Deficiency of mitogen-activated protein kinase phosphatase-1 results in iNOS-mediated hypotension in response to low-dose endotoxin

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Calvert TJ, Chioine LG, Liu Y, Nelin LD. Deficiency of mitogen-activated protein kinase phosphatase-1 results in iNOS-mediated hypotension in response to low-dose endotoxin. Am J Physiol Heart Circ Physiol 294: H1621–H1629, 2008. First published February 15, 2008; doi:10.1152/ajpheart.01008.2007.—Mitogen-activated protein kinase phosphatase-1 (MKP-1) is essential in limiting the proinflammatory response to lipopolysaccharide (LPS). We hypothesized that Mkp-1−/− mice would respond to low-dose LPS with a fall in blood pressure due to augmented expression of inducible nitric oxide (NO) synthase (iNOS). To test this hypothesis, Mkp-1−/− mice and their wild-type littermates were treated with 10 μg/kg iv LPS, and mean arterial blood pressure (MAP) and exhaled NO production (exNO) were measured. Tissues were harvested for an assessment of iNOS protein levels. Wild-type mice had no change in MAP or exNO during the experimental period, whereas Mkp-1−/− mice had a fall (P < 0.005) in MAP [79 ± 5% of baseline (BL)] and an increase (P < 0.01) in exNO (266 ± 50% of BL) after 150 min. The tissue levels of iNOS were greater in Mkp-1−/− than in wild-type mice. In additional experiments, 60 min after LPS treatment, Mkp-1−/− and wild-type mice were given Nω-nitro-arginine methyl ester (l-NAME) or aminoguanidine, and MAP and exNO were monitored for 90 min. Treatment with l-NAME prevented the LPS-induced increase in exNO and decrease in MAP but resulted in decreased exNO and elevated MAP in wild-type mice. Aminoguanidine prevented the increase in exNO and the fall in MAP caused by LPS in Mkp-1−/− mice, without significantly affecting MAP or exNO in wild-type mice. These results demonstrate that a deficiency of MKP-1 results in an exaggerated hypotensive response to LPS mediated by augmented iNOS expression. We speculate that defects in the Mkp-1 gene may increase susceptibility for the development of septic shock.

Sepsis represents a major challenge to the health care system, causing ~215,000 deaths annually in the United States (21). The immune response to sepsis involves an initial pathogen killing process, which is relatively rapidly dampened and followed by a repair response. If the pathogen killing response is not appropriately restrained, then severe host injury can ensue, including shock and organ damage. Although the mechanisms leading to hemodynamic disturbances and organ failure in patients with severe sepsis are not yet fully understood, proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, have been implicated in the pathophysiology (34). Cytokine production in response to microbial infection is dependent on a series of signal transduction events, which can be initiated by microbial components through Toll-like receptors (TLRs) (23). These signaling events have been extensively studied with regard to the cell wall component of gram-negative bacteria lipopolysaccharide (LPS) (3). The activation of TLR-4 by LPS triggers a cascade of signaling events that leads to the activation of a number of cell signaling cascades including the mitogen-activated protein kinase (MAPK) pathways. The MAPK pathways include the extracellular signal-regulated kinase (ERK), the c-Jun NH2-terminal kinase (JNK), and the p38 subfamilies (18). The MAPKs play a crucial role in mediating the induction of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, through multiple mechanisms involving both transcriptional and posttranscriptional regulatory events (18). Our laboratory has recently shown that the mitogen-activated protein kinase phosphatase-1 (MKP-1) plays a crucial role in the downregulation of MAPK signaling in LPS-stimulated macrophages (8, 29, 35). For example, our laboratory has recently shown that macrophages overexpressing MKP-1 have decreased total expression and a shorter duration of p38 and JNK activation following LPS stimulation (29, 35).

