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Protection Against Endotoxic Shock as a Consequence of Reduced Nitrosative Stress in MLCK210-Null Mice

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This study investigated the consequences of deletion of the long isoform of myosin light chain kinase (MLCK210) on the cardiovascular changes induced by the bacterial endotoxin lipopolysaccharide (LPS) and cecal ligation puncture using MLCK210$^{-/-}$ mice. Here, we provide evidence that deletion of MLCK210 enhanced survival after intraperitoneal injection of LPS or cecal ligation puncture. LPS-induced vascular hyporeactivity to vasoconstrictor agents was completely prevented in aorta from MLCK210$^{-/-}$ mice. This was associated with a decreased up-regulation of nuclear facor-κB expression and activity, inducible nitric-oxide synthase, and level of oxidative stress in the vascular media. Furthermore, LPS-induced increase of nitric oxide production in the circulation and tissues (including heart, liver, and lung) that was correlated with an increased expression of inducible nitric-oxide synthase was also reduced in MLCK210$^{-/-}$ mice. These data demonstrate a role for MLCK210 in endotoxin shock injury associated with oxidative and nitrosative stresses and vascular hyporeactivity. ***(Am J Pathol 2007, 170:439–446; DOI: 10.2353/ajpath.2007.060219)***

During severe sepsis, the cardiovascular system adopts a high cardiac output-low peripheral resistance hemodynamic profile whose vascular component includes arterial dilatation.[1,2] The increased cardiac output and peripheral dilatation may improve tissue perfusion and probably contribute to protection against multivisceral injury caused by sepsis. However, a prolonged or excessive drop in peripheral resistance may also cause progressive hypotension refractory to catecholamines and contribute to life-threatening cardiovascular failure.[3] Endothelial barrier dysfunction mainly through the release of oxidative metabolites by penetrant leukocytes also contributes to failure of organs such as lungs, heart, brain, and liver.[4–6] Data collected from clinical studies and different models of endotoxemia suggest that this phenomenon is related to activation of nuclear factor-κB (NF-κB)/Rel family, enabling the expression of several critical genes involved in the pathogenesis of septic shock: TNF-α, interleukins (IL-1, IL-2, IL-6, and IL-8), adhesion molecules (I-CAM-1 and E-selectin), cyclooxygenase-2, and inducible nitric-oxide synthase (iNOS).[6–9] An induction of iNOS and an overproduction of nitric oxide (NO) play a major role endotoxemia-induced vascular hyporeactivity in different experimental models[10,11] and in small vessels from patients with septic shock.[12]

The long form of the Ca^{2+}/calmodulin-dependent enzyme myosin light chain kinase (MLCK) plays a critical role in the mechanisms controlling barrier dysfunction.[13,14] A multifunctional role of MLCK has been reported such as vascular barrier regulation,[15,16] leukocyte diapedesis and cell migration,[17] and cellular apoptosis.[18] The isoform 210 of MLCK (MLCK210) differs both structurally and functionally when compared with smooth muscle MLCK (MLCK108).[19–21] However, the absence of specific inhibitor against this isoform of MLCK did not

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allow discriminating its precise function until recently. It has been shown that MLCK210 plays a central role in the control of permeability of tight junctions.[13,14] Wainwright et al[14] have shown that mice knockout for MLCK210 that still expressed MLCK108 are less susceptible to endotoxin-induced acute lung injury, and this strain shows enhanced survival during subsequent mechanical ventilation, suggesting a role for MLCK210 in pulmonary inflammation. However, no study has yet investigated its role in the cardiovascular perturbations and the up-regulation of NO production after endotoxic shock. In this study, we used genetic MLCK210$^{-/-}$ mice to study the role of MLCK210 in hemodynamic and vascular reactivity changes in mice subjected to endotoxic shock in two models of endotoxemia: lipopolysaccharide (LPS) injection and cecal ligation puncture (CLP). The effects of MLCK210 deletion on circulating and tissular nitrosative/oxidative stress were further characterized.

Materials and Methods

All animal experimentation was performed in accordance with institutional guidelines and protocols were approved by the French Animal Care Committee in accordance with European regulations. This study was performed in male 15- to 21-week-old MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice generated as previously described.[14]

LPS-Induced Endotoxic Shock

LPS (from *Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, MO) was administered at a dose of 40 mg/kg i.p. Control mice received equivalent volume of vehicle (0.9% NaCl solution). Before any sacrifice, mice were anesthetized by intraperitoneal injection with a mixture of ketamine (100 mg/kg), medetomidine (50 μg/kg), and heparin (500 U/kg).

