refractory hypotension and/or exaggerated oxidative stress were responsible for the mortality in HO-1 null mice. In these studies, we determined that after receiving LPS, HO-1-deficient mice had no difference in hypotension within the first 4 h, but after 24 h their blood pressure was significantly higher than that of wild-type mice receiving the same dose of LPS. Interestingly, even though blood pressure was higher, survival was worse in the HO-1-deficient mice. This increased mortality during endotoxemia in HO-1-deficient mice is related to increased oxidative stress and end-organ damage, not to refractory hypotension (48). These data suggested that although an exaggerated induction of HO-1 may participate in the hypotensive response to LPS, HO-1 expression is needed to resist oxidative stress and the subsequent tissue damage related to endotoxemia.

Another critical mediator up-regulated by endotoxin and proinflammatory cytokines is the inducible isoform of NOS (NOS2). NOS2 plays an important role in the pathophysiology of endotoxemia through overproduction of NO. NO is a labile and free-radical gas that acts as a potent vasodilator (27, 28). NO has been implicated in a number of pathophysiologic mechanisms of endotoxemia, including vasoplegia, induction and maintenance of an inflammatory response, and immune modulation. Administration of NO inhibitors in both animal experiments and human trials of sepsis has not proven consistently effective, perhaps related to the use of nonspecific inhibitors of NO production from all three NOS isoforms (NOS1 and NOS3, in addition to NOS2) (9). Mice with targeted disruption of NOS2 enabled the selective study of the inducible isoform. Studies comparing NOS2 knockout mice with wild-type mice have revealed a role for NOS2 expression in causing detrimental effects for the organism, such as LPS-induced hypotension and LPS-induced end-organ damage (32). Potentially beneficial effects from NOS2 expression have been identified as well; including decreased LPS-induced neutrophil adhesion to the endothelium and intracellular antimicrobial effects (32). The consequences of NOS2 deficiency on mortality in animals under conditions of endotoxemia vary depending on the experimental conditions (22, 24, 46); however, these variable results also reflect the balance of beneficial and harmful effects to the organism as a result of the level of NOS2 expression. Thus, we propose that modulation and decrease of NOS2 expression during endotoxemia, rather than complete elimination, are likely to be important for improving outcomes from this disease process.

COX-2 DEFICIENCY AND ENDOTOXEMIA

Alterations in HO-1 and NOS2 gene expression in the absence of COX-2

HO-1 and NOS2 are genes known to be induced by proinflammatory cytokines and during endotoxemia (37, 38, 40, 49). To assess further the regulation of these cytokine-inducible genes in the absence of COX-2, tissue levels of HO-1 and NOS2 mRNA were analyzed. Because damage to kidneys and

lungs during sepsis often contributes to the associated morbidity and mortality, these two organs were studied (Fig. 1). In wild-type mice, peak induction of HO-1 (50-fold in kidney and 13-fold in lung) and NOS2 (50-fold in kidney and sevenfold in lung) mRNA levels occurred 4 h after LPS administration. This peak induction of HO-1 and NOS2 corresponded to the peak induction of COX-2. Interestingly, the peak induction of HO-1 and NOS2 mRNA levels was delayed (12 h) in COX-2$^{-/-}$ mice. Moreover, in COX-2$^{-/-}$ mice, induction of HO-1 (threefold in kidney and threefold in lung) and NOS2 (13-fold in kidney and fourfold in lung) mRNA levels was markedly blunted (Fig. 1).

These data suggest a link between COX-2 products and gene regulation of HO-1 and NOS2. Previously, investigators have assessed the regulation of HO-1 induction by NO in the presence or absence of a COX-2 inhibitor *in vitro.* This study demonstrated that induction of HO-1 was actually increased (1), not decreased, in the presence of a COX-2 inhibitor. Moreover, interactions between the COX-2 and NOS2 pathways have not always been evident when using inhibitors of COX-2 (18). Thus, important differences may exist between cellular responses to an absolute deficiency in COX-2 *in vivo* and to pharmacological inhibition of COX-2 in experimental systems *in vitro*.

