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Original article

Nicotine reverses the enhanced renal vasodilator capacity in endotoxic rats: Role of α7/α4β2 nAChRs and HSP70

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ABSTRACT

Background: Nicotine alleviates renal inflammation and injury induced by endotoxemia. This study investigated (i) the nicotine modulation of hemodynamic and renal vasodilatory responses to endotoxemia in rats, and (ii) roles of α7 or α4β2-nAChRs and related HSP70/TNFα/iNOS signaling in the interaction.
Methods: Endotoxemia was induced by *ip* lipopolysaccharide (5 mg/kg/day, for 2 days) and changes in systolic blood pressure and vasodilator responsiveness of isolated perfused kidney to acetylcholine or 5′-N-ethylcarboxamidoadenosine (NECA, adenosine receptor agonist) were evaluated.
Results: Lipopolysaccharide had no effect on serum creatinine, reduced blood pressure, and increased renal vasodilations induced by acetylcholine or NECA in male and female preparations. Immunohistochemical analyses showed that lipopolysaccharide reduced renal HSP70 expression, but increased α7-nAChRs, α4β2-nAChRs and iNOS expressions. The co-administration of aminoguanidine (iNOS inhibitor), pentoxifylline (TNFα inhibitor), or nicotine attenuated lipopolysaccharide mediation of renal vasodilations and elevations in α7/α4β2-nAChR and iNOS expressions. Nicotine also reversed the downregulating effect of lipopolysaccharide on HSP70 expression. α7-nAChRs (methyllycaconitine citrate, MLA) or α4β2-nAChRs (dihydro-β-erythroidine, DHβE) blockade potentiated the lipopolysaccharide enhancement of renal vasodilations, and abolished the depressant effect of nicotine on lipopolysaccharide responses. A similar abolition of nicotine effects was seen after HSP70 inhibition by quercetin. Alternatively, lipopolysaccharide hypotension was eliminated in rats treated with DHβE/nicotine or quercetin/nicotine regimen in contrast to no effect for nicotine alone or combined with MLA.
Conclusions: These findings establish that nicotine offsets lipopolysaccharide facilitation of renal vasodilations possibly through a crosstalk between HSP70 and nAChRs of the α7 and α4β2 types.

Introduction

Sepsis is a life threatening medical condition in which high mortality rate has been linked to multiple organ dysfunction [1]. Among critically ill patients, sepsis is one of the most common contributing factors to acute kidney injury (AKI), accounting for approximately 50% of all cases of AKI in intensive care units [2]. Even in patients with less severe infections, the incidence of AKI is as high as 16–25% [3]. Compared with non-septic AKI, septic AKI is associated with greater burden of illness, aberrations in vital signs and markers of inflammation, hospital residence, and mortality rate [4]. Despite the numerous experimental studies found in the literature [1–4], the precise effect of sepsis on intrarenal hemodynamics remains unclear. Sepsis induced AKI is characterized by complex pathophysiology in which various immunologic, toxic and inflammatory mechanisms cause intrarenal hemodynamic derangements, endothelial dysfunction, tubular apoptosis and inflammation [5]. Increases in renovascular resistance and reactivity to vasoconstrictors, and consequent renal hypoperfusion are hallmarks of septic AKI [6–8]. Further, decreased renovascular responsiveness to vasodilators has also been reported [9–11]. Paradoxically, other experimental studies demonstrated sustained or even enhanced renal blood flow [12,13], reduced renal vasoconstrictor capacity [11,14–16] and facilitated renal vasodilator propensity in response to endotoxemia [17]. These apparently discrepant effects of endotoxemia could be attributed to differences in the septic model [18], lipopolysaccharide (LPS) serotype [19], or duration of the septic insult [13,20].

The cholinergic pathway regulates inflammation at both physiological and pathological states [21]. Nicotine or selective

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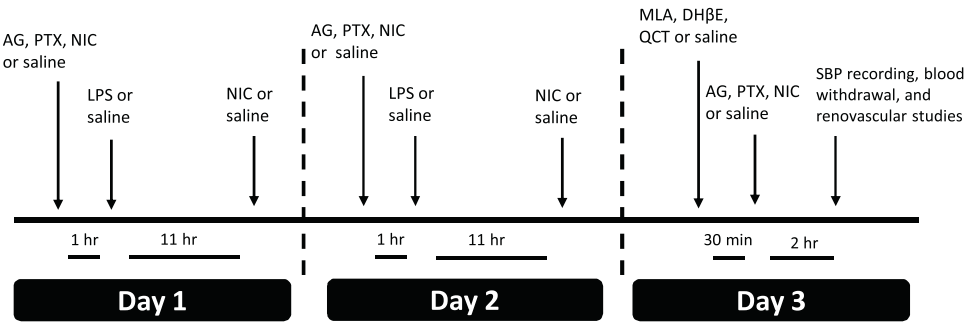

Fig. 1. Time schedules used for the investigation of the effect of *ip* nicotine (NIC, 0.5, 1, or 2 mg/kg), aminoguanidine (AG, iNOS inhibitor, 50 mg/kg), pentoxifylline (TNFα inhibitor, 3 mg/kg), or saline on lipopolysaccharide (LPS)-evoked changes in systolic blood pressure (SBP) and renal vasodilations caused by bolus injections of acetylcholine or 5′-N-ethylcarboxamidoadenosine in isolated perfused kidneys.

nAChR agonists exhibit antiinflammatory action in experimental inflammation models, including sepsis [22], ischemia reperfusion injury [23] and autoimmune arthritis [24]. The cholinergic pathway inhibits inflammation *via* suppressing the synthesis of proinflammatory cytokines [21,25] and nuclear translocation of NF-κB [26]. These effects are mediated primarily *via* activation of nAChRs, *e.g.* α7-nAChRs [27,28] and α4β2-nAChRs [29,30], inhibition of TNF-α/iNOS cascade [31,32] and induction of heme oxygenase [33] and HSP70 [34]. The latter is believed to mitigate inflammation and oxidative insults that are induced by iNOS and other inflammatory cytokines of endotoxemia [35–37]. In spite of the established role of nAChRs in inflammation and sepsis, no information is available to date regarding the role of homomeric and heteromeric nAChRs in hemodynamics and renovascular reactivity changes induced by endotoxemia.

Accordingly, pharmacologic studies were employed here to investigate the dose-related effects of nicotine (0.5, 1, or 2 mg/kg) on hemodynamic and renal vasodilator anomalies induced by short-term endotoxemia in rats. These dose regimens of nicotine have been found in a previous study from our laboratory [38] to produce plasma levels of cotinine, the principal metabolite and pharmacologically active form of nicotine, similar to those achieved after moderate cigarette smoking in humans [39,40]. The study was then extended to explore if these interactions could be modulated by α7/α4β2 nAChRs and related HSP70/TNFα/iNOS signaling. *In vitro* studies were undertaken in phenylephrine-preconstricted isolated perfused kidneys of rats to assess the effect of LPS in the absence or presence of nAChR modulators on renal vasodilations evoked by ACh or NECA. The latter is an adenosine analogue that, like ACh, causes nitric oxide-dependent vasorelaxation *via* the activation of adenosine A2 receptors [41,42].

Materials and methods

Animals

Age matched male and female Wistar rats (10–13 weeks old, 180–230 g, Animal Facility of Faculty of Pharmacy, Alexandria University, Egypt) were used. All experimental techniques and procedure were approved by the institutional Animal Care and Use Committee.

