Lumen LPS inhibits HCO$_3^-$ absorption in medullary thick ascending limb through TLR4-PI3K-Akt-mTOR-dependent inhibition of basolateral Na$^+$/H$^+$ exchange

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Abstract

Sepsis and endotoxemia induce defects in renal tubule function but the mechanisms are poorly understood. Recently we demonstrated that lipopolysaccharide (LPS) inhibits HCO$_3^-$ absorption in the medullary thick ascending limb (MTAL) through activation of different Toll-like receptor 4 (TLR4) signaling pathways in the basolateral and apical membranes. Basolateral LPS inhibits HCO$_3^-$ absorption through ERK-dependent inhibition of NHE3. Here, we examined the mechanisms of inhibition by lumen LPS. Adding LPS to the lumen decreased HCO$_3^-$ absorption by 29% in rat and mouse MTALs perfused in vitro. Inhibitors of phosphoinositide 3-kinase (PI3K) or its effectors Akt and mTOR eliminated inhibition of HCO$_3^-$ absorption by lumen LPS but had no effect on inhibition by bath LPS. Exposure to LPS for 15 min induced increases in phosphorylation of Akt and mTOR in microdissected MTALs that were blocked by wortmannin, consistent with activation of Akt and mTOR downstream of PI3K. The effects of lumen LPS to activate Akt and inhibit HCO$_3^-$ absorption were eliminated in MTALs from TLR4$^{-/-}$ and MyD88$^{-/-}$ mice but preserved in tubules lacking Trif or CD14. Inhibition of HCO$_3^-$ absorption by lumen LPS was eliminated under conditions that inhibit basolateral Na$^+$/H$^+$ exchange and prevent inhibition of HCO$_3^-$ absorption mediated through NHE1. Lumen LPS decreased basolateral Na$^+$/H$^+$ exchange activity through PI3K. We conclude that lumen LPS inhibits HCO$_3^-$ absorption in the MTAL through TLR4/MyD88-dependent activation of a PI3K-Akt-mTOR pathway coupled to inhibition of NHE1. Molecular components of the TLR4-PI3K-mTOR pathway represent potential therapeutic targets for sepsis-induced renal tubule dysfunction.

Keywords: sepsis; Na$^+$/H$^+$ exchange; acid-base balance; kidney; TLR4 signaling
Introduction

Sepsis is a major cause of morbidity and mortality in critically ill patients, resulting in more than 200,000 deaths per year in the United States and imposing a heavy burden on health care resources (1,61). Sepsis results when innate immune responses activated to defend against bacterial infection become dysregulated, leading to inflammatory cell and tissue injury and, ultimately, multiple organ failure (11). Renal insufficiency is among the most common and serious complications of sepsis, and the development of kidney dysfunction prolongs hospitalization and is associated with an unacceptably high mortality rate in septic patients (17,47,69,74,80). Sepsis and endotoxemia induce a variety of defects in renal tubule function that are associated with fluid and electrolyte imbalances that contribute to sepsis pathogenesis (18,47,80). These include a urinary concentrating defect (33,68), increased fractional excretion of sodium and potassium (68,79,85), impaired reabsorption of glucose (78), altered metabolism of glutamine (2), and hypotension (11,79,80), as well as the development of metabolic acidosis that contributes to multiple organ dysfunction (4,11,44,48,51,80) and is an independent risk factor for mortality in septic patients (23,52,66). The pathophysiological mechanisms underlying renal tubule dysfunction during sepsis are poorly understood. Possible contributing factors may include downregulation of transport proteins by systemic or locally produced inflammatory mediators and oxidative stress (68,79,84).

The Toll-like receptors (TLR) are a family of transmembrane receptors that recognize specific molecular patterns associated with a variety of microbial pathogens (42,43). Recognition of microbial components by TLRs triggers intracellular signaling pathways that activate early inflammatory responses, including induced expression of cytokines and other proinflammatory mediators (11,42,43). Recently we demonstrated that bacterial molecules can act directly through TLRs to impair the transport function of renal tubules, identifying a new pathophysiological mechanism that can contribute to renal tubule dysfunction during sepsis (29,30). In particular, absorption of $\text{HCO}_3^-$ by the medullary thick ascending limb (MTAL) is
inhibited by the Gram-negative bacterial molecule lipopolysaccharide (LPS) through activation of TLR4 and by Gram-positive bacterial molecules (lipoteichoic acid and peptidoglycan) through activation of TLR2 (29,30). The direct action of bacterial molecules to inhibit renal tubule HCO₃⁻ absorption would impair the ability of the kidneys to correct metabolic acidosis that contributes to sepsis pathogenesis (4,23,29,44,48,51,52,66,80). At present, the intracellular signaling pathways activated downstream of TLRs in nephron segments, and their pathophysiological significance for renal tubule function, are poorly defined. Despite improvements in the supportive care of septic patients, effective treatments for sepsis and sepsis-related kidney dysfunction are lacking and there is a critical need to identify key mediators and molecular targets that may lead to new and successful therapeutic strategies (17). Understanding the cellular and molecular events through which bacterial molecules impair the transport function of renal tubule segments may aid in identifying potential therapeutic targets to preserve kidney function and improve electrolyte and fluid balance in septic patients.

Our studies of the effects of LPS on MTAL transport uncovered a novel sidedness to TLR4 signaling in epithelial cells. LPS inhibits HCO₃⁻ absorption in the MTAL from either the basolateral or luminal cell surface through activation of TLR4; however, the signaling mechanisms are different. Basolateral LPS inhibits HCO₃⁻ absorption through activation of an ERK-dependent pathway (29,88). In contrast, inhibition of HCO₃⁻ absorption by lumen LPS is not affected by ERK inhibitors but is eliminated by the specific mTOR inhibitor rapamycin (29). Activation of phosphoinositide 3-kinase (PI3K) and its downstream effectors Akt and mTOR by LPS through TLR4 plays an important role in modulating a variety of immune cell defense mechanisms, including survival and proliferation, motility, phagocytosis, and cytokine production (3,20,21,36,82,93,94). We have shown previously that stimulation of PI3K-mTOR signaling inhibits HCO₃⁻ absorption in the MTAL (28). These findings raise the possibility that lumen LPS may inhibit HCO₃⁻ absorption in the MTAL through a PI3K-mTOR pathway. At present, however, whether LPS activates PI3K-mTOR signaling in renal tubules is not known. Whether
the PI3K pathway plays a role in coupling Toll-like receptors to renal tubule transport has not been investigated.

The MTAL participates in acid-base homeostasis by reabsorbing most of the filtered HCO$_3^-$ not reabsorbed by the proximal tubule (25). Absorption of HCO$_3^-$ by the MTAL depends on H$^+$ secretion mediated by the apical membrane Na$^+$/H$^+$ exchanger NHE3 (7,25,90). Recently we demonstrated that basolateral LPS decreases HCO$_3^-$ absorption by inhibiting NHE3 through TLR4-mediated ERK activation (88). The basolateral Na$^+$/H$^+$ exchanger NHE1 also is an important determinant of the rate of MTAL HCO$_3^-$ absorption. In particular, inhibition of NHE1 results secondarily in inhibition of apical NHE3, thereby decreasing HCO$_3^-$ absorption (27,32,86,87). We have shown that activation of PI3K-mTOR inhibits HCO$_3^-$ absorption in the MTAL through inhibition of NHE1 (28,32). Thus, it is conceivable that lumen LPS inhibits MTAL HCO$_3^-$ absorption through PI3K-mTOR-mediated inhibition of NHE1. LPS has been shown to modulate NHE1 in other cell systems, including immune cells, endothelial cells, and intestinal epithelial cells (5,8,14,58,65). Whether NHE1 is regulated by LPS or is a downstream target of TLR4 signaling in renal tubules has not been determined.