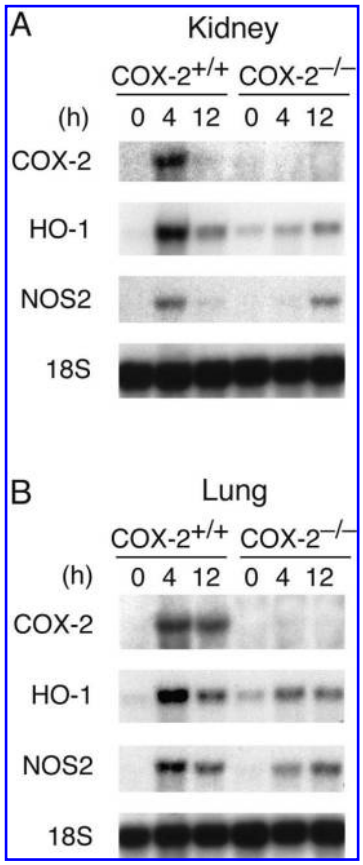

FIG. 1. Induction of HO-1 and NOS2 mRNA levels is altered in kidneys (A) and lungs (B) of COX-2-deficient mice. COX-2$^{+/+}$ and COX-2$^{-/-}$ mice were injected with LPS (10 mg/kg i.p.) and harvested at various time points (0, 4, and 12 h). Total RNA (10 μg/lane) harvested from the lungs was subjected to northern blotting. The membranes were hybridized with ^{32}P-labeled mouse COX-2 and rat HO-1 and NOS2 cDNA probes. RNA loading was assessed by hybridization with an 18S ribosomal probe. Data in this figure show a representative experiment that was performed three separate times. Adapted from (12) with permission.

Transcription factor binding and translocation is altered in the absence of COX-2

Transcription factors such as activator protein-1 (AP-1) (19) and nuclear factor-κB (NF-κB) (3) play a critical role in the signaling events that occur during the inflammatory response to endotoxin, and these factors play an important role in the regulation of HO-1 (37, 48, 49) and NOS2 (27, 39) gene expression, respectively. Thus, we investigated the effect of COX-2 deficiency on the binding of transcription factors to the 5′-flanking sequences of HO-1 and NOS2 genes. AP-1 binding activity was assessed by using a probe encoding the proximal AP-1 site of the −4-kb enhancer of the HO-1 promoter as described (47), and NF-κB binding activity was assessed by using a probe containing the upstream NF-κB site (−971 to −962) of the mouse NOS2 promoter. Nuclear extracts were obtained from kidney tissue of COX-2$^{+/+}$ and COX-2$^{-/-}$ mice that were given a vehicle or LPS for 2 and 4 h. Nuclear protein binding to the HO-1 and NOS2 probes was increased after 2 and 4 h of LPS stimulation in tissue from COX-2$^{+/+}$ mice (12). However, nuclear protein binding was reduced, and peak binding was delayed (particularly using the NOS2 probe) in tissue from COX-2$^{-/-}$ mice, similar to the mRNA response for these two genes. Incubation of the nuclear extract with an antibody specific for the p50 or p65 subunit of NF-κB produced a supershift (12). These data demonstrate that NF-κB subunits comprise the DNA–protein complex binding to the NOS2 probe. Similar supershift experiments were performed to determine nuclear proteins binding to the HO-1 probe. Electrophoretic mobility shift assay (EMSA) using an antibody against c-Jun and c-Fos revealed that AP-1 subunits comprise the DNA–protein complex binding to the HO-1 probe (12, 47).

To determine whether nuclear translocation of NF-κB was altered in the absence of COX-2, immunostaining for NF-κB subunit p65 was performed. LPS produced a striking translocation of p65 from the cytoplasm to the nucleus in kidney and lung tissue of wild-type mice (12). In contrast, COX-2$^{-/-}$ mice displayed marked reductions in LPS-induced nuclear translocation of p65 in kidney and lung tissue (12). Taken together, these data indicate that nuclear translocation and DNA binding of transcription factors important for the induction of NOS2 and HO-1 genes by inflammatory stimuli were also reduced, similar to mRNA levels, in the absence of COX-2.