Cecal Ligation and Puncture

CLP was performed as described elsewhere.[22] Briefly, MLCK210 $^{+/+}$ and their MLCK210$^{-/-}$ littermates were anesthetized by isoflurane. The cecum was exposed through a 1.0- to 1.5-cm abdominal midline incision and subjected to a 50 to 80% ligation of the distal half followed by double puncture with a G23 needle. A small amount of stool was expelled from the puncture to ensure patency. The cecum was then replaced into the peritoneal cavity and the abdominal incision closed with 5/0 Prolene thread (Ethicon, New Brunswick, NJ). We assessed survival after CLP for 7 days.

Telemetry Measurements: Mean Blood Pressure and Heart Rate

Mean blood pressure and heart rate were recorded using a telemetry system (Data Sciences, St. Paul, MN). This system consists of implantable radio frequency transmitters (TA11-PA20, 3.4 g) and a receiver placed under the cage of each animal.

Surgical Procedure

The mice were anesthetized with ketamine (100 mg/kg, i.p.) mixed with medetomidine (50 μg/kg, i.p.). The left carotid was isolated, the catheter linked to the transmitter was inserted into the carotid, and sutures were tied around the catheter and the carotid. The body of the transmitter was placed into a pocket subcutaneously along the animal's right flank. The skin incisions were then closed. The animals were kept under warming lights until they were fully conscious. Animals returned to their home cage under 12-hour light/dark conditions. All mice were allowed a 1-week recovery from surgery before any measurements were made.

Data Acquisition and Analysis

The mean blood pressure and heart rate were recorded over 30 seconds every 5 minutes during 5 days before the injection of LPS. Data were averaged for each mouse over 20-minute interval periods and averaged for each 20 minutes over the 5 days. Measurements were prolonged for 6 hours after the injection of LPS.

Aortic Preparations and Mounting

Segments of aortic rings (2 mm) with functional endothelium were mounted 4 hours after treating the mice with saline or LPS in a myograph filled with a physiological salt solution (PSS). PSS was maintained at 37°C and continuously bubbled with a 95% O_2/5% CO_2 mixture (pH 7.4), and mechanical activity was recorded isometrically. Concentration-response curves were constructed by cumulative application of phenylephrine in aorta with endothelium.

Ex vivo *NO Measurement with Spin Trapping and Electron Paramagnetic Resonance Studies*

Mice were injected with vehicle or LPS (40 mg/kg, i.p.). After 5.5 hours, *N*,*N*-diethyldithiocarbamate (DETC) and Fe^{2+}-citrate complex were separately injected into mice at the following doses: 400 mg/kg DETC (i.p.) and 40 mg/kg $FeSO_4$ $7H_2O$ + 200 mg/kg sodium citrate (s.c.). Animals were anesthetized and sacrificed 30 minutes later. Tissues (aorta, heart, liver, and lung) and blood samples were harvested and flash-frozen in liquid nitrogen for subsequent electron paramagnetic resonance assay. These studies were performed on a tabletop *x*-band spectrometer (MiniScope; Magnettech GmbH, Berlin, Germany). Recordings were made at 77 K using a Dewar flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 60 seconds of sweep time, and five scans.[23] Levels of NO were expressed as nanomoles per gram of frozen sample.

Western Blot Analysis

Proteins of lysates from aorta, heart, liver, and lung were subjected to SDS-PAGE using 7% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and were probed with monoclonal murine anti-iNOS antibody (Transduction Laboratories, Montluçon, France), polyclonal nuclear factor NF-κB p65 (Abcam, Cambridge, UK) or monoclonal anti-Iκ-B α-phosphorylated (USBiological, Swampscott, MA). Bound antibodies were detected with a secondary peroxidase-conjugated antibody (Promega, Madison, WI). The bands were visualized using the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) and quantified by densitometry.

