

Nicotinic Acetylcholine Receptor Agonists Attenuate Septic Acute Kidney Injury in Mice by Suppressing Inflammation and Proteasome Activity

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Abstract

Sepsis is one of the leading causes of acute kidney injury (AKI). Septic patients who develop acute kidney injury (AKI) are at increased risk of death. To date there is no effective treatment for AKI or septic AKI. Based on their anti-inflammatory properties, we examined the effects of nicotinic acetylcholine receptor agonists on renal damage using a mouse model of lipopolysaccharide (LPS)-induced AKI where localized LPS promotes inflammation-mediated kidney damage. Administration of nicotine (1 mg/kg) or GTS-21 (4 mg/kg) significantly abrogated renal leukocyte infiltration (by 40%) and attenuated kidney injury. These renoprotective effects were accompanied by reduced systemic and localized kidney inflammation during LPS-induced AKI. Consistent with these observations, nicotinic agonist treatment significantly decreased renal I κ B α degradation and NF κ B activation during LPS-induced AKI. Treatment of human kidney cells with nicotinic agonists, an NF κ B inhibitor (Bay11), or a proteasome inhibitor (MG132) effectively inhibited their inflammatory responses following stimulation with LPS or TNF α . Renal proteasome activity, a major regulator of NF κ B-mediated inflammation, was enhanced by approximately 50% during LPS-induced AKI and elevated proteasome activity was significantly blunted by nicotinic agonist administration *in vivo*. Taken together, our results identify enhanced renal proteasome activity during LPS-induced AKI and the suppression of both proteasome activity and inflammation by nicotinic agonists to attenuate LPS-induced kidney injury.

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Introduction

The kidney is a frequent target for sepsis-associated multi-organ injury, with increasing prevalence of acute kidney injury (AKI) observed among patients with severe sepsis (23%) and septic shock (51%) compared to patients with moderate sepsis (19%) (Reviewed in [1,2]). Indeed severe sepsis and septic shock are the leading causes of AKI in intensive care patients and may be responsible for more than 50% of cases of AKI in such patients. Septic patients who develop AKI have overall increased morbidity, require prolonged hospital stays, use more healthcare resources, and have increased mortality. The pathophysiology of septic AKI is complex, multi-factorial and distinct from non-septic AKI [2–5]. Epidemiological, clinical, and experimental studies support the concept that pro-inflammatory mediators produced during sepsis promote intrarenal hemodynamic changes, endothelial dysfunction, leukocyte infiltration, and damaging tissue inflammation that lead to renal failure. Unfortunately, despite several years of intense investigations and significant advances in medicine and therapeutics, there are still no effective treatments for septic AKI.

Experimental endotoxemia induced by LPS, the major endotoxin of gram negative bacteria, is the most frequently employed model to study septic AKI (LPS-induced AKI). This model produces consistent renal tissue damage that is similar to that observed in humans [6–9]. Lipopolysaccharide (LPS) disseminates to the kidney proximal tubules and microvilli of the brush border, and is found associated with epithelial cells and endothelial cells of the peritubular capillaries [10]. Localized LPS promotes TNF α production by resident renal tubular epithelial cells [11], mesangial cells [12], glomerular cells [13], as well as leukocytes which adhere to the endothelium [12]. Thus, renal TNF α is proposed to be responsible for some of the renal dysfunction observed in LPS-induced AKI [6;14] through both direct cellular injury [15] and the amplified induction of localized inflammatory mediators (via NF κ B activation), including cytokines and chemokines which stimulate the infiltration of damaging neutrophils and monocytes into the kidneys, particularly the renal cortex [6]. In addition, extrarenal and systemic TNF α contributes to kidney injury observed during septic-AKI [7]. Concurrent

vascular inflammation and dysfunction characterized by endothelial cell activation, microvascular injury, increased vascular permeability, intraglomerular thrombosis, and reduced renal perfusion are typically observed in sepsis-associated AKI [12,16].

Because the pathogenesis of sepsis-associated AKI is ultimately mediated through multiple pathways, a multi-targeted therapeutic approach is warranted. One recently described broad-based physiologic mechanism for regulating inflammation is the cholinergic anti-inflammatory pathway [17]. This pathway can be stimulated by the administration of nicotinic acetylcholine receptor (nAChR) agonists, including nicotine and GTS-21, to produce beneficial anti-inflammatory effects [18–22], even in the absence of an intact vagus nerve [23]. Recent studies show that nicotinic agonists abrogate renal damage during experimental ischemia-reperfusion injury [23,24]. While a localized anti-inflammatory effect was observed, the exact mechanism involved in mediating the anti-inflammatory effects of nicotinic agonists is not well understood. We investigated the effect of nicotinic agonists on renal NF κ B and proteasome activity, as well as renal damage during septic AKI.

Results

Nicotinic Agonists Attenuate LPS-induced AKI

Administration of either nicotine or GTS-21 during LPS-induced septic AKI model significantly suppressed kidney injury induced by LPS. Both nicotine and GTS-21 treatment reduced leukocyte infiltration (assessed by renal myeloperoxidase (MPO) levels) within the kidney by approximately 40%, during experimental LPS-AKI compared to saline treatment (Fig. 1A). Consistent with the decline in leukocyte trafficking to the kidneys, improved renal function (as suggested by BUN levels) was observed (Fig. 1B).

Nicotinic Agonists Regulate Systemic and Renal Inflammation During LPS-induced AKI

LPS produces renal damage by directly stimulating TNF α production within the kidney [6,11–13] and by increasing circulating TNF α levels [7]. Serum TNF α levels were enhanced during LPS-AKI and treatment with nicotinic agonists reduced serum TNF α levels (Fig. 2A). In addition, septic AKI was accompanied by significant kidney inflammation as measured by kidney TNF α , CCL2, and CXCL10 levels (Figs. 2B–D); renal concentrations of these pro-inflammatory mediators were blunted by treatment with nicotinic agonists (Figs. 2B–D).

Chemokine Production by Human Kidney Cells is Reduced by Nicotinic Agonists

To determine the potential cellular targets of nicotinic agonists within the kidney we examined the effect of nicotine and GTS-21 on both LPS- and TNF α -induced pro-inflammatory mediator production by several kidney cell populations, including human renal proximal tubular epithelial cells (HK-2 cells), human renal glomerular endothelial cells (HRGECs), and human mesangial cells (HMCs) (Fig. 3A–C). Stimulation with LPS induced significant production of CXCL8 by HK-2 cells, HRGECs, and HMCs, CXCL10 by HRGECs, and CCL2 by HRGECs and HMCs (Fig. 3). TNF α treatment induced significant production of both CXCL8 and CCL2 by HK-2 cells, HRGECs, and HMCs, and CXCL10 by HMCs (Fig. 3). Treatment of HK-2 cells with nicotinic agonists significantly reduced both LPS- and TNF α -induced CXCL8 and TNF α -induced CCL2 (Fig. 3A). We observed that treatment of HRGECs with either nicotine or GTS-21 (at optimal concentrations) significantly blocked LPS-induced CXCL8, CXCL10, and CCL2 production (Fig. 3B) and TNF α -induced CXCL8 and CCL2 production (Fig. 3B). Treatment of HMCs with nicotinic agonists significantly suppressed LPS-induced CXCL8 and CCL2 production (Fig. 3C) and TNF α -induced CXCL8, CXCL10, and CCL2 production (Fig. 3C). As expected, Bay 11, a cell-permeable inhibitor of I κ B α phosphorylation and hence, inhibitor of NF κ B activation, significantly reduced LPS- and TNF α -induced chemokine production by all three cell types (Fig. 3A–C). Treatment of HRGECs and HMCs with α bungarotoxin (α BGT), an α 7nAChR selective antagonist, prior to nicotinic agonists partly reversed their inhibitory effects on TNF- and LPS-induced CCL2 production (Fig. 3D).