The proinflammatory cytokines, such as TNF-α and IFN-γ, play an important role in the induction of inducible nitric oxide (NO) synthase (iNOS) protein expression during microbial infection (14, 25). MAPKs contribute to iNOS induction in LPS-stimulated RAW264.7 cells, a macrophage cell line (7, 25). Our laboratory has recently shown that MKP-1 overexpression blunts LPS-induced iNOS protein expression in RAW264.7 cells, whereas MKP-1 deficiency leads to augmented iNOS protein production in these cells (25). Our laboratory has also recently reported that Mkp-1−/− mice exhibit an exaggerated hypotensive response to intraperitoneal LPS challenge detected by tail-cuff systolic blood pressure measurements (36). Thus we hypothesized that MKP-1 is critical in limiting iNOS protein expression during microbial infection (14, 25).

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METHODS

Mice. The generation of MKP-1 knockout mice has been described previously (10). Cryopreserved embryos of the MKP-1 knockout mouse (−/−) and −/+ on a C57BL/6J mixed background were kindly provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Lawrenceville, NJ) and were regenereated into mice in Jackson Laboratory (Bar Harbor, ME). These mice were bred in-house to yield both wild-type and MKP-1−/− mice. All mice were maintained at 24°C with a relative humidity between 30% and 70% on a 12-h:12-h light-dark cycle. The mice were fed Harlan Teklad irradiated diet (Harlan Sprague-Dawley) ad libitum. All animals received humane care in accordance with the guidelines of the National Institutes of Health under a protocol approved by the Institutional Animal Care and Use Committee of the Columbus Children’s Research Institute.

Blood pressure measurements. The animals were anesthetized with 50 mg/kg ip pentobarbital sodium. An incision was made over the trachea, which was isolated and exposed. The trachea was cannulated using pulled polyethylene (PE)-90 tubing (BD Scientific, Franklin Lakes, NJ) and secured using a 4-0 silk suture (Ethicon, Piscataway, NJ). The tracheal cannula was connected to a rodent ventilator (Hugo/Saks) at a tidal volume of 5 ml/kg body wt and a respiratory rate of 50 breaths/min. The carotid artery was then isolated, and 6-0 Prolene (Ethicon) ties were placed around it. The distal tie was secured, a 32-gauge needle was used to pierce the artery, and pulled PE-50 tubing was inserted (BD) into the carotid artery. The proximal suture was then secured. The catheter was connected to a blood pressure transducer (Columbus Instruments, Columbus, OH), and the blood pressure was continuously monitored using Cardiomax hardware and software (Columbus Instruments) and a Dell laptop personal computer. The jugular vein was then exposed and isolated, and 6-0 Prolene ties were placed around it. The distal tie was secured, and a 32-gauge needle was used to introduce pulled PE-50 tubing. The jugular venous catheter was used for the administration of fluids and medications.

Exhaled NO measurements. Exhaled NO was measured as previously described (6, 26). The animals were ventilated with a NO free room air-gas mixture supplied from a mylar balloon attached to the inhalation port of the ventilator. The tidal volume and respiratory rate were held constant throughout the experimental period. During each experimental condition, exhaled gas was collected for the last 5 min of the experimental condition into a mylar balloon attached to the ventilator exhaust port. The gas collected in the mylar balloon was of the experimental condition into a mylar balloon attached to the experimental condition, exhaled gas was collected for the last 5 min were held constant throughout the experimental period. During each experimental condition, exhaled gas was collected for the last 5 min of the experimental condition into a mylar balloon attached to the ventilator exhaust port. The gas collected in the mylar balloon was analyzed using a chemiluminescence NO analyzer (Sievers, Boulder, CO). The analyzer was calibrated using a standard curve generated daily with authentic NO (1 parts per million in N2; Matheson, CO). The analyzer was calibrated using a standard curve generated with the primary antibody, iNOS (1:5,000; BD Transduction, San Diego, CA), eNOS (1:1,000; BD Transduction), or nNOS (1:5,000; BD Transduction) for 4 h and then washed three times with PBS-T with 1% nonfat dried milk. The membranes were then incubated with the biotinylated IgG secondary antibody (1:5,000; Vector, Burlingame, CA) for 1 h, washed, and then incubated with streptavidin-horseradish peroxidase conjugate (1:1,500; Bio-Rad) for 30 min. The protein bands were visualized using enhanced chemiluminescence (ECL reagent; Amer sham Pharmacia Biotech, Piscataway, NJ) and quantified using densitometry (Sigma Gel; Jandel Scientific, San Rafael, CA). To control for protein loading, the blots were stripped using a stripping buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-β-mercaptoethanol, and the blots were reprobed for β-actin (1:10,000; Abcam, Cambridge, MA) as described above.