Staining and Imaging by Confocal Microscopy

Vessels with endothelium were frozen and cut in 10-μm sections. After fixation, sections were incubated (2 hours at room temperature) in blocking buffer (5% nonfat dry milk in PBS). After three washes, tissue sections were incubated overnight (4°C) with polyclonal NF-κB p65 antibody (1:100). Three washes were followed by incubation (1 hour at room temperature) with secondary rabbit fluorescence-labeled antibody Alexa fluor-488 (1:100). In another set of experiments, the *in situ* production of superoxide was evaluated by the oxidative fluorescent dye hydroethidine following the previously described method.[24] Unfixed frozen sections of vessels were incubated with hydroethidine (3 μmol/L) for 30 minutes at room temperature in the dark. After washes, sections were mounted on glass slides. MRC-1024ES confocal equipment mounted on a Nikon Eclipse TE 300 inverted microscope was used for optical section of the tissue. Digital image recording was performed using LaserSharp software.

Statistical Analysis

Results are expressed as mean ± SEM of *n*, where *n* represents the number of mice. The survival curves are compared with a log-rank test. The hemodynamic parameters and the vascular reactivity were compared using a two-way analysis of variance with repeated measurements. Unpaired Student's *t*-test was used for Western blots. The levels of NO were compared using one-way analysis of variance followed by a Newman-Keuls multiple comparison posthoc test. In all cases, a *P* value less than 0.05 was considered to be significant.

Results

MLCK210 $^{-/-}$ Mice Are Protected Against Endotoxic Shock

We assessed the effect of LPS injection and CLP on survival of mice. MLCK210$^{+/+}$ and MLCK210$^{-/-}$ were subjected to intraperitoneal injection of LPS (40 mg/kg) and survival was assessed during the following 24 hours. Only 28% of MLCK210$^{+/+}$ mice survived compared with

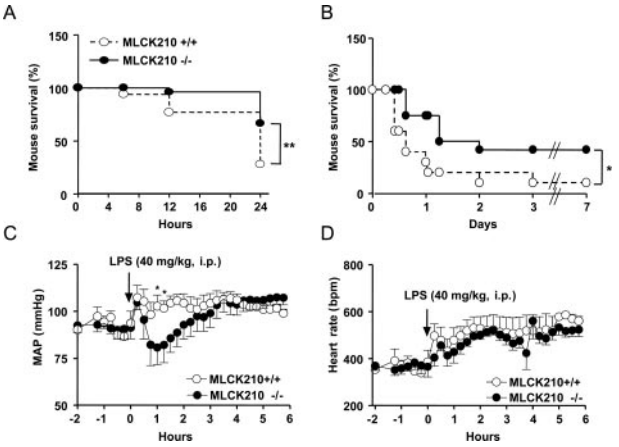


Figure 1. A: Survival curve of MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice during 24 hours after LPS (40 mg/kg, i.p.) injection. $^{**}P < 0.01$ was significant by log-rank test, $n = 11$ and 13 for MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice, respectively. **B:** Survival curve of MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice during 7 days after CLP ligation. $^{*}P < 0.05$ was significant by log-rank test, $n =$ 10 and 12 for MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice, respectively. Changes in hemodynamic parameters: MAP (**C**) and heart rate (**D**) in response to LPS. There was no difference between both strains. Data represented mean ± SEM of six or seven experiments. $^{*}P < 0.05$ was significantly different, MLCK210$^{+/+}$ versus MLCK210$^{-/-}$, by two-way analysis of variance with repeated measurements.

67% for MLCK210$^{-/-}$ (Figure 1A). In CLP model, we found a significant difference in survival between the two groups after 7 days (Figure 1B). Furthermore, MLCK210-deficient mice are resistant (Figure 1, A and B) to endotoxic shock lethality by systematically administered LPS for 24 hours and CLP for 7 days in the same manner, in the sense that the difference of percentage of survival is about 40% in the two models used. Mice were implanted with telemetric probes and their hemodynamic parameters [mean arterial pressure (MAP) and heart rate] were continuously measured before and after injection of LPS. These parameters were monitored for 6 hours to study the hyperdynamic and hyperkinetic states produced by this lethal dose of LPS. The MAP was not different between MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice before LPS injection (Figure 1C). The MAP of MLCK210$^{+/+}$ mice did not change significantly. In contrast, the MAP of MLCK210$^{-/-}$ decreased during the 1st hour and was significantly lower 1 hour after LPS injection compared with MLCK210$^{+/+}$ mice. However, there was no difference in MBP between the two strains 2 hours after LPS injection (Figure 1C). Whereas LPS induced a significant increase in heart rate, there was no difference between MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice (Figure 1D).