Drugs

Nicotine (Merck Schuchardt, Hohenbrunn, Germany), methyllycaconitine citrate (MLA), dihydro-β-erythroidine hydrobromide (DhβE), and quercetin (Tocris Bioscience Co, Bristol, UK), LPS (from E coli 0111:B4), pentoxifylline (PTX), aminoguanidine, 5′-N-ethylcarboxamidoadenosine (NECA), acetylcholine chloride, and phenylephrine hydrochloride (Sigma-Aldrich Co, St. Louis, MO, USA), thiopental sodium (Biochemie, Vienna, Austria), and heparin (5000 IU/ml; Nile pharmaceutical Co, Egypt) were purchased from commercial vendors. Nicotine, LPS, MLA, DhβE, aminoguanidine, and pentoxifylline were dissolved in saline while quercetin was dissolved in DMSO. Acetylcholine and NECA were prepared daily in distilled water and DMSO respectively.

Tail-cuff plethysmography

Systolic blood pressure (SBP) was measured in conscious rats just prior to sacrifice using tail cuff method as reported in previous studies [43]. A tail cuff ; pressure transducer (Pan Lab, Spain), and computerized data acquisition system with LabChart-7 pro software (Power Lab 4/30, model ML866/P, AD Instruments, Bella Vista, Australia) were employed. Heart rate (HR) was computed

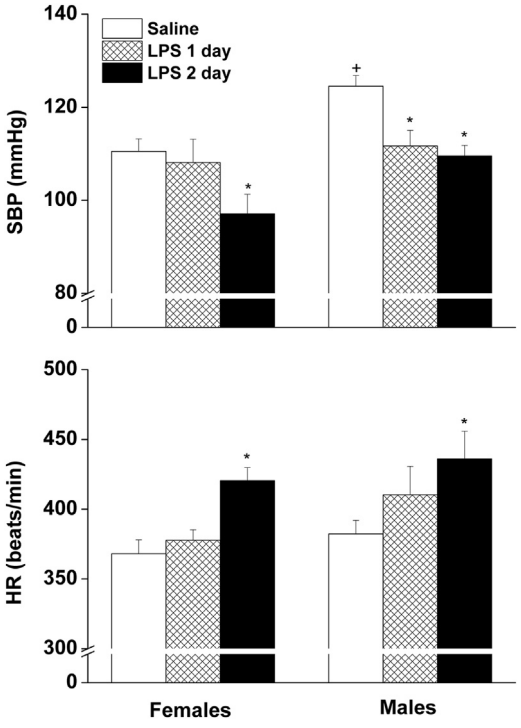

Fig. 2. Effect of one- or two-day *ip* lipopolysaccharide (LPS, 5 mg/kg/day) administration on tail-cuff measurements of systolic blood pressure (SBP) and heart rate (HR) in male and female Wistar rats. Separate groups of rats were used for each time period. After SBP measurements, rats were processed for *in vitro* measurements of renovascular reactivity as detailed in methods. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline values in the same sex, $^{+}p < 0.05$ *vs.* respective female values.

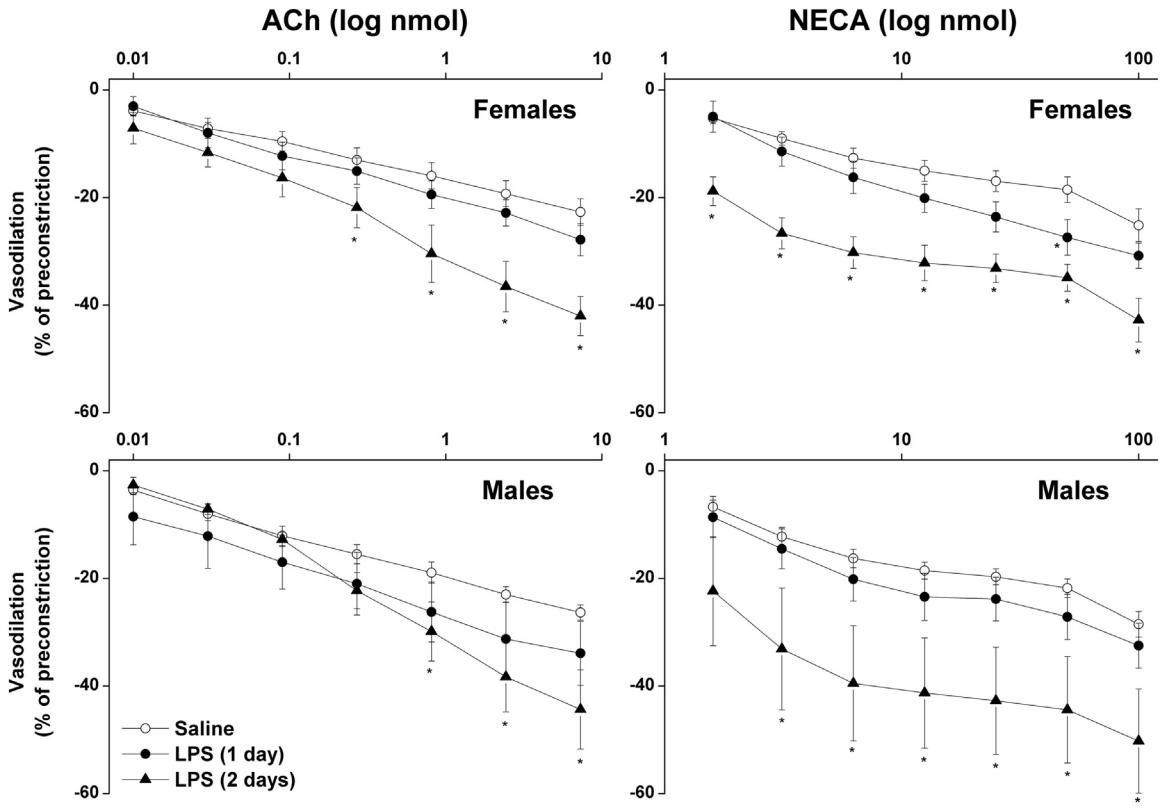

Fig. 3. Effect of one- or two-day *ip* lipopolysaccharide (LPS, 5 mg/kg/day) treatment on cumulative vasodilatory effects of acetylcholine (ACh, left panels) or 5′-N-ethylcarboxamidoadenosine (NECA, right panels) in phenylephrine-preconstricted isolated perfused kidneys of male or female Wistar rats. Vasodilatory responses are expressed as the percentage from phenylephrine-induced tone. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline.