Absence of COX-2 leads to a decreased inflammatory response after endotoxin administration

To determine further the impact of COX-2 on endotoxin-induced inflammatory responses, LPS was administered to COX-2$^{+/+}$ and COX-2$^{-/-}$ mice. Tissue from kidneys and lungs

was harvested 4 h, later and leukocyte infiltrates were assessed by immunohistochemical staining for CD45, a leukocyte common antigen (12). After LPS administration to COX-$2^{+/+}$ mice, inflammatory cell infiltrates were increased in kidney and lung tissue of wild-type mice. In contrast, COX-$2^{-/-}$ mice displayed marked reductions in LPS-induced CD45 staining in kidney and lung tissue (12). These data suggest that a marked reduction in tissue inflammation may contribute to the altered regulation of HO-1 and NOS2 by the proinflammatory stimulus LPS.

Another recognized consequence of septic shock is thrombotic complications. This was recently emphasized by the studies using activated protein C as a therapy for sepsis (4). Although vascular inflammation and hypoperfusion are initiators of *in situ* thrombosis, we did not see evidence of intravascular thrombosis in tissue from either COX-$2^{-/-}$ or COX-$2^{+/+}$ mice (12). Moreover, when levels of eicosanoids that may contribute to the thrombotic response in the two groups were assessed, the LPS-induced increase in thromboxane B_2 and prostaglandin E_2 in lung tissue of wild-type mice was blunted in COX-2 null mice.

Alteration in antiinflammatory, but not proinflammatory, cytokine expression in the absence of COX-2

To investigate further the mechanism(s) for COX-2 regulation of the inflammatory response to endotoxemia, peritoneal macrophages were harvested from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice and treated with LPS. mRNA levels for proinflammatory cytokines [such as IL-6, IL-1β, and tumor necrosis factor (TNF)-α] were induced by LPS stimulation at 4, 12, and 24 hours without significant differences between the expression of these cytokines in elicited macrophages from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice (Fig. 2A). Of interest, the induction of mRNA levels for the antiinflammatory cytokine IL-10 was markedly increased in COX-$2^{-/-}$ cells. Release of IL-10 protein by the LPS-exposed macrophages was also increased in the supernatant from COX-$2^{-/-}$ cells compared with COX-$2^{+/+}$ cells (Fig. 2B). These data suggest that the reduced inflammatory response in COX-$2^{-/-}$ mice may reflect an augmented induction of the antiinflammatory cytokine IL-10 in response to endotoxemia, rather than an altered expression of proinflammatory cytokines (IL-6, IL-1β, and TNF-α).

To understand the importance of this additional increase in IL-10 levels during endotoxemia, IL-10 was given to wild-type mice prior to a single administration of LPS (12). After 2 h, nuclear extracts were prepared from kidney tissue and EMSA was performed. NF-κB binding to the NOS2 5′-flanking sequence was reduced by IL-10 in a concentration-dependent manner (12). These data indicate that a further increase of IL-10 in COX-$2^{+/+}$ mice can inhibit NF-κB–DNA binding and contribute to a decreased inflammatory response as observed in the LPS-exposed COX-$2^{-/-}$ mice.

Absence of COX-2 improves survival in mice exposed to high doses of endotoxin

Finally, COX-$2^{-/-}$ mice and their COX-$2^{+/+}$ littermates were exposed to high doses of LPS (either 40 mg/kg i.p. on three consecutive days in Fig. 3A or a single dose of 100 mg/kg i.p. in Fig. 3B) and followed over a 96-h period. By 96 h in both groups of high-dose LPS, survival rates were ~50% in COX-$2^{+/+}$ mice. In contrast, none of the COX-$2^{-/-}$ mice died after administration of these same dosing regimens of LPS (100% survival at 96 h). These data suggest that the resistance of COX-$2^{-/-}$ mice to the detrimental consequences of endotoxemia, particularly the inflammatory response to endotoxin exposure, contributes to this improved survival.

