
The purpose of the present study was to determine whether activation of TLR2 by bacterial components alters HCO₃⁻ absorption by the MTAL. The results demonstrate that TLR2 is expressed in the basolateral membrane domain of the MTAL and that TLR2-specific agonists inhibit HCO₃⁻ absorption. The inhibition of HCO₃⁻ absorption by bacterial components acting through TLR2 is blocked by inhibitors of protein kinase C.
(PKC) and is additive to inhibition by LPS acting through TLR4. These results establish a role for TLR2 in the regulation of renal tubule ion transport and show that gram-positive and gram-negative bacterial molecules function independently through distinct receptor signaling pathways to impair the transport function of the MTAL. The ability of bacterial molecules to directly inhibit HCO₃⁻ absorption may contribute to and/or impair the ability of the kidneys to correct systemic acidosis during sepsis.

METHODS

Animals. Male Sprague-Dawley rats (50–90 g body wt) were purchased from Taconic (Germantown, NY). Male C57BL/6J (wild-type) and B6.129-Tlr2tm1K/J (TLR2−/−) mice (6 to 8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129-Tlr2tm1K/J mice do not produce TLR2 protein due to targeted disruption of the Tlr2 gene (85). Animals were maintained under pathogen-free conditions in microisolation cages and received standard rodent chow (NIH 31 diet, Ziegler) and distilled water up to the time of experiments. Body weight did not differ in wild-type and TLR2−/− mice (22 ± 1 g wild-type vs. 23 ± 1 g TLR2−/−). All protocols in this study were approved by the Institutional Animal Care and Use Committee 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 (31, 35, 82). Tubules were dissected from the inner stripe of the outer medulla at 10°C in control bath solution, transferred to a bath chamber (31, 35, 82). Tubules were dissected from the inner stripe of the outer medulla at 10°C in control bath solution, transferred to a bath chamber and imaged in a single session at identical settings of illumination, gain, and exposure time.

RESULTS

TLR2 is expressed in the basolateral membrane domain of the MTAL. To localize TLR2 in the MTAL, tubules microdissected from normal rats (Fig. 1, A–C) and from wild-type control (C57BL/6J) and TLR2−/− (B6.129-Tlr2tm1K/J) mice (Fig. 1, D and E) were stained with anti-TLR2 antibody and analyzed by confocal immunofluorescence microscopy. Staining for TLR2 was observed selectively in the basolateral membrane domain in both rat and mouse MTALs (Fig. 1, A, B, and D). The TLR2 staining was absent in the presence of specific blocking peptide (Fig. 1C) and in MTALs from the TLR2−/− mice (Fig. 1E). These results indicate that TLR2 is expressed in the MTAL and exhibits a selective basolateral location.

Pam₃CSK₄ inhibits HCO₃⁻ absorption in modulating TLR2. To examine a possible role for TLR2 in modulating MTAL transport, we examined the effect on HCO₃⁻ absorption of Pam₃CSK₄, a synthetic bacterial lipopeptide that activates cells as a specific TLR2 ligand (5, 17, 20, 57, 74, 75). Addition of Pam₃CSK₄ (1 µmol/ml) to the bath decreased HCO₃⁻ absorption by 25%, from 15.2 ± 0.6 to 11.4 ± 0.5 pmol·min⁻¹·mm⁻² (Fig. 2). The inhibition by Pam₃CSK₄ is rapid (<15 min), sustained for up to 60 min, and reversible. These results demonstrate that a TLR2 agonist directly alters the transport function of the MTAL.