The present study was designed to examine the signaling and transport mechanisms by which lumen LPS inhibits HCO$_3^-$ absorption in the MTAL. The results show that lumen LPS inhibits HCO$_3^-$ absorption through TLR4/MyD88-dependent activation of a PI3K-Akt-mTOR pathway coupled to inhibition of basolateral NHE1. These studies identify NHE1 as a target of TLR4 signaling in renal tubule cells and establish a role for the PI3K-Akt-mTOR signaling pathway in mediating LPS-induced renal tubule dysfunction. Lumen LPS inhibits HCO$_3^-$ absorption in the MTAL through signaling and transport mechanisms different from those that mediate inhibition by basolateral LPS, indicating that TLR4-induced cell signals that impact renal tubule function may be membrane specific.
Methods

Animals. Male Sprague Dawley rats (50-90 g body wt) were purchased from Taconic (Germantown, NY). Mice deficient in TLR4 (C57BL/10ScNJ; TLR4⁺⁻), MyD88 (B6.129P2(SJL)-Myd88<sup>tm1.1Defr</sup>/J; MyD88⁻⁻), Trif (C57BL/6J-Ticam<sup>1<sup>los2</sup>/J; Trif⁻⁻), and CD14 (B6.129S-Cd14<sup>tm1Fm</sup>/J; CD14⁻⁻), and wild-type control mice (C57BL/10SnJ for TLR4⁻⁻; C57BL/6J for all others) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were 6 to 12 weeks old. The animals were maintained under pathogen-free conditions in microisolator cages and received standard rodent chow (NIH 31 diet, Ziegler) and water up to the time of experiments. All protocols in this study were approved by the IACUC of The University of Texas Medical Branch.

Tubule perfusion and measurement of net HCO₃⁻ absorption. MTALs were isolated and perfused in vitro as previously described (24,29). Tubules were dissected from the inner stripe of the outer medulla at 10°C in control bath solution (see below), transferred to a bath chamber on the stage of an inverted microscope, and mounted on concentric glass pipets for perfusion at 37°C. The tubules were perfused and bathed in control solution that contained (in mM): 146 Na⁺, 4 K⁺, 122 Cl⁻, 25 HCO₃⁻, 2.0 Ca²⁺, 1.5 Mg²⁺, 2.0 phosphate, 1.2 SO₄²⁻, 1.0 citrate, 2.0 lactate, and 5.5 glucose (equilibrated with 95% O₂-5% CO₂, pH 7.45 at 37°C). Solutions containing LPS (ultra pure *E. coli* K12, InvivoGen) and other experimental agents were prepared as described (28,29,32,86,91). Akt inhibitor VIII (EMD Biosciences) was prepared as a stock solution in DMSO and diluted into bath solution to a final concentration of 5 µM. Experimental agents were added to the lumen and bath solutions as described in Results. In one series of HCO₃⁻ transport experiments (Fig. 8B), Na⁺ in the bath solution was replaced with N-methyl-D-glucammonium (NMDG⁺).

The protocol for study of transepithelial HCO₃⁻ absorption was as described (24,28,29). Tubules were equilibrated for 20-30 min at 37°C in the initial perfusion and bath solutions and the luminal flow rate (normalized per unit tubule length) was adjusted to 1.5-1.9 nl/min/mm. One to three 10-min tubule fluid samples were then collected for each period (initial,
The tubules were allowed to re-equilibrate for 5-10 min after an experimental agent was added to or removed from the lumen or bath solution. The absolute rate of HCO₃⁻ absorption (JHCO₃⁻, pmol/min/mm) was calculated from the luminal flow rate and the difference between total CO₂ concentrations measured in perfused and collected fluids (24). An average HCO₃⁻ absorption rate was calculated for each period studied in a given tubule.

When repeat measurements were made at the beginning and end of an experiment (initial and recovery periods), the values were averaged. Single tubule values are presented in Figures 1, 2, 3, 6, 7, and 8. Mean values ± SE (n = number of tubules) are presented in the text.

**Measurement of intracellular pH (pHi) and basolateral Na⁺/H⁺ exchange activity.** pHi was measured in isolated, perfused MTALs by use of the pH-sensitive dye BCECF and a computer-controlled spectrofluorometer (CM-X, SPEX Industries) coupled to the perfusion apparatus, as previously described (86,89). MTALs were perfused and bathed in Na⁺-free HEPES-buffered solution that contained (in mM): 145 NMDG⁺, 4 K⁺, 147 Cl⁻, 2.0 Ca²⁺, 1.5 Mg²⁺, 1.0 phosphate, 1.0 SO₄²⁻, 1.0 citrate, 2.0 lactate, 5.5 glucose, and 5 HEPES (equilibrated with 100% O₂; titrated to pH 7.4). Basolateral Na⁺/H⁺ exchange activity was determined by measurement of the initial rate of pHi increase after addition of 145 mM Na⁺ to the bath solution (Na⁺ replaced NMDG⁺), the intracellular buffering power, and cell volume, as described (28,86). Interruption of pHi recovery at various points along the recovery curve permits determination of the Na⁺/H⁺ exchange rate over a range of pHi values, with appropriate corrections for a variable background acid loading rate (86,89). The lumen solution contained ethylisopropyl amiloride (EIPA; 50 µM) to eliminate any contribution of apical Na⁺/H⁺ exchange to the Na⁺-induced changes in pHi. The Na⁺-dependent pHi recovery was inhibited > 90% by bath EIPA (50 µM) under all experimental conditions. Experimental agents were added to the lumen and bath solutions as described in Results.

**Immunoblotting.** Immunoblot analyses were carried out on the inner stripe of the outer medulla dissected from mouse kidneys as previously described (28,31,88). This tissue
preparation is highly enriched in MTALs and exhibits regulatory changes in signaling proteins that accurately reflect changes observed in the MTAL (26,28,88,91). The tissue was divided into samples of equal amount and incubated in vitro at 37° C in the same solutions used for HCO₃⁻ transport experiments. The specific protocols used for incubation are given in Results. Following incubation, the tissue samples were homogenized in ice-cold PBS and solubilized for 2 h at 4° C in RIPA buffer with protease inhibitors. Samples of equal protein content (50 µg/lane) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% BSA in TBS and incubated overnight at 4° C with antiphospho-Akt-Ser473 (1:2,000) or anti-Akt (1:2,000) antibodies, or with antiphospho-mTOR-Ser2448 (1:2,000) or anti-mTOR (1:2,500) antibodies (Cell Signaling Technology). After washing in TBS, horseradish peroxidase-conjugated anti-rabbit secondary antibody was applied and immunoreactive bands were detected by chemiluminescence (Luminol Reagent, Santa Cruz). Protein bands were quantified by densitometry (MetaMorph).

