Stress ›
The International Journal on the Biology of Stress
Volume 20, 2017 - Issue 1

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Original Research Report

Toll-like receptor 4 protects against stress-induced ulcers via regulation of glucocorticoid production in mice

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Pages 19-26 | Received 10 Sep 2015, Accepted 11 Aug 2016, Published online: 06 Dec 2016

Cite this article https://doi.org/10.1080/10253890.2016.1224843

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Abstract

Stress-induced gastric ulcer is an important life-threatening condition, while the molecular basis of its development is incompletely understood. Toll-like receptor 4 (TLR4), an innate immune pattern recognition receptor, can induce pro-inflammatory transcription, aggravating a stress ulcer. The present study found that TLR4 played a protective role in a mouse model of water immersion (23 °C) restraint stress. Wild-type (WT) and TLR4$^{-/-}$ male mice were respectively divided into five groups (5 per group), and exposed to the stressor for 0, 0.5, 1, 2, or 4 hours. Gastric ulcer index, determined post mortem, increased with time in both types of mice but was greater in TLR4$^{-/-}$ mice. Furthermore, increased serum cortisol and corticosterone concentrations were observed in WT mice only, and such increases were detected only in WT mice 4 h after

administration of cortisol alleviated the gastric injury in TLR4$^{-/-}$ mice. Western blotting showed expression in the adrenal of P450scc (CYP11A1), the first rate-limiting enzyme in the synthesis of steroids, was increased 4 h after water immersion restraint stress or LPS treatment in WT mice, but was conversely decreased in TLR4$^{-/-}$ mice after either stressor. Furthermore, in adrenal glands of TLR4$^{-/-}$ mice, structural distortion of mitochondria (which contain CYP11A1) was found with electron microscopy, and lack of lipid-storing droplets was found using light microscopy on adrenal cryosections stained with Oil red O. These data indicate that TLR4 plays a protective role in stress-induced gastric ulcer that is exerted via impacting synthesis of glucocorticoid in the adrenal gland.

Keywords:

Cortisol glucocorticoid LPS stomach ulcer stress TLR4

Introduction

Stress may affect different physiological functions of the gastrointestinal tract including gastric secretion, motility, permeability and barrier function, visceral sensitivity, and mucosal blood flow (Nardone & Compare, 2014). Many stressful situations could lead to gastric ulcers, especially in an intensive care unit where stress-induced ulcer is a common and major complication, often causing hemorrhage or even death (Ali & Harty, 2009). The mechanisms underlying ulcer development are multifactorial and not fully understood. Damage to the gastric mucosa occurs through a series of events starting with the breakdown of protective defenses, followed by the aggravation of ulceration by physiological factors, including hypo-perfusion and increased release of catecholamines and pro-inflammatory cytokines (Quenot et al., 2009).

Endogenous glucocorticoid (predominantly corticosterone in rats) released during acute stress has gastro-protective effects against stress-induced gastric injury (Filaretova et al., 2004). The hypothalamic-pituitary-adrenocortical (HPA) axis is rapidly activated in response to acute stress (Aguilera, 2011), and pretreatment with corticotropin-releasing factor (CRF) or preconditioning with a mildly stressful stimulus has gastroprotective functions that involve corticosterone (Filaretova et al., 2008, 2012).

increases as a function of decreased corticosterone level (Yigiter et al., 2010). Moreover, intra-hypothalamic dexamethasone implantation which induces long-lasting stress-induced glucocorticoid deficiency results in aggravation of stress-induced gastric injury (Filaretova & Filaretov, 1992). The gastroprotective effects of glucocorticoids during acute stress involve multiple mechanisms, including the maintenance of glucose homeostasis, gastric mucosal blood flow, mucus production, and attenuation of gastric motility and microvascular permeability (Filaretova et al., 2004; Kuo et al., 2015; Podvigina et al., 2009), as well as changes in the gastric microcirculation (Filaretova et al., 1999). Although circulating corticosterone is considered to be the main glucocorticoid in the regulation of stress in rodents, cortisol also has a corresponding response to stimulation by stress in mice (Gong et al., 2015). Whether it impacts stress-induced gastric ulcer in mice remains to be determined.