Statistical analysis. Values are means ± SE. One-way ANOVA was used to compare the data. Significant differences were identified using a Newman-Keuls post hoc test. For the Nω-nitro-l-arginine methyl ester (l-NNAME) and aminoguanidine dose-response data in wild-type mice, mean arterial blood pressure (MAP) and exNO after subsequent doses were also compared using a repeated-measures one-way ANOVA. The two regression lines in Fig. 7C were compared using covariance analysis and a t-test. SigmaStat 3.5 (Jandel Scientific) was the statistical program used to run these various tests. Differences were considered significant when P < 0.05.

RESULTS

Model development. To determine the effect of NOS blockade on MAP and exNO in anesthetized wild-type mice (n = 5), following a 30-min equilibration period, 2.5, 7.5, 25, and 75 mg/kg (cumulative doses) l-NNAME were administered intravenously every 30 min while continuously monitoring MAP. The exNO was measured for the last 5 min of the equilibration period, as well as from 25 to 30 min following the 2.5 and 25 mg/kg l-NNAME doses. A second group of anesthetized wild-type mice (n = 6) was given 0.1, 1, 10, and 100 mg/kg aminoguanidine (cumulative doses) administered intravenously every 30 min following a 30-min equilibration period. In the anesthetized wild-type mice, the mean MAP was 58.5 ± 3.4 mmHg for the l-NNAME-treated mice and 48.2 ± 1.3 mmHg for the aminoguanidine-treated mice. Treatment with l-NNAME caused a dose-dependent increase in mean MAP to 92.4 ± 6.8 mmHg after a dose of 7.5 mg/kg (Fig. 1A). Increasing the cumulative l-NNAME dose to 25 and 75 mg/kg had no further effect on MAP (Fig. 1A). However, treatment with aminoguanidine had no significant effect on MAP (Fig. 1A). Treatment with l-NNAME also resulted in a dose-dependent decrease in exNO from a mean of 8.8 ± 2.2 to 5.8 ± 1.4 pmol/min after 2.5 mg/kg l-NNAME and to 3.1 ± 1.4 pmol/min after 25 mg/kg l-NNAME (Fig. 1B). Aminoguanidine treatment had no significant effect on exNO, and mean exNO was 7.5 ± 2.1 pmol/min at baseline and 6.8 ± 2.3 pmol/min at 100 mg/kg aminoguanidine (Fig. 1B). These data suggest that aminoguanidine in this particular animal model does not affect NO production or MAP under basal conditions.

To determine whether changes in MAP caused by l-NNAME were correlated with changes in measured exNO caused by l-NNAME, the exNO was plotted against the MAP from the l-NNAME-treated mice (Fig. 1C). Given the wide physiological...
variability particularly in the exNO, the data are normalized as a percentage of baseline, and only the data wherein both exNO and MAP were measured were used (i.e., the data from 2.5 and 25 mg/kg l-NAME). There was a significant inverse correlation between exNO and MAP.

To determine whether there were differences in NO sensitivity due to MKP-1 deficiency, we instrumented Mkp-1−/− (n = 5) and wild-type mice (n = 3) as described in METHODS, and then 0.001, 0.01, and 0.1 nmol cumulative doses of the NO donor spermineNONOate were given intravenously every 30 min while measuring MAP and exNO as described in METHODS. SpermineNONOate caused a dose-dependent decrease in MAP (Fig. 2A) and a dose-dependent increase in exNO (Fig. 2B) in both Mkp-1−/− and wild-type mice. There were no differences in the response to the NO donor in Mkp-1−/− and wild-type mice. Thus these data suggest that Mkp-1−/− and wild-type mice have similar sensitivity to NO.