Confocal immunofluorescence microscopy. MTALs were studied by confocal microscopy as previously described (28,31,87,88). Mouse MTALs were microdissected and mounted on Cell-Tak-coated coverslips at 10° C. The tubules were then incubated at 37° C in a flowing bath using the same solutions as in HCO₃⁻ transport experiments. The specific protocols used for incubation are given in Results. Following incubation, the tubules were washed with PBS and fixed and permeabilized in acetone at −20° C for 10 min. The tubules were incubated in Image-iT FX signal enhancer (Invitrogen) for 30 min at room temperature, washed, and blocked in 10% goat serum in PBS for 1 hr at room temperature. The tubules were then incubated overnight at 4° C with antiphospho-Akt (1:200) or antiphospho-mTOR (1:50) antibody, washed, and then incubated for 1 hr at room temperature in Alexa 488-conjugated goat anti-rabbit IgG antibody (Invitrogen) in blocking buffer. Fluorescence staining was examined using a Zeiss laser-scanning confocal microscope (LSM510 UV META), as described (28,87,88). Tubules were imaged longitudinally and z-axis optical sections (0.4 µm) were obtained through...
a plane at the center of the tubule, which provides a cross-sectional view of cells in the lateral
tubule walls. For individual experiments, two to four tubules from the same kidney for each
experimental condition, or from wild-type and null-mutant mice, were fixed and stained
identically and imaged in a single session at identical settings of illumination, gain, and
exposure time. Fluorescence intensity of p-Akt or p-mTOR staining was quantified as
previously described (31,88). Two-dimensional image analysis was performed using
MetaMorph software in which a box (2.8 x 2.8 µm) was positioned on the cytoplasm in the mid-
region of the cell and pixel intensity per unit area was determined after background subtraction.
Three different cells were analyzed per optical section and three optical sections were analyzed
per tubule, one section at the center of the tubule and two sections positioned 0.12 µm above
and below the center section. The measurements were averaged to obtain a value for each
tubule. Fluorescence intensity for experimental groups was expressed as a percentage of the
control value measured in the same experiment. Mean values (n = number of tubules) were
used for statistical analysis.

*Analysis.* Results are presented as mean ± SE. Differences between means were
evaluated using Student’s *t* test for paired or unpaired data, or analysis of variance with
Newman-Keuls multiple range test, as appropriate. *P* < 0.05 was considered statistically
significant.
Results

Inhibitors of PI3K eliminate inhibition of $\text{HCO}_3^-$ absorption by lumen LPS. Under control conditions, adding LPS (250 ng/ml) to the lumen decreased $\text{HCO}_3^-$ absorption by 29% (from $16.3 \pm 0.8$ to $11.6 \pm 0.8$ pmol/min/mm) in MTALs from rats (Fig. 1A) and by 28% (from $15.1 \pm 0.7$ to $10.9 \pm 0.6$ pmol/min/mm) in MTALs from mice (Fig. 1C). The inhibition by LPS is rapid (<15 min), sustained for up to 60 min, and reversible. To determine the role of PI3K in the inhibition by LPS, we examined the effects of wortmannin and LY294002, two selective inhibitors of PI3K that block PI3K activation and PI3K-dependent transport regulation in the MTAL (26,28). The inhibition of $\text{HCO}_3^-$ absorption by lumen LPS was eliminated completely in rat and mouse MTALs bathed with wortmannin or LY294002 (Fig. 1, B and D). These results support an essential role for PI3K in mediating inhibition of $\text{HCO}_3^-$ absorption by lumen LPS.

Lumen addition of LPS had no effect on transepithelial voltage, an indirect measure of NaCl absorption rate, in rat MTAL (10.9 ± 0.2 mV, control vs. 10.8 ± 0.2 mV, lumen LPS, n = 5; P = NS).

Inhibitors of Akt and mTOR eliminate inhibition of $\text{HCO}_3^-$ absorption by lumen LPS. To define further the PI3K pathway responsible for inhibition of $\text{HCO}_3^-$ absorption by lumen LPS, we examined the role of two downstream mediators in PI3K signaling: Akt, a direct target of PI3K, and mTOR, an effector of PI3K-Akt signaling (20,60,93). Rat and mouse MTALs were bathed with either the selective Akt inhibitor VIII or the specific mTOR inhibitor rapamycin. The inhibition of $\text{HCO}_3^-$ absorption by lumen LPS was eliminated completely by the Akt inhibitor (Fig. 2A). Consistent with previous results (29), the inhibition by lumen LPS also was abolished by rapamycin (Fig. 2B). These results support the view that lumen LPS inhibits $\text{HCO}_3^-$ absorption in the MTAL through activation of a PI3K-Akt-mTOR signaling pathway.

Inhibitors of PI3K do not prevent inhibition of $\text{HCO}_3^-$ absorption by bath LPS. Previously we demonstrated that lumen and bath LPS inhibit $\text{HCO}_3^-$ absorption in the MTAL through different TLR4-dependent signaling pathways. In particular, inhibition by bath LPS is mediated
through activation of ERK and is not affected by rapamycin (29,88). To establish further that
LPS acts through different signaling pathways at the basolateral and apical membranes, we
examined the effect of PI3K inhibitors on inhibition of $\text{HCO}_3^-$ absorption by bath LPS. In tubules
bathed with either wortmannin or LY294002, adding LPS to the bath decreased $\text{HCO}_3^-$
absorption by 31% (from 15.7 ± 0.1 to 10.9 ± 0.2 pmol/min/mm) (Fig. 3A), a decrease similar to
that observed in the absence of the inhibitors (29,88). Thus, in contrast to their action to
eliminate inhibition of $\text{HCO}_3^-$ absorption by lumen LPS, inhibitors of PI3K have no effect on
inhibition by bath LPS. Conversely, adding LPS to the lumen decreased $\text{HCO}_3^-$ absorption by
28% (from 15.2 ± 0.4 to 10.9 ± 0.4 pmol/min/mm) in MTALs bathed with PD98059 or U0126
(Fig. 3B), inhibitors of ERK activation that eliminate inhibition of $\text{HCO}_3^-$ absorption by bath LPS
(29,88). These results confirm and extend our previous findings (29,88) demonstrating that LPS
inhibits $\text{HCO}_3^-$ absorption in the MTAL through distinct TLR4-dependent signaling pathways in
the apical and basolateral membranes: inhibition by lumen LPS is mediated through a PI3K-Akt-
mTOR pathway and is unaffected by inhibitors of ERK activation, whereas inhibition by
basolateral LPS is mediated through ERK and is unaffected by inhibitors of PI3K and mTOR.