MLCK210-deficient mice displayed lower mortality than MLCK210$^{+/+}$ mice in response to LPS and CLP. Minimal effects, if any, were seen in the hyperdynamic and hyperkinetic hemodynamic status after 6 hours of endotoxin treatment under the experimental conditions used.

Deletion of MLCK210 Prevents LPS-Induced Vascular Hyporeactivity and iNOS Expression in Aorta

In human septic shock and in animals injected with endotoxin, hyporeactivity to endogenous vasoconstrictor

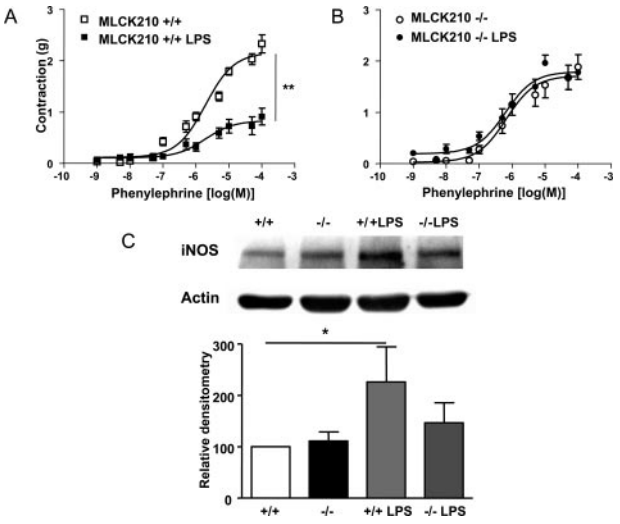


Figure 2. Concentration-response curves to phenylephrine in aortic rings with functional endothelium from MLCK$^{+/+}$ (**A**) and MLCK$^{-/-}$ (**B**) mice treated with LPS or untreated. $^{**}P < 0.01$ along the curve by two-way analysis of variance with repeated measurements. **C:** Western blot revealing expression of iNOS. $^{*}P < 0.05$ shows statistical significance between MLCK210$^{+/+}$ versus MLCK210$^{+/+}$ + LPS using unpaired Student's *t*-test. Data are presented as mean ± SEM of $n = 5$ to 9 mice.

agonists has been reported, and this results in hypotension and multiple organ failure in this disease. Thus, it was important to test whether deletion of MLCK210 prevents LPS-induced vascular hyporeactivity. MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice were systemically injected with saline or LPS. Then aortic rings from treated animals were mounted on a myograph, and mechanical activity in response to the vasoconstrictor agonist phenylephrine was recorded. The agonist concentration-response curves for MLCK210$^{+/+}$ (Figure 2A) and MLCK210$^{-/-}$ (Figure 2B) mice treated with saline are indistinguishable, showing that deletion of MLCK210 did not affect the contractile response to phenylephrine. Vascular reactivity to phenylephrine was decreased in aortic rings prepared from MLCK210$^{+/+}$ mice treated with LPS compared with those taken from MLCK210$^{-/-}$ mice treated with saline (Figure 2A). However, LPS treatment did not affect the response to phenylephrine in vessels from MLCK210$^{-/-}$ mice (Figure 2B), suggesting that the deletion of this enzyme completely prevented the vascular hyporeactivity induced by LPS.

Interestingly, iNOS expression was detected in aorta taken from both MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice (Figure 2C). Whereas LPS induced a significant increase of iNOS expression in aortas from MLCK210$^{+/+}$ animals, it had no effect on those taken from MLCK210$^{-/-}$ mice.