Fig. 1. Inhibition of nitric oxide (NO) synthase (NOS) with Nω-nitro-l-arginine methyl ester (l-NAME), but not with aminoguanidine, results in increased blood pressure and decreased exhaled NO production (exNO). A: mean arterial blood pressure (MAP) in response to increasing doses of l-NAME (black circles) or aminoguanidine (gray squares) in anesthetized wild-type (WT) mice (n = 5 in each group). L-NAME (0, 2.5, 7.5, 25, and 75 mg/kg cumulative dose) or aminoguanidine (0, 0.1, 1, 10, and 100 mg/kg cumulative dose) was administered intravenously every 30 min, following a 30-min equilibration period [baseline (BL)]. B: exNO rates measured during BL or after administration of either l-NAME or aminoguanidine in these anesthetized mice. C: exNO was inversely correlated with MAP in the L-NAME-treated anesthetized WT mice. The 2 points where both exNO and MAP were measured in these 5 L-NAME-treated WT mice are plotted, and the BL point for each of the animals (100, 100) was excluded. The solid line represents the linear regression fit of the data (y = −0.67x + 140, r = −0.64, P < 0.05). *P < 0.05, different from BL; #P < 0.05, different from previous dose.

Fig. 2. Mitogen-activated protein kinase phosphatase-1 (Mkp-1)−/− knockout (KO) and WT mice have similar sensitivities to NO. Instrumented WT animals were given 0.001, 0.01, and 0.1 nmol iv cumulative doses of the NO donor spermineNONOate every 30 min while measuring MAP and exNO. A: the MAP response to increasing doses of the NO donor, spermineNONOate, in KO (n = 5; black circles) and WT (n = 3; white circles) mice. SpermineNONOate caused a dose-dependent decrease in MAP, and there was no difference in the MAP response to spermineNONOate between KO and WT mice. B: the exNO response to increasing doses of the NO donor, spermineNONOate, in KO (n = 5; black circles) and WT (n = 3; white circles) mice. As expected, the NO donor spermineNONOate caused a similar dose-dependent increase in exNO in both KO and WT mice. *P < 0.05, different from BL same genotype.
The effect of LPS on blood pressure and NO production. To determine the effect of MKP-1 deficiency on LPS-induced alterations in MAP and exNO, the following studies were done. First, we sought a dose of LPS that would have minimal effect in the wild-type mice. Our laboratory has previously found that 1.5 mg/kg LPS given intraperitoneally to wild-type mice had little effect on mortality (36). Therefore, we started with intravenous doses of 1 mg/kg and found that anesthetized wild-type mice given 1 mg/kg iv (n = 3) had a dramatic fall in MAP and an increase in exNO and died before the completion of the experimental protocol. Furthermore, anesthetized wild-type mice given either 0.5 (n = 2) or 0.1 mg/kg iv (n = 2) LPS also had a dramatic fall in MAP and an increase in exNO but died within 60 min of LPS injection [mean time to death, 61 ± 12 (SD) min with 1 mg/kg, 52 min with 0.5 mg/kg, and 58 min with 0.1 mg/kg]. Wild-type mice given 0.01 mg/kg iv LPS did not die during the 150-min experimental period. Therefore, a dose of 0.01 mg/kg iv LPS was used in all subsequent studies.

To determine the role of MKP-1 in LPS-induced alterations in blood pressure and exNO, we studied wild-type (n = 6) and Mkp-1−/− (n = 6) mice. Low-dose LPS challenge had little effect on MAP in wild-type mice (Fig. 3A). On the other hand, in Mkp-1−/− mice, MAP was significantly lower at 120 and 150 min after treatment with low-dose LPS (Fig. 3A). Although low-dose LPS had little effect on exNO in wild-type mice (Fig. 3B), it resulted in a significantly higher exNO in Mkp-1−/− mice beginning at 120 min and continuing at 150 min after treatment (Fig. 3B).

To determine whether exNO correlated with MAP in these LPS-challenged mice, exNO was plotted against MAP for measurements made at 120 and 150 min in both wild-type and Mkp-1−/− mice (Fig. 3C). There was a significant inverse correlation (r = −0.61; P < 0.002) between exNO and MAP, which suggests that changes in MAP caused by low-dose LPS were due to increases in NO production.