LPS induces PI3K-dependent phosphorylation of Akt and mTOR in the MTAL. To
determine whether LPS activates Akt and mTOR downstream of PI3K in the MTAL, we
examined the effects of LPS on Akt and mTOR phosphorylation. MTALs dissected from mice
were incubated in the absence and presence of LPS for 15 min, stained with antiphospho-Akt
(p-Akt) or antiphospho-mTOR (p-mTOR) antibody, and then analyzed by confocal
immunofluorescence (Fig. 4, A and B). Stimulation with LPS increased p-Akt staining 1.3 ± 0.1-
fold and increased p-mTOR staining 1.2 ± 0.1-fold. The LPS-induced increases in Akt and
mTOR phosphorylation were eliminated by wortmannin (Fig. 4A). The effects of LPS on Akt
and mTOR phosphorylation were examined further by immunoblot analysis of inner stripe
tissue. The results in Figure 4, C and D show that LPS increased Akt and mTOR
phosphorylation without a change in total Akt or total mTOR level, and that the LPS-induced Akt
and mTOR phosphorylation was again inhibited by wortmannin. These results demonstrate that LPS activates Akt and mTOR in the MTAL and that this activation depends on PI3K. These findings support a role for PI3K-Akt-mTOR signaling in mediating inhibition of HCO$_3^-$ absorption by LPS in the MTAL.

**LPS-induced Akt activation is mediated through TLR4.** Previously we demonstrated that lumen LPS inhibits HCO$_3^-$ absorption in the MTAL through activation of its cell-surface receptor TLR4 (29). To determine whether TLR4 mediates activation of PI3K-Akt, the effects of LPS on Akt phosphorylation were examined in MTALs from wild-type and TLR4$^{-/-}$ mice (Fig. 5). Treatment with LPS for 15 min increased Akt phosphorylation 1.3 ± 0.1-fold in MTALs from wild-type mice but had no effect on Akt phosphorylation in MTALs from TLR4$^{-/-}$ mice. These results indicate that the PI3K-dependent activation of Akt by LPS in the MTAL is mediated through TLR4. The inability of LPS to activate PI3K-Akt in MTALs from the TLR4$^{-/-}$ mice can explain our previous finding that lumen LPS fails to inhibit HCO$_3^-$ absorption in TLR4$^{-/-}$ MTALs (29).

**Inhibition of HCO$_3^-$ absorption by lumen LPS is mediated through MyD88.** Binding of LPS to the TLR4 receptor complex generates intracellular signals through two distinct pathways mediated by recruitment of the adaptor molecules MyD88 and Trif (42,43). We have shown previously that TLR4-dependent inhibition of HCO$_3^-$ absorption by basolateral LPS is dependent on MyD88 (88). To determine the role of these adaptors in the inhibition of HCO$_3^-$ absorption by lumen LPS, MTALs from MyD88$^{-/-}$ and Trif$^{-/-}$ mice were perfused in vitro. Adding LPS to the lumen had no effect on HCO$_3^-$ absorption in MTALs from MyD88$^{-/-}$ mice (Fig. 6A). In contrast, lumen LPS decreased HCO$_3^-$ absorption by 26% in MTALs from Trif$^{-/-}$ mice. These results demonstrate that inhibition of HCO$_3^-$ absorption by lumen LPS depends on MyD88.

To examine the role of MyD88 in LPS-induced PI3K signaling, the effects of LPS on Akt phosphorylation were studied in MTALs from wild-type and MyD88$^{-/-}$ mice by confocal immunofluorescence. As shown in Fig. 6B, LPS increased Akt phosphorylation 1.4 ± 0.1-fold in MTALs from wild-type mice but had no effect on phosphorylation of Akt in MTALs from MyD88$^{-/-}$
mice. Taken together, these results demonstrate that the TLR4-dependent effect of lumen LPS to inhibit HCO$_3^-$ absorption through PI3K-Akt activation is mediated through MyD88.

**Inhibition of HCO$_3^-$ absorption by lumen LPS does not require CD14.** CD14 is a membrane-anchored LPS binding protein that enhances LPS signaling by facilitating transfer of LPS to the TLR4 signaling complex (43,95). We have shown previously that CD14 is not required for inhibition of HCO$_3^-$ absorption by basolateral LPS in the MTAL (31). However, TLR4-induced activation of PI3K-Akt has been shown to depend on CD14 in macrophages (72,77). To determine whether CD14 is involved in mediating inhibition of HCO$_3^-$ absorption by lumen LPS, tubules from CD14$^{-/-}$ mice were perfused in vitro. Adding LPS to the lumen decreased HCO$_3^-$ absorption by 31% (from 13.5 ± 0.4 to 9.3 ± 0.5 pmol/min/mm) in CD14$^{-/-}$ MTALs (Fig. 7). This decrease is similar to that observed in MTALs from wild-type controls (C57BL/6J, Fig. 1C). Thus, CD14 is not required for TLR4-dependent inhibition of HCO$_3^-$ absorption by lumen LPS.

**Inhibition of HCO$_3^-$ absorption by lumen LPS is eliminated by inhibitors of basolateral Na$^+$/H$^+$ exchange.** We have shown previously that activation of PI3K-mTOR inhibits HCO$_3^-$ absorption in the MTAL through inhibition of the basolateral membrane NHE1 Na$^+$/H$^+$ exchanger (28,32,86). To test whether NHE1 is involved in inhibition by lumen LPS, the effects of lumen LPS on HCO$_3^-$ absorption were examined in the presence of 10 µM bath amiloride and in the absence of bath Na$^+$, two conditions that inhibit basolateral Na$^+$/H$^+$ exchange and prevent inhibition of HCO$_3^-$ absorption mediated through NHE1 (27,32,86,87). The results in Figure 8 show that the effect of lumen LPS to inhibit HCO$_3^-$ absorption was eliminated completely in tubules bathed with amiloride or studied in a Na$^+$-free bath. These results support the view that inhibition of HCO$_3^-$ absorption by lumen LPS is mediated through inhibition of basolateral Na$^+$/H$^+$ exchange.

**Lumen LPS inhibits basolateral Na$^+$/H$^+$ exchange through a PI3K-dependent pathway.** Further studies were carried out to examine whether lumen LPS decreases HCO$_3^-$ absorption...
through primary inhibition of basolateral Na\(^+\)/H\(^+\) exchange. The effect of lumen LPS on basolateral Na\(^+\)/H\(^+\) exchange activity was examined in the absence and presence of wortmannin in the bath solution. Lumen LPS decreased basolateral Na\(^+\)/H\(^+\) exchange activity over the range of pH\(_i\) values studied (Fig. 9A). Overall, lumen LPS decreased the basolateral Na\(^+\)/H\(^+\) exchange rate by 38\% (control vs. lumen LPS, Fig. 9B). The effect of lumen LPS to decrease basolateral Na\(^+\)/H\(^+\) exchange activity was eliminated in tubules bathed with wortmannin (Fig. 9, A and B). These results indicate that lumen LPS inhibits basolateral Na\(^+\)/H\(^+\) exchange in the MTAL through a PI3K-dependent pathway.
Discussion