TLR4 is an innate immune pattern recognition receptor, which is part of the Interleukin-1 Receptor/Toll-like Receptor Superfamily containing a toll-like/IL-1 Receptor (TIR) domain and a Leucine-rich repeat motif in the extracellular domain (Nardone & Compare, 2014). As the receptor for lipopolysaccharide (LPS), TLR4 can be activated by the LPS-lipopolysaccharide-binding protein (LBP)-myeloid differentiation protein 2 (MD2) complex, thus passing this signal to the downstream nuclear factor (NF)-kb pathway (Akira & Takeda, 2004). Several studies have reported that downregulation of NF-kb or inflammation mitigates gastric ulcer (Kang et al., 2014; Mahmoud-Awny et al., 2015; Sinha et al., 2015). Hence it has been speculated that TLR4 signaling may promote stress-induced gastric injury because of its function in modulating immune activity. However, previous research reported that TLR4 activation can stimulate the HPA axis (Monroe & Simons, 1991). TLR4 signaling can also induce the upregulation of CRF (CRH) gene expression in paraventricular nucleus neurons in the hypothalamus (Loum-Ribot et al., 2006). Thus TLR4 activation is considered to give protection against stress-induced gastric ulcer through mediation by the synthesis and release of glucocorticoid.

Taken together, the above findings indicate that the relationship between the TLR4 and stress ulcer is still unclear. Our preliminary study has shown activation of the TLR4 downstream NF-kB pathway could accelerate the pathogenesis of stress-induced gastric damage (Jia et al., 2007a). Herein, we hypothesized that activation of TLR4 also could enhance glucocorticoid synthesis, thus playing a protective role in the pathological ulceration process. In this study, functions of TLR4 in gastric injury and synthesis of glucocorticoid during stress and effects of cortisol on stress ulcer in mice were

Methods

Animals and management

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Military Medical University in Shanghai (IACUC-2012-XuSG) in accordance with the U.S.A. National Institutes of Health guidelines for the care and use of laboratory mice. TLR4 knockout ($TLR4^{-/-}$; C57BL/10ScNJU) and wild-type (WT;C57BL/6_129) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). The $TLR4^{-/-}$ mice were genetically identified in the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, Jiangsu, China). Male WT and $TLR4^{-/-}$ mice bred under specific pathogen-free conditions aged 6–8 weeks were used for experiments. Mice were housed three to five per cage and acclimatized to standard laboratory conditions (lights on between 08:00 h and 19:00 h, temperature 20 ± 1 °r, free access to food and water) for 7 days before the experiment.

Induction of ulcers by exposure to combined water immersion and restraint stress

WT and $TLR4^{-/-}$ mice were fasted for 24 h before experiments but given free access to tap water, and kept in two different cages with adequate capacity respectively. WT and $TLR4^{-/-}$ mice were restrained in perforated centrifuge tubes (50 ml) and immersed in 23 °C water in a 20-cm deep constant temperature water tank to the depth of the xiphoid process for 0, 0.5, 1, 2, and 4 h as described previously (Jia et al., 2007b). After the indicated times, mice were surgically anesthetized by intraperitoneal injection of sodium pentobarbital (1% w/v, 50 mg/kg), and blood samples were collected from the inferior vena cava and the stomach was removed to assess gastric damage. Blood samples were allowed to clot for 30 min at room temperature before centrifugation for 15 min at 1000 × g. Serum was removed and assayed immediately or stored at −80 °C until assay.

Evaluation of the gastroprotective effects of exogenous cortisol

WT and $TLR4^{-/-}$ mice were randomized (n = 5 per group) and injected subcutaneously 15 min prior to stress exposure with 4 mg/kg cortisol (0.4 mg/ml, Tocris Bioscience,

treatment and vehicle groups, respectively. A control group was untreated. After 4 h of water immersion restraint stress (Jia et al., 2007b), mice were anesthetized with sodium pentobarbital (as above) for blood sampling and stomach removal for the estimation of cortisol level and gastric injury, respectively.