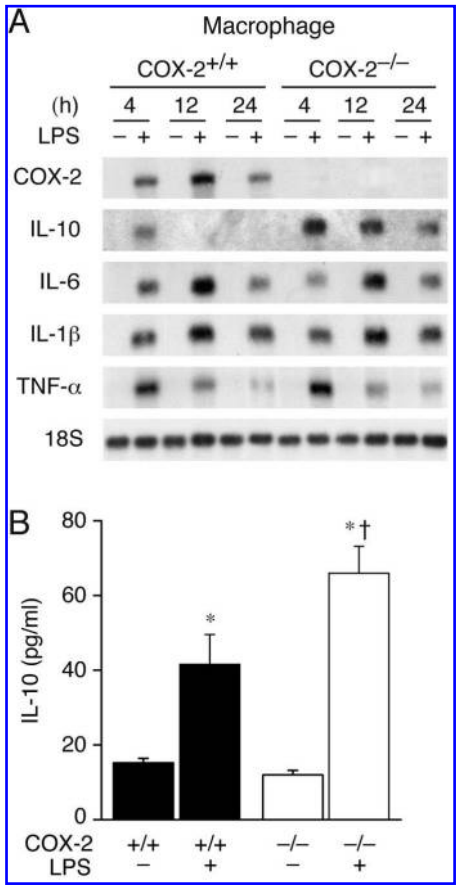

FIG. 2. LPS-induced expression of pro- and antiinflammatory cytokines in the presence or absence of COX-2. Peritoneal macrophages were harvested from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice. (**A**) The macrophages were exposed to LPS (500 ng/ml), and total RNA was harvested after 4, 12, and 24 h of stimulation. Northern blotting was performed using 2 μg/lane of RNA. The membranes were hybridized with ^{32}P-labeled cDNA probes for COX-2, IL-10, IL-6, IL-1β, and TNF-α. RNA loading was assessed by hybridization with an 18S ribosomal probe. Data in this figure show a representative experiment that was performed in duplicate. (**B**) The macrophages were exposed to LPS (500 ng/ml), and after 48 h an enzyme-linked immunosorbent assay for IL-10 was performed on aliquots of supernatant from the cells. For vehicle controls $n = 3$, and for LPS-stimulated groups $n = 6$. *$p < 0.05$ compared with vehicle control, †$p < 0.05$ compared with COX-$2^{+/+}$ cells receiving LPS. Adapted from (12) with permission.

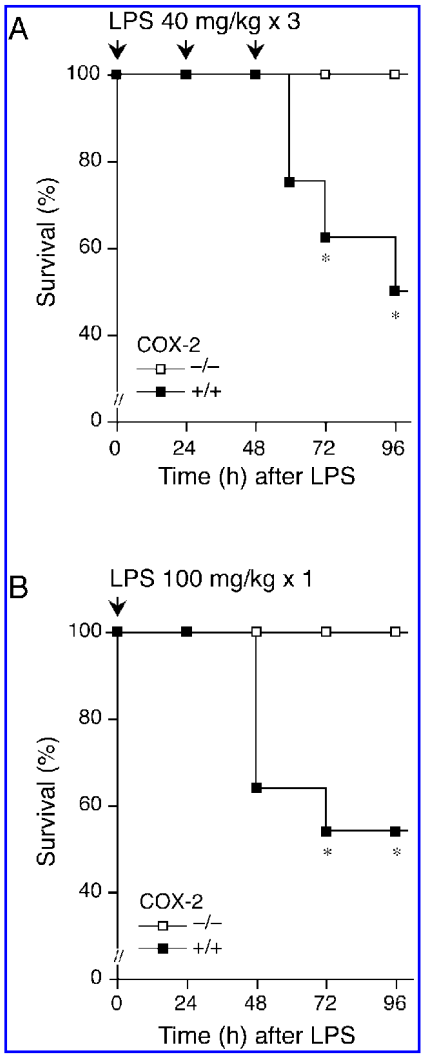

FIG. 3. Absence of COX-2 improves survival after LPS administration in mice. Wild type (+/+) or homozygous (−/−) mice targeted for disruption of COX-2 were administered *E. coli* LPS at (**A**) 40 mg/kg i.p. on three consecutive days (+/+, $n = 11$; −/−, $n = 6$) or (**B**) 100 mg/kg i.p. in a single dose (+/+, $n = 8$; −/−, $n = 7$), and survival was monitored over a 96-h period. Data are expressed as percentage of mice alive at each time point. *$p < 0.05$ compared with COX-2$^{-/-}$ mice. Adapted from (12) with permission.