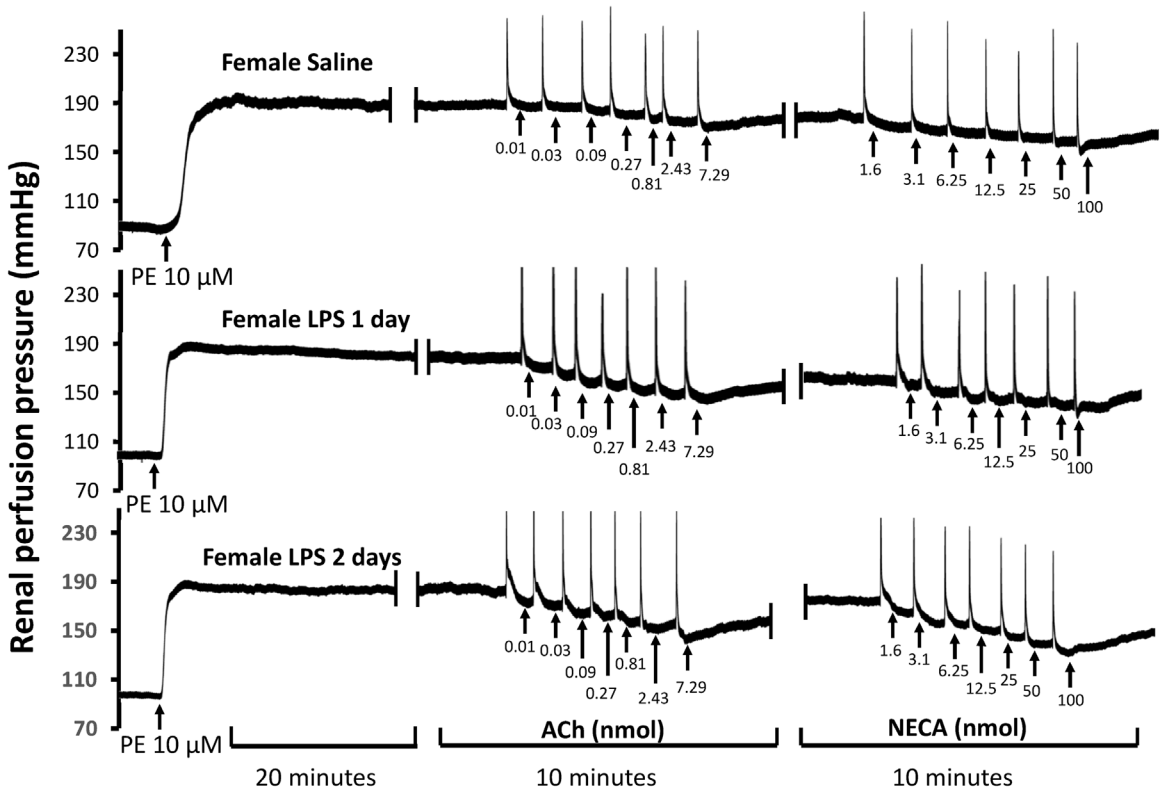

Fig. 4. Representative tracings of cumulative renal vasodilations induced by acetylcholine (ACh) or 5′-N-ethylcarboxamidoadenosine (NECA) in phenylephrine-preconstricted isolated perfused kidneys of female rats treated with lipopolysaccharide (LPS, 1 or 2 days) or saline.

from BP waveforms and displayed on another channel of the recording system. Rats were allowed to acclimate to the tail cuff procedure for at least two sessions on two consecutive days prior to the day of actual SBP recording. SBP measurement was repeated 3 consecutive times and values were averaged for each individual rat. SBP and HR values of rats in each particular group were averaged to get the mean values for the whole group.

The rat isolated perfused kidney

The left kidney from each rat was isolated and perfused according to the technique described in previous studies [44,45]. In order to evaluate the renal vasodilatory responses to the endothelium dependent vasodilator acetylcholine or the adenosine analogue NECA, the renal vascular tone was first elevated by a continuous infusion of the α_1-adrenoceptor agonist phenylephrine (10 μM), then cumulative dose response curves to bolus injections of acetylcholine (ACh, 0.01–7.29 nmol) and NECA (1.6–100 nmol) were constructed. Each dose of acetylcholine or NECA was injected when the vasodilatory response to the preceding dose has plateaued.

Measurement of serum creatinine

Blood samples (approximately 2 ml) were withdrawn from rats under thiopental anesthesia, just prior to sacrifice and kidney isolation using retro-orbital bleeding technique [46]. The retro-orbital venous plexus puncture was performed using a glass capillary tube and blood was allowed to drain into a collection tube. Blood was allowed to clot at room temperature for 15 min, then centrifuged at 1200 *g* for 10 min. The resultant supernatant layer (serum) was aspirated, transferred into Eppendorf tubes and stored at - 80 °C till the time of analysis. Serum creatinine was measured colorimetrically [47].

Immunohistochemistry

The technique described in our previous studies [48,49] was employed for the determination of protein expression of α7/α4β2-nAChR, HSP70, and iNOS in glomerular and tubular renal tissues. Briefly, 4-μm thick kidney sections were laid on positively charged microscope slides (Thermo Scientific®, Berlin, Germany), placed in xylene for deparaffinization, and then gradually rehydrated in ethanol (100, 95, and 70%) and phosphate buffered saline (PBS). Heat-induced epitope retrieval was carried out by immersing the slides in citrate buffer (pH 6, Thermo Scientific) and incubating them in the microwave at power 100 and 30 for 1 and 9 min, respectively. Hydrogen peroxide (3%) was used to quench endogenous peroxidases. Sections were incubated overnight with monoclonal antibody for α7-nAChR, HSP 70, or iNOS (1:200 dilution, Bioss Inc, USA), 30 min with HRP-secondary antibody (EnVision™ FLEX, Dako Agilent, CA, USA), and chromogen 3,3′-diaminobenzidine (DAB) for protein visualization. Afterwards, sections were counterstained with haematoxylin and dehydrated with ethanol (95 and 100%) and xylene. The immunohistochemical signals were quantified with computer-assisted microscopy (Optika® Microscopes, Italy) and ImageJ software (version 1.51 w, National Institutes of Health, Bethesda, Maryland, USA). Images of glomeruli and tubules were analyzed using Fiji development of imageJ [50]. Each image was subjected to the plug-in "color deconvolution" using H-DAB as vector, where the immunostained image was separated into 3 panels representing colors of hematoxylin, DAB and background. Panel 2 (DAB) was then selected and the threshold was set to be between 0 (zero = deep brown, highest expression) and 135. Eventually, area fraction was measured which depict the percentage of image area that have been highlighted after adjusting the threshold. Since multiple pictures were captured from same slide, the average % area was calculated for each slide.

Protocols and experimental groups

Hemodynamic and renovascular effects of LPS

Six groups of rats (3 males and 3 females, n = 6–8 each) were utilized to investigate the effect of endotoxemia on hemodynamics and renal vasodilations. Rats from either sex were randomly assigned to receive *ip* injections of (i) saline, (ii) a single dose of LPS (5 mg/kg) [51,52], or (iii) two doses of LPS on 2 consecutive days (5 mg/kg/day). For male or female rats in the first 2 groups, tail-cuff SBP and renovascular studies were performed 24 h after LPS or saline administration. Rats in the third group (males or females) were processed for SBP and renovascular studies 24 h after the second LPS dose. Afterward, rats were anesthetized by *ip* injection of thiopental (50 mg/kg) and blood samples were withdrawn for future serum analyses. The left kidney was then isolated, perfused and preconstricted with phenylephrine in order to evaluate the renal vasodilatory responses to cumulative bolus additions of ACh (0.01–7.29 nmol) and NECA (1.6–100 nmol). The right kidney was harvested, paraffin molded and used for immunohistochemical determinations of nAChRs, HSP70, and iNOS expression. A timeline for the sequence of experimental events employed in this study is shown in Fig. 1.

Impact of TNFα or iNOS inhibition on hemodynamic and renal effects of endotoxemia

Because the results of the preceding experiment showed that the hemodynamic and renal consequences of LPS were similar in the two rat sexes, all subsequent studies were carried out in female rats only. Two groups of female rats (n = 6 each) were employed in

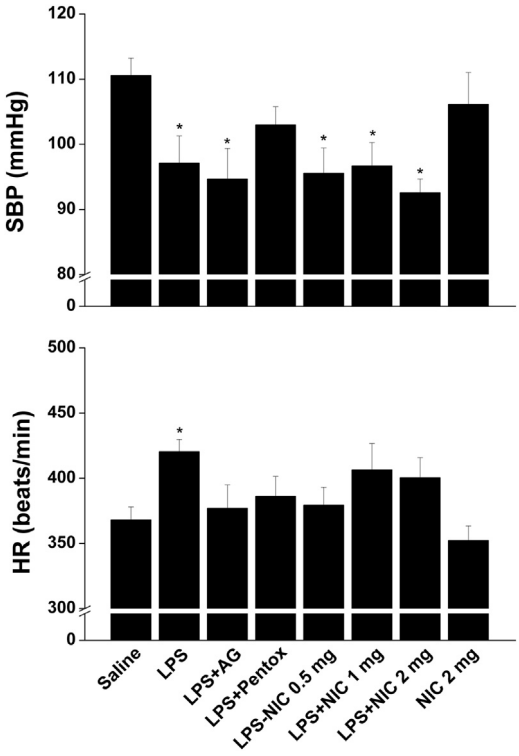

Fig. 5. Effects of *ip* nicotine (NIC, 0.5, 1, or 2 mg/kg), aminoguanidine (AG, iNOS inhibitor, 50 mg/kg) or pentoxifylline (Pentox, TNFα inhibitor, 3 mg/kg) on lipopolysaccharide (LPS)-evoked changes in SBP and HR measured by tail-cuff plethysmography in female Wistar rats. Saline and LPS values presented in this figure have also been used in Fig. 2. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline.

this experiment to assess the role of TNFα and iNOS in the LPS responses. One hour prior to LPS administration, rats were treated with pentoxifylline (TNFα inhibitor, 3 mg/kg) [53] or aminoguanidine (iNOS inhibitor, 50 mg/kg) [53]. A third dose of each inhibitor was given on the experiment day (day 3), 2 h before SBP measurement and *in vitro* experimentation.