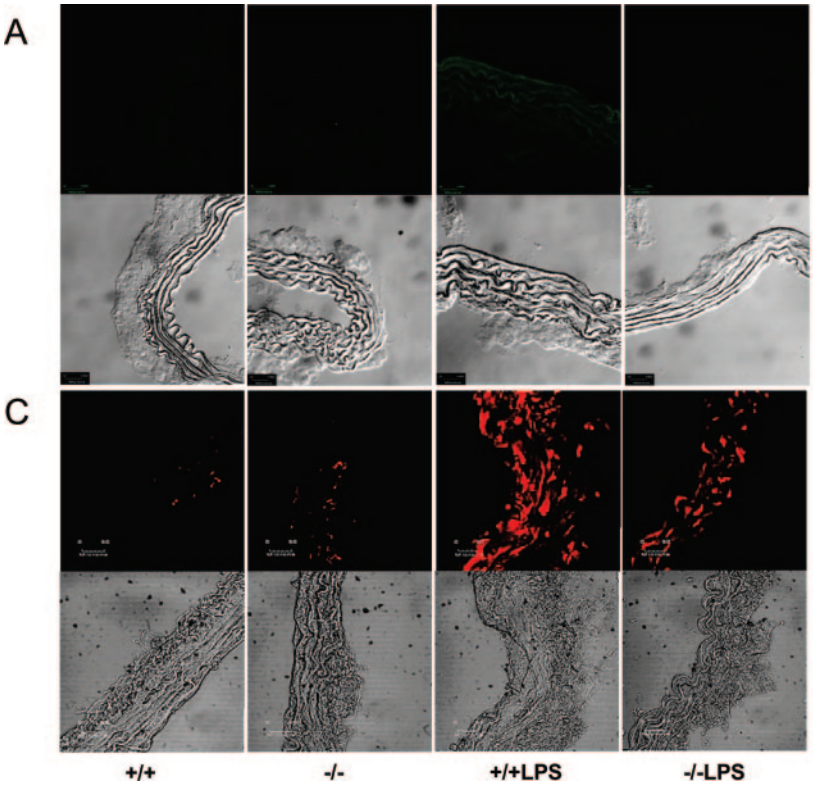
Deletion of MLCK210 Protects Against LPS-Induced NF-κB Activation and Increase of Oxidative Stress in the Aorta

Stimulation of LPS receptor, *Toll*-like receptor 4, induces NF-κB activation. NF-κB family members are heterodimers of p65/RelA and p50/NF-κB, but only the p65 subunit has transactivation domains capable of initiating transcription on proinflammatory genes that code for various cytokines and enzymes such as iNOS. The latter is responsible for the overproduction of circulating and tissular NO including the aorta and is involved in vascular hyporeactivity to vasoconstrictor agonist after LPS treatment. Therefore, we assessed the effect of the deletion of MLCK210 on LPS-induced NF-κB activation within the aorta. For this purpose, immunohistochemical staining and Western blot of the p65 subunit of NF-κB as an index of activation of NF-κβ were performed. In addition, Western blot of Iκ-B α-phosphorylated was conducted to confirm NF-κβ activation. No specific staining was found in aortas taken from MLCK210$^{+/+}$ and MLCK210$^{-/-}$ treated with saline (Figure 3A). The treatment with LPS induced a marked aortic staining of p65/RelA subunit of NF-κB in the medial and in the adventitial layers of aorta from MLCK210$^{+/+}$ mice. This staining was markedly reduced in aortic rings taken from LPS-treated MLCK210$^{-/-}$ mice. As for immunostaining studies, LPS induced a significant increase of p65/RelA subunit of NF-κB expression in aortas taken from MLCK210$^{+/+}$ mice but not in those MLCK210-null mice (Figure 3B). Furthermore, LPS treatment significantly increased the expression of Iκ-B α-phosphorylated by 1.8 ± 0.17-fold ($n = 5$, $P < 0.01$) in vessels from MLCK210$^{+/+}$ but not in those from MLCK210$^{-/-}$ (1.02 ± 0.04, $n = 5$, NS) mice.

On LPS treatment, increase in oxidative stress also plays a central role in sepsis. We assessed the level of oxidative stress in the aorta using the oxidative fluorescent dye hydroethidine to demonstrate the presence of O_2^-. Hydroethidine is cell-permeable and is oxidized in the presence of O_2^- to give fluorescent ethidium bromide. Aorta taken from MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice treated with saline displayed comparable and low ethidium bromide fluorescence (Figure 3C). After LPS treatment, aorta taken from MLCK210$^{+/+}$ mice showed a marked increase in ethidium bromide fluorescence. Although ethidium bromide fluorescence was observed in aorta taken from LPS-treated MLCK210$^{-/-}$ mice, the apparent signal intensity was much lower than that obtained in aorta from MLCK210$^{+/+}$ (Figure 3C). These results are consistent with the hypothesis that deletion of MLCK210 reduces LPS-induced NF-κB activation and increase of oxidative stress in the aorta.

