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Involvement of Toll-Like Receptor 4 in Decreased Vasopressor Response Following Trauma/Hemorrhagic Shock

OBJECTIVES: Refractory vascular failure due to the inability of vascular smooth muscle to respond to vasoconstrictors such as phenylephrine is a final common pathway for severe circulatory shock of any cause, including trauma/hemorrhagic shock. Increased inflammation, Toll-like receptor 4 activation, and decreased response of the alpha-1 adrenergic receptors which control vascular tone have been reported in trauma/hemorrhagic shock.

HYPOTHESIS: In trauma/hemorrhagic shock, Toll-like receptor 4 activation contributes to vascular failure via decreased bioavailability of adrenergic receptors.

DESIGN AND MEASUREMENTS: Trauma/hemorrhagic shock was induced in Wistar rats (laparotomy combined with mean arterial pressure at 40 mm Hg for 90 min followed by 2 hr resuscitation with Lactated Ringers solution). To inhibit Toll-like receptor 4, resatorvid (TAK-242) and resveratrol were used, and plasma was collected. Smooth muscle cells were incubated with lipopolysaccharide (10 ng/mL) or plasma. Inflammatory cytokines were screened using dot-blot. Toll-like receptor 4 and nuclear factor κ B activation and cellular localization of the alpha-1 adrenergic receptor were measured by immunofluorescence imaging and Western blot analysis. Clustered regularly interspaced short palindromic repeats/Cas9 was used to knock out Toll-like receptor 4, and calcium influx following stimulation with phenylephrine was recorded.

MAIN RESULTS: Trauma/hemorrhagic shock caused a decreased response to phenylephrine, whereas Toll-like receptor 4 inhibition improved blood pressure. Trauma/hemorrhagic shock plasma activated the Toll-like receptor 4/nuclear factor κ B pathway in smooth muscle cells. Double labeling of Toll-like receptor 4 and the alpha-1 adrenergic receptor showed that these receptors are colocalized on the cell membrane. Activation of Toll-like receptor 4 caused cointernalization of both receptors. Calcium influx was impaired in cells incubated with trauma/hemorrhagic shock plasma but restored when Toll-like receptor 4 was knocked out or inhibited.

CONCLUSIONS: Activation of the Toll-like receptor 4 desensitizes vascular smooth muscle cells to vasopressors in experimental trauma/hemorrhagic shock by reducing the levels of membrane alpha-1 adrenergic receptor.

KEY WORDS: adrenergic receptor; biological availability; shock hemorrhagic; Toll-like receptor 4; vascular smooth muscle; vasoconstrictor

Trauma/hemorrhagic shock (T/HS) is a major cause of death worldwide and is the leading cause of death in those 1–44 years in the United States (1, 2). During late stages of ongoing hemorrhage, vasodilatory shock and hypotension may ensue that are refractory to fluid resuscitation or blood transfusion (3).

Vasodilatory shock includes multiple etiologies (e.g., late hemorrhagic, septic, cardiogenic, neurogenic, and anaphylactic shock) and ultimately results

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in uncontrolled vessel relaxation. The mechanism is multifactorial and includes activation of vasodilatory pathways and vascular hyporesponsiveness to vaso-pressors, leading to arterial hypotension, multiple organ failure, and death (4–7).

Contraction of arteriolar smooth muscle cells (SMCs) is mediated primarily by α -1 adrenergic receptors (α 1ARs), G-protein–coupled receptors that are important mediators of the sympathetic nervous system involved in vasoconstriction. α 1ARs are activated by catecholamines such as phenylephrine and norepinephrine, and their activation leads to signal transduction events, including activation of phospholipase C and generation of inositol triphosphate and diacylglycerol, which cause increases in intracellular calcium and vessel constriction (6).

Recalcitrant hypotension following T/HS is implicated in the release of inflammatory mediators which lead to multiple organ dysfunction syndrome, similar to what is seen in septic patients (7, 8). Indeed, inhibition of inflammation prevents end organ damage in a rat model of T/HS (9, 10). A pivotal mediator of inflammatory cytokine release seen in T/HS is activation of the nuclear factor κ B (NF- κ B) transcription pathway, which correlates with the severity of the acute inflammatory response (11, 12). When activated, the associated protein nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α undergoes phosphorylation, leading to the translocation of p65 into the nucleus, where it promotes the transcription of many inflammatory cytokines (13). Specifically for the α 1AR, studies have shown a dysfunctional contractile response in SMCs following activation of NF- κ B, whereas inhibition of this pathway restores normal contractility (14, 15).

A putative activator of the NF- κ B is the Toll-like receptor 4 (TLR4) (16, 17). TLR4 is implicated in poor outcomes from shock, and its inhibition serves to mitigate its deleterious effects in several experimental models (10, 18, 19). When activated (e.g., by Lipopolysaccharides [LPS]), the receptor initiates a signal transduction cascade to induce inflammation leading to increased production of inflammatory cytokines such as interleukin-6, tumor necrosis factor- α , and others (13, 20) and is rapidly endocytosed into endosomes (21, 22).

Although ample evidence describes TLR4 activation by bacterial substances such as LPS, recent data show

activation of TLR4 in conditions where no bacterial infection is present, where it is involved in mediating inflammation and organ damage, and its inhibition results in a favorable outcome (10, 16, 17). In these cases, the activating agents are damage-associated molecular pattern molecules, endogenous molecules released after cellular damage or stress, such as fibrinogen, high mobility group box-1 (HMGB1), and others (23–26).

A role for TLR4 and NF- κ B activation in mediating the α 1AR response was suggested in shock. Inhibition of NF- κ B served to restore the otherwise decreased levels of α 1AR following circulatory failure, and Eritoran, a TLR4 inhibitor, prevented LPS-induced loss of contractility in a mouse model of septic shock (9, 18).

Given the demonstrated contribution of TLR4 to the outcomes of circulatory shock and the modulation of the adrenergic response by this receptor, we sought to determine the mechanism for decreased α 1AR response seen following experimental T/HS in rats. A decreased translation and expression of the α 1AR, as is seen hours into septic shock, cannot fully explain the rapid decrease in vascular response to vasopressors seen in T/HS. Thus, we tested the hypothesis that activation of TLR4 during T/HS will result in a rapid decrease in the bioavailability of α 1AR on the cell surface, rendering the vascular system resistant to vasoconstrictor administration.