Kidney dysfunction accentuates the pathogenesis and lethality of sepsis by inducing abnormalities in fluid, electrolyte, and metabolic homeostasis (18,47,80). Alterations in renal tubule function caused by endotoxemia and sepsis include impaired urinary concentrating ability, increased fractional excretion of sodium and glucose, hypovolemia, and altered glutamine metabolism (2,33,68,78,79,85). These functional defects can occur in the absence of changes in blood pressure, global renal blood flow, GFR, and/or renal oxygenation, suggesting that primary abnormalities in renal tubule function unrelated to changes in hemodynamic factors may play an important role (2,68,79). Recently we demonstrated that Gram-negative and Gram-positive bacterial molecules act directly through TLRs to impair renal tubule transport, identifying a mechanism that could contribute to renal tubule dysfunction during sepsis unrelated to circulatory abnormalities or oxidative stress (29,30). LPS inhibits HCO$_3^-$ absorption in the MTAL from either the luminal or basolateral cell surface through activation of TLR4 (29,88), effects that would negatively impact the sepsis state by impairing the ability of the kidneys to correct metabolic acidosis that contributes to organ dysfunction and mortality in septic patients (4,23,44,48,51,52,66). In the present study, we show that lumen LPS decreases HCO$_3^-$ absorption in the MTAL through TLR4/MyD88-dependent activation of a PI3K-Akt-mTOR signaling pathway coupled to inhibition of the basolateral Na$^+$/H$^+$ exchanger NHE1 (Figure 10). These signaling and transport mechanisms differ from those that mediate inhibition of HCO$_3^-$ absorption by basolateral LPS.

PI3K and its downstream effectors Akt and mTOR regulate diverse events in cells of the immune system, including proliferation, survival, differentiation, chemotaxis, phagocytosis, and production of inflammatory mediators (20,21,82,93). The PI3K pathway is an important mediator of LPS-induced innate immune responses and can function either as a positive or negative regulator of TLR-triggered cellular responses (21). In some systems, activation of PI3K by LPS promotes immune cell responses such as the production of cytokines and...
chemokines, phagocytosis, and survival (3,15,21,36,54,67,94). Conversely, a negative
regulatory role for PI3K signaling is supported by observations that TLR4-induced production of
proinflammatory mediators is increased when PI3K or mTOR is inhibited, suggesting that PI3K
functions to prevent excessive and potentially harmful inflammatory responses (21,35,49,92,93).
LPS- and TLR4-mediated activation of PI3K has been shown to depend on the adaptor protein
MyD88 (3,49,54,67), but a role for MyD88-independent activation through Trif also has been
suggested (3). At present, the role of PI3K signaling in the innate immune response of renal
tubules is undefined. Inhibitors of PI3K were found to reduce bacterial invasion and bacteria-
induced increases in transepithelial resistance in a collecting duct cell line (10). However,
whether PI3K is activated in response to LPS in renal tubules, and the role of PI3K signaling in
TLR4-mediated renal tubule responses, are unknown. Results of the current study show that
lumen LPS stimulates PI3K signaling through TLR4 and MyD88 in the MTAL and that this PI3K
activation impairs the ability of the MTAL to absorb $\text{HCO}_3^-$ . These results identify the PI3K
pathway as a component of TLR4-induced cell signaling that impairs the transport function of
MTAL cells.

The effect of lumen LPS to inhibit $\text{HCO}_3^-$ absorption in the MTAL through PI3K depends
on downstream activation of Akt and mTOR. Receptor-activated PI3K signaling involves PIP$_3$-
dependent recruitment and activation of PDK1, which directly phosphorylates and activates Akt
(20). Activation of Akt, in turn, leads to downstream signaling through mTOR. mTOR kinase
exists in two functionally distinct multiprotein complexes: mTORC1 contains mTOR in complex
with raptor and LST8, whereas mTORC2 contains mTOR, rictor, LST8, and SIN1 (50,60,93).
Akt stimulates mTORC1 through two mechanisms: 1) by directly phosphorylating TSC2, which
negates the inhibitory effect of the TSC1-TSC2 complex on mTORC1, and 2) by
phosphorylating and inhibiting PRAS40, which prevents its ability to suppress mTORC1
signaling (60,93). Rapamycin inhibits the activity of mTORC1 in part by disrupting the
interaction between mTOR and raptor (46,71); in contrast, rapamycin has no direct effect on the
activity of mTORC2 (50,59,76). Two observations in the current study support the view that lumen LPS inhibits HCO₃⁻ absorption in the MTAL through activation of mTORC1: 1) the inhibition is blocked by rapamycin, and 2) LPS increased phosphorylation of mTOR on Ser2448, a phosphorylation site reported as a selective marker for mTORC1 activation (12). Our results do not rule out a possible role for mTORC2 in facilitating the LPS-induced inhibition. Akt is a downstream target of mTORC2, and direct phosphorylation of Akt on Ser473 by mTORC2 is required for full Akt activation (16,50,55). In the MTAL, activation of Akt by LPS involves increased phosphorylation at the Ser473 site. Thus, it is possible that activation of mTORC2 by LPS may enhance Akt activity and Akt regulation of its downstream targets, thereby contributing indirectly to LPS-induced activation of mTORC1 (16,60) and inhibition of HCO₃⁻ absorption. Our results show that the ability of lumen LPS to impair MTAL transport is prevented by selective inhibitors of PI3K, Akt, or mTORC1, identifying multiple sites within the TLR4-induced PI3K pathway as potential targets for intervention.