Endotoxemia model

WT and TLR4$^{-/-}$ mice were randomized (n = 5 per group) and injected intraperitoneally with LPS (0.2 mg/ml, 2 mg/kg) (Sigma-Aldrich Corp., St. Louis, MO) or isotonic saline and killed 4 h later to determine the effect of TLR4 (receptor for LPS) on the regulation of endogenous glucocorticoids.

Corticotropin (adrenocorticotropic hormone, ACTH) test

WT and TLR4$^{-/-}$ mice were randomized and injected intraperitoneally with corticotropin (5 IU/ml, 50 IU/kg) (Shanghai NO.1 Biochemical & Pharmaceutical Co., LTD, Shanghai, China) or isotonic saline and killed after 4 h to measure serum cortisol and corticosterone.

Assessment of gastric damage

Stomachs were removed and filled with 2 ml of 1% formalin and immersed in formalin for 24 h. The stomachs then were cut along the length of the greater curvature and examined for mucosal lesions. The total length (mm) of linear hemorrhagic erosions was measured macroscopically as the ulcer index (UI) (mm) by an investigator blinded to the treatment conditions (Shimozawa et al., 2006). Part of each stomach tissue sample was used for pathological examination.

Measurement of serum cortisol, corticosterone, LPS, and lipopolysaccharide-binding protein (LBP) concentrations

Samples were allowed to clot for 30 min at room temperature in a serum separator tube and centrifuged for 15 min at 1000 × g. Cortisol and corticosterone concentrations in serum samples were measured using a cortisol EIA Kit (sensitivity: 0.11 ng/ml; coefficient of variation: intra-assay range 5.4%–9.2%, and inter-assay range 9.3%–21.2%) (R&D Systems, Minneapolis, MN) and a Corticosterone Parameter Assay Kit (sensitivity: 0.047 ng/ml; coefficient of variation: intra-assay range 4.5%–7.5%, and inter-assay range 5.6%–7.1%) (R&D Systems, Minneapolis, MN). LPS and LBP

immunosorbent assay kits (sensitivity: 0.039 ng/ml; coefficient of variation: intra-assay <8%, and inter-assay <10%) (Cusabio, Wuhan, China) and the mouse LBP enzyme-linked immunosorbent assay kits (sensitivity: 3.12 ng/ml; coefficient of variation: intra-assay <8%, and inter-assay <10%) (Cusabio, Wuhan, China).

Western blotting

Whole adrenal glands were lysed in protein extraction buffer [20 mM Tris (pH7.5), 150 mM NaCl, 1%Triton X-100, 1 mM EDTA] followed by centrifugation at 12,000 × g for 15 min at 4 °C. The supernatant was removed and total protein was determined (Bradford assay). Samples containing 40 µg protein were loaded, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (90 V), and blotted onto nitrocellulose membrane. The blots were probed with anti-CYP11A1 antibody (1/1000; Cell Signaling Technology) and with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1/2000; Santa Cruz Biotechnology). After extensive washing, protein bands were visualized by chemiluminescence staining. Blots were also probed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1/1000; Sigma–Aldrich) to confirm equal loading of protein. Expression of proteins was then calculated semiquantitatively as integrated optical density (IOD) ratio of CYP11A1 to GAPDH.

Electron microscopy

Adrenal glands of WT and TLR4$^{-/-}$ mice (3 per group) without any treatment were removed and fixed in 0.1-M phosphate buffer (pH 7.3) with 2% (v/v) formaldehyde and glutaraldehyde. Tissue sections were post-fixed for 90 min (2% OsO_4 in 0.1M cacodylate buffer, pH 7.3), dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) for 15 min at each step, and embedded in epoxy resin. Ultrathin sections (70–90 nm) were stained with uranyl acetate and lead citrate and examined at 80 kV using a CM 10 electron microscope (Philips, Best, Netherlands) to assess mitochondrial structure in cells of the zona fasciculata, which produce glucocorticoids from pregnenolone via action of lCYP11A1 located in the inner membrane of mitochondria to convert cholesterol to pregnenolone (Halkerston et al., 1961; Koritz & Kumar, 1970; Stone & Hechter, 1955).