Inhibition by Pam₃CSK₄ is eliminated in MTALs from TLR2−/− mice. To establish the functional significance of TLR2 for ion transport regulation, the effects of Pam₃CSK₄ on HCO₃⁻ absorption were examined in MTALs from wild-type control and TLR2−/− mice (Fig. 3). The basal rate of HCO₃⁻ absorption was similar in MTALs from wild-type and TLR2−/− mice. Similar to results obtained in the rat, addition of Pam₃CSK₄ to the bath decreased HCO₃⁻ absorption in MTALs from wild-type mice from 16.4 ± 0.7 to 10.8 ± 1.0 pmol·min⁻¹·mm⁻² (Fig. 3A). In contrast, addition of Pam₃CSK₄ to the bath had no effect on HCO₃⁻ absorption in MTALs from TLR2−/− mice (17.4 ± 0.5 pmol·min⁻¹·mm⁻²).
pmol·min⁻¹·mm⁻¹, control vs. 17.5 ± 0.6 pmol·min⁻¹·mm⁻¹, Pam3CSK₄; Fig. 3B). These results identify a role for TLR2 in the regulation of renal tubule transport and support the conclusion that TLR2 is the signaling receptor that mediates inhibition of HCO₃⁻ absorption by Pam3CSK₄.

Lipoteichoic acid and peptidoglycan inhibit HCO₃⁻ absorption in the MTAL. To assess further the significance of TLR2 for MTAL transport regulation, we examined the effects of the TLR2 agonists lipoteichoic acid and peptidoglycan. Lipoteichoic acid and peptidoglycan are components of the cell wall of gram-positive bacteria and function as TLR2 ligands structurally distinct from bacterial lipoproteins (2, 4, 40, 54, 57, 69, 72, 87). Addition of lipoteichoic acid (1 g/ml) to the bath decreased HCO₃⁻ absorption by 26%, from 14.7 ± 0.3 to 10.9 ± 0.2 pmol·min⁻¹·mm⁻¹ (Fig. 4A). Similarly, addition of peptidoglycan (10 g/ml) to the bath decreased HCO₃⁻ absorption by 22%, from 15.8 ± 0.4 to 12.4 ± 0.3 pmol·min⁻¹·mm⁻¹ (Fig. 4B). The inhibition by both compounds is reversible and occurs with a time course similar to that observed with Pam3CSK₄. These results support further the view that bacterial components recognized by TLR2 directly modify the transport function of the MTAL.

LPS and Pam3CSK₄ inhibit HCO₃⁻ absorption through distinct signaling pathways. Previously, we demonstrated that absorption of HCO₃⁻ by the MTAL is inhibited directly by LPS, the dominant cell wall molecule of gram-negative bacteria. The inhibition by LPS is mediated through activation of its cell-surface receptor TLR4 (34). Further studies were carried out to test whether the inhibition by bath LPS through TLR4 and the inhibition by Pam3CSK₄ through TLR2 may be mediated through a common signaling pathway. The effects of LPS and Pam3CSK₄ on HCO₃⁻ absorption were examined in the
absence and presence of U0126, a MEK1/2 inhibitor that selectively blocks ERK activation and ERK-mediated inhibition of HCO$_3^-$ absorption in the MTAL (33, 82, 83). Consistent with previous results (34), bath LPS decreased HCO$_3^-$ absorption by 33 ± 3% under control conditions and this inhibition was eliminated completely by U0126 (Fig. 5A). In contrast, bath Pam$_3$CSK$_4$ decreased HCO$_3^-$ absorption by 27 ± 1% in the absence and 31 ± 2% in the presence of U0126 (Fig. 5B). Thus, U0126 had no effect on the inhibition by bath Pam$_3$CSK$_4$. These results indicate that agonists of TLR4 and TLR2 in the basolateral membrane inhibit HCO$_3^-$ absorption in the MTAL through different signal transduction pathways.

Inhibition by Pam$_3$CSK$_4$ is eliminated by inhibitors of PKC. Previous reports showed that stimulation of TLR2 leads to the induction of innate immune responses through the activation of PI3K- and PKC-dependent signaling pathways (8, 18, 27, 39, 64, 72, 77, 86). To examine the role of PI3K in the inhibition of HCO$_3^-$ absorption by Pam$_3$CSK$_4$, MTALs were bathed with LY294002 or wortmannin, inhibitors that selectively block PI3K-mediated regulation of HCO$_3^-$ absorption in the MTAL (33). In the presence of LY294002 or wortmannin, Pam$_3$CSK$_4$ decreased HCO$_3^-$ absorption by 25%, from 15.8 ± 0.6 to 11.9 ± 0.4 pmol·min$^{-1}$·mm$^{-1}$ (Fig. 6A). Thus, the inhibition by Pam$_3$CSK$_4$ is not mediated through PI3K.