To further examine the effects of low-dose LPS on NO production in these mice, the protein levels in the aorta, heart, kidney, liver, and lungs for iNOS, eNOS, and nNOS 150 min after vehicle or LPS treatment were determined. In the vehicle-treated mice, there were no readily detectable levels of iNOS protein in any organ studied in either the Mkp-1−/− or wild-type animals (Fig. 4A). The levels of iNOS protein from the aorta, heart, and lungs were significantly greater in the LPS-treated Mkp-1−/− than in the LPS-treated wild-type mice (Fig. 4B). There was no significant difference in iNOS protein levels in the kidneys between the groups, and interestingly, the liver levels of iNOS protein were actually lower in the LPS-treated Mkp-1−/− than in the LPS-treated wild-type mice (Fig. 4B). To determine whether other NOS isoforms may be involved in the responses we saw, we measured eNOS and nNOS protein levels in these same organs. In animals treated with vehicle, there were no differences in the levels of eNOS protein between Mkp-1−/− and wild-type mice, except that the wild-type mice had greater eNOS protein levels than did Mkp-1−/− mice in the kidneys (eNOS/β-actin, 1.33 ± 0.24 wild-type vs. 0.50 ± 0.14 Mkp-1−/− mice, P < 0.05). In Mkp-1−/− mice treated with LPS, there were significantly greater eNOS protein levels in the aorta than in the LPS-treated wild-type animals, and there were no other statistically significant differences between LPS-treated Mkp-1−/− and wild-type mice (Fig. 4C). In animals treated with vehicle, there were no differences between Mkp-1−/− and wild-type mice in nNOS protein levels in any of the organs studied. In mice treated with LPS, the nNOS levels were lower than those in vehicle-treated animals in the aorta, kidney, liver, and lungs (Fig. 4D). Wild-type mice...
treated with LPS had significantly greater kidney nNOS protein levels than did LPS-treated Mkp-1−/− mice, whereas LPS-treated Mkp-1−/− mice had significantly greater lung nNOS protein levels than did LPS-treated wild-type mice (Fig. 4D). These results are consistent with the premise that iNOS is the major NOS isoform contributing to the LPS-induced decrease in MAP and increase in exNO following low-dose LPS in the Mkp-1−/− mice.

The effect of nonselective NOS inhibition on LPS-induced blood pressure changes. To determine whether a nonselective NOS inhibitor, l-NAME, could prevent the fall in MAP seen in Mkp-1−/− mice after LPS treatment, wild-type and Mkp-1−/− mice were given 7.5 mg/kg l-NAME 60 min after receiving 0.01 mg/kg LPS. In the low-dose LPS-treated wild-type mice, there was a significant increase in MAP following the administration of l-NAME (Fig. 5A). In the low-dose LPS-treated Mkp-1−/− mice, there was no significant change in MAP following l-NAME administration (Fig. 5A). Thus l-NAME treatment prevented the LPS-induced fall in MAP in the Mkp-1−/− mice. Although MAP at 150 min in the Mkp-1−/− mice was not different from baseline, MAP was lower at 150 min in the Mkp-1−/− (127 ± 15% of baseline MAP) than in the wild-type (163 ± 3% of baseline MAP) due to the increase in MAP seen in the wild-type mice. These alterations in MAP correlated with lower exNO rates after l-NAME treatment. In wild-type mice, there was a decrease in exNO rates following l-NAME treatment (Fig. 5B), which correlated with an increase in MAP. In the Mkp-1−/− mice, the LPS-induced increase in exNO was abolished by l-NAME (Fig. 5B), resulting in the maintenance of baseline MAP.