Kidney Cells Express α 4, α 7, and β 2 nAChR Subunits

While nicotine is a non-specific nAChR agonist, GTS-21 is a partial selective agonist of the α 7nAChR but can also bind to α 4 β 2nAChRs. Using QPCR methods we detected the presence of α 7, α 4, and β 2 nAChR subunits in normal mouse kidneys. When expressed as a ratio of specific nAChR subunit mRNA:HPRT1 (housekeeping gene) mRNA, α 4, α 7, and β 2 nAChR subunit expressions were 2.292 ± 0.21 ; 1.628 ± 0.054 ; 1.334 ± 0.091 , respectively. We used the human kidney cells/cell lines to explore the expression of these nAChR subunits by various kidney cell types using flow cytometry methods. All three cell types expressed all three nAChR subunits, with α 7nAChR expression the highest for both HK-2 (proximal

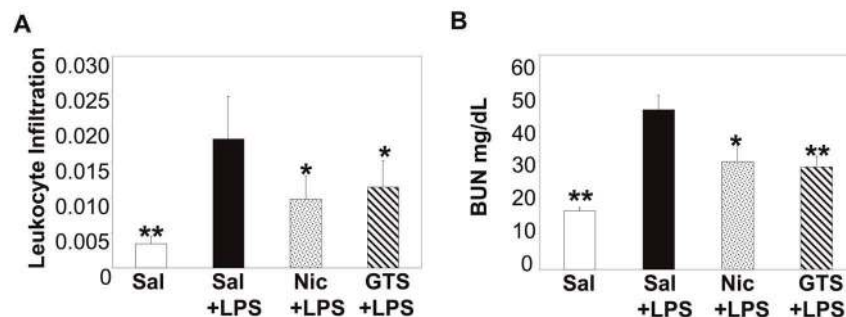


Figure 1. Nicotinic agonists protect against kidney damage during LPS-induced septic AKI. (A–B) Mice were treated with saline alone or LPS (5 mg/kg) plus either saline, nicotine (1 mg/kg), or GTS-21 (4 mg/kg, i.p.). (A) Renal leukocyte infiltration was determined by measuring kidney MPO levels by ELISA. (B) BUN levels (mg/dL) were assessed 24 hrs post LPS. Data are shown as mean \pm SD. * p < 0.05, ** p < 0.001 vs. Sal+LPS-treated mice. n = 5 for saline alone group and n = 8 for all other groups. doi:10.1371/journal.pone.0035361.g001

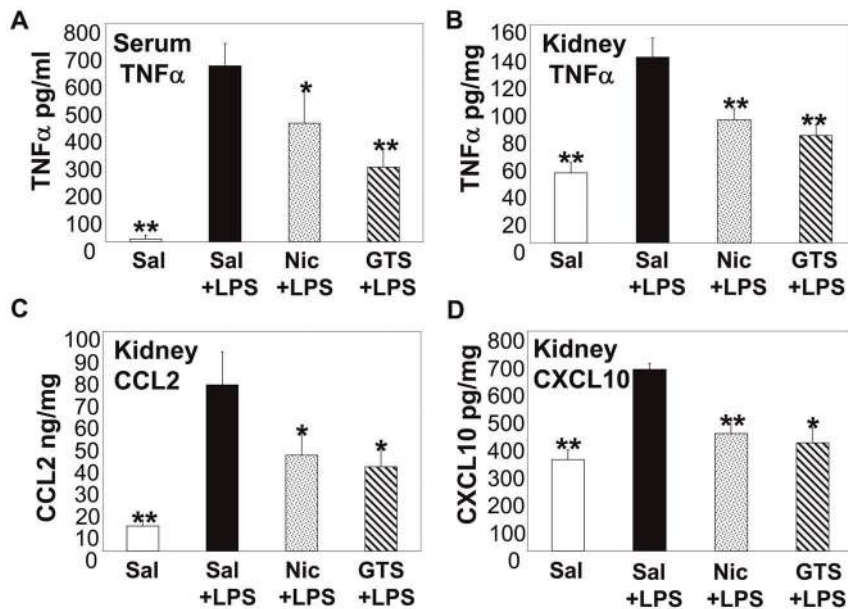


Figure 2. Nicotinic agonist administration reduces systemic and local renal inflammation during septic AKI. Mice were treated with saline alone (Sal, i.p.) or LPS (5 mg/kg, i.p.) plus either saline (i.p.), nicotine (Nic, 1 mg/kg, i.p.), or GTS-21 (GTS, 4 mg/kg, i.p.). Serum TNF α levels (A) and renal TNF α , CCL2 and CXCL10 levels (B–D) were determined by ELISA. Data are shown as mean \pm SD, as pg/ml (A) or corrected for protein concentration, pg/mg kidney tissue (B–D). * p <0.05, ** p <0.001 vs. Sal+LPS-treated mice. n =5 for saline alone group and n =8 for all other groups. doi:10.1371/journal.pone.0035361.g002

tubule epithelial cells) and HRGECs (renal glomerular endothelial cells) (Fig. 4).

Nicotinic Agonists Suppress LPS-induced AKI-associated NF κ B Activation

NF κ B activation within the kidney regulates the production of numerous pro-inflammatory mediators during septic AKI [25]. Next, we examined the effects of nicotinic agonist administration on I κ B α degradation within the kidney under normal conditions and during LPS-induced AKI. Nicotine treatment alone (in the absence of LPS) slightly enhanced renal I κ B α levels by 10 \pm 4.9% (mean \pm SD) (not significant); whereas treatment with GTS-21 alone significantly increased renal I κ B α expression by 33 \pm 8.9% (P <0.05) when compared to saline-treatment alone. LPS-induced AKI was accompanied by renal I κ B α degradation which was significantly reduced by treatment of mice with nicotinic agonists (Fig. 5A). Consistent with these observations, LPS treatment significantly enhanced NF κ B activation within the kidney which was attenuated by treating the mice with nicotinic agonists (Fig. 5B).

Proteasome Inhibition Suppresses Inflammatory Mediator Production by Human Kidney Cells

Because NF κ B-mediated inflammation is regulated via I κ B α degradation by the ubiquitin-proteasome system, we investigated the effect of a widely used proteasome inhibitor, MG132, on LPS- and TNF α -induced chemokine production by various human kidney cells. Renal epithelial cells, glomerular endothelial cells and mesangial cells were treated with either vehicle or MG132 (10 μ M) prior to LPS (1 μ g/ml) or TNF α (10 ng/ml) addition. After 18 hours, cell supernatants were assayed for CXCL8 and CCL2 (Fig. 6). Similar to nicotinic agonists and the NF κ B inhibitor, Bay11, MG132 treatment effectively inhibited LPS- and TNF α -stimulated chemokine production by all three human kidney cells (Fig. 6).

LPS-AKI is Accompanied by Enhanced Kidney Proteasome Activity which can be Reversed by Nicotinic Agonists

Nuclear NF κ B translocation leading to the production of inflammatory mediators depends, in part, on the degradation of phosphorylated I κ B α by the ubiquitin-proteasome system. Because LPS-AKI is characterized by renal NF κ B activation, we compared the proteasome activity of kidney tissues obtained from LPS-AKI mice vs. saline-treated (control) mice. LPS-AKI mice exhibited significantly enhanced renal proteasome activity compared to saline control mice (Fig. 7A). Using the septic AKI model, treatment with either nicotine or GTS-21 significantly attenuated both ATP-independent and ATP-dependent renal proteasome activity induced by LPS *in vivo* (Fig. 7A). Next, we examined the effect of exogenously added nicotinic agonists on renal proteasome activity *ex vivo* using renal homogenates. The addition of nicotine (10⁻⁶ M–10⁻⁴ M) or GTS-21 (10⁻⁷ M–10⁻⁵ M) significantly inhibited renal proteasome activity *ex vivo* in the absence (Fig. 7B) and presence of ATP (Fig. 7C), supporting a direct inhibitory effect of nicotinic agonists on renal proteasome activity.