Effect of nicotine on endotoxic manifestations

Three groups of female rats (n = 6–8 each) were used to test whether concomitant nicotine administration would abrogate hemodynamic and renovascular changes caused by endotoxemia. Along with the two LPS doses administered on two successive days, a total of five *ip* nicotine doses (0.5, 1, or 2 mg/kg) [38] were given every 12 h. The last nicotine dose was injected on the third day, 2 h ahead of SBP measurement and *in vitro* experimentation. A fourth group was added to test the effect of 2 mg/kg dose of nicotine in the absence of LPS.

Role of nAChRs and HSP70 in the nicotine-endotoxemia interaction

Three groups of female rats (n = 6–8) were used to test the effect of prior pharmacologic blockade of α7 (MLA, 2 mg/kg) [54] or α4β2 nAChRs (DhβE, 2 mg/kg) [55] or inhibition of HSP70 (quercetin, 50 mg/kg) [56] on nicotine (2 mg/kg)-LPS interaction. Another 3 groups of female rats were employed that received the same drug regimens, but replacing nicotine with saline. As detailed above, SBP and HR were measured by the tail-cuff technique on the third day. Rats were then anesthetized by *ip* injection of thiopental (50 mg/kg) and blood samples were withdrawn for future serum analyses. Then, the left kidney was isolated, perfused and preconstricted with phenylephrine in order to evaluate the renal vasodilatory responses to acetylcholine and NECA. The right kidney was harvested for immunohistochemical determination of the renal expression of nAChRs, HSP70, and iNOS.

Statistical analysis

Values are expressed as means ± SEM. ACh and NECA vasodilatory responses were expressed as the percentage from phenylephrine induced preconstriction. Furthermore, the area under the curve (AUC) was calculated for individual experiments to indicate the cumulative vasodilatory effect of acetylcholine and NECA. AUC was computed using trapezoidal integration and zero line as the baseline [43,45]. The Repeated measures analysis of variance followed by the Tukey's *post hoc* test was used to test for statistical significance with the level of significance set at $p < 0.05$. Analyses were performed using GraphPad prism software, version. 6.01.

Results

Compared with control values, serum creatinine levels were not affected by the 2-day regimen of LPS in male (0.19 ± 0.04 *vs.* 0.18 ± 0.02 mg/dl) or female rats (0.24 ± 0.02 *vs.* 0.26 ± 0.02 mg/dl). Likewise, under a constant flow rate of 5 ml/min, the average basal renal perfusion pressure in isolated perfused kidneys (100 mmHg) and the elevation in renal perfusion pressure caused by continuous infusion of 10 μM phenylephrine (80 mmHg) were not statistically different in various experimental groups (data not shown).

Effects of LPS on SBP and renal vasodilations

The effects of LPS or an equal volume of saline on tail cuff measurement of SBP and HR in male and female rats are depicted

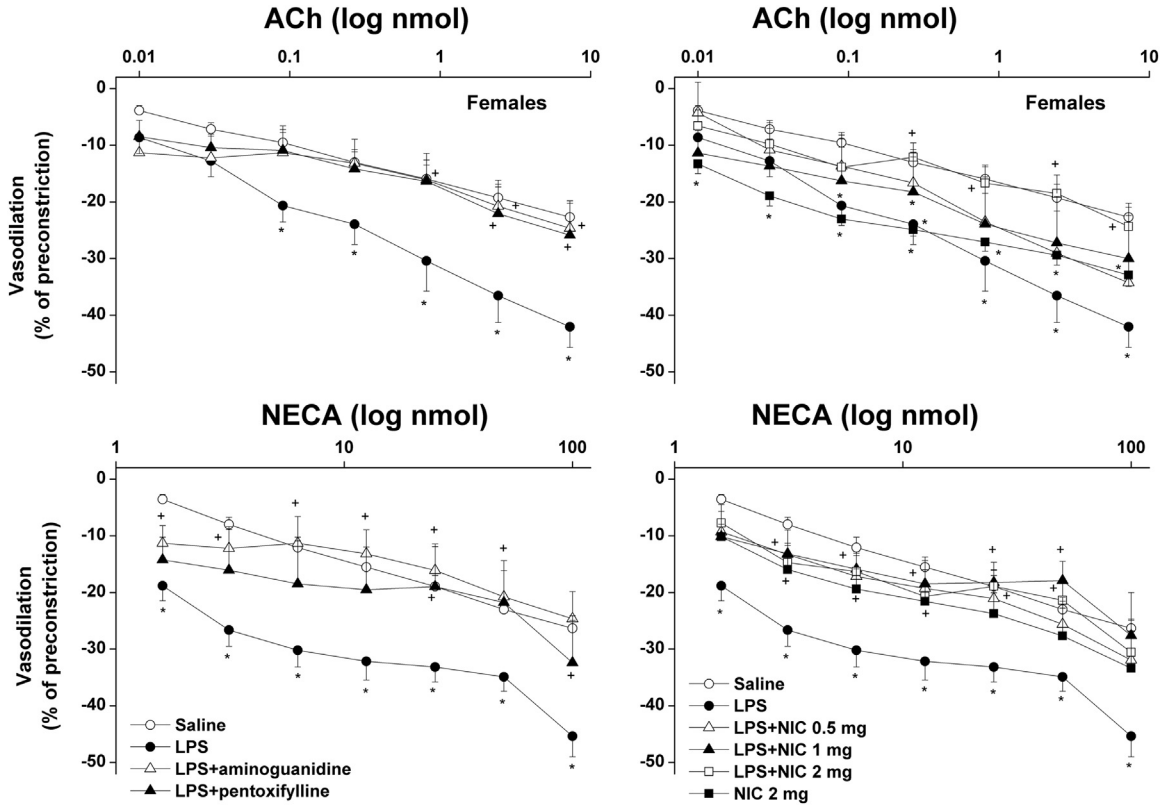

Fig. 6. Effects of aminoguanidine (iNOS inhibitor, 50 mg/kg) or pentoxifylline (TNFα inhibitor, 3 mg/kg) (left panels) or nicotine (NIC, 0.5, 1, or 2 mg/kg, right panels) on lipopolysaccharide (LPS, 5 mg/kg/day for 2 days)-evoked increases in acetylcholine (ACh) or 5′-N-ethylcarboxamidoadenosine (NECA) vasodilations in isolated perfused kidneys of female Wistar rats. Saline and LPS values presented in this figure have also been used in Fig. 3. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* respective saline values, $^{+}p < 0.05$ *vs.* respective LPS values.

in Fig. 2. Compared with respective saline values, the 2-day LPS administration (5 mg/kg/day) in rats of either sex produced significant decreases and increases in SBP and HR, respectively. SBP was also significantly decreased in male, but not female, rats when measured one day after LPS administration. A similar pattern was observed when the effect of LPS on the renal vasodilatory capacity in perfused phenylephrine-preconstricted kidneys was investigated. Compared with respective control rats, significantly greater vasodilatory responses to cumulative bolus injections of ACh (0.01–7.29 nmol) and NECA (1.6–100 nmol) were caused by LPS in isolated perfused kidneys of male and female rats (Fig. 3). Fig. 4 shows representative tracings of the cumulative renal vasodilatory responses elicited by ACh or NECA in isolated perfused kidneys of female rats treated with LPS or saline.