Deletion of MLCK210 Leads to a Reduced Efficacy of LPS to Increase Tissular Expression of iNOS and the Associated Overproduction of Circulating and Tissular NO

We looked at the consequence of MLCK210 deletion both on tissue iNOS expression and circulating and tissue NO production. As shown in Figure 4, basal iNOS expression was detected in tissues taken from both MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice. Whereas LPS induced a sharp increase in iNOS expression in heart, liver, and lung taken from MLCK210$^{+/+}$ animals, it had no effect in tissues taken from MLCK210$^{-/-}$ mice. With regard to NO production, both circulating and tissular lev-



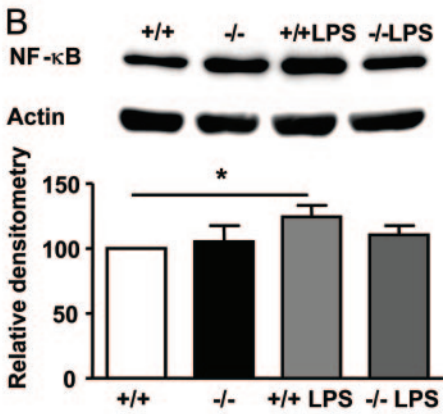


Figure 3. A: Immunohistochemical staining for NF-κB p65 of mouse aorta sections from wild-type MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice treated with LPS or untreated. Green fluorescence was linked to secondary Alexa 488-anti-rabbit-conjugated antibody. **B:** Western blot expression of NF-κB p65 in aorta from MLCK210$^{+/+}$and MLCK210$^{-/-}$ mice treated with LPS or not. $^{*}P < 0.05$ shows statistical significance between MLCK210$^{+/+}$ versus LPS-treated MLCK210$^{+/+}$ by unpaired Student's *t*-test. Data are presented as mean ± SEM of $n = 5$ mice. **C:** *In situ* detection of superoxide anions in mouse aorta sections from MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice treated with LPS or untreated. Sections of aorta were labeled with the oxidative dye hydroethidine (red fluorescence when oxidized to ethidium bromide by superoxide anions). The fluorescent images are correlated to corresponding phase-contrast images.

els of NO were not significantly different between the two strains treated with saline (Figure 5). Also in both strains, LPS treatment induced an increase of NO levels in blood, heart, liver, and lung. Even though LPS-induced increase in NO was lower in heart from MLCK210$^{-/-}$ versus MLCK210$^{+/+}$, this decrease was not statistically significant (Figure 5B). Interestingly, LPS was less effective in enhancing circulating and tissular (blood, liver, and lung) NO production in MLCK210$^{-/-}$ compared with MLCK210$^{+/+}$ mice (Figure 5, A, C, and D). Altogether, these data strongly demonstrate that MLCK210 plays an important role in the mechanism leading to an overproduction of NO through induction of iNOS on LPS treatment.

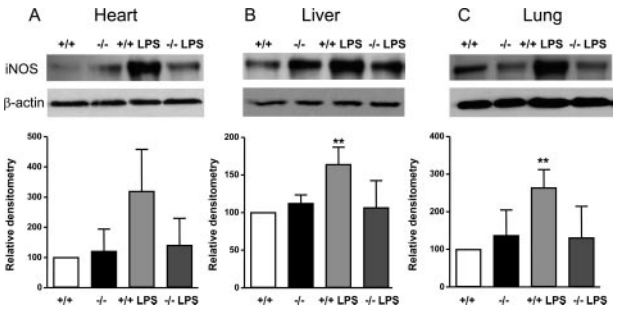


Figure 4. Western blot revealing expression of iNOS in the heart (**A**), liver (**B**), and lung (**C**) of wild-type (WT) and knockout (KO) mice treated with LPS or untreated. The level of β-actin expression for each corresponding sample is also shown. Histograms show densitometric analysis of iNOS expression. $^{**}P < 0.01$ shows statistical significance between MLCK210$^{+/+}$ versus MLCK210$^{+/+}$ + LPS by unpaired *t*-test.