TLR2 signaling in several cell systems leads to activation of PKC. The role of PKC in mediating Pam$_3$CSK$_4$-induced inhibition of HCO$_3^-$ absorption in the MTAL was examined using the selective PKC inhibitors chelerythrine Cl and bisindolylmaleimide (32, 38, 78). As shown in Fig. 6B, the effect of Pam$_3$CSK$_4$ to inhibit HCO$_3^-$ absorption was eliminated completely by the PKC inhibitors (15.1 ± 0.3 pmol·min$^{-1}$·mm$^{-1}$, inhibitors vs. 15.0 ± 0.3 pmol·min$^{-1}$·mm$^{-1}$, inhibitors + Pam$_3$CSK$_4$). These results support an essential role for PKC in mediating the inhibition of HCO$_3^-$ absorption by Pam$_3$CSK$_4$ through TLR2.

Inhibition by TLR2 agonists is additive to inhibition by LPS. Based on the preceding results, further experiments were carried out to determine whether inhibition of HCO$_3^-$ absorption by TLR2 agonists is additive to inhibition by LPS. In tubules bathed with LPS, adding Pam$_3$CSK$_4$, lipoteichoic acid, or peptidoglycan to the bath decreased HCO$_3^-$ absorption by 25%, from 11.8 ± 0.3 to 8.8 ± 0.4 pmol·min$^{-1}$·mm$^{-1}$ (Fig. 7A and B). Similarly, in tubules bathed with Pam$_3$CSK$_4$, adding LPS to the bath decreased HCO$_3^-$ absorption by 33%, from 11.0 ± 0.4 to 7.4 ± 0.3 pmol·min$^{-1}$·mm$^{-1}$ (Fig. 7C), an inhibition similar to that observed with bath LPS in the absence of Pam$_3$CSK$_4$ (Fig. 5A) (34). In the combined presence of a TLR2 and TLR4 agonist, the HCO$_3^-$ absorption rate is reduced by ~50% compared with the rate measured in tubules not exposed to bacterial stimuli.¹ These results demonstrate that agonists of TLR2 and TLR4 function independently through the activation of distinct pathways to inhibit HCO$_3^-$ absorption in the MTAL.

¹ The initial control rate of HCO$_3^-$ absorption was measured in 6 of 10 experiments in Fig. 7 before the addition of bacterial compounds. For those tubules, the initial HCO$_3^-$ absorption rate was 15.9 ± 0.4 pmol·min$^{-1}$·mm$^{-1}$ compared with a rate of 8.1 ± 0.5 pmol·min$^{-1}$·mm$^{-1}$ measured in the combined presence of a TLR2 agonist plus LPS (49% inhibition). This inhibition is in good agreement with that predicted by adding the individual inhibitory effects of TLR2 agonists (25%) and LPS (33%) when these agents are studied in separate tubules.
The development of kidney dysfunction during sepsis predicts a poor outcome and the risk for mortality doubles when renal insufficiency accompanies sepsis (43, 48, 56, 62, 67). Sepsis and endotoxemia induce a variety of functional defects within the nephron in association with alterations in fluid and electrolyte balance, including impaired urinary concentrating ability, increased fractional excretion of sodium, hypotension, and metabolic acidosis (10, 12, 22, 24, 36, 59, 65–67, 80). The mechanisms that underlie the development of kidney dysfunction during sepsis are incompletely understood. We examined the possibility that bacterial molecules act directly through TLRs to alter renal tubule function. In recent studies, we demonstrated that gram-negative bacterial LPS decreases HCO₃⁻/H₁₅₂₀₂ absorption in the MTAL through activation of TLR4 (34). In the present study, we demonstrate that MTAL HCO₃⁻ absorption is inhibited by bacterial lipoproteins and gram-positive bacterial cell wall molecules that activate TLR2. These studies establish that both TLR2 and TLR4 play a role in modulating renal tubule ion transport and that bacterial components can impair renal tubule function directly through interaction with these cell surface receptors. We show further that the inhibition of HCO₃⁻ absorption by bacterial components acting through TLR2 is additive to inhibition by LPS acting through TLR4 and that TLR2 and TLR4 agonists impair MTAL transport through the activation of different signal transduction pathways. Thus, gram-positive and gram-negative bacteria, which account for most cases of clinical sepsis, can function directly and independently to impair renal tubule function as a result of their specific molecular patterns activating different intracellular signaling pathways through distinct TLRs.