Effects of nicotine or TNFα or iNOS inhibition on hemodynamic and renal effects of LPS

The effects of concurrent administration of nicotine (0.5, 1, or 2 mg/kg) or pharmacologic inhibition of TNFα (pentoxifylline, 3 mg/kg) or iNOS (aminoguanidine, 50 mg/kg) on the hemodynamic and renal vasodilatory actions of LPS in female rat

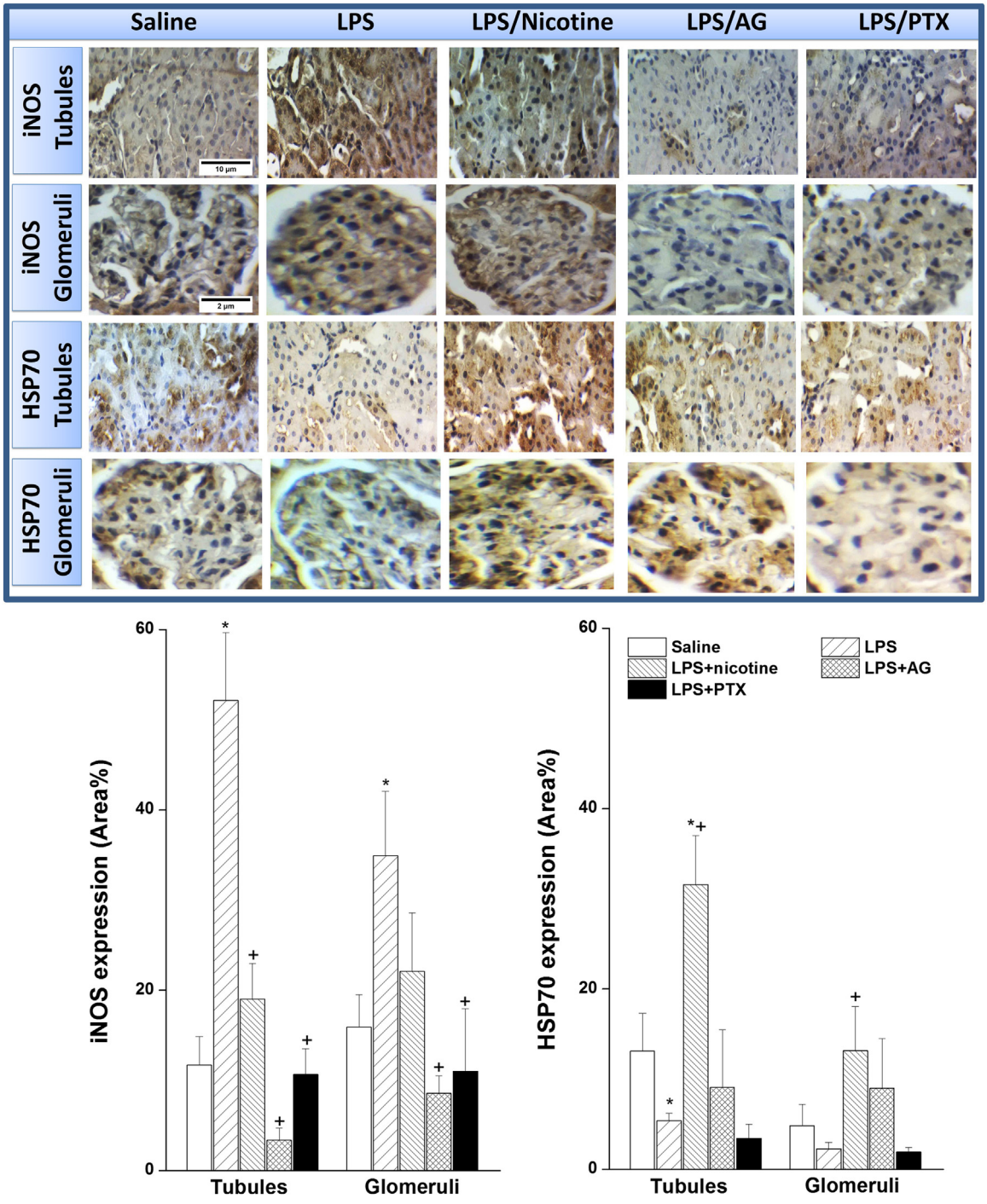

Fig. 7. Effects of nicotine (NIC, 2 mg/kg), aminoguanidine (iNOS inhibitor, 50 mg/kg), or pentoxifylline (TNFα inhibitor, 3 mg/kg) on lipopolysaccharide (LPS, 5 mg/kg/day for 2 days)-evoked changes in the immunohistochemical iNOS and HSP70 protein expressions in renal cortical glomeruli and medullary tubules of female Wistar rats. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline values, $^{+}p < 0.05$ *vs.* LPS values. Representative images of immunostained tissues are also shown (400×).

preparations are depicted in Figs. 5 and 6. Whereas each of the above treatments abolished the tachycardic effect of LPS, the LPS-evoked reduction in SBP was only blunted upon co-treatment with pentoxifylline (Fig. 5). By contrast, the increases caused by LPS in ACh and NECA vasodilations in perfused phenylephrine-preconstricted kidneys were reversed and restored to control values after concomitant administration of pentoxifylline, aminoguanidine, or nicotine (Fig. 6).

The protein expressions of iNOS, HSP70, and α7/α4β2 nAChRs in cortical glomerular and tubular tissues of female rats were measured by immunohistochemistry and illustrated in Figs. 7 and 8. LPS treatment for 2 days caused significant increases in glomerular and tubular protein expressions of iNOS, α7-nAChR, or α4β2-nAChR (Figs. 7,8). To the contrary, HSP70 expression was reduced by LPS in tubular tissues whereas glomerular HSP70 expression remained unaltered (Fig. 7). These molecular effects of LPS disappeared upon concurrent administration of nicotine, aminoguanidine, or pentoxifylline (Figs. 7,8).

Pharmacologic nAChRs or HSP70 inhibition modulates nicotine-LPS interaction

The effects of blockade of α7 (MLA, 2 mg/kg) or α4β2 nAChRs (DHβE, 2 mg/kg) or inhibition of HSP70 (quercetin) on hemodynamic