Discussion

We provide evidence that MLCK210-deficient mice display a resistance to LPS- and CLP-induced death. Deletion of MLCK210 prevents vascular hyporeactivity, NF-κB

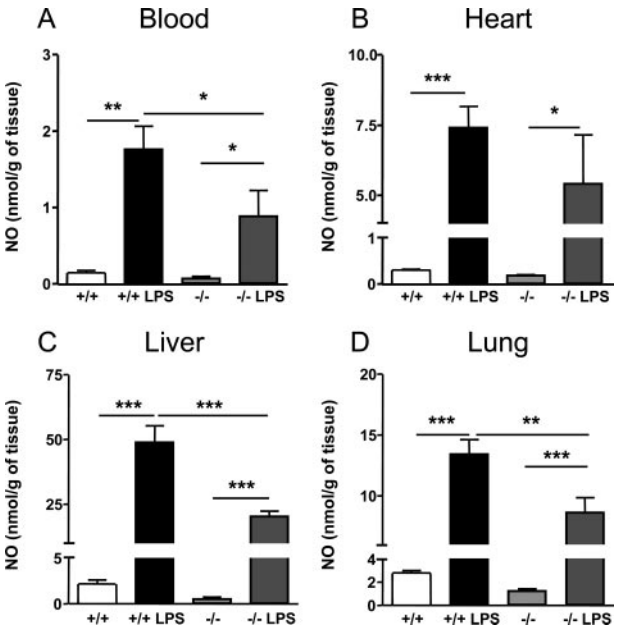


Figure 5. Levels of NO measured in blood (**A**), heart (**B**), liver (**C**), and lung (**D**) from MLCK210$^{+/+}$ and MLCK210$^{-/-}$ mice injected with vehicle or LPS. Bars represent mean ± SEM ($n = 3$ or 6 per group): $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by one-way analysis of variance followed by Newman-Keuls post hoc test.

activation and increase of nitrosative/oxidative stress. In addition, deletion of MLCK210 leads to a reduced efficacy of LPS to increase tissular iNOS expression within the heart, liver, and lung and the associated over-production of circulating and tissular NO. The convergent results from the present study and those from Wainwright et al[14] reporting reduced lethal complications of mechanical ventilation after an endotoxin shock in the same model demonstrate that MLCK210 plays an *in vivo* role in susceptibility to sepsis and its consequences in both cardiovascular and lung injuries.

Very recently, we reported that deletion of MLCK210 does not affect physiological cardiovascular parameters *in vivo* such as blood pressure, heart rate, heart chamber size, contractility, or ECG electrophysiological characteristics. In contrast, MLCK210 plays a role in the release of endothelial factors in response to flow in resistance arteries without affecting response to vasoconstrictor or vasodilator agents in conductance vessels such as the aorta.[25] Thus, MLCK210 may not play a significant role in cardiovascular parameters under physiological conditions, although the presence of a compensatory mechanism masking the effects of this kinase cannot be excluded.

We have used two models of endotoxemia (LPS and CLP) to study the effect of MLCK210 deletion on death following endotoxic shock. CLP was used as a model of polymicrobial sepsis and focused on hemodynamic parameters, organ failure, and focal gene expression. It is believed that CLP mimics sepsis symptoms in terms of the kinetics of cytokine expression much more accurately than the LPS model. We found less mortality in MLCK210-null mice compared with wild type in the two models studied. Because the time of death studied in CLP model was over 7 days, it is likely that MLCK-null mice are really protected from death. Furthermore, MLCK210-deficient mice are resistant both to endotoxic shock lethality by systematically administered LPS for 24 hours and CLP for 7 days to the same manner, in the sense that the difference of percentage of survival is about 40% in the two models used. Altogether, these data demonstrate protection after MLCK210 deletion in the two models of sepsis studied.

The response to endotoxin-induced septic shock has been tested in a number of genetically modified mice, including those for iNOS,[26] TNF-α,[27] and polyADP-ribose polymerase 1 (PARP-1).[28] The iNOS$^{-/-}$ mice were not resistant to LPS, and TNF$\alpha^{-/-}$ mice were resistant to low but not to high doses of endotoxin. As expected through the complex mechanisms by which LPS mediates its deleterious actions, one can advance the hypothesis of NO- and TNF-α-independent pathways to LPS-induced death. The better protection by PARP-1 deletion has been reported to its action upstream of the source of free radical generation during inflammation. Thus, deletion of PARP-1 leads to the abrogation of NF-κB activation and down-regulation of iNOS.[28,29] In the present study, we report that deletion of MLCK210 leads to a reduced susceptibility to endotoxin-induced death associated with decreased NF-κB activation and nitrosative/oxidative stress in the vessel wall. In addition, MLCK210$^{-/-}$ mice display down-regulation of iNOS in heart, liver, and lung and reduced overproduction of circulating and tissular NO. Thus, MLCK210 may intervene upstream at the early phase of LPS-induced NF-κB activation and cytokine releases by a mechanism similar to PARP-1 via its functional association with NF-κB activation and the consequences on systemic inflammatory process. Indeed, MLCK210 has been reported to contain amino acid sequence motifs associated with subcellular targeting or protein-protein interactions in the proteome.[30,31] This domain of the enzyme plays a role as a cellular organizer, providing integration among diverse protein including cytoskeletal proteins[21] and possibly NF-κB. Further studies are needed to demonstrate a direct association between MLCK210 and NF-κB. Congruent with that hypothesis, MLCK activity has been shown to drive TNFα-dependent NF-κB activation and amplification of pro-survival signals in endothelial cells.[32]