Discussion

The prevalence of AKI during sepsis (septic-AKI) significantly increases during the progression of sepsis with 19%, 23% and 51% observed among patients with moderate sepsis, severe sepsis, and septic shock, respectively (Reviewed in [1]). Among hospitalized patients, the mortality in patients with AKI complicating severe sepsis is an abysmal 70% compared to 39% in patients with non-septic AKI [26]. Unfortunately, there is no treatment for AKI or septic-AKI other than supportive care and treatment of the underlying disorders. Because nicotinic agonists exert beneficial anti-inflammatory activity [18–22], we examined the effects of nicotinic agonist treatment during experimental LPS-induced septic AKI. We found that administration of nicotine or GTS-21

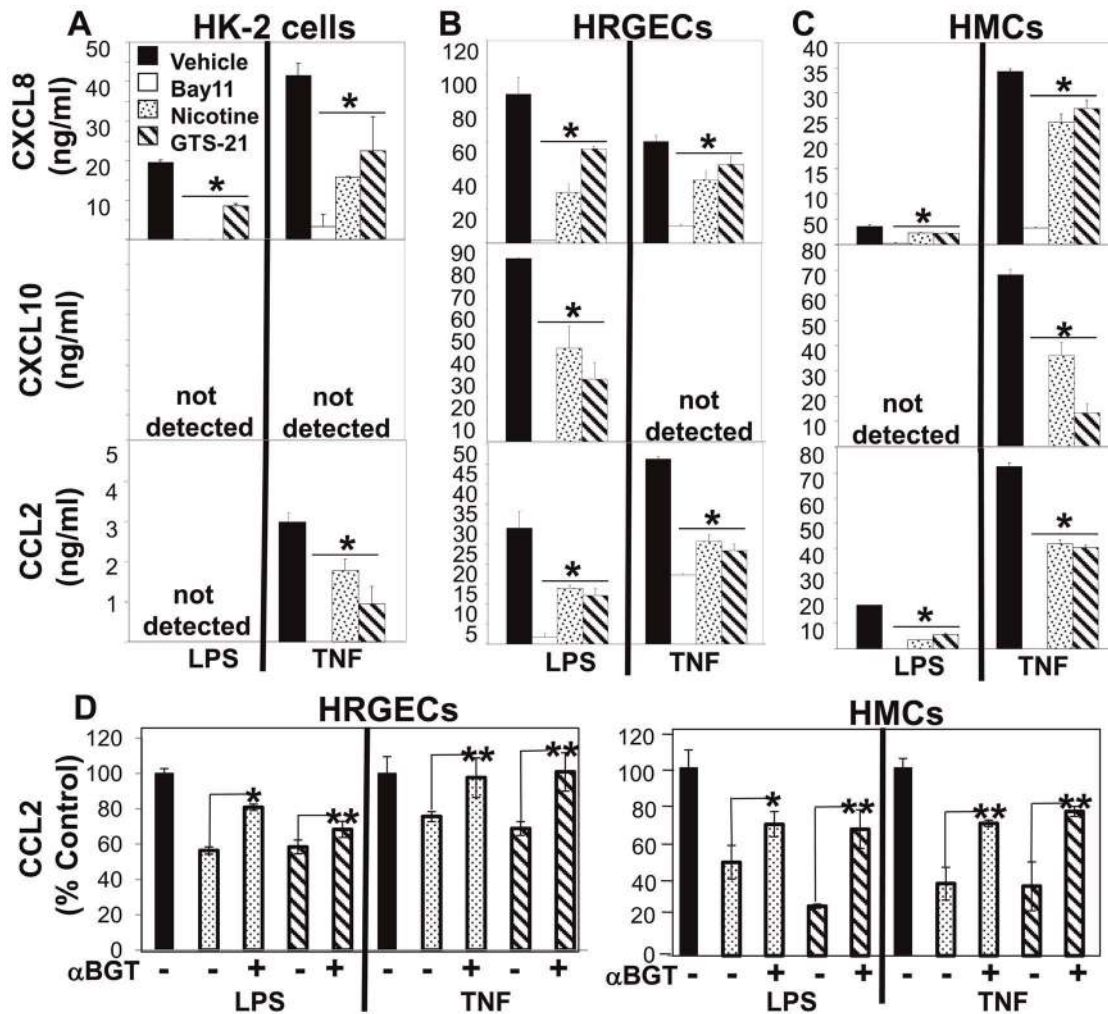


Figure 3. Nicotinic agonists suppress inflammatory responses by human kidney cell populations. (A) Human HK-2 renal tubular epithelial cells, (B) human renal glomerular endothelial cells (HRGECs), and (C) human mesangial cells (HMCs) were treated with either vehicle (solid bars), Bay 11, an NF κ B inhibitor (20 μ M, open bars), nicotine (10 μ M, speckled bars), or GTS-21 (1 μ M, hatched bars) and then either LPS (1 μ g/ml), or TNF α (20 ng/ml). Supernatants (n=3 wells per condition) were analyzed for CXCL8 (upper panel), CXCL10 (middle panel), and CCL2 (lower panel) by ELISA. Data are shown as cytokine levels (pg/ml) (mean \pm SEM) of 3 independent experiments. *p<0.05 vs. LPS- or TNF α -treated control (vehicle) cells. (D) HRGECs and HMCs were treated with saline or α BGT (2 μ M) prior to treatment with nicotine (10 μ M, speckled bars), or GTS-21 (1 μ M, hatched bars), and then either LPS (1 μ g/ml) or TNF α (20 ng/ml) (solid bars), as indicated. Supernatants (n=3 wells per condition) were analyzed for CCL2. Data are shown as % control with 100% saline+LPS or saline+TNF. *p<0.05 and **p<0.01 comparing \pm α BGT. doi:10.1371/journal.pone.0035361.g003

during LPS-induced AKI significantly reduced the infiltration of leukocytes into the kidneys and reduced BUN levels (suggestive of reduced kidney injury) (Fig. 1). Using the LPS-AKI model and various human kidney cells we explored the mechanisms by which nicotinic agonists attenuate septic AKI.

Both LPS and TNF α have been found in the glomeruli of rats following LPS administration [10,13]. LPS-induced AKI is characterized by renal damage mediated, in part, through localized renal TNF α production [6,14] and enhanced circulating TNF α [27]. TNF α is produced by isolated glomeruli following treatment with LPS [13], as well as several kidney cell types (e.g. mesangial and tubular epithelial cells) [11,12]. Local renal TNF α production promotes cytotoxic and inflammatory responses within the kidneys that trigger the generation of additional cytotoxic and chemotactic mediators. Like TNF α , both CCL2 and CXCL10 have been implicated in renal inflammation. Localized CCL2 production activates tubular epithelial cells to promote tubulointerstitial damage and recruits monocytes which further amplify

renal inflammation [28]. CXCL10 is chemotactic for T cells (which have been found to be important mediators of renal injury [29,30]) and promotes T cell-endothelial cell interactions [31]. Using the LPS-AKI model, we found that administration of nicotinic agonists blunted kidney inflammation (e.g. TNF α , CCL2, and CXCL10, Fig. 2). To explore which kidney cell types might be targets of nicotinic agonists, we treated human renal tubular epithelial, glomerular endothelial, and mesangial cells with nicotinic agonists during LPS or TNF α stimulation *ex vivo*. HK-2 renal epithelial cells, which retain the phenotypic expression and functional characteristics of human proximal tubules [32,33], produced significant amounts of CXCL8, but not CXCL10 or CCL2 following LPS stimulation; LPS-induced CXCL8 was significantly reduced by treatment with nicotinic agonists (Fig. 3A). Likewise, TNF α induced CXCL8 and low levels of CCL2 production by HK-2 cells which were significantly blocked by nicotinic agonists (Fig. 3A). We found that nicotinic agonists also significantly suppressed pro-inflammatory chemokine production