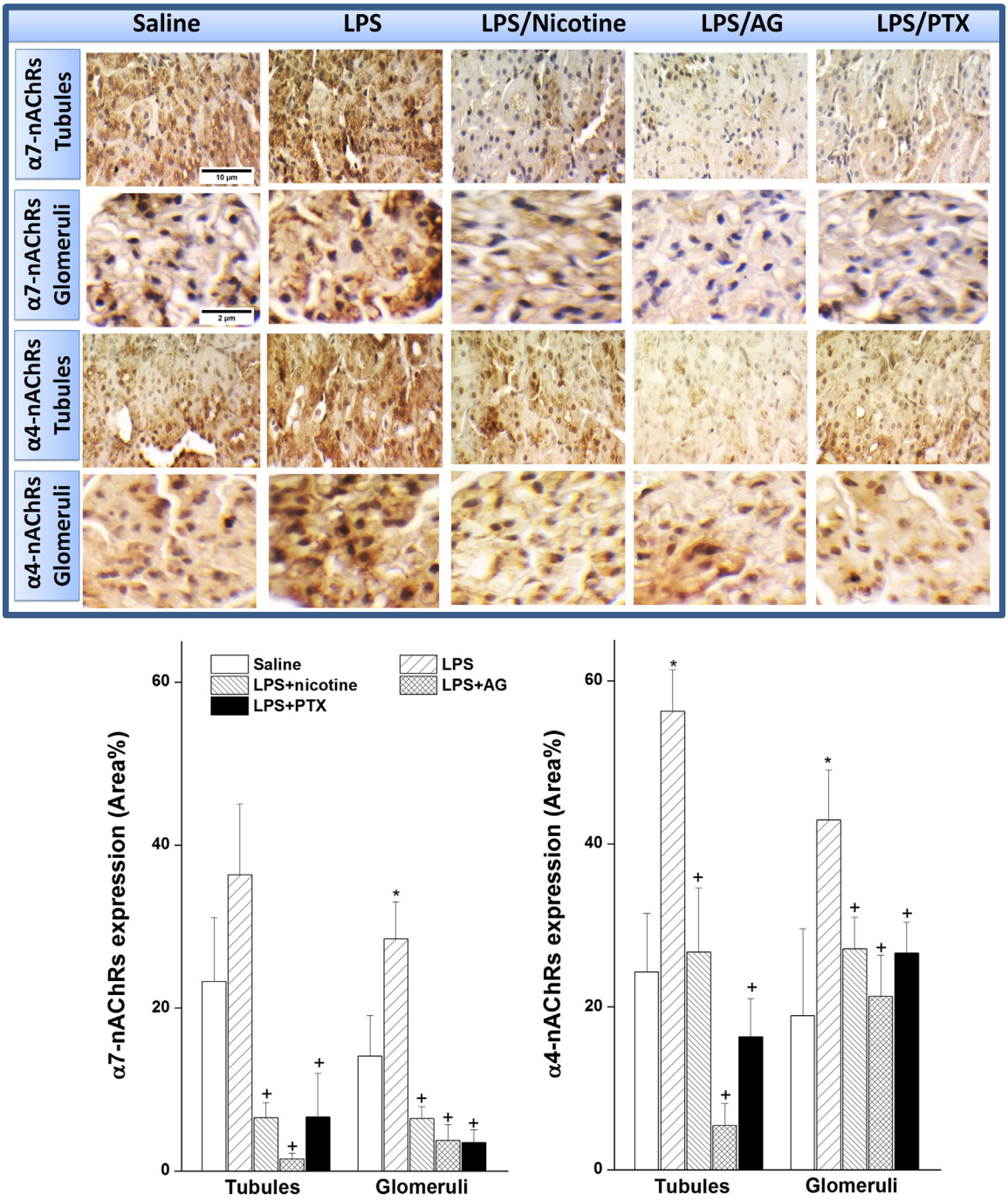

Fig. 8. Effects of nicotine (NIC, 2 mg/kg), aminoguanidine (iNOS inhibitor, 50 mg/kg), or pentoxifylline (TNFα inhibitor, 3 mg/kg) on lipopolysaccharide (LPS, 5 mg/kg/day for 2 days)-evoked changes in the immunohistochemical α7 and α4β2 nAChRs protein expressions in renal cortical glomeruli and medullary tubules of female Wistar rats. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline values, $^{+}p < 0.05$ *vs.* LPS values. Representative images of immunostained tissues are also shown (400×).

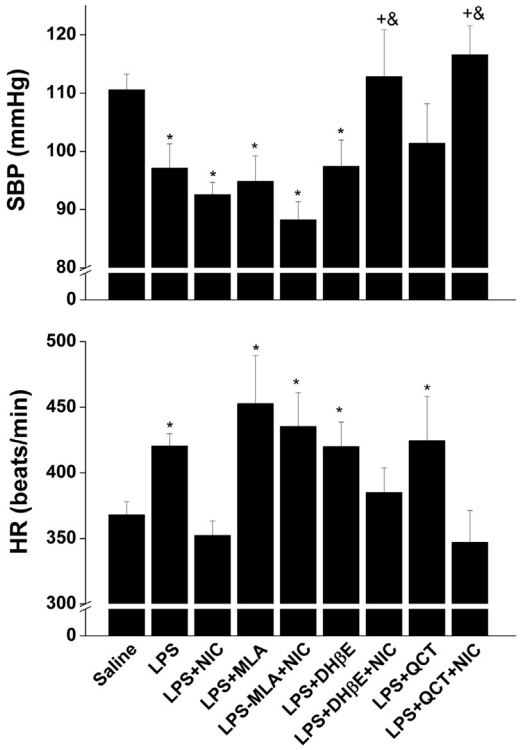

Fig. 9. Effects of nicotine (NIC, 2 mg/kg), methyllycaconitine citrate (MLA, α7nAChR antagonist, 2 mg/kg), DHβE (α4β2nAChR antagonist, 2 mg/kg), quercetin (QCT, HSP-70 inhibitor, 50 mg/kg), or their combinations on lipopolysaccharide (LPS)-evoked hypotension and tachycardia in female Wistar rats. Saline and LPS values presented in this figure have also been used in Figs. 2 and 5. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline values, $^{+}p < 0.05$ *vs.* LPS values, $^{\&}p < 0.05$ *vs.* LPS + NIC values.

and renal interactions between nicotine (2 mg/kg) and LPS in female rats were studied. While, LPS hypotensive was preserved in rats treated with nicotine, MLA or DHβE, it was eliminated in rats treated with the combined DHβE/nicotine or quercetin/nicotine regimen (Fig. 9). The concurrent administration of MLA or DHβE potentiated the enhancing action of LPS on renal vasodilator responses to ACh (Fig. 10A, B), but not NECA (Fig. 11A, B), and significantly increased AUCs of the dose-vasodilatory response curves of ACh (Fig. 10D). In addition, MLA or DHβE abolished the depressant effect of nicotine on LPS mediation of these responses. Similarly, the inhibitory effect of nicotine on renal vasodilation was blunted upon co-administration of quercetin (Figs. 10,11).

Discussion

The present study reports on short-term effects of nicotine on hypotensive and renal vasodilatory influences of endotoxemia and its modulation by nAChRs and HSP70/TNFα/iNOS signaling in rats. The data showed that LPS lowered BP, enhanced renal vasodilatory responses to ACh and NECA, and caused opposite changes in renal expression of HSP70 (decreases) and α7-nAChRs/α4β2-nAChRs/iNOS (increases). Such responses, except hypotension, were largely eliminated after co-exposure to nicotine, pentoxifylline (TNFα inhibitor), or aminoguanidine (iNOS inhibitor). Moreover, the counteraction by nicotine of the facilitatory action of LPS on the renal vasodilator capacity disappeared after selective blockade of α7-nAChRs (MLA) or α4β2-nAChRs (DHβE) or inhibition of HSP70 (quercetin). Together, these data suggest a possible role for the interplay between α7/α4β2nAChRs and HSP70 in the correcting effects of nicotine on the renal vasodilatory and inflammatory states of endotoxemia.

Endotoxemia variably affects renal and extra-renal vasoreactivity, depending on factors such as the duration of LPS exposure and serotype of E. coli LPS employed. Impaired renovascular reactivity to vasodilators is mostly observed during the early phase of endotoxemia [57,58]. Studies in renal, carotid and hindquarter vascular beds showed that cholinergic vasodilator responsiveness is reduced after 6 h and augmented after 24 h of LPS exposure [9,59]. Gardiner et al. [13,20] demonstrated progressive and time-related increases in renal vascular conductance and blood flow after LPS infusion. Heyman et al. [19] reported that while LPS 0127:B8 serotype attenuates renal medullary vasodilation, LPS serotype 0111:B4 produces predominant renal vasodilation. In accordance with this view, we showed here that cumulative vasodilatory responses elicited by ACh or NECA were significantly augmented in male and female rats treated with the LPS serotype 0111:B4 for two consecutive days. Mechanistically, the delayed enhancement of renal vasodilation by endotoxemia may be triggered by machinaries that function to counterbalance the initial renal vasoconstriction and reduced renal perfusion [57]. Pathophysiologically, the excessive renal arteriolar vasodilation and subsequent decreases in glomerular capillary pressure and filtration rate have been causally related to septic kidney dysfunction [5,60,61].