Adrenal neutral lipid visualization

Frozen sections (7 µm, transverse sections) were prepared on a Leica CM3050-S

visualization. Nuclei were detected using a hematoxylin stain.

Statistical analysis

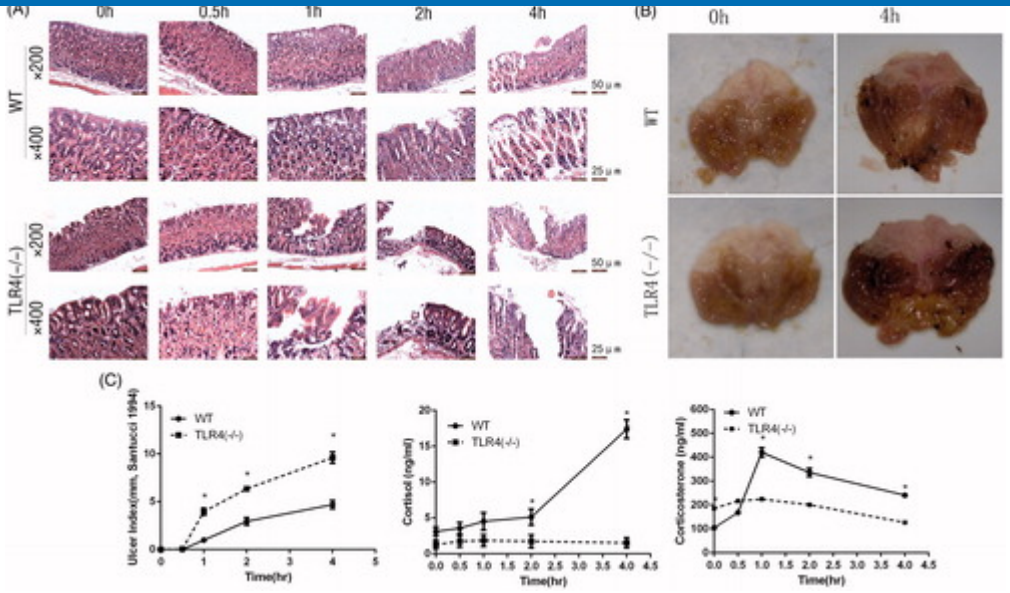
Data were analyzed by two-way ANOVA, one-way ANOVA, or Tukey's test of variance using SPSS v.20.0 software (SPSS Inc., Chicago, IL). Differences between groups were calculated using the least significant differences method, and significance level was defined as $p < .05$. Results are expressed as mean ± SEM.

Results

TLR4 deficiency exacerbates stress-induced gastric mucosal injury and modulates serum glucocorticoid concentrations

Gastric mucosa. Gastric mucosal injury was exacerbated in WT mice as a function of water immersion restraint time. Cellular swelling, hypochromia of the cytoplasm, stenosis, and erythropenia in the mucosa appeared starting 0.5 h after stress application. Atrophy and exfoliation of mucosal cells, and increased gastric stromal cell erosion was observed at 1 h. After 2 h, mucosal erosion became more severe, and ulceration appeared at 4 h. The pathological changes induced by water immersion restraint stress were more severe in TLR4$^{-/-}$ than in WT mice (Figure 1(A)), and more ulcers were observed grossly in the gastric mucosa of the mutants after 4 h (Figure 1(B)), which was associated with a higher UI at successive time points (two-way ANOVA, $F_{\text{mice-type}} = 145.8$, $p_{\text{mice-type}} < .001$; $F_{\text{time}} = 215.2$, $p_{\text{time}} < .001$; $F_{\text{mice-type} \times \text{time}} = 26.8$, $p_{\text{mice-type} \times \text{time}} < .001$; one-way ANOVA after two-way ANOVA, df = (9,40), $F = 123.7$, $p < .001$; Tukey's test, $p_{\text{WT0h/TLR4}-/-0\,\text{h}} = 1.000$, $p_{\text{WT0.5\,h/TLR4}-/-0.5\,\text{h}} = 1.000$, $p_{\text{WT1h/TLR4}-/-1\,\text{h}} < .001$, $p_{\text{WT2h/TLR4}-/-2\,\text{h}} < .001$, $p_{\text{WT4h/TLR4}-/-4\,\text{h}} < .001$) (Figure 1(C)).