The effect of selective iNOS inhibition on LPS-induced blood pressure and exNO changes. To determine whether treatment with the putative NOS inhibitor aminoguanidine could prevent the drop in MAP and the increase in exNO seen in the low-dose LPS-challenged Mkp-1−/− mice, wild-type and Mkp-1−/− mice were given 10 mg/kg aminoguanidine 60 min after receiving 0.01 mg/kg LPS. In the wild-type mice given low-dose LPS and 10 mg/kg iv aminoguanidine, there was no significant increase in MAP (Fig. 6A). The exNO rate at 150 min was not different from baseline in the wild-type mice (Fig. 6B). The administration of 10 mg/kg iv aminoguanidine at 60 min prevented the fall in MAP and the increase in exNO caused by low-dose LPS in the Mkp-1−/− mice (Fig. 6). In fact, there was no difference in MAP or exNO between the wild-type and Mkp-1−/− mice at 150 min.
To determine whether the putative iNOS inhibitor aminoguanidine could reverse the changes in MAP and exNO seen 150 min after low-dose LPS in Mkp-1−/− mice, wild-type and Mkp-1−/− mice were given increasing doses of aminoguanidine starting 150 min after receiving 0.01 mg/kg LPS. At 150 min before beginning aminoguanidine administration, the wild-type mice had a MAP and exNO that were not different from their baseline values (Fig. 7). On the other hand, Mkp-1−/− mice had a significantly lower MAP and greater exNO than they did at baseline (Fig. 7). In the wild-type mice, aminoguanidine in doses of 0.001, 0.01, 0.1, 1, and 10 mg/kg resulted in a trend toward an increase in MAP, although this increase did not reach statistical significance (Fig. 7A). The exNO fell in the wild-type mice with cumulative doses of aminoguanidine of 0.01 mg/kg and greater (Fig. 7B). However, the exNO in the Mkp-1−/− mice was significantly greater than that of the wild-type mice at every aminoguanidine dose tested (Fig. 7B). To determine the correlation between exNO and MAP, the values for all of the aminoguanidine doses were plotted for wild-type and Mkp-1−/− mice (Fig. 7C). The wild-type and Mkp-1−/− mice had a significant inverse correlation between exNO and MAP, although there was no statistically significant difference between the two regression lines for Mkp-1−/− and wild-type mice. These data demonstrate that when NO production as measured by exNO was reduced by aminoguanidine, there was an increase in MAP.

**DISCUSSION**

The main findings of this study were 1) exhaled NO inversely correlated with MAP in wild-type mice given L-NAME; 2) low-dose LPS resulted in a fall in MAP and an increase in exNO only in the Mkp-1−/− mice, which was associated with an increase in iNOS protein levels; 3) L-NAME prevented the LPS-induced fall in MAP and the increase in exNO in Mkp-1−/− mice and caused an increase in MAP and a decrease in exNO in wild-type mice; 4) aminoguanidine...
Fig. 7. Selective NOS inhibition dose dependently rescued the LPS-induced fall in MAP and increase in exNO in KO mice. A: MAP in WT (n = 5; white circles) and KO (n = 5; black circles) mice in response to increasing doses of aminoguanidine. The animals were given LPS (0.01 mg/kg iv; BL), and 150 min (labeled as 0 mg/kg aminoguanidine on graph) later the dose-response experiments were started. Aminoguanidine was administered in cumulative doses to a maximum dose of 10 mg/kg iv every 15 min. *P < 0.05, KO different from WT same dose; §P < 0.01, time 0 different from BL same genotype. B: exNO rates in the animals shown in A. *P < 0.05, KO different from WT mice same dose; #P < 0.05, different from previous dose same genotype; $P < 0.01, time 0 different from BL same genotype. C: exNO was inversely correlated to MAP both in WT (white circles) and KO (black circles) mice treated with aminoguanidine. The dashed line is the regression line fit to the data from the WT mice (y = -0.56x + 121.5, r = -0.44, P < 0.005). The solid line is the regression line fit to the data from the KO mice (y = -0.59x + 148.8, r = -0.37, P < 0.05). The slopes of the regression lines were not different between WT and KO mice.

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prevented the LPS-induced fall in MAP and increase in exNO in Mkp-1−/− mice, without significantly affecting MAP in wild-type mice; and 5) aminoguanidine actually reversed the LPS-induced fall in MAP and the increase in exNO in Mkp-1−/− mice after the drop in MAP had occurred. Taken together, these findings demonstrate that Mkp-1−/− mice respond to low-dose LPS with an increase in iNOS protein levels and NO production, which resulted in a fall in MAP, whereas wild-type mice are relatively unaffected by the same dose of LPS. These findings are consistent with our hypothesis and demonstrate the central role of MKP-1 in preventing iNOS overexpression in response to LPS.