The TLRs are a family of closely related transmembrane receptors that participate in innate immunity by recognizing distinct microbial structures (2, 40). To date, at least 13 members of the TLR family have been identified in mammalian cells (40). In response to pathogen recognition, TLRs activate intracellular signal transduction pathways that lead to activation of specific kinases and transcription factors and the production of proinflammatory mediators (2, 22, 40). TLR2 plays a major role in the recognition of gram-positive bacterial components, including lipoprotein, lipoteichoic acid, and peptidoglycan, and is important in host defense against gram-positive bacterial infection (2, 15, 40, 73, 87). Surprisingly, however, despite the fact that gram-positive bacteria account for at least half of sepsis cases (3, 14, 22, 50), the role of TLR2 in sepsis-induced kidney injury is undefined.

Our results indicate that TLR2 plays a direct role in modulating the transport function of renal tubules. The ability of the MTAL to absorb HCO₃⁻ is decreased by the synthetic bacterial lipopeptide Pam₃CSK₄ and by a purified preparation of the gram-positive bacterial cell wall glycolipid lipoteichoic acid, two TLR2-specific ligands (2, 5, 17, 20, 54, 57, 69, 74, 75). The inhibition by Pam₃CSK₄ is eliminated in MTALs from TLR2⁻/⁻ mice, confirming that TLR2 is the receptor responsible for the transport inhibition. MTAL HCO₃⁻ absorption is also inhibited by peptidoglycan, an additional gram-positive cell wall molecule reported to be recognized by TLR2 (2, 4, 57, 69, 73, 87). The direct action of bacterial molecules to impair luminal acidification in renal tubules may accentuate the pathogenicity of sepsis through several mechanisms. The development of systemic metabolic acidosis contributes to multiple organ dysfunction (particularly instability of the cardiovascular...
system) during sepsis (12, 22, 44, 59, 67) and is independently associated with increased mortality in septic patients (30, 45). The inhibition of renal tubule acid secretion by bacterial components mediated through TLR2 would exacerbate and impair the correction of sepsis-induced acidemia. Within the kidney, the relative alkalization of the luminal fluid would promote a variety of pathogenic processes, including increased attachment of bacteria to renal tubule cells that is critical for bacterial colonization (68), increased bacterial cell growth (29, 41, 68), the formation of bacteria-associated phosphate and calcium stones (61), and resistance to certain antibiotics (88). Thus, the ability of bacterial molecules to directly inhibit MTAL HCO₃⁻ absorption through TLR2 can adversely affect the severity and progression of sepsis at both the systemic and kidney levels.