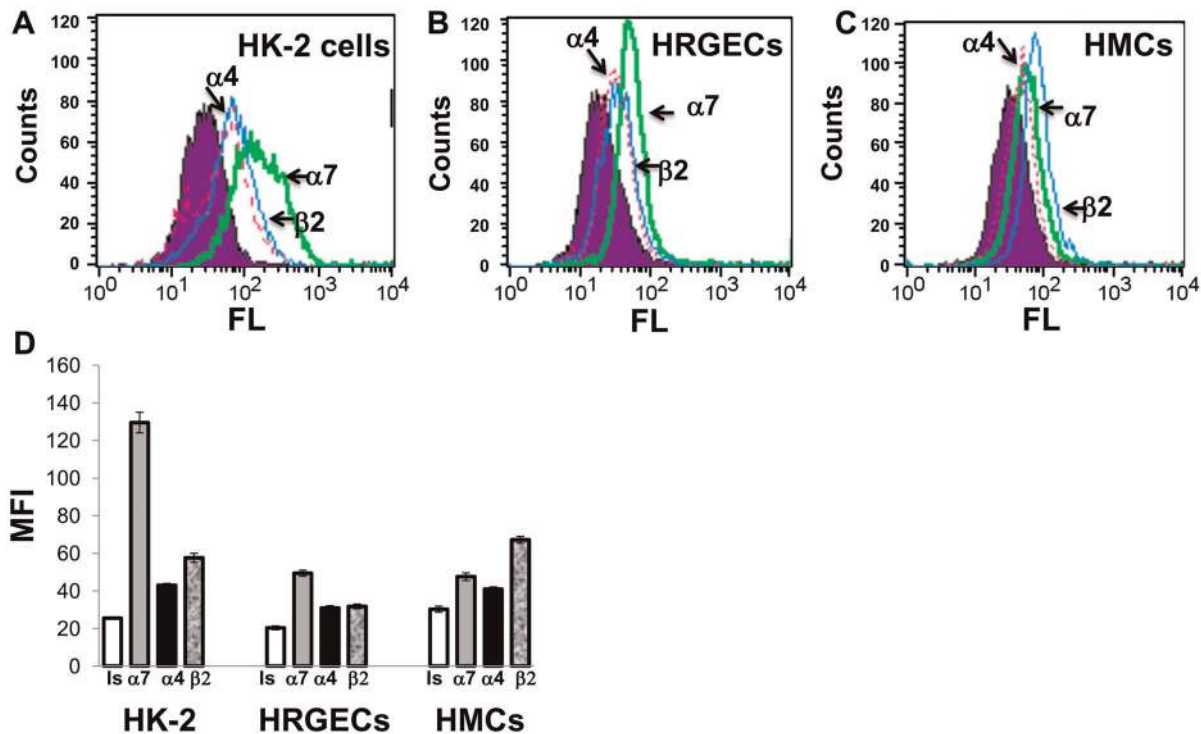


Figure 4. Human Kidney Cells Express nAChR subunits. To assess nAChR protein levels, (A) HK-2, (B) HRGECs, and (C) HMCs were analyzed by flow cytometry using specific antibodies against the $\alpha 7$ nAChR (green, thick solid line), $\alpha 4$ nAChR (pink, dotted line), and $\beta 2$ nAChR (blue, thin solid line) vs. isotype control (shaded plot). Histogram plots (FL-nAChR subunits vs. counts) are shown for each cell type. (D) Graph shows the average geometric mean MFI (\pm SD) for each cell type and each nAChR subunit (isotype control = Is; $\alpha 7$ nAChR = $\alpha 7$; $\alpha 4$ nAChR = $\alpha 4$; and $\beta 2$ nAChR = $\beta 2$). $n = 3$ samples per each condition and cell type. doi:10.1371/journal.pone.0035361.g004

by primary human renal glomerular endothelial cells following LPS or TNF α stimulation (Fig. 3B). Mesangial cells are localized around the blood vessels of the kidney at the mesangium where they function like smooth muscle cells to regulate capillary blood flow. Treatment of mesangial cells with nicotinic agonists significantly suppressed both LPS- and TNF α -induced inflammatory chemokine production (Fig. 3C). Similar to previous studies linking the inhibition of NF κ B activation to reduced leukocyte

infiltration during sepsis and endotoxic shock [34], we observed that both TNF α - and LPS-stimulated chemokine production by renal cells was blocked by the NF κ B inhibitor Bay 11 (Fig. 3).

NF κ B activation within the kidneys during septic AKI has been reported [35,36] and appears to play a critical role in the regulation of numerous pro-inflammatory mediators during infection and endotoxemia [25], as well as the promotion of tubular damage in LPS-induced AKI [36]. The I κ B α inhibitory

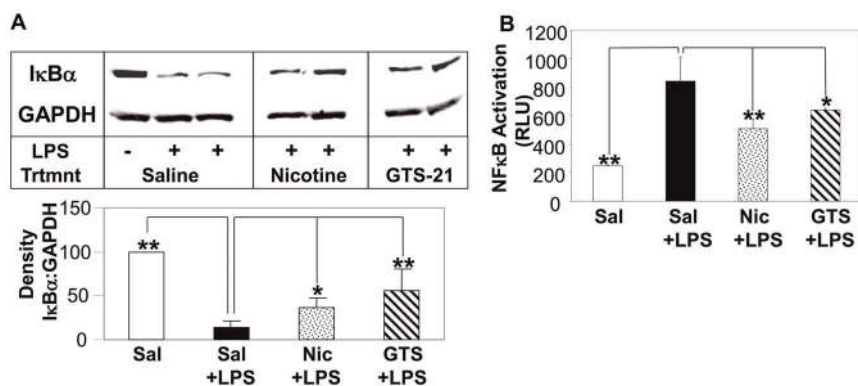


Figure 5. Nicotine and GTS-21 treatment inhibits I κ B α degradation and NF κ B activation within kidneys during septic AKI. Mice were treated with saline alone (i.p.) or LPS (5 mg/kg, i.p.) plus either saline, nicotine (1 mg/kg, i.p.), or GTS-21 (4 mg/kg, i.p.). Kidney tissues were analyzed for (A) I κ B α degradation and (B) NF κ B activation. (A) Cytoplasmic I κ B α and GAPDH levels were assessed by Western blotting (representative blot is shown) and the density of the bands were analyzed. The data are shown as the ratio of I κ B α :GAPDH densities. (B) NF κ B activation was determined as described in the Methods section. Data are shown as NF κ B activation (expressed as relative light units, RLU)(mean \pm SD). * $p < 0.05$, ** $p < 0.001$ vs. Sal+LPS. $n = 5$ for saline alone group and $n = 8$ for all other groups. doi:10.1371/journal.pone.0035361.g005

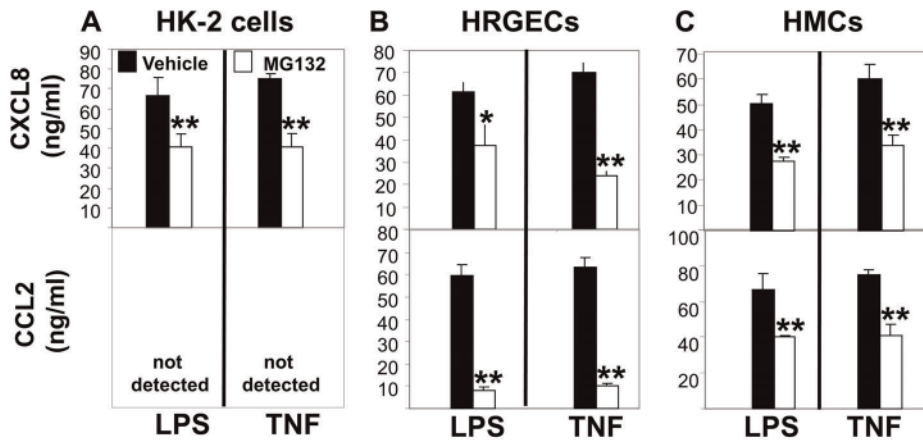


Figure 6. Proteasome inhibition blunts inflammatory responses by human kidney cell populations. (A) Human HK-2 renal tubular epithelial cells, (B) human renal glomerular endothelial cells (HRGECs), and (C) human mesangial cells (HMCs) were treated with either vehicle (filled bars) or proteasome inhibitor MG132 (10 μ M, open bars) and then either LPS (1 μ g/ml), or TNF α (20 ng/ml). Cell-free supernatants were analyzed for CXCL8 (upper panel) or CCL2 (lower panel) by ELISA 18 hrs later. Data are shown as cytokine levels (pg/ml) (mean \pm SEM) of 3 independent experiments. * p <0.05, ** p <0.001 vs. LPS- or TNF α -treated (vehicle) cells. doi:10.1371/journal.pone.0035361.g006