METHODS

Animals

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of California, San Diego (approval number: S16062). Animals were housed in a vivarium with controlled temperature (20–22°C) and humidity (70–75%), and light-dark cycles were maintained according to standardized procedures for animal housing. Food and water were supplied ad libitum, and the health of the animals was visually verified before the experiments. Fifteen 250–400 g male Wistar rats (Harlan Laboratories, Indianapolis, IN) were used for the experiments. The TLR4 inhibitors resatorvid (TAK-242) (1 mg/kg; Sigma) and resveratrol (10 mg/kg) were administered to the experimental groups ($n = 5$) before induction of T/HS. A control group was subjected to T/HS without the administration of TLR4 inhibitors.

Anesthesia was induced by 5% isoflurane and maintained with 1.5% through a nose cone at a rate of 0.8 L/min at 21% FIO₂. The right femoral vein and artery were cannulated for blood withdrawal, drug administration, and monitoring of blood pressure (Power Laboratory acquisition system; ADInstruments, Dunedin, New Zealand). Body temperature was maintained at 37°C by a water-heated platform. T/HS was induced by laparotomy followed by blood withdrawal (0.5 mL/min) to a target mean arterial pressure (MAP) of 40 mm Hg. Pressure was maintained between 35 and 45 mm Hg for 1.5 hours followed by reperfusion with Lactated Ringers (LR) solution (2 mL/min) to achieve a MAP of 65 mm Hg, and the animals were monitored for an additional 120 minutes. To measure acute vascular response to an $\alpha 1$ agonist, following 20 minutes of baseline pressure measurement (animals under anesthesia without any interaction or intervention), a single bolus of phenylephrine (4 μ g/kg) was IV infused, and blood pressure increase was recorded. The effect lasted for ~10 seconds, and after 1 minute, the blood pressure returned to preinfusion values. This procedure was repeated 120 minutes following reperfusion. Animals were subsequently euthanized, and plasma was collected.

Cell Culture

Rat SMCs (SMCs; Cell-applications, San Diego, CA) were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were maintained at 37°C in a CO₂ incubator with a controlled atmosphere composed of 95% air and 5% CO₂. Before experiments, the complete media was replaced with serum-free media for 2 hours. Cells were incubated with plasma (1:4 plasma/media) from the experimental and control groups. For in vitro activation of TLR-4, cells were incubated with LPS (10 ng/mL) in serum-free media. To knock out TLR4, single guide RNA clustered regularly interspaced short palindromic repeats All-in-One Lentivirus set (ABM, Vancouver, BC, Canada) was used. Briefly, vectors were amplified in DH5 α cells and purified using a miniprep kit (Qiagen, Valencia, CA). SMCs were transfected using lipofectamine 2000 (Invitrogen, Waltham, MA) and selected using puromycin (0.3 μ g/mL). TLR4 deletion was confirmed by Western blot.

Western Blot

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes in transfer buffer (20% methanol, 25 mmol/L tris, 192 mmol/L glycine). Signal was detected using the chemiluminescence reagents of the EZ-ECL kit (Pierce, Waltham, MA). The following antibodies were used: mouse anti-TLR4 (Abcam, Waltham, MA), rabbit anti- $\alpha 1$ AR (Abcam), rabbit anti-NF- κ B P65 (Cell Signaling, Danvers, MA), goat antiphospho-inhibitor of nuclear factor kappa B (I κ B) (Cell Signaling), and goat anti-I κ B (Cell Signaling).

Immunofluorescent Imaging

Cells were fixed for 10 minutes in 2% paraformaldehyde, blocked in 5% normal donkey serum in 0.1% Tris Buffered Saline Buffer with Tween 20 for 1 hour followed by overnight incubation with primary antibody. Following washes, slides were incubated with fluorescein isothiocyanate/Texas Red secondary antibodies and imaged by fluorescent microscopy.

Cytokine Detection

Cytokines in the plasma of rats before and after T/HS were evaluated using the Proteome Profiler Rat Cytokine Array Kit (R&D Systems, Minneapolis, MN). The panel consists of 29 cytokines and is semiquantitative by the use of a reference between samples. HMGB1 levels were measured using a commercial enzyme-linked immunosorbent assay (LSBio, Seattle, WA).

Calcium Imaging

Cells were incubated for 2 hours with plasma from the experimental groups. Following the addition of phenylephrine (1 ng/mL), intracellular calcium was measured using the Fluo-4 AM tracer kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR).

Statistics

Values are presented as means \pm sd. Data were analyzed using two-way analysis of variance (ANOVA) for repeated measurements. When appropriate, post hoc analyses were performed with the Tukey multiple comparisons test. Bands and fluorescence intensities were measured using the NIH Image-J software. A paired or

unpaired two-tailed Student *t* test was used for comparisons between groups as applicable. Results were determined to be significant at *p* value of less than 0.05.

RESULTS

Decreased Blood Pressure Response to Vasopressors After T/HS

To validate vasopressor resistance in our model, the vascular response to phenylephrine was measured in terms of increase in systolic blood pressure (SBP) (Fig. 1). Phenylephrine induced a rapid increase in SBP before T/HS, which then returned to higher levels than baseline for up to 30 seconds (Fig. 1A). Analyzing the difference in peak response, phenylephrine caused SBP to increase by an average of 32 mm Hg before T/HS but only by 20 mm Hg following reperfusion (*p* < 0.05) (Fig. 1B).

Plasma From T/HS Activates NF- κ B in SMCs

T/HS plasma incubated with SMCs caused a rapid phosphorylation of I κ B which was evident after 10 minutes, peaked after 20 minutes, and remained elevated over 30 minutes. In contrast, when incubated with control plasma, increase in phospho-I κ B was detected only at 20 minutes and returned to baseline after 30 minutes. For each time point measured, the increase in I κ B phosphorylation was higher in T/HS plasma-treated cells

(Fig. 2A). In addition, translocation of p65 to the nuclei was induced by T/HS but not control plasma, as evidence by immunofluorescence imaging (Fig. 2B) and Western blot analysis of p65 nuclei extracts (Fig. 2C).

Activation of TLR4 Induces Endocytosis of the α 1AR

SMCs were incubated with control and T/HS plasma, as well as LPS, a specific activator of TLR4. In untreated cells, colabeling of TLR4 and α 1AR showed colocalization that was mostly confined to the cell membrane (Fig. 3A). Coculture with LPS or T/HS plasma caused internalization of receptors as seen on fluorescent imaging (Fig. 3A). To confirm α 1AR internalization following TLR4 stimulation, we stimulated SMCs with either T/HS plasma or LPS and measured the expression of the membrane versus cytosolic α 1AR. As shown in Figure 3B–E, in resting cells, α 1AR is mainly expressed in the membrane with low intracellular expression. Both T/HS plasma and LPS caused an increase in intracellular expression concomitantly with reduced membrane expression (*p* < 0.05) vs untreated control), confirming α 1AR internalization following TLR4 stimulation. It should be emphasized, that although internalization was evident following treatment with both activators, the ratio of extra- versus intracellular expression was lower in LPS-treated cells compared with both control- and T/HS-treated cells.