Figure 1. Effect of TLR4 deficiency on stress-induced gastric mucosal injury. (A) Histological analysis of gastric mucosa of WT and TLR4$^{-/-}$ mice (n = 5 each) exposed for indicated times to water immersion restraint stress. (B) Gastric mucosa of mice (n = 5 each) stressed for 4 h. (C) Stress-induced gastric mucosal damage quantified as gastric ulcer index (UI); these values, along with serum cortisol and corticosterone concentrations, are shown as the mean ± SEM (n = 5 mice per group). *$p < .05$ (one-way ANOVA, Tukey's test) versus stressed WT mice at each time point.



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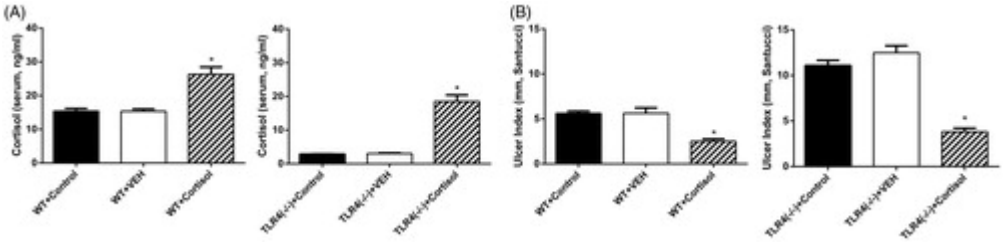
Serum cortisol. Serum cortisol concentrations were increased in WT mice starting from 2 h after the application of the stressor and peaked at the 4-h time point (two-way ANOVA, $F_{\text{mice-type}} = 109.4$, $p_{\text{mice-type}} < .001$; $F_{\text{time}} = 29.8$, $p_{\text{time}} < .001$; $F_{\text{mice-type} \times \text{time}} = 30.7$, $p_{\text{mice-type} \times \text{time}} < .001$; one-way ANOVA after two-way ANOVA, df = (9,40), F = 39.0, $p < .001$; Tukey's test, $p_{\text{WT0h/WT0.5 h}} = 1.000$, $p_{\text{WT0.5 h/WT1h}} = .39$, $p_{\text{WT1h/WT2h}} = .001$, $p_{\text{WT2h/WT4h}} = .005$). In contrast, cortisol concentration remained lower in $TLR4^{-/-}$ mice during exposure to stress (one-way ANOVA after two-way ANOVA, df = (9, 40), F = 39.0, $p < .001$; Tukey's test, $p_{\text{WT0h/TLR4}-/-\text{0 h}} = .8$, $p_{\text{WT0.5 h/TLR4}-/-\text{0.5 h}} = .8$, $p_{\text{WT1h/TLR4}-/-\text{1 h}} = .3$, $p_{\text{WT2h/TLR4}-/-\text{2 h}} = .042$, $p_{\text{WT4h/TLR4}-/-\text{4 h}} < .001$) (Figure 1(C)).