Nicotine exerts beneficial effects in inflammatory and septic disorders [62,63]. Evidence suggests that nicotine prevents translocation of NF-κB [64] and suppresses endotoxin induced production of proinflammatory cytokines [65,66]. Although the antiinflammatory effect of nicotine has been the subject of rigorous investigation in recent years, the precise underlying mechanisms remain largely unknown. Accordingly, the current work opted to investigate dose-related effects of nicotine on endotoxic renovascular responses and its modulation by α7 or α4β2 nAChRs and related HSP70/TNFα/iNOS signaling. We demonstrated that nicotine abolished the LPS augmentation of renal vasodilation capacity and this was associated with, and possibly attributed to, the reversal of LPS-induced increases in iNOS expression in renal tubular and glomerular tissues. Because these functional and molecular effects of nicotine in endotoxic rats were reproduced after replacing nicotine with iNOS inhibitor aminoguanidine or TNFα inhibitor pentoxifylline, it is conceivable that the counteraction of the endotoxic inflammatory response accounts for the observed favorable renal effects of nicotine.

HSP70 is an intracellular protein that promotes protein refolding and mitigates inflammation and oxidative insults that are induced by iNOS and other inflammatory cytokines over the course of endotoxemia [35–37]. Here we report two observations that implicate HSP70 in the nicotine counteraction of endotoxic manifestations. First, there was the observation that nicotine not only abolished the reductions in tubular HSP70 expression caused by LPS, but it also increased the tubular HSP70 abundance to levels that were well above those seen in control rats (see Fig. 7). Interestingly, nicotine also caused proportionally similar increases in glomerular HSP70 expression, despite the inability of LPS on its own to significantly alter glomerular HSP70 expression. These molecular data are complemented by pharmacological studies in which HSP70 inhibition by quercetin mitigated the counteracting effect of nicotine on LPS mediation of renal vasodilations. Overall, the current data suggest a contributory role for HSP70 in the renoprotective action of nicotine against endotoxemia.

Receptor antagonist studies of the current investigation underscore a fundamental role for α7 and α4β2 nAChRs in the renoprotective effect of nicotine during endotoxemia. This is because the ameliorating action of nicotine on the LPS-evoked increases in renal vasodilatory capacity was lost after selective blockade of α7 or α4β2 nAChRs by MLA and DHβE, respectively. Equally important, in nicotine-untreated rats, the co-exposure to LPS plus MLA or DHβE produced exaggerated increases in ACh, but

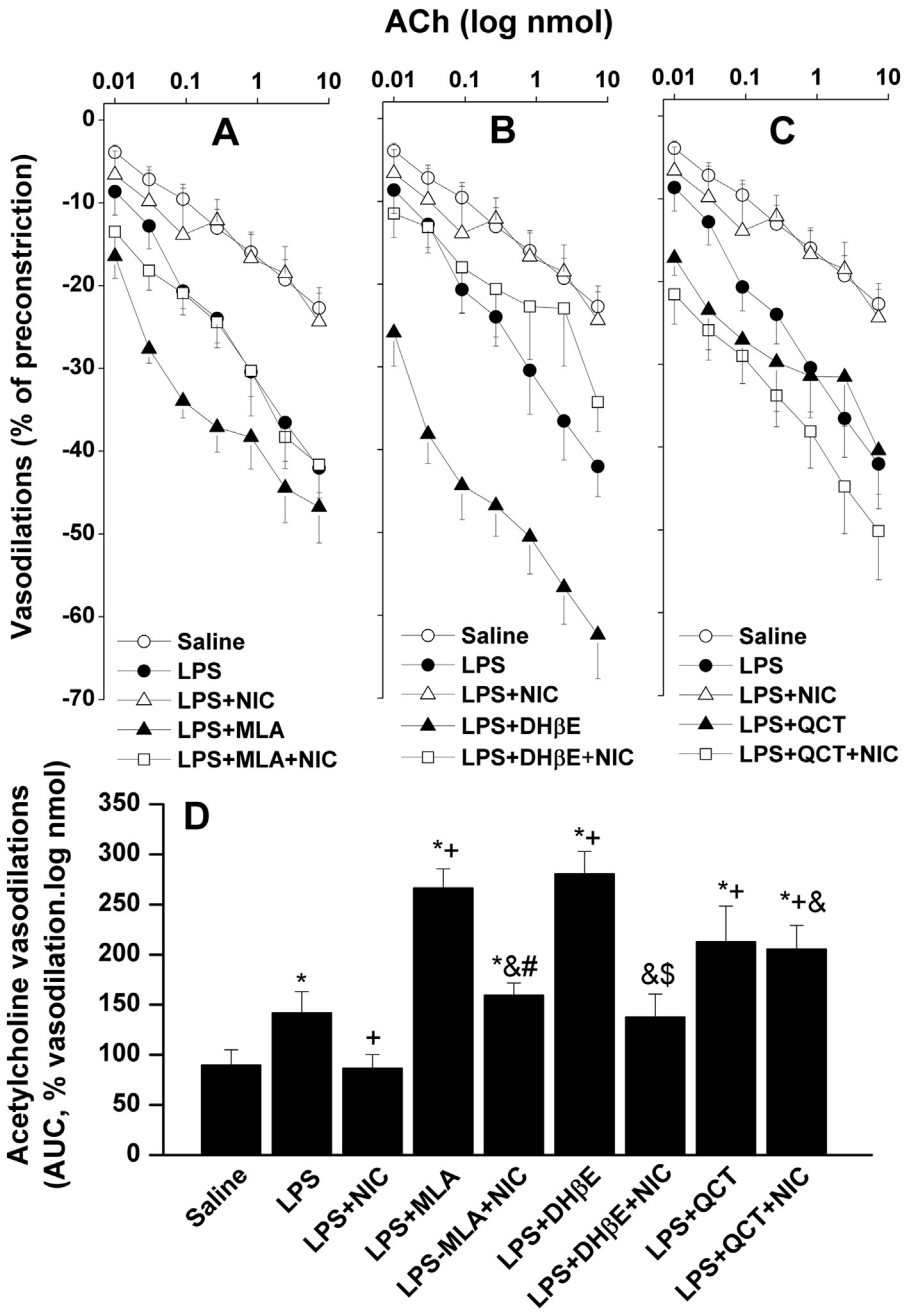

Fig. 10. Effects of nicotine, methyllycaconitine citrate (MLA, α7nAChR antagonist, 2 mg/kg, panel A), DHβE (α4β2nAChR antagonist, 2 mg/kg, panel B), or quercetin (QCT, HSP70 inhibitor, 50 mg/kg, panel C) on lipopolysaccharide (LPS)-evoked increases in renal acetylcholine (ACh) vasodilations in female Wistar rats. Areas under the curves (AUC) of the cumulative renal vasodilatory effect of ACh are shown in panel D. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline values, $^{+}p < 0.05$ *vs.* LPS values, $^{\&}p < 0.05$ *vs.* LPS + NIC values, $^{\#}p < 0.05$ *vs.* LPS + MLA values, $^{\$}p < 0.05$ *vs.* LPS + DHβE values.

not NECA, vasodilations to levels that far exceeded those caused by LPS alone (Figs. 10D and 11D). It appears, therefore, that the presence of intact and functional α7 or α4β2 nAChRs serves probably to tonically restrain the influences of LPS on renal vasodilatory responses of cholinergic origin. In fact, the facilitated α7/α4β2 nAChRs expression in glomerular and tubular tissues of LPS-treated rats might constitute an adaptational mechanism to offset the inflammatory response to endotoxemia. Such presumed compensatory increases in nAChR abundance has been validated in a variety of inflammatory conditions such as endotoxemia [67] and renal ischemia-reperfusion injury [68]. Remarkably, in the nicotine-protected endotoxic rats, which exhibited improved renovascular and inflammatory profiles, near-control expression levels of renal α7/α4β2 nAChRs were noted. Remarkably, protein expression studies were performed in the current study in both glomerular and tubular tissues of the kidney. Although molecular changes in glomerular tissues are more influential in defining the LPS interaction with renal vasculature, evidence obtained from animal and human studies suggest that tubular changes can as well account for renal and microcirculatory responsiveness to injurious signals [69,70].