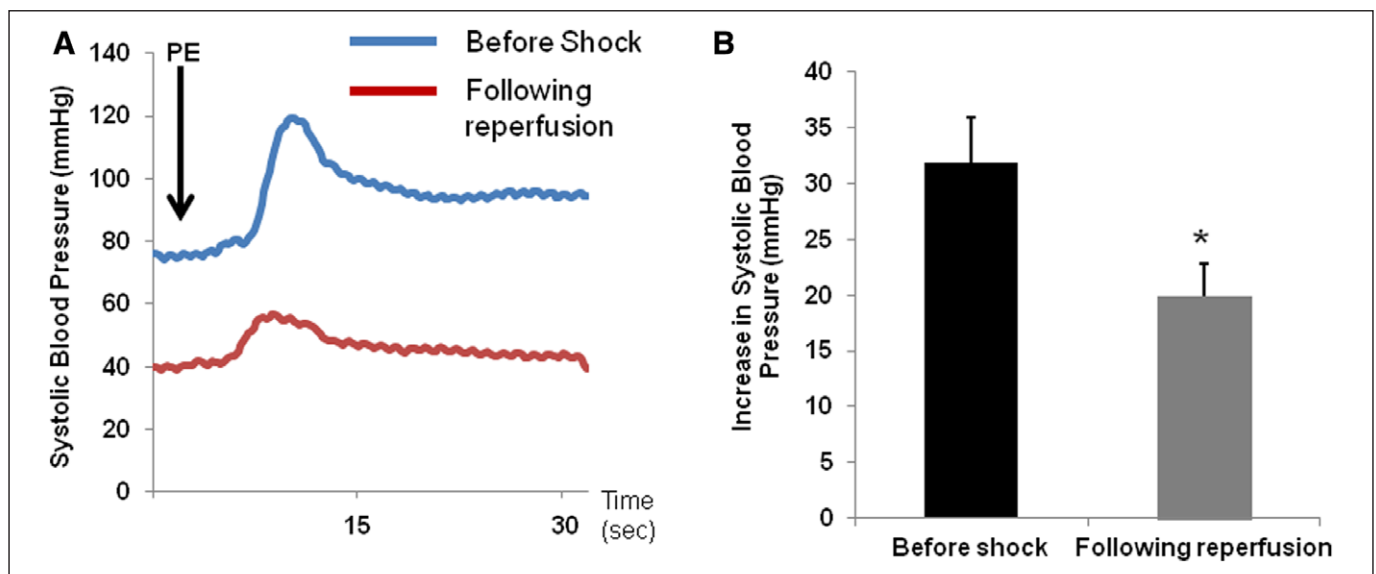


Figure 1. Trauma/hemorrhagic shock (T/HS) induces vasopressor resistance. **A**, A representative systolic blood pressure (SBP) response to a single phenylephrine (PE) dose before T/HS and following reperfusion. **B**, Difference in SBP response to PE following T/HS. PE (2 μ g/kg) was injected before T/HS and 2 hr following resuscitation with Lactated Ringers solution. **p* < 0.05 SBP following resuscitation versus before shock; *n* = 5.