Serum corticosterone. Serum corticosterone concentrations were increased by the stressor from 0.5 h, peaked at 1 h in WT mice and then decreased in both types of mice (two-way ANOVA, $F_{\text{mice-type}} = 84.4$, $p_{\text{mice-type}} < .001$; $F_{\text{time}} = 86.9$, $p_{\text{time}} < .001$; $F_{\text{mice-type} \times \text{time}} = 62.3$, $p_{\text{mice-type} \times \text{time}} < .001$; one-way ANOVA after two-way ANOVA, df = (9,40), F = 75.7, $p < .001$; Tukey's test, $p_{\text{WT0h/WT0.5 h}} = .006$, $p_{\text{WT0.5 h/WT1h}} < .001$, $p_{\text{WT1h/WT2h}} < .001$, $p_{\text{WT2h/WT4h}} < .001$, $p_{\text{TLR4}-/-\text{0 h/TLR4}-/-\text{0.5 h}} = .6$, $p_{\text{TLR4}-/-\text{0.5 h/TLR4}-/-\text{1 h}} = 1.000$, $p_{\text{TLR4}-/-\text{1 h/WT2h}} = .9$, $p_{\text{TLR4}-/-\text{2 h/WT4h}} = .001$). The corticosterone concentrations showed a higher basal value in $TLR4^{-/-}$ mice but increased less than in the WT with exposure to the stressor (one-way ANOVA after two-way ANOVA, df = (9, 40), F = 75.7, $p < .001$; Tukey's test, $p_{\text{WT 0 h/TLR4}-/-\text{ 0 h}} < .001$, $p_{\text{WT 0.5 h/TLR4}-/-\text{ 0.5 h}} = .08$, $p_{\text{WT 1 h/TLR4}-/-\text{ 1 h}} < .001$, $p_{\text{WT 2 h/TLR4}-/-\text{ 2 h}} < .001$, $p_{\text{WT 4 h/TLR4}-/-\text{ 4 h}} < .001$) (Figure 1(C)).

Administration of cortisol alleviates stress-induced gastric mucosal injury

As expected, serum cortisol concentrations were increased in WT and TLR4$^{-/-}$ mice upon injection of exogenous cortisol as compared to the vehicle-injected and uninjected control groups (WT: $F = 2.98$, $df = (2, 12)$ $p = .0001$; TLR4$^{-/-}$: $F = 67.18$, $df = (2, 12)$, $p < .0001$) (Figure 2(A)) and was associated with a lower UI (WT: $F = 19.02$, $df = (2, 12)$ $p = .0002$; TLR4$^{-/-}$: $F = 60.13$, $df = (2, 12)$, $p < .0001$) (Figure 2(B)). Hence, cortisol administration alleviated stress-induced gastric mucosal damage, in both WT and TLR4$^{-/-}$ mice.

Figure 2. Effect of cortisol administration on stress-induced ulceration. WT and TLR4$^{-/-}$ mice were evaluated for (A) serum cortisol concentrations, and (B) gastric ulcer index (UI) after pretreatment with cortisol. Results represent mean ± SEM ($n = 5$ mice per group). *$p < .05$ (one-way ANOVA, Tukey's test) versus vehicle group (dimethylsulfoxide in saline, 1:200) and control groups (without any treatment).



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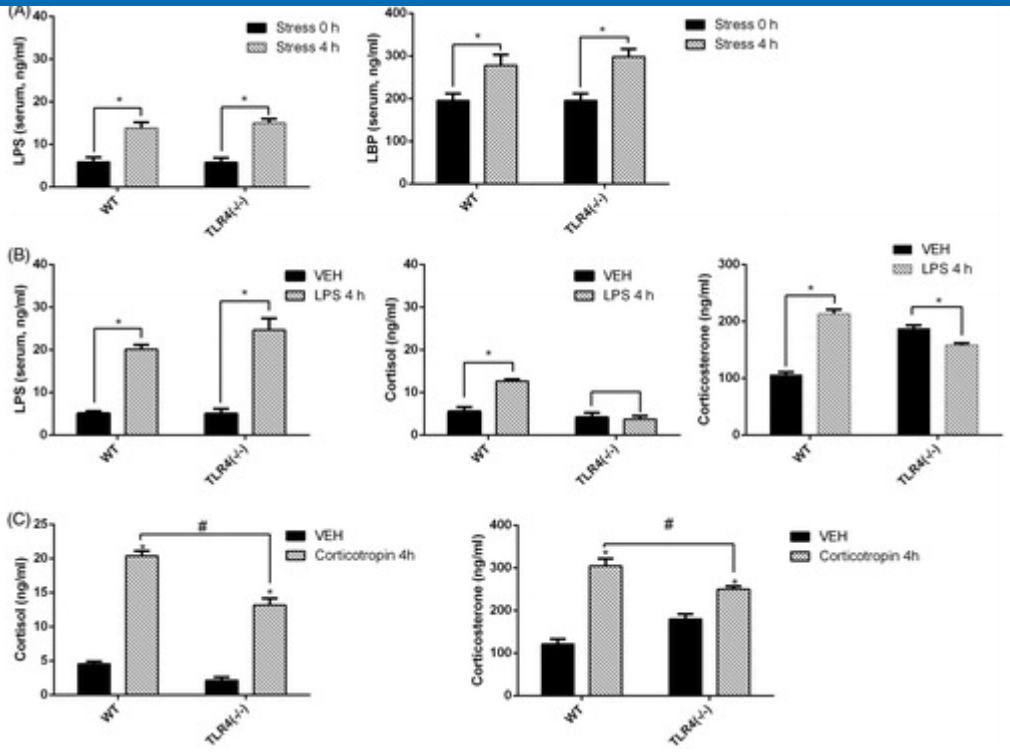
LPS and TLR4 modulate serum glucocorticoid concentrations upon exposure to stress