SUMMARY

Sepsis is a frequent and severe systemic inflammatory response to infection (6, 36) that is associated with excess morbidity and mortality. Despite intense efforts to improve therapeutic strategies, the incidence of sepsis continues to rise (2, 45). Thus, further understanding of the endogenous counterregulatory pathways that are dysregulated during sepsis may yield insight into new therapeutic strategies. Studies reviewed in this article suggest that a deficiency in COX-2 has a dramatic protective effect, improving survival in endotoxemic mice (Fig. 3) and reducing several parameters of the inflammatory response, including leukocyte infiltration into vital organs and activation of transcription factors important for signaling events during endotoxemia (12). This is particularly important for the transcription factor NF-κB, which has been shown to play a critical role in the pathogenesis and mortality of sepsis (5). Whereas COX-2 expression did not alter the LPS-initiated expression of proinflammatory cytokines, the induction of the antiinflammatory cytokine IL-10 was increased in macrophages from COX-2-deficient mice (Fig. 2). Moreover, exogenous administration of IL-10 reduced NF-κB–DNA binding in COX-2$^{+/+}$ mice in a concentration-dependent manner (12). The subsequent reduced inflammatory response also resulted in a blunted and delayed induction of the cytokine-responsive genes HO-1 and NOS2 in the absence of COX-2 (Fig. 1).

Results from our experiments provide several lines of evidence demonstrating that COX-2 is a critical component of the lethal response associated with endotoxemia in mice (12). In addition to a role in LPS responses, our data suggest a link between COX-2 products and gene regulation of HO-1 and NOS2 (Fig. 4). The findings reviewed above suggest that COX-2-deficient mice are resistant to many of the detrimental consequences of endotoxemia and death, in part, by a compensatory increase in IL-10 that counterbalances the proinflammatory host response. Although the blunted and delayed induction of HO-1 and NOS2 by LPS in the absence of COX-2 may result indirectly from an overall reduction in the systemic inflammatory response, further studies will need to be performed to determine whether regulation of HO-1 and NOS2 in COX-2$^{-/-}$ mice has a direct effect on the improved survival. Prior studies have demonstrated that an absolute deficiency in HO-1 (48) or NOS2 (32) has detrimental consequences during endotoxemia. Furthermore, it has been suggested that HO-1 is a downstream effector of IL-10 contributing to its antiinflammatory response (23). These data suggest that an overwhelming decrease or absence of HO-1 should not lead to improved outcome during endotoxemia. Our data would also suggest that expression of IL-10 and HO-1 is not closely associated in the absence of COX-2 *in vivo,* as has been shown in wild-type mice (23).

Beyond the inflammatory response, it is also known that both the HO-1 and NOS2 pathways contribute to the hypotension associated with endotoxemia (24, 39, 46, 48, 49). Thus, from our current data we cannot exclude the possibility that a reduction, instead of an absence, of HO-1 and NOS2 during the early stages of endotoxemia may have resulted in a preserved blood pressure with better organ perfusion contributing to the beneficial outcome in these COX-2$^{-/-}$ mice. We are presently investigating this concept. Nevertheless, our present working hypothesis is that an exaggerated induction of NOS2 during endotoxemia contributes to disease morbidity related to hypotensive and inflammatory responses (Fig. 4). In regard to HO-1 and endotoxemia, we believe that induction of HO-1 is playing a cytoprotective role to counterbalance the acute inflammatory response of endotoxin exposure (Fig. 4). However, before we can definitively come to these conclusions, further studies need to be performed including genetic depletion or overexpression of HO-1 and NOS2 in COX-2$^{-/-}$ mice. These types of studies will help us