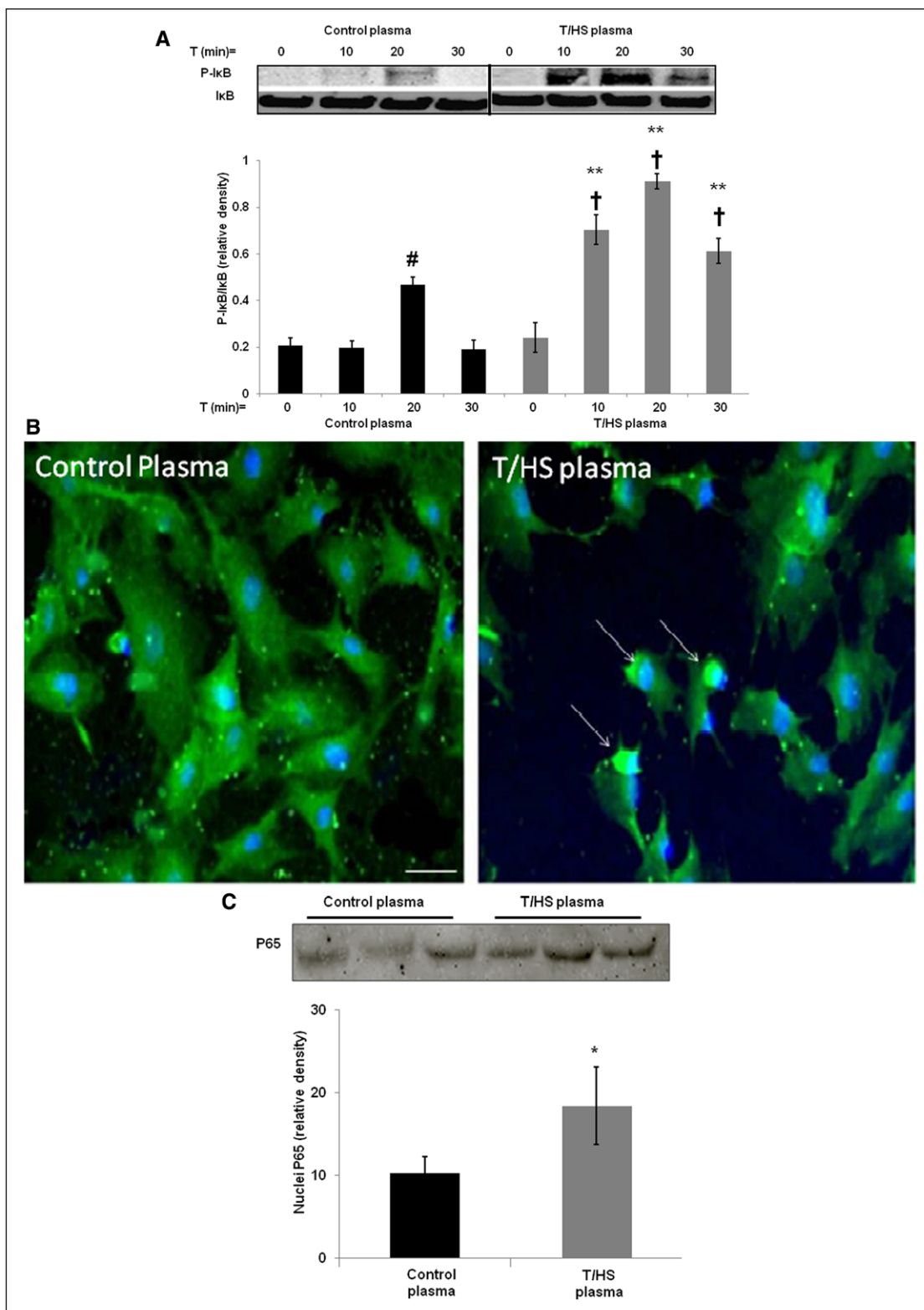


Figure 2. The nuclear factor κ B pathway is activated by trauma/hemorrhagic shock (T/HS) plasma. **A**, Smooth muscle cells were treated with plasma from T/HS and control group. I κ B phosphorylation was measured at selected time points, and results are presented as mean of the relative density units of the ratio between the phosphorylated and nonphosphorylated forms. [†] $p < 0.01$ for T/HS treated cells at 10, 20, and 30 min post stimulation versus unstimulated cells; [#] $p < 0.05$ for cells treated with control plasma at 20 min versus unstimulated cells; [†] $p < 0.01$ T/HS plasma versus control plasma at corresponding time points. $n = 3$ for all groups. **B**, Immunofluorescence labeling of P65 demonstrating nuclei localization following treatment with T/HS plasma (white arrows; bar = 10 μ m). **C**, Western blot analysis of P65 in nuclei extracts from cells treated with T/HS plasma versus controls. $p < 0.05$, $n = 3$.

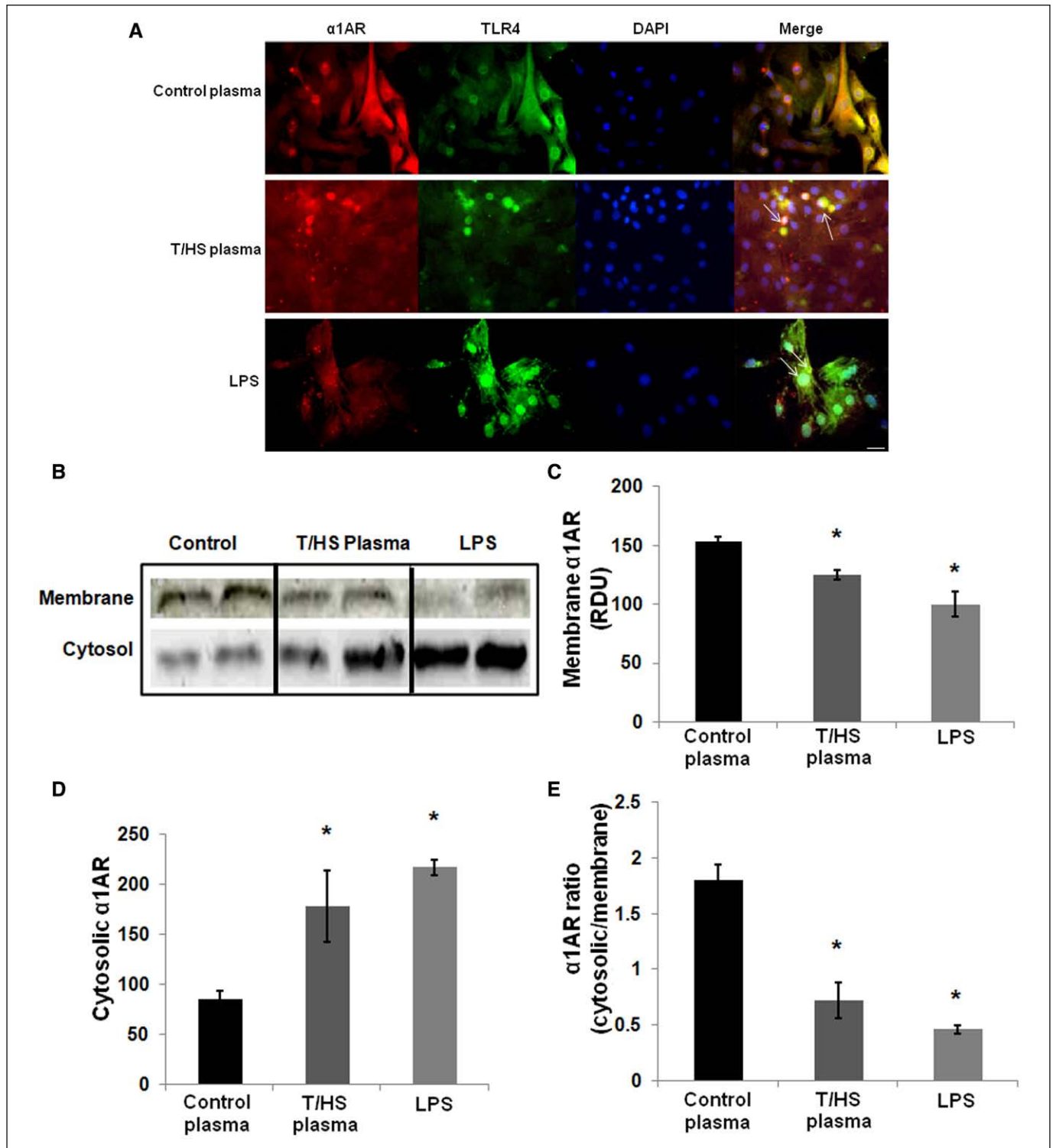


Figure 3. A, Double immunofluorescence imaging of the alpha-1 adrenergic receptor (α1AR) and Toll-like receptor 4 (TLR4) in cells treated with lipopolysaccharide (LPS) or plasma from trauma/hemorrhagic shock (T/HS) or control rats. *Red* indicates the localization of the α1AR; *green* indicates the localization of the TLR4, and *blue* (DAPI) was used to stain the cell nuclei. Colocalization (*white arrows*) is indicated in *yellow* in the merged images, bar = 10 μm. **B**, Representative Western blot for detection of α1AR in membrane and cytosolic fractions of cells treated with control and T/HS plasma as well LPS. **C** and **D**, Western blot analysis for the levels of membrane (**C**) and cytosolic (**D**) α1AR in the experimental groups. **E**, Ratio between membrane and cytosolic expression of the α1AR. Data are expressed as mean density units ± sd. * $p < 0.05$ for T/HS and LPS versus control; $n = 6$. DAPI = 4',6-diamidino-2-phenylindole, RDU = relative density unit.