Serum LPS and LBP concentrations increased markedly in WT mice after 4 h of exposure to water immersion restraint stress (WT: $F = 20.04$, $p = .0021$; TLR4$^{-/-}$: $F = 6.03$, $p = .039$. All df are (3, 16)) (Figure 3(A)).

Figure 3. Effect of stress, lipopolysaccharide (LPS), or corticotropin on serum LPS, lipopolysaccharide-binding protein (LBP), and glucocorticoid concentrations. (A) Serum LPS and LBP concentrations after a 4-h exposure to water immersion restraint stress or not. (B) Serum LPS, cortisol, and corticosterone concentrations in mice treated with LPS or vehicle control (isotonic saline) for 4 h. (C) Serum cortisol and corticosterone concentrations in WT and TLR4$^{-/-}$ mice after a corticotropin (5 IU/ml, 50 IU/kg) test. Results represent mean ± SEM ($n = 5$). *$p < .05$ (one-way ANOVA, Tukey's test) versus vehicle group. #$p < .05$ (one-way ANOVA, Tukey's test) between linked groups.



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Cortisol and corticosterone concentrations were measured in mice pretreated with LPS (1% w/v, 2 mg/kg) for 4 h. Serum LPS concentration increased compared to the vehicle-treated group in both WT and TLR4$^{-/-}$ mice (WT: $F = 123.4$, $p < .0001$; TLR4$^{-/-}$: $F = 35.14$, $p = .0004$. All df are (3, 16)), while cortisol concentrations were elevated in LPS-treated as compared to vehicle-treated WT but not TLR4$^{-/-}$ mice (WT: $F = 33.03$, $p = .0004$; TLR4$^{-/-}$: $F = .091$, $p = .77$. All df are (3, 16)). The corticosterone concentrations were elevated also in WT, but declined in TLR4$^{-/-}$ mice compared to the vehicle-treated group (WT: $F = 146.4$, $p < .0001$; TLR4$^{-/-}$: $F = 18.37$, $p = .003$. All df are (3, 16)) (Figure 3(B)).

The corticotropin test showed the concentrations of cortisol and corticosterone increased in both types of mice, but the increase was less in TLR4$^{-/-}$ mice (Figure 3(C)).