subunit of NF κ B is regulated by the ubiquitin-proteasome system [37], the major non-lysosomal mechanism responsible for degrading regulatory cellular proteins, as well as misfolded, damaged, and/or unnecessary proteins following ubiquitin tagging [38;39]. In the absence of LPS, there was a slight increase in renal I κ B α levels observed following the injection of nicotine alone and a significant increase in renal I κ B α levels following the injection of GTS-21 alone, suggesting that nicotinic agonists might either increase renal I κ B α expression or prevent its degradation. Nicotinic agonist administration reduced I κ B α degradation within the kidneys and significantly suppressed renal NF κ B activation *in vivo* (Fig. 5), supporting the concept that targeting the NF κ B pathway will protect against kidney damage associated with septic-AKI, as previously described [40,41]. While skeletal muscle biopsies obtained from hospitalized patients with sepsis and multiple organ failure exhibit increased proteasome activity compared to healthy controls [42], the assessment of kidney proteasome activity during septic AKI has not been reported. Treatment of several human kidney cells, including tubular epithelial cells, endothelial cells, and mesangial cells with a widely known proteasome inhibitor, MG132, suppressed both TNF α - or LPS-stimulated chemokine production (Fig. 6). In addition, we found that LPS-induced septic AKI was characterized by enhanced ATP-independent and ATP-dependent renal proteasome activity *in vivo* (Fig. 7A) and this activity was significantly reduced by nicotinic agonist administration *in vivo* (Fig. 7A). Likewise, renal proteasome activity (ATP-independent and ATP-dependent) was inhibited by exogenously added nicotine or GTS-21 (Fig. 7B–C). Thus, our data support the localized regulation of proteasome inhibition and inflammation by nicotinic agonists. These findings are consistent with previous findings revealing that nicotinic agonists suppressed local inflammation in the absence of an intact vagus nerve [18]. The inhibition of kidney proteasome activity *ex vivo* by nicotine and GTS-21 suggests that nicotinic agonists directly block proteasome activity. Based on the preservation of the I κ B α -NF κ B complex via proteasome inhibition and the subsequent blockade of NF κ B-mediated inflammation, proteasome inhibitors have emerged as potential therapies for treating several inflammatory conditions, including adjuvant-induced arthritis [43], acute pancreatitis [44], hypercholesterol-

emia-associated renal disease [45], and endotoxemia lethality [46,47].

Nicotinic acetylcholine receptors (nAChRs) are comprised of α 1– α 10, γ , δ , and ϵ subunits, and β 1–4 subunits. Outside of the neuromuscular junction nAChRs form heteropentameric receptors comprised of α and β subunits, and homomeric non- β -receptors comprised of either α 7 and α 9 subunits [48,49]. Previous studies by our group have described the constitutive expression of the α 2, α 3, α 5, α 7, α 9, α 10, β 1, β 2 and β 4 nAChR subunits in rat kidney [50]. Using immunohistochemical staining methods, we showed that the rat kidneys express the α 2, α 3, and α 7 nAChR subunits (with endothelial cells specifically expressing the α 7nAChR) [50]. While nicotine can bind to: α 2 β 2, α 4 β 2, α 3 β 2, α 4 β 4, α 2 β 4, α 3 β 4, and α 7 subunits, GTS-21, an α 7 selective partial agonist, binds α 7 and α 4 β 2 nAChRs. However, GTS-21 appears to only activate the α 7nAChRs [51]. In addition, GTS-21 has higher efficacy for rodent α 7nAChRs than human α 7nAChRs [52]. Based on our results, we evaluated the expression of the α 7, α 4, and β 2 subunits in mouse kidneys and human renal cells. Mouse kidneys express mRNA transcripts for the α 7, α 4, and β 2 nAChR subunits. In addition, we examined the expression of nAChR by the human kidney cells used in this study. While all three cell types expressed the α 7, α 4, and β 2 nAChR subunits, both HK-2 cells (human proximal tubular epithelial cell line) and HRGECs expressed the highest level of α 7nAChR (Fig. 4). From these results, we demonstrate the expression of the α 7nAChR (a homopentameric receptor), but it is difficult to confirm expression of the α 4 β 2nAChR by these cells because the α 4 and β 2 subunits can form heteropentameric nAChRs with various β subunits and α subunits, respectively. Treatment of HRGECs and HMCs with α BGT, an α 7nAChR selective antagonist, prior to treatment with nicotinic agonists significantly blocked their inhibitory effects on TNF- and LPS-induced CCL2 production (Fig. 3D), supporting the role of α 7nAChRs in mediating the anti-inflammatory effects of nicotine and GTS-21.

Proteasomes are very large protein complexes composed of a core of stacked rings (20 S proteasome) flanked by regulatory particles that facilitate protein entry into the 'degradation chamber' [53]. The complexity of proteasomes implies potentially multiple mechanisms for their functional regulation. Drews et al demonstrated that the regulation of cardiac proteasomes involved

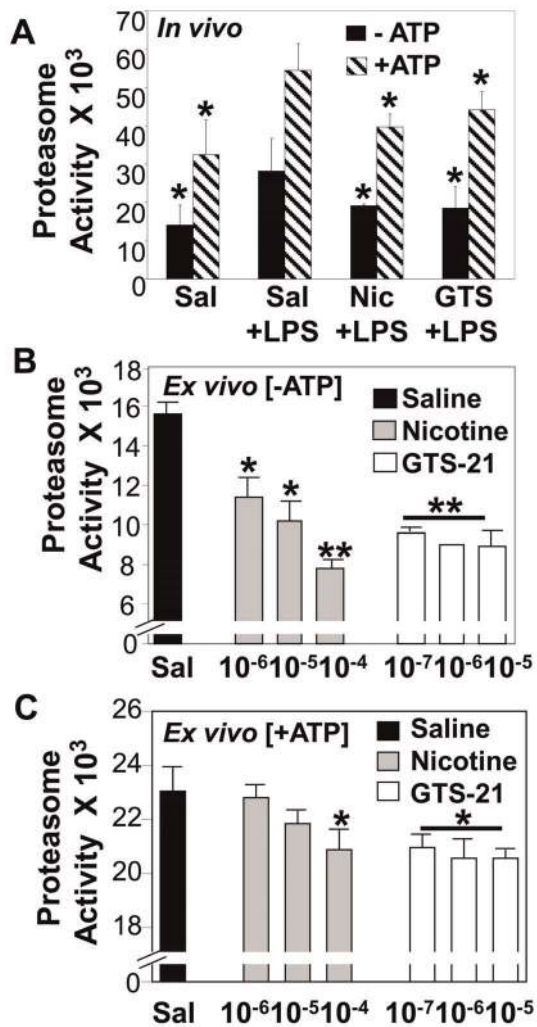


Figure 7. Septic AKI is characterized by dysregulated renal proteasome activity which is attenuated by nicotinic agonists. (A) Nicotinic agonist treatments vs. saline+LPS-treatment *in vivo*: Using the LPS-AKI model, mice were treated with saline, nicotine (1 mg/kg, i.p.) or GTS-21 (4 mg/kg, i.p.) (n=5 per group); 1 hr post saline or LPS treatment, the effect of saline vs. nicotinic agonist treatment on LPS-induced renal proteasome activity (\pm ATP, 38 μ M) was determined. * p <0.05 vs. Sal+LPS. (B-C) Nicotinic agonist treatments vs. saline treatment *ex vivo*: The effects of nicotine and GTS-21 (at the indicated molar concentrations) vs. saline treatment on proteasome activity in the absence of exogenously added ATP (B) and in the presence of exogenously added ATP (C) were assessed *ex vivo* using mouse kidney homogenates. Each experiment was repeated and the data are shown as proteasome activity (mean \pm SD). * p <0.05 and ** p <0.01 vs. Sal (vehicle control). doi:10.1371/journal.pone.0035361.g007

in left ventricular hypertrophy is linked to the desensitization of the beta adrenergic pathway [54]. Interestingly, previous studies support the negative regulation of proteolysis by the cholinergic neurotransmitter acetylcholine. Blockade of acetylcholine release increased protein degradation by proteasomes in *C. elegans* while stimulation of post-synaptic nAChRs via acetylcholine suppressed proteolysis [55]. Similarly, nicotine-mediated proteasome inhibition in the brain (*in vivo* and *ex vivo*) has been reported. Rezvani and co-workers found that nicotine treatment reduced proteasome activity (by directly interacting with the 20 S proteasome), thereby affecting synaptic plasticity [56]. Together, these data support the

hypothesis that neural cholinergic inputs (e.g. acetylcholine) and nicotinic agonists regulate proteasome activity by interacting (either directly or indirectly) with one or more components of the proteasome complex. Based on our data supporting the role of α 7nAChRs, we postulate that increased intracellular cAMP levels following α 7nAChR ligation [57] augments I κ B α levels to effectively reduce NF κ B activation [58–60]. Consistent with this concept, our data show that in the kidney, nicotinic agonists increase the cytosolic availability I κ B α protein and attenuate NF κ B activation by inhibiting proteasomal degradation of I κ B α . Future studies will investigate the potential mechanism(s) whereby nicotinic agonists and acetylcholine produced by electrical stimulation of the vagus nerve lead to the inhibition of proteasome activity.