TLR4 Inhibition Increases Calcium Influx Upon α 1AR Stimulation

We used calcium imaging to measure the response of SMCs to stimulation with phenylephrine following incubation with T/HS or control plasma. Decreased calcium labeling intensity was recorded when cells were preincubated with T/HS plasma compared with control plasma (24.6 ± 9.3 vs 49.9 ± 17.5 mean fluorescent intensity [MFI]; $p < 0.01$) (**Fig. 4, A and B**). Knocking out TLR4 (TLR4^{-/-}) served to restore the otherwise decreased calcium labeling due to T/HS plasma (36.9 ± 12.8 MFI; $p < 0.05$ vs T/HS plasma). Last, we treated the cells with resveratrol to block TLR4 (19, 27). As with the TLR4^{-/-}, resveratrol restored calcium labeling intensity in cells treated with T/HS plasma (43.8 ± 17.6 MFI; $p < 0.01$ vs T/HS).

TLR4 Inhibition Improves Blood Pressure Following T/HS

We administered resveratrol as well as TAK-242 prior to induction of T/HS and measured SBP following a single phenylephrine bolus during resuscitation. SBP values were higher in the TLR4 inhibited groups compared with the control ($p < 0.05$ TAK-242 vs LR; $p < 0.01$ resveratrol vs LR) (**Fig. 4C**). TAK-242 improved SBP during resuscitation, and the values had a downward trend up to 220 minutes. This decrease in SBP was not apparent in the resveratrol group ($p < 0.05$ resveratrol vs TAK-242) which remained stably increased.

Inflammatory Cytokines Are Elevated Following T/HS

To address possible activators of TLR4 in T/HS, we screened for inflammatory cytokines as well as measured levels of HMGB1 (**Fig. 5**). Of 21 cytokines detectable on our dot-blot screen, we found 10 to be significantly increased following T/HS (Figure 5, **A and B**). In addition, HMGB1 significantly increased from time 0 to 220 minutes after reperfusion (1.05 ± 0.25 pg/mbbL vs 1.81 ± 0.3 pg/mL respectively; $p < 0.01$) (**Fig. 5C**).

DISCUSSION

In the present study, we sought to determine a role for TLR4 in vasopressor resistance following T/HS. Our results indicate that T/HS induces activation of the TLR4/NF- κ B pathway and promotes cointernalization

of the receptor together with the α 1AR, thus reducing its bioavailability. Inhibition of TLR4 served to restore α 1AR response to the vasopressor phenylephrine, explaining, at least in part, vasopressor resistance seen following severe T/HS.

Hypotension refractory to fluids and vasopressor agents is a universal phenomenon in advanced shock states. Among the mechanisms contributing to resistant hypotension is adrenergic receptor desensitization in vascular smooth muscle (4, 5). In agreement, we found that following resuscitation, the vascular response to phenylephrine was markedly reduced compared with the preshock response, confirming vasopressor resistance in our model.

Data suggest involvement of inflammation and activation of the NF- κ B pathway in end organ damage following T/HS, where activation of NF- κ B in SMCs promotes dysfunctional contraction, and inhibition of this pathway restores normal contractility (14, 15). Thus, we focused our efforts to study whether inflammation can also be a link between T/HS and vasopressor resistance.

We found that the NF- κ B pathway is activated in SMCs by T/HS plasma, as evidenced by increased phosphorylation of I κ B and nuclear translocation of p65 (13). Our results are in agreement with others who have shown different mechanisms for NF- κ B activation and subsequent inflammation in T/HS, even before resuscitation, such as oxidative stress, cytokine release, and others (11, 12, 28).

Of specific interest in shock is activation of the TLR4, as its inhibition serves to mitigate its deleterious effects in several experimental models (10, 18, 19). We thus used TLR4 internalization as a marker for the receptor activation in SMCs and found that T/HS plasma, as for LPS, induced rapid internalization of the receptor into the cell cytosol.

We found that activation of TLR4 induces desensitization of the α 1AR, thereby explaining in part the decreased response to phenylephrine following T/HS. In other experimental models of shock, activation of TLR4, NF- κ B, and inflammatory cytokines have been described as promoting down-regulation of the α 1AR, decreasing overall sensitivity to vasopressors (4, 29, 30). However, this down-regulation is recorded hours into shock and cannot explain the short time-frame for decreased vasopressor response following T/HS. Thus, we hypothesized that another mechanism