The present finding that the hypotensive response elicited by LPS, unlike its tachycardic or renal vasodilatory effects, was preserved after concurrent administration of nicotine deserves two comments. First, it raises the possibility that the effects of LPS on SBP and renal vasodilation might be mediated *via* distinct

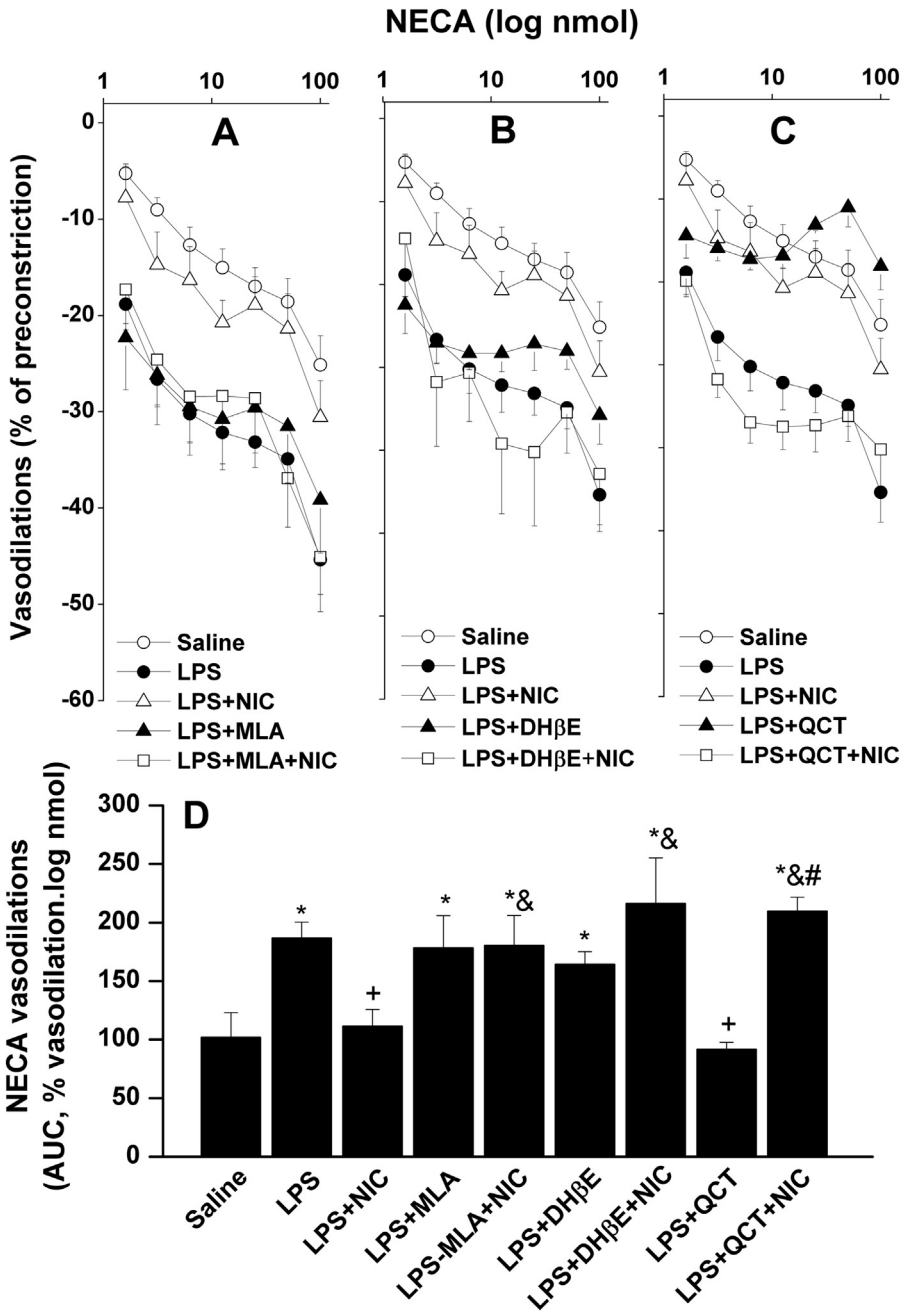

Fig. 11. Effects of nicotine, methyllycaconitine citrate (MLA, α7nAChR antagonist, 2 mg/kg, panel A), DHβE (α4β2nAChR antagonist, 2 mg/kg, panel B), or quercetin (QCT, HSP70 inhibitor, 50 mg/kg, panel C) on lipopolysaccharide (LPS)-evoked increases in renal 5′-N-ethylcarboxamidoadenosine (NECA) vasodilations in female Wistar rats. Areas under the curves (AUC) of the cumulative renal vasodilatory effect of NECA are shown in panel D. Each value is the mean ± SEM of 6–8 animals. $^{*}p < 0.05$ *vs.* saline values, $^{+}p < 0.05$ *vs.* LPS values, $^{\&}p < 0.05$ *vs.* LPS + NIC values, $^{\#}p < 0.05$ *vs.* LPS + QCT values.

cellular mechanisms. Blood pressure homeostasis, unlike renal control, is a highly complex process, encompassing a multitude of peripheral and central machineries. Moreover, the abolition of the tachycardic action of LPS, which is mostly a reflex response to the evoked hypotension [71], could be related to the ability of nicotine to suppress the arterial baroreceptor activity [72,73]. Indeed, our observation that the hypotensive action of LPS was reduced in rats treated with pentoxifylline but not aminoguanidine implies a potential role for iNOS-independent inflammatory events in the hypotensive response. Having said that, it cannot be argued that the dose of aminoguanidine employed here (50 mg/kg) might not be adequate for effective iNOS inhibition because the same dose did abolish the enhancing effect of LPS on renal vasodilations in this study and eliminated the hypotension seen during early hours of endotoxemia in our previous study [53]. The second comment relates to the observation that nicotine reversed the hypotensive action of LPS only when combined with DHβE but not MLA (Fig. 9). It is tempting to speculate that functional nAChRs of the α4β2 type acts preferentially to block the pressor effect of nicotine. More studies are obviously needed to investigate the reason(s) of these interactions.

It is imperative to comment on the relative non-specificity of the pharmacological agents used in this study. Aminoguanidine has multiple biological effects such as inhibition of iNOS [74], amine oxidase [75] and advanced glycosylation end products [76]. Alternatively, in addition to TNFα inhibition [53], PTX is a

nonselective inhibitor of phosphodiesterases [77]. Quercetin, a naturally occurring flavonoid of plant origin, has a wide array of biological effects including antiinflammatory and anti-carcinogenic actions [78]. Paradoxically, other studies have highlighted a key role for HSP70 inhibition evoked by quercetin in promoting toxic and inflammatory effects of sepsis [79,80]. The potential contributions of these off-target effects for aminoguandine, PTX, or quercetin to reported data of the current study cannot be overlooked.

In conclusion, the present study showed that nicotine offsets the renovascular vasodilatory and molecular effects of endotoxemia. Pharmacologic and immunohistochemical studies reveal a crosstalk between α7/α4β2 nAChRs and HSP70 in modulating the advantageous counteractive effects of nicotine on inflammatory and renovascular consequences of endotoxemia. Clinically, the data advocate potential therapeutic relevance for nicotine or perhaps selective α7 or α4β2 nAChRs agonists in the control of altered renal hemodynamics and vasodilator capacity observed in septic patients.

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Conflict of interest

None

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