TLR2 is expressed constitutively in renal tubule segments of rat, mouse, and human kidney, including segments of the proximal tubule and thick ascending limb (1, 23, 25, 42, 46, 49, 70, 84). Our results show that TLR2 is expressed in the basolateral membrane domain of the rat and mouse MTAL. This finding is consistent with the study of Shigeoka et al. (70), which reported that TLR2 was localized to the basolateral membrane of renal tubule cells in mouse outer medulla. The selective expression of TLR2 in the basolateral membrane differs from the expression of TLR4, which is localized in both basolateral and apical membrane domains of the MTAL (34). The luminal location of TLR4 is thought to be important for its crucial role in defense against ascending urinary tract infections, which are due predominantly to gram-negative bacteria (19, 25, 37, 58, 60). The coexpression of TLR2 and TLR4 in the basolateral membrane indicates that these two receptors are situated to monitor the composition of the interstitial fluid. This would enable the MTAL to recognize and respond rapidly to gram-positive and gram-negative bacteria that spread from the intravascular compartment to the interstitial space in the renal outer medulla during hematogenous infection. This would initiate innate immune responses that may aid in eliminating the invading bacteria. However, bacterial recognition also triggers intracellular signals that impair renal tubule function (Figs. 2 and 4) (34) and that may lead to inflammatory responses that contribute to sepsis-induced acute kidney injury. Activation of TLR2 by bacterial lipopeptide stimulated the production of proinflammatory cytokines and chemokines in cultured renal tubule epithelial cells (20, 79), consistent with a potential role for TLR2 in mediating kidney inflammation during sepsis. TLR2 on renal cells has been shown to play an important role in mediating inflammatory kidney injury in a variety of noninfectious conditions, including acute ischemia-reperfusion injury (42, 47, 52, 70, 84), nephrotoxic antibody-induced glomerulonephritis (16), obstructive nephropathy (46), and possibly exposure to nephrotoxic drugs (1, 49). Inflammatory injury in these conditions is associated with increased levels of TLR2 protein in the renal tubules, including increased expression of TLR2 in the thick ascending limb during ischemia-reperfusion and cyclosporine-induced renal injury (42, 46, 49, 84). TLR2-mediated inflammation and injury in these conditions are thought to involve receptor activation by endogenous “danger” molecules that are released by damaged cells (6, 42, 46, 49, 70, 71). These molecules, which include heat shock proteins, extracellular matrix components such as hyaluronic and biglycan, and the nuclear protein HMGB1, accumulate in the interstitial space during cellular injury and are recognized by TLR2 to result in the initiation of inflammatory responses (13, 46, 51, 52, 71). Our results raise the possibility that activation of TLR2 on the basolateral membrane of the MTAL by endogenous ligands may also contribute directly to alterations in renal tubule transport during these pathological conditions. Whether TLR2-induced inflammatory responses may play a role in kidney injury during sepsis remains to be determined.

A unique feature of TLR2 is its ability to interact functionally and physically with other TLRs (TLR1 and TLR6) to recognize different bacterial lipoproteins (2, 57). In particular, TLR2 cooperates with TLR1 to recognize Pam₃CSK₄ and other triacylated lipopeptides (17, 40, 75). Studies using cells from TLR2-deficient mice and TLR reconstitution experiments showed that expression of TLR2 is essential for responses to Pam₃CSK₄ and that coexpression of TLR1 significantly enhances Pam₃CSK₄-induced TLR2 activation (17, 75). Alternatively, TLR2 cooperates with TLR6 to discriminate diacylated lipopeptides (17, 40, 54, 57, 74) and recent evidence suggests that the responses of TLR2 to peptidoglycan and lipoteichoic acid are enhanced by interaction with TLR6 (54, 57, 74). The mechanisms involved in these cooperative interactions are incompletely understood but are thought to involve the heterodimerization of TLR2 with TLR1 or TLR6 (17, 40, 54, 57, 74, 75). TLR1 and TLR6 were detected in mouse renal tubule epithelial cells in culture (79), but to our knowledge neither receptor has been localized in native renal tubules. Whether TLR1 and/or TLR6 is coexpressed with TLR2 in the basolateral membrane of the MTAL and functions to enhance TLR2 signaling in response to bacterial structures, or to confer specificity of TLR2 ligand recognition, will be important areas for future investigation.

Our results demonstrate further that the inhibition of HCO₃⁻ absorption by bacterial molecules acting through TLR2 is baseline to inhibition by gram-negative LPS acting through TLR4. This finding has important implications for the pathogenesis of renal tubule dysfunction during septic conditions in which a mixture of gram-positive and gram-negative bacteria is responsible for the sepsis response. These include sites of infection involving the gastrointestinal tract (peritonitis), lung (pneumonia), and skin (burn wounds). Our results indicate that gram-positive and gram-negative bacterial components can act independently through different TLRs to impair renal tubule function during polymicrobial sepsis. The ability of TLR2 and TLR4 agonists to inhibit HCO₃⁻ absorption additively in the MTAL is a result of their activating different intracellular signaling pathways. Consistent with previous results (34), the inhibition of HCO₃⁻ absorption by basolateral LPS is eliminated by U0126, which selectively blocks ERK activation and ERK-mediated inhibition of HCO₃⁻ absorption in the MTAL (33, 82, 83). In contrast, U0126 had no effect on the inhibition by Pam₃CSK₄. Thus, the recognition of specific bacterial pathogens by TLR4 and TLR2 results in inhibition of HCO₃⁻ absorption through the activation of distinct downstream mediators.