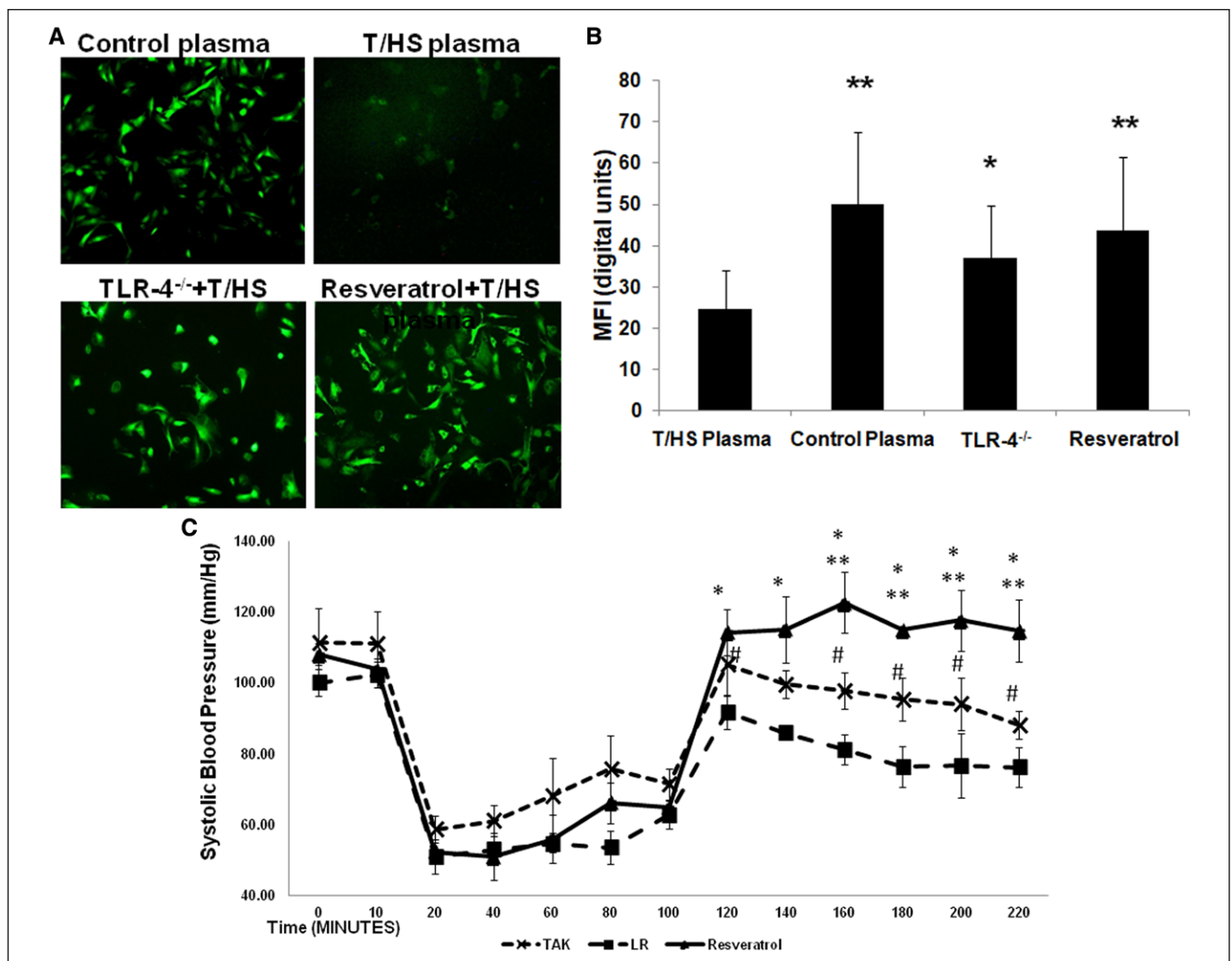


Figure 4. Toll-like receptor-4 (TLR-4) inhibition restores calcium influx. **A**, Representative fluorescence labeling of calcium influx as detected by the Fluo-4 AM tracer (Molecular Probes, OR). Smooth muscle cells were incubated with plasma from control, trauma/hemorrhagic shock (T/HS), and T/HS animals treated with resveratrol. In addition, TLR-4 was knocked out, and cells were incubated with T/HS plasma. **B**, Image analysis of calcium influx. Data are presented as digital units of the mean fluorescent intensity (MFI) \pm sd. Results are derived from three experiments and calculated for $n = 100$ cells. $^{**}p < 0.01$ T/HS versus control, resveratrol and TLR-4^{-/-}; $^{*}p < 0.05$ TLR-4^{-/-} and resveratrol versus T/HS plasma. **C**, Systolic blood pressure values following resuscitation in rats pretreated with the TLR4 inhibitors resatorvid (TAK-242) and resveratrol. $^{*}p < 0.05$ TAK-242 versus Lactated Ringers (LR); $^{*}p < 0.01$ resveratrol versus LR; $^{*}p < 0.05$ resveratrol versus TAK-242; $n = 5$ for resveratrol and LR, $n = 3$ for TAK-242.

might be dependent on the spatial location of the $\alpha 1$ AR and TLR4. We found that both receptors are colocalized on the membrane of SMCs, and once TLR4 is activated, it internalizes together with the $\alpha 1$ AR into intracellular vesicles. Colocalization promoting cointernalization has been previously described for other receptors; the adenosine A2A receptor cointernalizes with the dopamine D2 receptor, and activation of either will decrease their activity (31). Likewise, the angiotensin II AT1 receptor and the bradykinin B2 receptor colocalize in SMC membranes, and their cointernal-

ization reduces B2 receptor bioavailability (32, 33). Finally, CD13, a membrane metalloproteinase, is cointernalized with TLR4 and modulates the receptor activity (34). Thus, it is plausible that the $\alpha 1$ AR, due to its cellular localization with TLR4, is cointernalized once the receptor is activated, explaining the reduced vasoconstrictor response in T/HS.

To further characterize the physiologic relevance of reduced $\alpha 1$ AR bioavailability following TLR4 stimulation, we measured intracellular calcium flux as a marker for cell activation following stimulation with