The Na⁺/H⁺ exchanger isoform NHE1 is expressed ubiquitously in the plasma membrane of nonpolarized cells and in the basolateral membrane of epithelial cells, where it plays essential roles in a variety of cell processes, including maintenance of intracellular pH and cell volume, proliferation, survival, and migration (62,70). In the MTAL, we have identified a novel role for basolateral NHE1 in the regulation of HCO₃⁻ absorption. Inhibition of NHE1 with amiloride or nerve growth factor, or by NHE1 knockout, results secondarily in inhibition of apical NHE3, thereby decreasing HCO₃⁻ absorption (27,28,32,86). NHE1 modulates NHE3 activity by regulating the organization of the actin cytoskeleton (87). The results of the present study indicate that lumen LPS inhibits HCO₃⁻ absorption in the MTAL through coupling of the PI3K-Akt-mTOR pathway to inhibition of NHE1. This conclusion is supported by several observations, including: 1) lumen LPS decreases basolateral Na⁺/H⁺ exchange activity through a PI3K-dependent pathway, 2) bath amiloride, which specifically inhibits HCO₃⁻ absorption in the MTAL through inhibition of NHE1 (27,32,86,87), eliminates inhibition by lumen LPS, and 3)
rapamycin blocks PI3K-induced inhibition of basolateral Na\(^+\)/H\(^+\) exchange in the MTAL (28). In addition, we have shown that rapamycin has no effect on the cytoskeleton-mediated regulatory interaction between the basolateral NHE1 and apical NHE3 Na\(^+\)/H\(^+\) exchangers (28); thus, the role of the PI3K-mTOR pathway in inhibition of HCO\(_3\)\(^-\) absorption is to mediate inhibition of basolateral Na\(^+\)/H\(^+\) exchange. Lumen LPS inhibits NHE1 when exchanger activity is studied independently of the activity of other transporters and in the absence of a change in the driving force for the exchanger, indicating that the LPS-induced PI3K-mTOR pathway is coupled directly to inhibition of NHE1. These results are consistent with our previous studies demonstrating that NGF inhibits MTAL HCO\(_3\)\(^-\) absorption through PI3K-mTOR-dependent inhibition of NHE1 (28,32). NHE1 has been identified as a target for LPS-induced regulation in other cell systems and has been ascribed a role in a variety of immunoregulatory cell responses, including cytokine and chemokine production, migration, survival, and phagocytosis (5,8,14,22,58,65). We show that NHE1 is a downstream target of LPS-induced TLR4 signaling in renal tubule cells and that LPS impairs the absorptive function of the MTAL through inhibition of this exchanger. NHE1 and TLR4 are expressed throughout the mammalian nephron, including segments of the proximal tubule, thick ascending limb, and collecting duct (6,9,19,32,45,56,57,73,96,97). Our findings raise the possibility that LPS and sepsis could impair a variety of processes in renal tubule cells that depend on NHE1, including intracellular pH regulation and cell survival. NHE1 has been shown to play a role in defense against apoptosis in renal proximal tubule cells (40). Thus, a direct action of LPS to inhibit NHE1 through TLR4 could aid in promoting renal tubule epithelial cell death in the septic state. We have shown previously in the MTAL that basolateral LPS acts directly through TLR4-mediated ERK activation to inhibit NHE3, the apical membrane Na\(^+\)/H\(^+\) exchanger isoform that mediates NaHCO\(_3\) and NaCl absorption by renal and intestinal epithelial cells (70,88). Thus, LPS can induce signals that impair the function of both the NHE1 and NHE3 exchanger isoforms in renal tubule cells.
The specific mechanisms by which the PI3K-Akt-mTOR pathway inhibits NHE1 in the MTAL are undefined. There is precedent for regulation of NHE1 by PI3K and Akt in other systems. PI3K was shown recently to inhibit NHE1 in proximal tubule cells through generation of PIP3, which reduces NHE1 activity by competitively inhibiting the stimulatory effect of PIP2 (40). This mechanism is unlikely to contribute to our results because it does not depend on mTOR and, thus, should not be sensitive to rapamycin. In addition, it involves direct interactions of locally-produced membrane phospholipids with NHE1 in the plasma membrane. In our experiments, PI3K signals generated at the apical membrane through TLR4 are transmitted through Akt and mTOR activation to inhibit NHE1 in a different (basolateral) cell membrane. Akt has been reported in different cell types to stimulate (5,63) or inhibit (81) NHE1 activity, and both effects were attributed to direct phosphorylation of NHE1 by Akt on Ser648 (63,81). This mechanism also is unlikely to account for our results because the regulation of NHE1 by direct Akt phosphorylation is not affected by rapamycin (63). Our current and previous (28) results in the MTAL provide the first evidence of a role for mTOR in regulation of NHE1 activity, and identify activation of mTOR as a mechanism involved in PI3K-Akt-dependent inhibition of NHE1. The sensitivity of the LPS-induced transport inhibition to rapamycin supports a specific role for mTORC1 in NHE1 regulation. The molecular mechanisms by which activation of mTOR leads to inhibition of NHE1 remain to be determined. One possibility is that the inhibition is mediated by activation of ribosomal protein S6 kinase (S6K), a major downstream effector of mTORC1 (60). S6K is a member of the AGC subfamily of serine/threonine kinases that includes several well-defined regulators of epithelial transport proteins, including protein kinases A, C, and G, serum-and glucocorticoid-induced protein kinase, and p90 ribosomal S6 kinase (39,64). We have shown previously that S6K is activated downstream of PI3K-mTOR in the MTAL (28). Moreover, LPS stimulates phosphorylation of mTOR at Ser2448, a site directly phosphorylated by S6K (38). Thus, the role of S6K in mediating mTOR-dependent regulation of NHE1 warrants further investigation.
In the MTAL, the PI3K-mTOR pathway can be targeted specifically to either inhibit basolateral NHE1 or stimulate apical NHE3, depending on the physiological stimulus (26,28). The results of the present study reveal an additional level of complexity in this signal specificity whereby PI3K signals activated through TLR4 at the apical membrane are targeted not to stimulate NHE3 in the same membrane, but rather are targeted transcellularly to inhibit NHE1 in the basolateral membrane. The molecular mechanisms that underlie the targeting of PI3K signals to specific effectors in different membranes, and the potential importance of this signal targeting in determining the immunoregulatory responses of renal tubule cells to LPS, will be important areas for future study. It is noteworthy in this context that activation of PI3K-Akt was reported to mediate inhibition of NaCl absorption by endothelin-1 in rat thick ascending limbs (37). In contrast, we found no effect of lumen LPS on transepithelial voltage, a correlate of NaCl absorption rate, in the MTAL. These findings suggest that targeting of the PI3K-Akt pathway to regulate NaCl absorption also may be stimulus specific in MTAL cells.

The molecular mechanisms by which LPS activates different TLR4 signaling pathways at the apical and basolateral membranes of the MTAL are incompletely understood; however, it is possible to comment on some potential contributing factors. First, both the TLR4-mediated effects of LPS to stimulate PI3K at the apical membrane and ERK at the basolateral membrane are eliminated in MyD88-deficient MTALs but preserved in MTALs lacking Trif (Fig. 6 ; 88). Thus, MyD88 is essential for both the lumen and basolateral LPS responses, indicating that the different LPS-induced signaling pathways are not the result of differential activation of MyD88-dependent and MyD88-independent (Trif-dependent) TLR4 pathways at the different membranes. Second, LPS-induced activation of TLR4 signaling, including PI3K-Akt (72,77), has been shown to depend on CD14, a membrane-anchored glycoprotein that binds LPS and enhances LPS signaling by facilitating its interaction with the TLR4 receptor complex (43,95). CD14 was reported recently to play a role in LPS-induced TLR4 signaling in the renal proximal tubule (41). Our results show, however, that inhibition of HCO$_3^-$ absorption by both lumen and
bath LPS is fully preserved in MTALs from CD14−/− mice (Fig. 7; 31). Thus, the activation of
different TLR4 signaling pathways by LPS does not depend on CD14. Finally, a mechanism
that may contribute to the different LPS signaling pathways involves a selective interaction of
TLR4 with TLR2 in the basolateral membrane. We have shown that TLR2 is co-expressed with
TLR4 in the basolateral membrane, but not in the apical membrane, of MTAL cells (29,30) and
that TLR4 and TLR2 are physically associated in the region of the kidney enriched in MTALs
(31). Moreover, the effects of basolateral LPS to activate ERK and inhibit HCO₃⁻ absorption
require both TLR4 and TLR2 (31,88), whereas the PI3K-dependent inhibition of HCO₃⁻
absorption by lumen LPS requires TLR4 but not TLR2 (29,31). These findings suggest that the
ability of LPS to activate different TLR4 signaling pathways at the apical and basolateral
membranes involves a novel interaction between TLR4 and TLR2 in the basolateral membrane
(31). The molecular basis for the requirement of TLR2 in LPS-induced TLR4 signaling remains
to be determined.