Our results indicate that PKC is an essential component of the TLR2 signaling pathway that leads to inhibition of HCO₃⁻ absorption in the MTAL. The inhibition by Pam₃CSK₄ was eliminated by chelerythrine Cl and bisindolylmaleimide, two selective and chemically unrelated PKC inhibitors with differ-
ent mechanisms of action (38, 78). PKC has been shown to play a role in mediating innate immune responses induced by TLR2 in a variety of systems, including macrophages, neutrophils, and intestinal epithelial cell lines (18, 27, 64, 76, 77, 86). These responses are associated with TLR2-mediated activation of specific PKC isoforms, including PKC-α, -δ, -ε, and -ζ (18, 27, 64, 77, 86). We showed that the MTAL constitutively expresses members of the conventional (α, δII), novel (δ, ε), and atypical (ζ) PKC subfamilies and that PKC-dependent transport regulation in the MTAL may involve the activation of specific PKC isoforms (9). It will be important in future studies to determine the role of distinct PKC isoforms in TLR2 signaling in the MTAL, the mechanisms involved in TLR2-induced PKC activation (phosphorylation, subcellular relocalization), and the upstream activators and downstream substrates leading to PKC-dependent inhibition of HCO₃⁻ absorption. Our results provide new evidence of a role for PKC in TLR2-induced signaling in renal epithelial cells. These findings raise the possibility that the PKC pathway may be a therapeutic target for modulating TLR2 signaling in renal tubules and, potentially, for suppressing TLR2-dependent signals that lead to inflammatory kidney injury (16, 25, 46, 47, 49, 70).

TLR2 contains a consensus binding site for the p85 subunit of PI3K and activation of PI3K through TLR2 stimulation plays a role in the induction of inflammatory responses in immune cells (8, 39, 72). TLR2 signaling also involves ERK activation in other cell systems, including renal tubule epithelial cells (11, 17, 52). Activation of the PI3K and ERK pathways has been shown to inhibit HCO₃⁻ absorption in the MTAL (33, 82, 83). However, the results of experiments using highly selective inhibitors indicate that the PI3K and ERK pathways are not involved in mediating TLR2-dependent inhibition of HCO₃⁻ absorption in the MTAL. Thus, either activation of PI3K and ERK is not a component of Pam₃CSK₄-induced TLR2 signaling in the MTAL or activation of these pathways through TLR2 is not coupled to the inhibition of HCO₃⁻ absorption.

In summary, our data provide new evidence of a role for TLR2 in the regulation of renal tubule ion transport. TLR2, which plays a major role in recognition of gram-positive bacteria (2), is expressed in the basolateral membrane of the MTAL. Absorption of HCO₃⁻ by the MTAL is inhibited by bacterial components recognized by TLR2, including the bacterial lipopeptide Pam₃CSK₄ and the gram-positive bacterial cell wall molecules lipoteichoic acid and peptidoglycan. Inhibition of HCO₃⁻ absorption by gram-positive bacterial components acting through TLR2 is additive to inhibition by LPS acting through TLR4. This is a result of the TLR2 and TLR4 agonists activating different intracellular signal transduction pathways: inhibition of HCO₃⁻ absorption through TLR2 is blocked by inhibitors of PKC, whereas inhibition through TLR4 is blocked by inhibitors of ERK. Thus, gram-positive and gram-negative bacterial molecules can act independently during sepsis to impair renal tubule function. Understanding the distinct molecular components of the TLR2 and TLR4 pathways that are triggered by different bacterial molecules to inhibit MTAL transport, and the ability to manipulate these pathways, will be important for therapeutic strategies aimed at treating and preventing renal tubule dysfunction during sepsis.

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**DISCLOSURES**

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