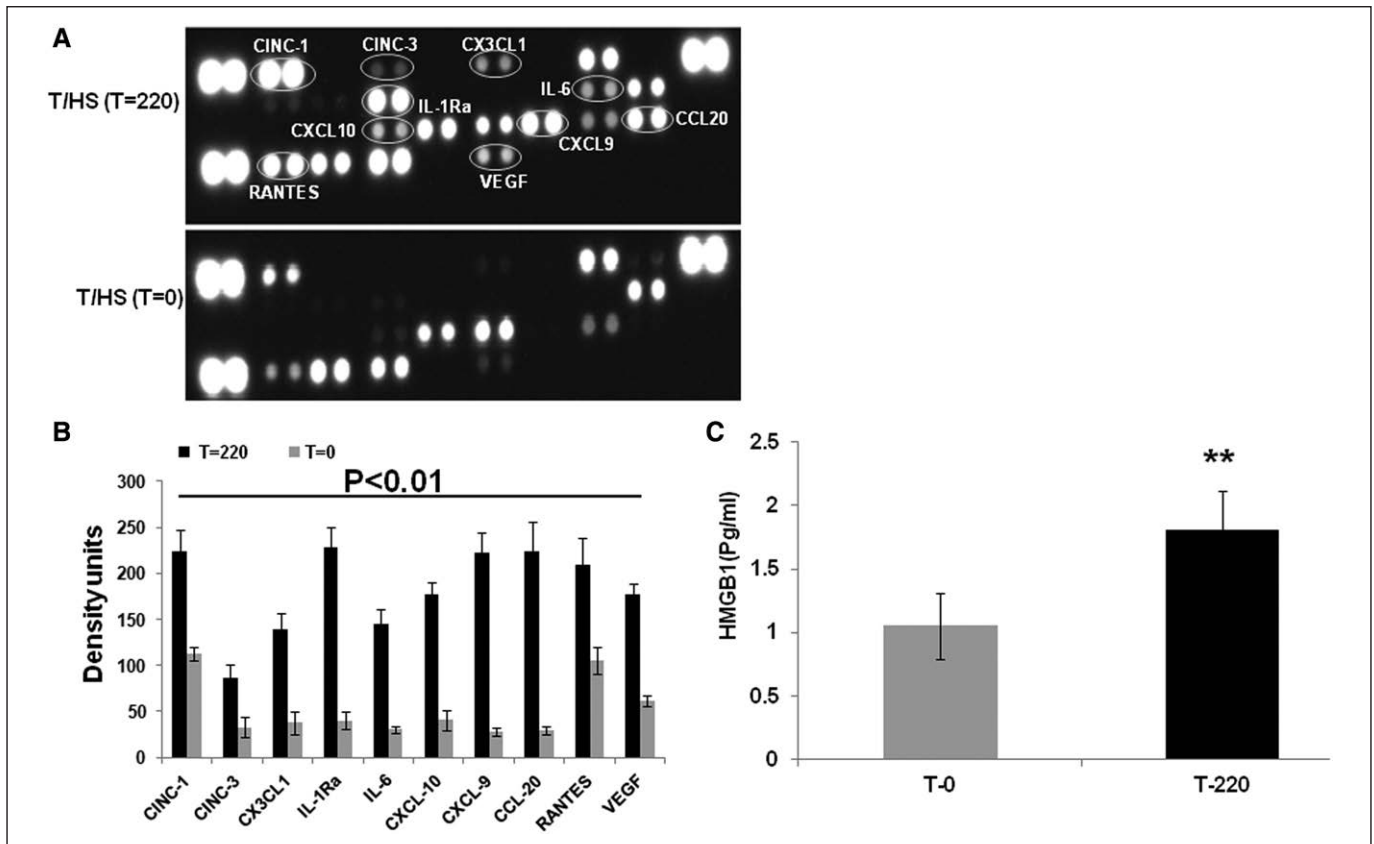


Figure 5. Trauma/hemorrhagic shock (T/HS) promotes secretion of cytokines. **A** and **B**, A Representative dot-blot for inflammatory cytokines screen in response to T/HS. Plasma was collected before shock and 220 min following resuscitation with LR. From the panel available, we found 10 cytokines that were at least $\times 2.5$ higher after shock. Data are presented as the mean density units \pm SD; $^{**}p < 0.01$ plasma at T = 220 min versus plasma at baseline; $n = 3$. **C**, Levels of high mobility group box-1 (HMGB1) were measured by enzyme-linked immunosorbent assay, $^{**}p < 0.01$ plasma at T = 220 min versus plasma at baseline; $n = 5$. IL = interleukin.

phenylephrine (35). Using the Fluo-4 calcium indicator for its ability to allow stable calcium imaging under normal imaging techniques, we found increased signal in cells treated with control plasma that was markedly decreased when cells were preincubated with T/HS plasma. Knocking out TLR4 as well as pharmacologic inhibition of the receptor with resveratrol (19, 27) served to restore calcium signaling. Other studies have shown that cytokine-induced calcium influx is inhibited in pulmonary artery SMCs treated with a TLR4 inhibitor (36), supporting our data pointing to decreased response to vasopressors following TLR4 stimulation.

Given our findings showing TLR4 dependent reduction in $\alpha 1$ AR bioavailability and response to phenylephrine, we examined in vivo the effect of TLR4 inhibition on blood pressure response to phenylephrine following T/HS. We used resveratrol and TAK-242, which were reported to have beneficial effects in other models of shock through inhibition

of TLR4 and inflammation (19, 37). We found that TLR4 inhibition increased SBP response, with resveratrol being the most effective. The superiority of resveratrol over the specific TLR4 inhibitor TAK-242 may be explained by its additional attributes such as antidiabetic, anti-inflammatory, antioxidant, and cardioprotective properties that may be beneficial in shock (38). Nevertheless, by using two different pharmacologic inhibitors, our results unequivocally show that vasopressor resistance is significantly decreased by TLR4 inhibition.

In an attempt to address possible TLR4 activators following T/HS, we screened plasma before and after T/HS for increased inflammatory cytokines. We detected over 10 different inflammatory mediators that were over two-fold expressed in plasma following T/HS. Of specific interest, CXC motif chemokine-10 and HMGB1 have been shown to act as TLR4 agonists (25, 39), whereas HMGB1 suppresses vascular reactivity in septic shock (40).

To summarize, our studies in this T/HS rat model suggest a role for TLR4 in desensitization of the $\alpha 1$ AR through a mechanism that involves cointernalization of both receptors and reduced bioavailability of the $\alpha 1$ AR on the cell membrane. This decreased expression explains, at least in part, the decreased vasopressor response seen in these animals following experimental T/HS. Some limitations are associated with our study: the relatively small number of animals and cell culture experiments need to be broadened to fully understand the kinetics and exact mechanism by which TLR4 affects the bioavailability of the $\alpha 1$ AR. Furthermore, LPS as a result of bacterial translocation is increased following shock (41), and its levels were not controlled in the conditioned media. However, inhibition of TLR4 activation improved vasopressor response, and hence supports future studies aimed at evaluating the therapeutic value of TLR4 inhibition in T/HS. Furthermore, studies are needed to identify potential activators of TLR4 in T/HS. Inhibition of such mediators and their subsequent effects in T/HS remain to be elucidated.