During bacterial infection via the bloodstream or the ascending urinary tract, LPS gains
access to both the luminal and basolateral surfaces of renal tubules in the medulla and cortex.
This may occur through several mechanisms, including translocation of bacteria across the
renal tubule and vascular walls, processes that promote contiguous spread of bacteria and the
formation of locally proliferating microcolonies in the luminal and interstitial compartments
(10,13,75). Interactions of LPS with the tubule cells may occur through LPS as a major
component of the outer membrane of the bacterial cell wall, through free LPS released locally
by dividing or damaged bacterial cells, or through circulating LPS filtered at the glomerulus. Our
finding that LPS activates different TLR4 signaling pathways at the apical and basolateral
membranes of MTAL cells raises questions regarding the immunoregulatory functions and
pathophysiological significance of the different pathways. Both the apical TLR4-PI3K and the
basolateral TLR4-ERK pathways are coupled to inhibition of HCO₃⁻ absorption in the MTAL and
thus can play a role in mediating tubule dysfunction during infection. Additional important
questions include whether the different apical and basolateral TLR4 pathways may play distinct roles in the renal tubule innate immune response or inflammatory kidney injury, and whether the pathways may be differentially regulated in inflammatory disease states. Activation of TLR4 on renal tubule cells by LPS or endogenous ligands plays an important role in mediating inflammatory kidney injury in a variety of infectious and non-infectious conditions, including sepsis and endotoxemia, ischemia-reperfusion, diabetes, glomerular diseases, and nephrotoxic injury (34,45,53,56,57,83,96,97). Understanding the molecular components and immunoregulatory functions of membrane-specific TLR4 signaling pathways in the MTAL may advance the goal of selective targeting of TLR4 pathways to enhance beneficial responses that improve host defense and suppress harmful pathways that lead to renal tubule dysfunction and inflammatory kidney injury.

In summary, the results of the present study demonstrate that lumen LPS inhibits HCO$_3^-$ absorption in the MTAL through TLR4/MyD88-dependent activation of a PI3K-Akt-mTOR (mTORC1) signaling pathway. This pathway is coupled to inhibition of the basolateral Na$^+/H^+$ exchanger NHE1, which results in inhibition of HCO$_3^-$ absorption. The results identify the PI3K-Akt-mTOR pathway as a signaling intermediate that couples the TLR4 receptor complex to modulation of renal tubule transport and suggest that molecular components of this pathway represent potential therapeutic targets for sepsis-induced renal tubule dysfunction. Lumen LPS inhibits HCO$_3^-$ absorption through signaling and transport mechanisms that differ from those activated by basolateral LPS, which inhibits HCO$_3^-$ absorption through TLR4-ERK-dependent inhibition of NHE3 (29,88). Understanding the molecular events by which LPS activates different TLR4 signaling pathways at the apical and basolateral membranes of the MTAL, and the specific immunoregulatory functions of these pathways, may lead to new approaches for targeting TLR4 signals to preserve renal tubule function and reduce kidney injury during sepsis and other inflammatory conditions.
Footnote

1. Chronic treatment with rapamycin (≥ 24 hr) has been shown to inhibit mTORC2 function by disrupting mTORC2 assembly (12,76). The effects of prolonged rapamycin treatment on the response of the MTAL to LPS were not examined in our experiments.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: B.A.W. and T.G. performed experiments; B.A.W. and D.W.G. analyzed data; B.A.W. and D.W.G. interpreted results of experiments; B.A.W. and D.W.G. prepared figures; D.W.G. drafted manuscript; D.W.G. and B.A.W. approved final version of manuscript; D.W.G. and B.A.W., conception and design of research.
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**Figure Legends**

**Figure 1.** Inhibitors of phosphoinositide 3-kinase (PI3K) eliminate inhibition of HCO$_3^-$ absorption by lumen LPS. Medullary thick ascending limbs (MTAL) from Sprague-Dawley rats and C57BL/6J mice were isolated and perfused in vitro. Tubules were studied in control solution (A and C), or bathed with 100 nM wortmannin or 20 µM LY294002 (B and D), and then LPS (250 ng/ml) was added to and removed from the lumen solution. Absolute rates of HCO$_3^-$ absorption (JHCO$_3^-$) were measured as described in Methods. Data points are average values for single tubules. Lines connect paired measurements made in the same tubule. P values are for paired $t$ test. NS, not significant. Mean values are given in Results.

**Figure 2.** Inhibitors of Akt and mTOR eliminate inhibition of HCO$_3^-$ absorption by lumen LPS. MTALs from rats and mice were bathed with 5 µM Akt inhibitor VIII (A) or 20 nM rapamycin (Rap) (B), and then LPS was added to and removed from the lumen solution. JHCO$_3^-$, data points, lines, and P values are as in Figure 1. NS, not significant.

**Figure 3.** Inhibition of HCO$_3^-$ absorption by bath LPS is unaffected by inhibitors of PI3K. A. MTALs were bathed with wortmannin or LY294002, and then LPS was added to and removed from the bath solution. B. MTALs were bathed with 15 µM PD98059 or 15 µM U0126, and then LPS was added to and removed from the lumen solution. Experiments were performed on MTALs from rats. JHCO$_3^-$, data points, lines, and P values are as in Figure 1. Mean values are given in Results.

**Figure 4.** LPS increases phosphorylation of Akt and mTOR in the MTAL. A: MTALs dissected from mice were incubated in vitro at 37°C in the absence (Control) and presence of wortmannin (Wort) for 15 min, then treated with LPS for 15 min. The tubules were fixed and stained with antiphospho-Akt-Ser473 (p-Akt) or antiphospho-mTOR-Ser2448 (p-mTOR) antibody and analyzed by confocal immunofluorescence as described in Methods. Images are Z-axis sections (0.4 µm) taken through a plane at the center of the tubule showing a cross-sectional view of cells in the lateral tubule walls (28,87,88). LPS increased p-Akt and p-mTOR labeling.
and these increases were eliminated by wortmannin. Images are representative of at least ten
tubules of each type. Scale bar = 5 µm. B: The intensity of p-Akt and p-mTOR staining for
experiments in A was quantified as described in Methods and is presented as a percentage of
the control level. Bars are means ± SE. * P < 0.05 vs. control. C: Inner stripe tissue was
incubated in vitro at 37° C in the absence (Control) and presence of wortmannin (Wort) for 15
min, then treated with LPS for 15 min. Cell lysates were immunoblotted with p-Akt or p-mTOR
antibody to analyze Akt and mTOR phosphorylation, and with anti-Akt or anti-mTOR antibody
for total Akt and total mTOR levels. Blots are representative of 4 independent experiments. D:
p-Akt and p-mTOR levels normalized for total Akt and total mTOR were determined for
experiments in C by densitometry. p-Akt/Akt and p-mTOR/mTOR ratios are presented as a
percentage of control values measured in the same experiment. Bars are means ± SE. * P <
0.05 vs. control.

Figure 5. LPS-induced Akt phosphorylation is mediated through TLR4. A: MTALs dissected
from wild-type and TLR4−/− mice were incubated in vitro for 15 min at 37° C in the absence
(Control) and presence of LPS. The tubules were then fixed and stained with p-Akt antibody
and analyzed by confocal immunofluorescence as in Fig. 4A. LPS increased Akt
phosphorylation in MTALs from wild-type mice but had no effect in MTALs from TLR4−/− mice.
Images are representative of six tubules of each type. B: The intensity of p-Akt staining was
quantified for experiments in A as described in Methods and is presented as a percentage of
control level measured in the same experiment. * P < 0.05 vs. control.