In summary, previous studies highlight the broad-based therapeutic targeting of inflammation using nicotinic agonists via the regulation of NF κ B activation [18,21] (and Fig. 5) and STAT3 activation [50,61]. Herein we demonstrate the suppression of LPS- and TNF α -induced inflammatory mediator production by resident kidney cells by nicotinic agonists, enhanced renal proteasome activity during LPS-AKI, and the attenuation of renal injury in LPS-induced AKI following treatment with nicotinic agonists (which suppressed both ATP-dependent and ATP-independent proteasome activity). Together, these studies support targeting multiple pathways of inflammation and proteasome activity using nicotinic agonists to protect against renal inflammation and injury observed during septic AKI.

Materials and Methods

Antibodies and Reagents

Anti-I κ B α and GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA). The α 7nAChR (319) and α 4nAChR (286) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the β 2nAChR (270) monoclonal antibody was purchased from Covance (Princeton, NJ). LPS (*E. coli* 011:B5), nicotine, and ATP were purchased from Sigma-Aldrich (St. Louis, MO). α Bungarotoxin (α BGT) was purchased from Tocris (Minneapolis, MN) and Bay 11-7082 (Bay 11), an NF κ B inhibitor, and MG132, a proteasome inhibitor, were purchased from Calbiochem (San Diego, CA). GTS-21 was provided by Dr. Y Al-Abed (The Feinstein Institute); GTS-21 was synthesized as described previously by a reaction of anabaseine dihydrochloride with 2,4-dimethoxybenzaldehyde according to [62] and confirmed using NMR and LC-MS analyses. Suc-LLVY-AMC was purchased from Enzo Life Science (Farmingdale, NY). Lactacystin was purchased from Boston Biochemical (Cambridge, MA).

Cell cultures. HK-2 (human renal proximal epithelial tubule) cells, purchased from ATCC (Manassas, VA), were grown in DMEM 10%FBS, penicillin, streptomycin, and glutamine. Normal human mesangial cells (HMCs) (Sciencell, Carlsbad, CA) and human renal glomerular endothelial cells (HRGECs) (Sciencell) were grown according to the manufacturers' recommendations. For activation assays, cells were grown to confluency in 96-well plates and treated with vehicle or nicotinic agonists (used at optimal doses determined from our previous studies [61] or NF κ B inhibitor Bay 11 (25 μ M or MG132 (10 μ M) for 30–45 min prior to stimulation with either LPS (1 μ g/ml) or TNF α (20 ng/ml) (n = 3 per condition). After a 20 hr incubation period, cell-free supernatants were collected and assayed for CXCL8, CCL2, and/or CXCL10 levels by ELISA (R&D Systems). Each experiment was repeated twice and the combined data are shown as mean (\pm SEM). In a separate set of

experiments, HRGECs and HMCs were incubated with α BGT (2 μ M) for 30 min before the addition of either nicotine (10 μ M, speckled bars), or GTS-21 (1 μ M, hatched bars), followed by stimulation with either LPS (1 μ g/ml, solid bar) or TNF α (20 ng/ml, solid bar) (n = 3 per condition). After an overnight stimulation, cell-free supernatants were analyzed for CCL2, as described above. Each experiment was repeated twice and the combined data are shown as mean %control (\pm SEM), with 100% being saline+LPS or saline+TNF. For the assessment of nAChR subunit expression, flow cytometry methods were used to detect the expression of specific nAChR subunits, as previously described [63]. Cultured cells were harvested using a non-enzymatic cell dissociation buffer (Cellstripper, Cellgro, Manassas, VA); all samples were blocked with 10% serum and then incubated with primary antibodies specific for α 7nAChR (319), α 4nAChR (286), and β 2nAChR (270) subunits, according to the manufacturers' directions. Antibody binding was revealed using biotinylated anti-rat IgM+IgG (SouthernBiotech, Birmingham, AL), followed by avidin-FITC (BD Biosciences). Rat Ig specific isotype controls (BD Biosciences) were used as negative controls and live fluorescent cells (without debris or aggregates) were analyzed on a FACSCalibur flow cytometer (BD). Data were analyzed using CellQuest data. Data are shown for each cell type as raw mean fluorescence intensity (FL) vs. counts and as the geometric mean fluorescence intensity or MFI (\pm SD) for each subunit for n = 3 samples.

Animals

Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research prior to experimentation (IACUC#2005-049). All animals were housed, maintained, and manipulated according to the guidelines described in the National Research Council's Guide for the Care and Use of Laboratory Animals.

Animal model of LPS-induced AKI. Swiss Webster mice (female, ages 8–10 wks, Taconic Farms, n = 5–8 per group) were injected with LPS (5 mg/kg, i.p.) immediately after saline (200 μ l, i.p.), nicotine (1 mg/kg, i.p.), or GTS-21 (4 mg/kg, i.p.). These doses were based on our previously published studies [22]. Mice not euthanized prior to 6 hrs were treated with either additional saline (200 μ l, i.p.), nicotine (1 mg/kg, i.p.), or GTS-21 (4 mg/kg, i.p.) at 6 and 18 hrs post LPS. Mice were euthanized by CO₂ asphyxiation at indicated time points following the injection of LPS. Blood was collected immediately by cardiac puncture and serum was isolated and assessed for blood urea nitrogen (BUN) levels (measured by the Core Laboratories of the North Shore-LIJ Health System) or stored at -80° C. Cortical kidney samples were collected and either immediately flash-frozen in liquid nitrogen (cytokine and myeloperoxidase (MPO) assays) or immediately processed for the isolation of nuclear and cytoplasmic fractions for NF κ B activation studies (see below).

Assessment of Systemic and Renal Inflammation During LPS-induced AKI

Mouse serum TNF α levels were assessed at 2 hr post LPS injection and kidney cytokine/chemokine levels were assessed at 3 hrs post LPS. Frozen cortical renal tissue specimens (200 μ g) were homogenized in 200 μ l lysis buffer (Tris buffered saline pH 7.3 containing 0.5% TritonX-100 and protease inhibitor cocktail) on ice using a Dounce homogenizer; supernatants were collected after centrifugation. Serum and homogenates (n = 5–8 mice per group) were assayed for cytokines and chemokines by ELISA and protein levels by BioRad protein assay (assays

performed in triplicate). Data are expressed as mean concentrations adjusted for protein levels.

Analysis of Renal Leukocyte Infiltration During LPS-induced AKI

Mice were euthanized 3 hr post saline or LPS (in the presence or absence of nicotinic agonists) for assessment of leukocyte influx (n = 5–8 per group). Frozen cortical kidney specimens were assessed for leukocyte infiltration using an ELISA assay for mouse myeloperoxidase (MPO), according to the manufacturer's directions (Cell Sciences, Canton, MA). Briefly, kidney samples (20 mg) were homogenized on ice in 200 μ l lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.4, 10% glycerin, and protease inhibitor cocktail). After two centrifugation steps, lysates (1:4 dilution) were assessed for MPO levels by ELISA, using a standard curve ranging from 0–500 ng/ml.

I κ B α Assessment and NF κ B Activation Studies

Nuclear and cytoplasmic extracts were isolated from fresh cortical kidney specimens (n = 5–8 mice per group) collected 3 hrs post saline or LPS treatment (in the presence and absence of nicotinic agonist administration) using a nuclear extraction kit (Active Motif, Carlsbad, CA). Nuclear extracts were analyzed for NF κ B activation using the TransAM NF κ B p65/NF κ B p50 Chemi Transcription Factor Assay Kit (Active Motif), according to the manufacturer's instructions. I κ B α protein levels (and GAPDH levels) in the cytoplasmic extracts were determined by Western blotting using specific anti-I κ B α and GAPDH antibodies (a representative blot is shown). The density of the bands was determined using the NIH image program and the ratios of I κ B α :GAPDH densities are shown.