The endothelium is the primary target of LPS-induced vascular inflammation, and there is much evidence showing that MLCK210 localized in endothelial cells play a significant role in the maintenance of endothelial barrier function.[5,14] Studies from Wainwright et al[14] have noted the participation of MLCK210 in the inflammatory response to lung injury, and they have proposed a mechanism associated with protection of endothelial barrier function that leads to a decrease of neutrophils in lung from MLCK210$^{-/-}$ mice. In conjunction with these results, Eutamene et al[33] report that the inhibition of MLCK inhibits the neutrophilic inflammation caused by LPS and reduces *in vitro* phosphorylation of MLCK after LPS exposure. Thus, the regulation of tight junction in the lung involving MLCK is important for the inflammatory reaction and the influx of inflammatory cells. Epithelial MLCK is also essential for intestinal barrier dysfunction. Indeed, genetic knockout of MLCK210 prevented epithelial MLC phosphorylation, tight junction disruption, protein leak, and diarrhea following T cell activation.[13] In addition, it has been reported that the inhibition of MLCK prevented LPS-induced bacterial translocation through the gastrointestinal barrier, colonic inflammation, and visceral hyperalgesia. The mechanism involves protection of colonocyte tight junction morphological changes, increased MLC phosphorylation, and mucosal release of proinflammatory cytokines.[34] This latter study did not discriminate which isoform of MLCK was implicated, but it is clear that MLCK plays a significant role in the early phase of LPS-induced inflammatory response with cytokine release in colonic mucosa. The present study extends and demonstrates the role of MLCK210 by genetic knockout approaches in the resistance of MLCK210$^{-/-}$ mice to endotoxin shock in terms of increased tissular oxidative and nitrosative stresses linked to an increase of neutrophil adhesion, motility, and penetration of the endothelial cell layer leading to a massive cytokine release on LPS stimulation.

With regard to cytokine release, a novel interaction between endothelial MLCK and macrophage migration inhibitory factor has been reported,[35] and this might provide a linkage between MLCK activation, NF-κB-dependent transcription, and E-selectin expression in TNF-α-

challenged bovine lung endothelium. TNF-α-induced cytoskeletal rearrangement driven by MLCK activity is necessary for TNF-α-dependent NF-κB activation. Such an effect might partly explain the decreased susceptibility to endothelial injury by LPS and its consequences on vascular wall such as reduced vascular hyporeactivity or reduced tissular inflammation through increase of iNOS and NO production via NF-κB activation. It is interesting to note a shift between mean arterial pressure and vascular hyporeactivity within the aorta after LPS administration. Changes in mean arterial pressure are mainly controlled by changes in vascular resistance. Former papers from our previous laboratory looking at vascular reactivity in conductance (aorta) and resistance (ie, small femoral and mesenteric arteries) show that within the frame of 4-hour treatment with LPS, hyporeactivity to vasoconstrictor agents occurs in the aorta but not in resistance arteries.[10,36,37] Thus, vascular hyporeactivity associated with iNOS induction within the aorta precedes the decrease of the mean arterial pressure. Nevertheless, it is clearly demonstrated that MLCK210 deletion protects against endotoxic shock in the two models of sepsis studied (LPS and CLP models) in terms of mortality.

In conclusion, this study provides evidence that MLCK210 is involved in lethal complications as well as in the vascular reactivity changes associated with endotoxic shock. MLCK210 is linked to LPS-induced up-regulation of NF-κB and increased oxidative and nitrosative stress. Thus, these findings help to design new therapeutic strategies for the treatment of septic shock, based on pharmacological inhibition of MLCK210, allowing the effective down-regulation of oxidative and nitrosative stress associated with this life-threatening disease.

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