Figure 6. Inhibition of HCO3− absorption by lumen LPS and LPS-induced Akt activation are
mediated by MyD88. A: MTALs from MyD88−/− and Trif−/− mice were perfused in vitro under
control conditions and then LPS was added to and removed from the lumen solution. JHCO3−,
data points, lines, and P values are as in Fig. 1. NS, not significant. C57BL/6J mice (Fig. 1C)
are wild-type controls. B: MTALs from wild-type and MyD88−/− mice were incubated in vitro for
15 min at 37° C in the absence (Control) and presence of LPS. The tubules were stained with
p-Akt antibody and analyzed by confocal immunofluorescence as in Fig. 4A. The effect of LPS to increase Akt phosphorylation was eliminated in MTALs from MyD88<sup>-/-</sup> mice. Images are representative of six tubules of each type. C: The intensity of p-Akt staining was quantified for experiments in B as described in Methods and is presented as a percentage of control level measured in the same experiment. * P < 0.05 vs. control. Control fluorescence intensity did not differ in wild-type and MyD88<sup>-/-</sup> tubules.

**Figure 7.** CD14 is not required for inhibition of HCO<sub>3</sub><sup>-</sup> absorption by lumen LPS. MTALs from CD14<sup>-/-</sup> mice were perfused in vitro under control conditions and then LPS was added to and removed from the lumen solution. JHCO<sub>3</sub><sup>-</sup>, data points, lines, and P value are as in Figure 1. Mean values are given in Results. C57BL/6J mice (Fig.1C) are wild-type controls.

**Figure 8.** Inhibition of HCO<sub>3</sub><sup>-</sup> absorption by lumen LPS is eliminated by inhibitors of basolateral Na<sup>+</sup>/H<sup>+</sup> exchange. MTALs were studied with 10 µM amiloride in the bath (A) or in a Na<sup>+</sup>-free bath (B), conditions that inhibit basolateral Na<sup>+</sup>/H<sup>+</sup> exchange (27,32,86,88). LPS was then added to and removed from the lumen solution. In B, Na<sup>+</sup> in the bath was replaced with NMDG<sup>+</sup>; the lumen was perfused with control solution containing 146 mM Na<sup>+</sup> (27,86). JHCO<sub>3</sub><sup>-</sup>, data points, lines, and P values are as in Fig. 1. NS, not significant.

**Figure 9.** Lumen LPS inhibits basolateral Na<sup>+</sup>/H<sup>+</sup> exchange through a PI3K-dependent pathway. A: MTALs from rats were studied under control conditions, with LPS in the lumen, or with LPS in the lumen plus wortmannin in the bath for 15-20 min. Basolateral Na<sup>+</sup>/H<sup>+</sup> exchange rates (JNa<sup>+</sup>/H<sup>+</sup>) were determined from initial rates of pH<sub>i</sub> increase measured after addition of Na<sup>+</sup> to the bath solution (see Methods). Data points are from 10 control tubules, 9 tubules with LPS, and 7 tubules with wortmannin + LPS. B: Mean basolateral Na<sup>+</sup>/H<sup>+</sup> exchange rates for conditions in A. * P < 0.05 vs. other conditions. Wortmannin alone has no effect on basolateral Na<sup>+</sup>/H<sup>+</sup> exchange activity.

**Figure 10.** Model for inhibition of HCO<sub>3</sub><sup>-</sup> absorption by lumen LPS in the MTAL. Lumen LPS decreases HCO<sub>3</sub><sup>-</sup> absorption by inhibiting NHE1 through activation of a PI3K-Akt-mTOR
(mTORC1) signaling pathway. Inhibition of basolateral NHE1 results secondarily in inhibition of apical NHE3, thereby decreasing $\text{HCO}_3^-$ absorption (27,32,86,88). The LPS-induced PI3K-Akt activation is mediated through TLR4 and the adaptor molecule MyD88. Arrows do not necessarily imply direct relationships; regulatory steps may involve additional signaling components. See text for details.
Figure 1
Figure 2

![Graphs showing Akt Inhibitor and mTOR Inhibitor effects on JHCO$_3^-$ production in rat and mouse cells.](image)

**A** Akt Inhibitor

- Akt Inhibitor
- Akt VIII
- Akt VIII + Lumen LPS

**B** mTOR Inhibitor

- mTOR Inhibitor
- Rap
- Rap + Lumen LPS

*P = NS*
Figure 3

A  PI3K Inhibitor
    Bath LPS

B  ERK Inhibitor
    Lumen LPS

JHCO₃⁻, pmol/min/mm

P<0.001

wortmannin
LY294002

PD98059
U0126
Figure 4

A  MTAL

Control  LPS  Wort  Wort + LPS

p-Akt

p-mTOR

B

Fluorescence intensity, % of Control

0  50  100  150

Control  LPS  Control  LPS

p-Akt

p-mTOR

C  Inner Stripe

Control  LPS  Wort  Wort + LPS

p-Akt

Akt

p-mTOR

mTOR

D

p-Akt

p-mTOR

p-Akt/Akt, % of Control

0  50  100  150

Control  LPS

p-mTOR/mTOR, % of Control

0  50  100  150

Control  LPS
Figure 5

A

Wild-type

TLR4\textsuperscript{-/-}

Control

LPS

B

Fluorescence Intensity, % of Control

Wild-type

TLR4\textsuperscript{-/-}

Control

LPS

*p-Akt*
Figure 6

A) MyD88<sup>−/−</sup> and Trif<sup>−/−</sup> mice were treated with LPS. JHCO<sub>3</sub><sup>−</sup> levels were measured in the control and LPS-treated groups. MyD88<sup>−/−</sup> mice showed a significant increase in JHCO<sub>3</sub><sup>−</sup> levels compared to control mice, while Trif<sup>−/−</sup> mice did not show a significant change.

B) Immunohistochemical analysis of p-Akt expression in control and LPS-treated mice. Wild-type mice showed a decrease in p-Akt expression after LPS treatment, while MyD88<sup>−/−</sup> mice did not show a significant change.

C) Bar graph showing the fluorescence intensity of p-Akt in control and LPS-treated groups. Wild-type mice showed a significant increase in p-Akt expression after LPS treatment, while MyD88<sup>−/−</sup> mice did not show a significant change.
Figure 7

CD14^−/−

P<0.001

JHCO₃^−, pmol/min/mm

Control | Lumen | Control

20 15 10 5 0
Figure 8

A

Bath Amiloride

P = NS

B

0 Na⁺ Bath

P = NS
Figure 9

A

B

Intracellular pH

JNa⁺/H⁺, pmol/min/mm

Control
Lumen LPS
Wort + Lumen LPS

*
Figure 10

LPS

\[ \text{TLR4} \]

Apical membrane

MyD88

wortmannin
LY294002

PI3K

Akt inhibitor VIII

Akt

Akt

mTORC1

mTORC2

NHE1

NHE3

\[ \downarrow \text{HCO}_3^- \text{Absorption} \]