In this study, we found that changes in the measured exNO rates inversely correlated with changes in the measured MAP. The exNO has been used as a measure of pulmonary NO production both experimentally (1, 2, 6, 26) and clinically (30). However, the finding that exNO correlates with changes in systemic blood pressure suggests that the measurement of exNO can be a marker of systematically relevant NO production. Interestingly, Pedoto et al. (28) found that in rats given LPS, the exNO rates did not correlate with MAP, despite the finding that MAP decreased and exNO increased with LPS treatment. However, in a subsequent study the same group (27) found that the increase in exNO was temporally related to the fall in blood pressure in LPS-treated rats. A temporal relationship between changes in exNO and MAP has also been described in rabbits given nitroglycerin (2) or a cyclooxygenase inhibitor (1). A recent case report found an inverse relationship between exNO and MAP in a patient with hepatopulmonary syndrome treated with either curcumin or terlipressin (4). The advantage of exNO measurements as an index of NO production is that NO can be determined in real time, whereas other measures of NO production require invasive sampling and later processing, i.e., plasma nitrite/nitrate or tissue NOS activities.

The fall in MAP was temporally correlated with the increase in exNO in the Mkp-1−/− mice given low-dose LPS. This suggests that the iNOS induction by intravenous LPS was involved in the fall in MAP. Further support for a role for iNOS protein induction comes from the immunoblot data, which demonstrates greater levels of iNOS protein in the aorta, heart, and lungs from Mkp-1−/− than in those from wild-type mice. The site of iNOS protein induction is consistent with a central role of MKP-1 in preventing iNOS overexpression in the aorta, heart, and lungs after the administration of 5 mg/kg LPS intraperitonally. The central role of iNOS in the hemodynamic alterations seen with LPS treatment has been described. For example, MacMicking et al. (19) found that wild-type mice had a 65% decrease in MAP 210 min after 1 mg/kg LPS administration, whereas the same dose of LPS had no significant effect on MAP in iNOS−/− mice. Hallemansch et al. (13) found that conscious iNOS−/− mice had no decrease in blood pressure following 10 mg/kg ip LPS, whereas wild-type mice had a significant fall in blood pressure. Likewise, Carnio et al. (5) also found that iNOS−/− mice, unlike wild-type mice, did not have a fall in MAP following LPS. Interestingly, although iNOS−/− mice are protected from septic shock, they are not protected from inflammatory injury or mortality. In fact, iNOS−/− mice are more susceptible to bacterial and viral pathogens than are wild-type mice (19, 22), demonstrating the critical role of early iNOS upregulation in host defense follow-
ing infection. In this regard, one might speculate that MKP-1-deficient mice would initially fight off infection more effectively with the upregulation of iNOS but would then be highly likely to develop septic shock due to iNOS overproduction.

The role of iNOS in the fall in MAP following low-dose LPS is further supported by the data using the nonspecific NOS inhibitor l-NAME. When l-NAME was given 60 min after the administration of LPS, there was no significant fall in MAP or exNO at either 120 or 150 min after an LPS administration in Mkp-1−/− mice. Interestingly, there was a significant increase in MAP starting 30 min after l-NAME and a significant fall in exNO starting 60 min after l-NAME in the wild-type mice. A hypertensive response to l-NAME in wild-type rodents has been well documented.