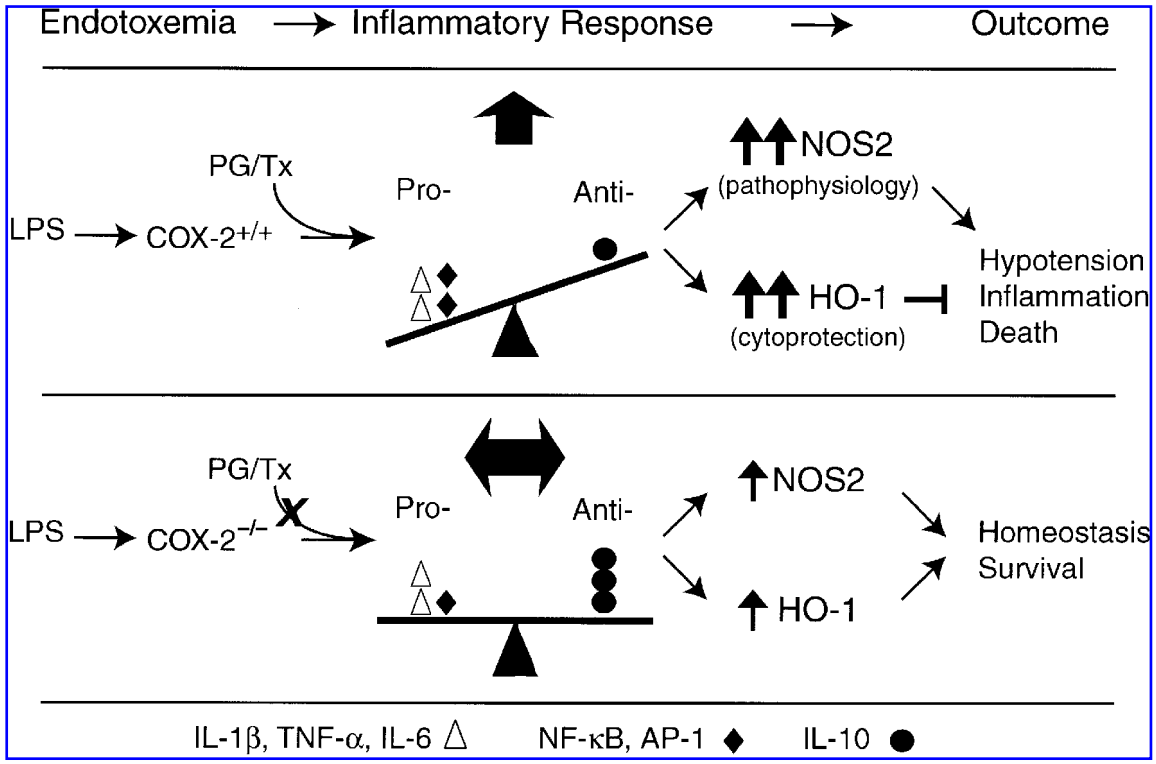

FIG. 4. Schematic diagram of the role of COX-2 in the response to endotoxemia in mice. Our data suggest that a deficiency in COX-2, and the resulting blockade (**X**) in prostaglandin (PG) and thromboxane (Tx) production, have a protective effect to improve survival in endotoxemic mice and reduce several parameters of the inflammatory response, including translocation and DNA binding of transcription factors (NF-κB and AP-1, depicted by ◆) important for signaling events during endotoxemia. LPS induction of proinflammatory cytokines (IL-6, IL-1β, and TNF-α, depicted by △) was not different between COX-$2^{+/+}$ and COX-$2^{-/-}$ mice; however, induction of the antiinflammatory cytokine IL-10 (depicted by ●) was exaggerated in the absence of COX-2. This compensatory IL-10 antiinflammatory response counterbalances the proinflammatory responses to endotoxemia and helps to restore homeostasis in COX-$2^{-/-}$ mice. Our working hypothesis is that an exaggerated induction of NOS2 (↑↑) during endotoxemia contributes to disease morbidity related to hypotensive and inflammatory responses, whereas the induction of HO-1 (↑↑) is playing a cytoprotective role to counterbalance the acute inflammatory response of endotoxin exposure.

determine the physiologic role of a blunted and delayed expression of HO-1 and NOS2 during endotoxemia in the absence of COX-2.

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ABBREVIATIONS

AP-1, activating protein-1; CO, carbon monoxide; COX, cyclooxygenase; EMSA, electrophoretic mobility shift assay; HO, heme oxygenase; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; TNF, tumor necrosis factor.

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