Assessment of nAChR mRNA Expression by Mouse Kidney Tissue Using QPCR

RNA was isolated from 100 mg of normal mouse kidney tissue using the RNeasy[®] Mini kit (Qiagen, Valencia CA) with DNase treatment, according to the manufacturer's directions. The purity/concentration of total RNA was assayed using the NanoDrop spectrophotometer (Wilmington, DE). QPCR was used to assess specific nAChR mRNA expression using the following primers (and Roche Universal library probes): α 7nAChR: forward: 5'-ccctggcttgcgtgatt-3', reverse: 5'-gcatgaagacagtcagagaagtaa-3' (probe 27); α 4nAChR: forward: 5'-cgtccagtcattcagacc-3', reverse: 5'-atggccagctattccagtc-3' (probe 29); β 2 nAChR: forward: 5'-actctatggcgtgctgcttc-3', reverse: 5'-tcctctgtgctcagcccaaaa-3' (probe 76); HPRT1 forward: 5'-tcctctcagaccgctttt-3', reverse: 5'-cctgttcacatcgctaac-3' (probe 95). Briefly, PCR reactions were performed in quadruplicate using the Eurogentec One step RT qPCR mastermix, 100 ng RNA and the Roche 480 Light Cycler using the following conditions: 48 $^{\circ}$ C for 30 min, 95 $^{\circ}$ C for 10 min followed by 45 cycles of 95 $^{\circ}$ C for 15 secs and 60 $^{\circ}$ C for 1 min. Relative gene expression was calculated using the comparative Ct ($\Delta\Delta$ Ct) method as target gene to HPRT1 (housekeeping gene) for normalizing transcript levels [64].

Renal Proteasome Activity Assays

The effect of nicotinic agonists on kidney proteasome activity was determined using the Suc-LLVY-AMC (chymotrypsin-like) substrate in the absence and presence of exogenously added ATP, as previously described [65;66]. For *in vivo* proteasome inhibition assays, mice (5 per group) were treated with saline, GTS-21 (4 mg/kg, i.p.) or nicotine (1 mg/kg, i.p.). Cortical kidney sections were isolated from mice, flash-frozen, and stored at -80° C; frozen

kidneys were homogenized in freshly prepared assay buffer (50 mM HEPES, pH 7.5, 20 mM KCl, 5 mM MgCl₂, 1 mM DTT) and used immediately. Briefly, 5 µg kidney homogenates (per well, with each sample run in triplicate) was incubated with vehicle or lactacystin (15 µM) for 20 min at RT prior to addition of assay buffer containing either 0 or 38 µM ATP. The reaction was initiated with the addition of substrate (Suc-LLVY-AMC) and incubated for 30 min at 37°C. This concentration of ATP (38 µM) was chosen because it produced maximal stimulation of proteasome activity using mouse renal homogenates (5 µg). The assay was terminated by the addition of 150 µl cold ethanol and read in a VictorX3 fluorescence plate reader (Perkin Elmer, Ex 360/Ex440 nm). Proteasome activity (chymotrypsin-like activity in fluorescence units) produced (\pm exogenously added ATP) was derived by subtracting the level of fluorescence obtained within 30 min in the presence of lactacystin from the values obtained without lactacystin after normalization. For the *ex vivo* assays, cortical kidney homogenates (5 µg) were incubated with nicotinic agonists (as indicated) or lactacystin (15 µM) for 20 min at RT prior to the addition of ATP (or no ATP) and substrate, as described above. The data from three separate experiments were

averaged and results are shown as the mean proteasome activity (\pm SD).

Statistics

Data are expressed as mean \pm SD (or \pm SEM), as indicated. For data with multiple comparisons, data were analyzed by one way ANOVA followed by the Dunnett's test (to compare each treatment to control value). When only two samples were compared, Student's t tests were used for single comparisons between treatment and control. P values <0.05 were considered significant.

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Author Contributions

Conceived and designed the experiments: CNM PKC MY SRP YAA. Performed the experiments: OD XX PKC. Analyzed the data: CNM PKC SR. Contributed reagents/materials/analysis tools: YAA. Wrote the paper: CNM PKC MY.

References

- Schrier RW, Wang W (2004) Acute renal failure and sepsis. *N Engl J Med* 351: 159–169.
- Zarjou A, Agarwal A (2011) Sepsis and acute kidney injury. *J Am Soc Nephrol* 22: 999–1006.
- Schrier RW, Wang W, Poole B, Mitra A (2004) Acute renal failure: definitions, diagnosis, pathogenesis, and therapy. *J Clin Invest* 114: 5–14.
- Ricci Z, Ronco C (2009) Pathogenesis of acute kidney injury during sepsis. *Curr Drug Targets* 10: 1179–1183.
- Wan L, Bagshaw SM, Langenberg C, Saotome T, May C, et al. (2008) Pathophysiology of septic acute kidney injury: what do we really know? *Crit Care Med* 36: S198–S203.
- Cunningham PN, Dyanov HM, Park P, Wang J, Newell KA, et al. (2002) Acute renal failure in endotoxemia is caused by TNF acting directly on TNF receptor-1 in kidney. *J Immunol* 168: 5817–5823.
- Cunningham PN, Wang Y, Guo R, He G, Quigg RJ (2004) Role of Toll-like receptor 4 in endotoxin-induced acute renal failure. *J Immunol* 172: 2629–2635.
- Copeland S, Warren HS, Lowry SF, Calvano SE, Remick D (2005) Acute inflammatory response to endotoxin in mice and humans. *Clin Diagn Lab Immunol* 12: 60–67.
- Remick DG, Ward PA (2005) Evaluation of endotoxin models for the study of sepsis. *Shock* 24 Suppl 1: 7–11.
- Kang YH, Falk MC, Bendley TB, Lee CH (1995) Distribution and role of lipopolysaccharide in the pathogenesis of acute renal proximal tubule injury. *Shock* 4: 441–449.
- Jevnikar AM, Brennan DC, Singer GG, Heng JE, Maslinski W, et al. (1991) Stimulated kidney tubular epithelial cells express membrane associated and secreted TNF alpha. *Kidney Int* 40: 203–211.
- Kita T, Tanaka N, Nagano T (1993) The immunocytochemical localization of tumour necrosis factor and leukotriene in the rat kidney after treatment with lipopolysaccharide. *Int J Exp Pathol* 74: 471–479.
- Fouqueray B, Philippe C, Herbelin A, Perez J, Ardaillou R, et al. (1993) Cytokine formation within rat glomeruli during experimental endotoxemia. *J Am Soc Nephrol* 3: 1783–1791.
- Knotek M, Rogachev B, Wang W, Eceder T, Melnikov V, et al. (2001) Endotoxemic renal failure in mice: Role of tumor necrosis factor independent of inducible nitric oxide synthase. *Kidney Int* 59: 2243–2249.
- Bertani T, Abbate M, Zoja C, Corna D, Perico N, et al. (1989) Tumor necrosis factor induces glomerular damage in the rabbit. *Am J Pathol* 134: 419–430.
- Gupta A, Rhodes GJ, Berg DT, Gerlitz B, Molitoris BA, et al. (2007) Activated protein C ameliorates LPS-induced acute kidney injury and downregulates renal iNOS and angiotensin 2. *Am J Physiol Renal Physiol* 293: F245–F254.
- Tracey KJ (2007) Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest* 117: 289–296.
- Saced RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhanch D, et al. (2005) Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. *J Exp Med* 201: 1113–1123.
- de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, et al. (2005) Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol* 6: 844–851.
- van Westerloo DJ, Giebelen IA, Florquin S, Bruno MJ, Larosa GJ, et al. (2006) The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. *Gastroenterology* 130: 1822–1830.
- Pavlov VA, Ochani M, Yang LH, Gallowitsch-Puerta M, Ochani K, et al. (2007) Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. *Crit Care Med* 35: 1139–1144.
- Huston JM, Rosas-Ballina M, Xue X, Dowling O, Ochani K, et al. (2009) Cholinergic neural signals to the spleen down-regulate leukocyte trafficking via CD11b. *J Immunol* 183: 552–559.
- Yeboah MM, Xue X, Duan B, Ochani M, Tracey KJ, et al. (2008) Cholinergic agonists attenuate renal ischemia-reperfusion injury in rats. *Kidney Int* 74: 62–69.
- Sadis C, Teske G, Stokman G, Kubjak C, Claessen N, et al. (2007) Nicotine protects kidney from renal ischemia/reperfusion injury through the cholinergic anti-inflammatory pathway. *PLoS ONE* 2: e469.
- Schor N (2002) Acute renal failure and the sepsis syndrome. *Kidney Int* 61: 764–776.
- Hoste EA, Lameire NH, Vanholder RC, Benoit DD, Decruyenaere JM, et al. (2003) Acute renal failure in patients with sepsis in a surgical ICU: predictive factors, incidence, comorbidity, and outcome. *J Am Soc Nephrol* 14: 1022–1030.
- De Vriese AS (2003) Prevention and treatment of acute renal failure in sepsis. *J Am Soc Nephrol* 14: 792–805.
- Viedt C, Orth SR (2002) Monocyte chemoattractant protein-1 (MCP-1) in the kidney: does it more than simply attract monocytes? *Nephrol Dial Transplant* 17: 2043–2047.
- Monteiro RM, Camara NO, Rodrigues MM, Tzelepis F, Damiao MJ, et al. (2009) A role for regulatory T cells in renal acute kidney injury. *Transpl Immunol* 21: 50–55.
- Liu M, Chien CC, Grigoryev DN, Gandolfo MT, Colvin RB, et al. (2009) Effect of T cells on vascular permeability in early ischemic acute kidney injury in mice. *Microvasc Res* 77: 340–347.
- Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, et al. (1993) Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J Exp Med* 177: 1809–1814.
- Racusen LC, Monteil C, Sgrignoli A, Lucskay M, Marouillat S, et al. (1997) Cell lines with extended in vitro growth potential from human renal proximal tubule: characterization, response to inducers, and comparison with established cell lines. *J Lab Clin Med* 129: 318–329.
- Ryan MJ, Johnson G, Kirk J, Fuerstenberg SM, Zager RA, et al. (1994) HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int* 45: 48–57.
- Sheehan M, Wong HR, Hake PW, Malhotra V, O'Connor M, et al. (2002) Parthenolide, an inhibitor of the nuclear factor-kappaB pathway, ameliorates cardiovascular derangement and outcome in endotoxic shock in rodents. *Mol Pharmacol* 61: 953–963.
- Pocock J, Gomez-Guerrero C, Harendza S, Ayoub M, Hernandez-Vargas P, et al. (2003) Differential activation of NF-kappaB, AP-1, and C/EBP in endotoxin-tolerant rats: mechanisms for in vivo regulation of glomerular RANTES/CCL5 expression. *J Immunol* 170: 6280–6291.
- Meyer-Schwesinger C, Dehde S, von RC, Gatzemeier S, Klug P, et al. (2009) Rho kinase inhibition attenuates LPS-induced renal failure in mice in part by