The putative iNOS-specific inhibitor aminoguanidine given 60 min after LPS also prevented the fall in MAP and increase in exNO in Mkp-1−/− mice. In Wistar rats, aminoguanidine in one study (33) prevented LPS-induced hypotension, whereas in another study (24) it had little effect on LPS-induced hypotension. In mice, aminoguanidine has been shown to decrease plasma nitrates and improve mortality following LPS treatment (15, 31). In our study, aminoguanidine given to hypotensive Mkp-1−/− mice (i.e., 150 min after LPS challenge) restored MAP and exNO values, a finding that is consistent with our hypothesis and demonstrates the importance of iNOS induction in the hypertensive response following LPS challenge. Although a study of a double knockout mouse, wherein both MKP-1 and iNOS are knocked out (Mkp-1−/−iNOS−/−), may give a more definitive answer than using putative-specific pharmacological inhibitors for iNOS, those studies are beyond the scope of this article. It should be noted that when aminoguanidine was given to wild-type mice after 150 min (when the LPS-induced response was established in the Mkp-1−/− mice), there was a clear trend (although it did not reach statistical significance) toward a dose-dependent increase in MAP and there was a decrease in exNO. Indeed, when all of the individual exNO versus MAP points for the wild-type animals studied with increasing doses of aminoguanidine after LPS challenge were plotted (Fig. 7C), there was a significant inverse correlation between exNO and MAP. We did find detectable iNOS protein in the wild-type mice, although in a smaller quantity than in the Mkp-1−/− mice. This finding is consistent with the notion that in the wild-type mice treated with low-dose LPS, there was a role for iNOS induction in the regulation of systemic blood pressure and exNO, although it was smaller than that seen in the Mkp-1−/− mice.

The hemodynamic and exNO findings demonstrate the central role that MKP-1 plays in limiting iNOS protein production following LPS stimulation. These results are compatible with our previous studies wherein 1.5 mg/kg ip LPS resulted in greater plasma nitrite/nitrate levels and lower tail-cuff systolic blood pressure in Mkp-1−/− than in wild-type mice (36). MKP-1 prefers JNK and p38 as substrates, and in cultured macrophages, MKP-1 peaks 60 min after LPS stimulation and correlates with JNK and p38 dephosphorylation (8, 29, 35). Deficiency in MKP-1 results in greater and/or more sustained p38 and JNK activities due to less efficient dephosphorylation of these kinases (8, 35). Interestingly, Kan et al. (16) found that the LPS-induced iNOS mRNA induction in the lungs of mice was inhibited by pretreatment with the p38 inhibitor SB-203580. Moreover, it has recently been reported that LPS stimulates iNOS expression via the activation of NF-κB in RAW264.7 macrophages (9) and that p38 activation is involved in this signaling pathway (10). Thus alterations in p38 and JNK phosphorylation are likely to be directly responsible for the observed increases in iNOS protein levels in Mkp-1−/− mice, possible through a process that also involves NF-κB signaling.

Interestingly, the levels of eNOS protein rose significantly after LPS treatment in the aorta of the Mkp-1−/− mice, whereas eNOS protein levels did not change significantly in the aorta of wild-type mice. Our findings in wild-type mice are consistent with previous studies examining eNOS protein levels after LPS challenge (11, 12). The role of the MAPK in alterations in eNOS protein levels has not been extensively studied. It has been found in cell culture models that stimuli that upregulate MAPK activation also increase eNOS protein levels (17, 32). Our data suggest that in the presence of the normal phosphatase activity of MKP-1, there is no increase in aortic eNOS levels following LPS; however, when the normal phosphatase activity of MKP-1 is removed, there are elevated levels of aortic eNOS protein following LPS challenge, although further studies are necessary to elucidate the exact role of MKP-1 in eNOS induction due to LPS challenge. However, the increased levels of eNOS protein may contribute to the LPS-induced fall in MAP and increase in exNO seen in the Mkp-1-deficient mice after LPS challenge. The data from the experiments where aminoguanidine was given 150 min after LPS (Fig. 7) support this concept, since although the aminoguanidine did reverse the fall in MAP and the increase in exNO in the Mkp-1−/− mice, the MAP was lower and exNO was greater in the Mkp-1−/− than in the wild-type mice.

In conclusion, we found that the fall in MAP in Mkp-1−/− mice following low-dose endotoxin administration was due to iNOS induction, resulting in increased NO production. Although there are many protein phosphatases that can dephosphorylate p38 and JNK, MKP-1 is clearly the primary phosphatase responsible for the attenuation of these kinases. A critical physiological function of MKP-1 during gram-negative bacterial infection is to limit iNOS induction following endotoxin exposure, thereby preventing the development of septic shock. Thus MKP-1 may represent a novel target to be explored for the development of new therapies for septic shock. Furthermore, we speculate that polymorphisms in the Mkp-1 gene may underlie the susceptibility of some patients to develop septic shock following gram-negative sepsis.

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