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REFERENCES

- Owattanapanich N, Chittawatanarat K, Benyakorn T, et al: Risks and benefits of hypotensive resuscitation in patients with traumatic hemorrhagic shock: A meta-analysis. *Scand J Trauma Resusc Emerg Med* 2018; 26:107
- Scerbo MH, Holcomb JB, Taub E, et al: The trauma center is too late: Major limb trauma without a pre-hospital tourniquet has increased death from hemorrhagic shock. *J Trauma Acute Care Surg* 2017; 83:1165–1172
- Dalibon N, Schlumberger S, Saada M, et al: Haemodynamic assessment of hypovolaemia under general anaesthesia in pigs submitted to graded haemorrhage and retransfusion. *Br J Anaesth* 1999; 82:97–103
- Levy B, Collin S, Sennoun N, et al: Vascular hyporesponsiveness to vasopressors in septic shock: From bench to bedside. *Intensive Care Med* 2010; 36:2019–2029
- Levy B, Fritz C, Tahon E, et al: Vasoplegia treatments: The past, the present, and the future. *Crit Care* 2018; 22:52
- Graham RM, Perez DM, Hwa J, et al: $\alpha 1$ -adrenergic receptor subtypes. Molecular structure, function, and signaling. *Circ Res* 1996; 78:737–749
- Shenkar R, Coulson WF, Abraham E: Hemorrhage and resuscitation induce alterations in cytokine expression and the development of acute lung injury. *Am J Respir Cell Mol Biol* 1994; 10:290–297
- Eltzschig HK, Carmeliet P: Hypoxia and inflammation. *N Engl J Med* 2011; 364:656–665
- Yang L, Dong X: Inhibition of inflammatory response by crocin attenuates hemorrhagic shock-induced organ damages in rats. *J Interferon Cytokine Res* 2017; 37:295–302
- Korff S, Loughran P, Cai C, et al: Eritoran attenuates tissue damage and inflammation in hemorrhagic shock/trauma. *J Surg Res* 2013; 184:e17–e25
- Altavilla D, Saitta A, Guarini S, et al: Oxidative stress causes nuclear factor-kappaB activation in acute hypovolemic hemorrhagic shock. *Free Radic Biol Med* 2001; 30:1055–1066
- Guarini S, Altavilla D, Cainazzo MM, et al: Efferent vagal fibre stimulation blunts nuclear factor-kappaB activation and protects against hypovolemic hemorrhagic shock. *Circulation* 2003; 107:1189–1194
- Liu T, Zhang L, Joo D, Sun S-C: NF- κ B signaling in inflammation. *Signal Transduct Target Ther.* 2017;2:17023.
- Park M, Choi S, Kim S, et al: NF- κ B-responsive miR-155 induces functional impairment of vascular smooth muscle cells by downregulating soluble guanylyl cyclase. *Exp Mol Med* 2019; 51:1–12
- Shi XZ, Lindholm PF, Sarna SK: NF-kappa B activation by oxidative stress and inflammation suppresses contractility in colonic circular smooth muscle cells. *Gastroenterology* 2003; 124:1369–1380
- Küper C, Beck FX, Neuhofer W: Toll-like receptor 4 activates NF- κ B and MAP kinase pathways to regulate expression of proinflammatory COX-2 in renal medullary collecting duct cells. *Am J Physiol Renal Physiol* 2012; 302:F38–F46
- Kawai T, Akira S: Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med* 2007; 13:460–469
- Ehrentauf S, Frede S, Stapel H, et al: Antagonism of lipopolysaccharide-induced blood pressure attenuation and vascular contractility. *Arterioscler Thromb Vasc Biol* 2007; 27:2170–2176
- Wang B, Bellot GL, Iskandar K, et al: Resveratrol attenuates TLR-4 mediated inflammation and elicits therapeutic potential in models of sepsis. *Sci Rep* 2020; 10:18837
- Kagan JC, Su T, Horng T, et al: TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* 2008; 9:361–368
- Tan Y, Zononi I, Cullen TW, et al: Mechanisms of toll-like receptor 4 endocytosis reveal a common immune-evasion strategy used by pathogenic and commensal bacteria. *Immunity* 2015; 43:909–922

22. Zanoni I, Ostuni R, Marek LR, et al: CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 2011; 147:868–880
23. Reino DC, Palange D, Feketeova E, et al: Activation of toll-like receptor 4 is necessary for trauma hemorrhagic shock-induced gut injury and polymorphonuclear neutrophil priming. *Shock* 2012; 38:107–114
24. Fan J, Li Y, Levy RM, et al: Hemorrhagic shock induces NAD(P) H oxidase activation in neutrophils: Role of HMGB1-TLR4 signaling. *J Immunol* 2007; 178:6573–6580
25. Yang WS, Han NJ, Kim JJ, et al: TNF- α activates high-mobility group box 1 - Toll-like receptor 4 signaling pathway in human aortic endothelial cells. *Cell Physiol Biochem* 2016; 38:2139–2151
26. Yu L, Wang L, Chen S: Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med* 2010; 14:2592–2603
27. Tong W, Chen X, Song X, et al: Resveratrol inhibits LPS-induced inflammation through suppressing the signaling cascades of TLR4-NF- κ B/MAPKs/IRF3. *Exp Ther Med* 2020; 19:1824–1834
28. Altavilla D, Saitta A, Guarini S, et al: Nuclear factor-kappaB as a target of cyclosporin in acute hypovolemic hemorrhagic shock. *Cardiovasc Res* 2001; 52:143–152
29. Schmidt C, Kurt B, Höcherl K, et al: Inhibition of NF-kappaB activity prevents downregulation of alpha1-adrenergic receptors and circulatory failure during CLP-induced sepsis. *Shock* 2009; 32:239–246
30. Burgdorff AM, Bucher M, Schumann J: Vasoplegia in patients with sepsis and septic shock: Pathways and mechanisms. *J Int Med Res* 2018; 46:1303–1310
31. Hillion J, Canals M, Torvinen M, et al: Coaggregation, coin-ternalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J Biol Chem* 2002; 277:18091–18097
32. Quitterer U, AbdAlla S: Vasopressor meets vasodepressor: The AT1-B2 receptor heterodimer. *Biochem Pharmacol* 2014; 88:284–290
33. Quitterer U, Pohl A, Langer A, et al: A cleavable signal peptide enhances cell surface delivery and heterodimerization of Cerulean-tagged angiotensin II AT1 and bradykinin B2 receptor. *Biochem Biophys Res Commun* 2011; 409: 544–549
34. Ghosh M, Subramani J, Rahman MM, et al: CD13 restricts TLR4 endocytic signal transduction in inflammation. *J Immunol* 2015; 194:4466–4476
35. Meininger GA, Zawieja DC, Falcone JC, et al: Calcium measurement in isolated arterioles during myogenic and agonist stimulation. *Am J Physiol* 1991; 261:H950–H959
36. Dai M, Xiao R, Cai L, et al: HMGB1 is mechanistically essential in the development of experimental pulmonary hypertension. *Am J Physiol Cell Physiol* 2019; 316:C175–C185
37. Sha T, Sunamoto M, Kitazaki T, et al: Therapeutic effects of TAK-242, a novel selective Toll-like receptor 4 signal transduction inhibitor, in mouse endotoxin shock model. *Eur J Pharmacol* 2007; 571:231–239
38. Rauf A, Imran M, Suleria HAR, et al: A comprehensive review of the health perspectives of resveratrol. *Food Funct* 2017; 8:4284–4305
39. Schulthess FT, Paroni F, Sauter NS, et al: CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. *Cell Metab* 2009; 9:125–139
40. Nishiike S, Hiramatsu T, Shiraishi M, et al: Relationship between vascular reactivity and expression of HMGB1 in a rat model of septic aorta. *J Anesth* 2013; 27:684–692
41. Jiang J, Bahrami S, Leichtfried G, et al: Kinetics of endotoxin and tumor necrosis factor appearance in portal and systemic circulation after hemorrhagic shock in rats. *Ann Surg* 1995; 221:100–106