- attenuation of NF-kappaB p65 signaling. *Am J Physiol Renal Physiol* 296: F1088–F1099.
37. Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, et al. (1995) Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev* 9: 1586–1597.
 38. Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* 67: 425–479.
 39. Hochstrasser M (1996) Protein degradation or regulation: Ub the judge. *Cell* 84: 813–815.
 40. Leelahavanichkul A, Yasuda H, Doi K, Hu X, Zhou H, et al. (2008) Methyl-2-acetamidoacrylate, an ethyl pyruvate analog, decreases sepsis-induced acute kidney injury in mice. *Am J Physiol Renal Physiol* 295: F1825–F1835.
 41. Lutz J, Luong IA, Strobl M, Deng M, Huang H, et al. (2008) The A20 gene protects kidneys from ischaemia/reperfusion injury by suppressing pro-inflammatory activation. *J Mol Med* 86: 1329–1339.
 42. Klaude M, Fredriksson K, Tjader I, Hammarqvist F, Ahlman B, et al. (2007) Proteasome proteolytic activity in skeletal muscle is increased in patients with sepsis. *Clin Sci (Lond)* 112: 499–506.
 43. Ahmed AS, Li J, Ahmed M, Hua L, Yakovleva T, Ossipov MH, et al. (2010) Attenuation of pain and inflammation in adjuvant-induced arthritis by the proteasome inhibitor MG132. *Arthritis Rheum* 62: 2160–2169.
 44. Letoha T, Feher LZ, Pecze L, Somlai C, Varga I, et al. (2007) Therapeutic proteasome inhibition in experimental acute pancreatitis. *World J Gastroenterol* 13: 4452–4457.
 45. Chade AR, Herrmann J, Zhu X, Krier JD, Lerman A, et al. (2005) Effects of proteasome inhibition on the kidney in experimental hypercholesterolemia. *J Am Soc Nephrol* 16: 1005–1012.
 46. Qureshi N, Perera PY, Shen J, Zhang G, Lensch A, et al. (2003) The proteasome as a lipopolysaccharide-binding protein in macrophages: differential effects of proteasome inhibition on lipopolysaccharide-induced signaling events. *J Immunol* 171: 1515–1525.
 47. Qureshi N, Vogel SN, Van WC, III, Papsian CJ, Qureshi AA, et al. (2005) The proteasome: a central regulator of inflammation and macrophage function. *Immunol Res* 31: 243–260.
 48. Lindstrom J (1997) Nicotinic acetylcholine receptors in health and disease. *Mol Neurobiol* 15: 193–222.
 49. D'hoedt D, Bertrand D (2009) Nicotinic acetylcholine receptors: an overview on drug discovery. *Expert Opin Ther Targets* 13: 395–411.
 50. Yeboah MM, Xue X, Javdan M, Susin M, Metz CN (2008) Nicotinic acetylcholine receptor expression and regulation in the rat kidney after ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 295: F654–F661.
 51. Briggs CA, McKenna DG (1998) Activation and inhibition of the human alpha7 nicotinic acetylcholine receptor by agonists. *Neuropharmacology* 37: 1095–1102.
 52. Papke RL, Porter Papke JK (2002) Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. *Br J Pharmacol* 137: 49–61.
 53. Lecker SH, Mitch WE (2011) Proteolysis by the ubiquitin-proteasome system and kidney disease. *J Am Soc Nephrol* 22: 821–824.
 54. Drews O, Tsukamoto O, Liem D, Streicher J, Wang Y, et al. (2010) Differential regulation of proteasome function in isoproterenol-induced cardiac hypertrophy. *Circ Res* 107: 1094–1101.
 55. Szewczyk NJ, Hartman JJ, Barmada SJ, Jacobson LA (2000) Genetic defects in acetylcholine signalling promote protein degradation in muscle cells of *Caenorhabditis elegans*. *J Cell Sci* 113 (Pt 11): 2003–2010.
 56. Rezvani K, Teng Y, Shim D, De BM (2007) Nicotine regulates multiple synaptic proteins by inhibiting proteasomal activity. *J Neurosci* 27: 10508–10519.
 57. Andersson U, Tracey KJ (2011) Reflex Principles of Immunological Homeostasis. *Annu Rev Immunol*: Mar 24. [Epub ahead of print].
 58. Mustafa SB, Olson MS (1998) Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP. *J Biol Chem* 273: 5073–5080.
 59. Farmer P, Pugin J (2000) beta-adrenergic agonists exert their “anti-inflammatory” effects in monocytic cells through the IkappaB/NF-kappaB pathway. *Am J Physiol Lung Cell Mol Physiol* 279: L675–L682.
 60. Kamthong PJ, Wu M (2001) Inhibitor of nuclear factor-kappaB induction by cAMP antagonizes interleukin-1-induced human macrophage-colony-stimulating-factor expression. *Biochem J* 356: 525–530.
 61. Chatterjee PK, Al-Abed Y, Sherry B, Metz CN (2009) Cholinergic agonists regulate JAK2/STAT3 signaling to suppress endothelial cell activation. *Am J Physiol Cell Physiol* 297: C1294–C1306.
 62. Zoltewicz JA, Prokai-Tatrai K, Bloom L, Kem W (1993) Long range transmission of polar effects of cholinergic 3-arylideneanabases. Conformations calculated by molecular modelling. *Heterocycles* 35: 171–179.
 63. Su X, Matthay MA, Malik AB (2010) Requisite role of the cholinergic alpha7 nicotinic acetylcholine receptor pathway in suppressing Gram-negative sepsis-induced acute lung inflammatory injury. *J Immunol* 184: 401–410.
 64. Cikos S, Bukovska A, Koppel J (2007) Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. *BMC Mol Biol* 8: 113.
 65. Powell SR, Davies KJ, Divald A (2007) Optimal determination of heart tissue 26S-proteasome activity requires maximal stimulating ATP concentrations. *J Mol Cell Cardiol* 42: 265–269.
 66. Reinheckel T, Sitte N, Ullrich O, Kuckelkorn U, Davies KJ, et al. (1998) Comparative resistance of the 20 S and 26 S proteasome to oxidative stress. *Biochem J* 335 (Pt 3): 637–642.