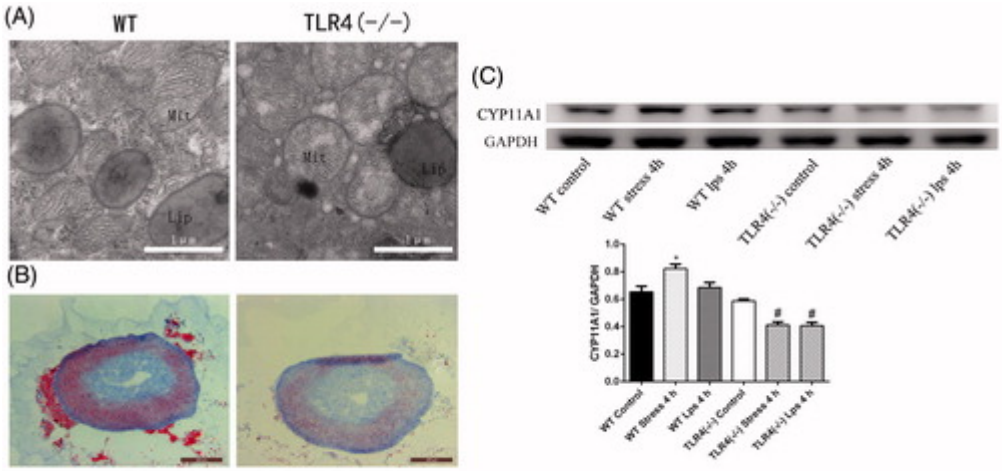
Together, these findings indicate that the stress-induced increases in glucocorticoid concentration are due to increased LPS/TLR4 signaling.

TLR4$^{-/-}$deficiency causes disorganization of mitochondrial structure, lack of lipid droplets, and down expression of CYP11A1 in adrenal cortex

In WT mice, the oval mitochondria of zona fasciculata cells were filled with parallel, tubulovesicular cristae in a homogeneous arrangement. In contrast, mitochondria in

microscopy, obvious reduction in density of lipid-storing droplets was seen in Oil red O-stained whole adrenal frozen section in unstressed TLR4$^{-/-}$ mice (Figure 4(B)).

Figure 4. Ultrastructure, lipid storage, and steroidogenic enzyme content in WT and TLR4$^{-/-}$ mouse adrenal glands. (A) Electron micrographs of adrenocortical cells in the zona fasciculata. Mitochondria (MIT), smooth endoplasmic reticulum, and liposomes (LIP) are visible in the cytoplasm of WT and TLR4$^{-/-}$ mice. Note the round shape, disorganized arrangement of tubulovesicular cristae, and presence of metrical vacuoles in the mutant. Scale bar 1 µm. (B) Cryosections of whole adrenal gland stained with Oil red O in WT and TLR4$^{-/-}$ mice without any treatment showing fewer red-stained lipid-droplets in TLR4 mutant mice. Scale bar 200 µm. (C) Western blotting of adrenal gland protein samples and the corresponding integrated optical density (IOD) ratio values (CYP11A1/glyceraldehyde 3-phosphate dehydrogenase [GAPDH]). The expression of CYP11A1 (P450scc) increased 4 h after stress or lipopolysaccharide (LPS) treatment in WT but decreased in TLR4$^{-/-}$ mice. Results represent mean ± SEM (n = 5 mice per group). *p < .05 versus WT vehicle group (isotonic saline). #p < .05 (one-way ANOVA, Tukey's test) versus TLR4$^{-/-}$ vehicle group (isotonic saline).



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Western blotting of the protein samples from the adrenal glands showed the expression of CYP11A1 protein was increased 4 h after water immersion restraint stress or LPS treatment in WT but was decreased in TLR4$^{-/-}$ mice (ANOVA, WT: F = 5.88, df = (2, 12), p = .017; TLR4$^{-/-}$: F = 24.41, df = (2, 12), p < .0001) (Figure 4(C)). Thus, in the absence of TLR4, adrenocortical mitochondrial structure and CYP11A1 protein expression were compromised, which may be an underlying cause of low secretion of glucocorticoids during stress.

Discussion

The activation of the inflammatory response and ensuing release of pro-inflammatory cytokines are features of stress-induced peptic ulcers (McGettrick & O'Neill, 2004). The innate inflammatory/immune response is activated after exposure to stressful stimuli. Cold restraint stress can induce a rapid activation of nuclear factor (NF)-κB in rats, the inhibition of which alleviates inflammation and injury (Jia et al., 2007a). The deletion of the NF-κB p50 subunit can alleviate gastric injury by inhibiting NF-κB activation and consequent inflammation (Ye et al., 2013). We previously demonstrated that the activation of the mitogen-activated protein kinase (MAPK) p38 by reactive oxygen species may also play a role in stress-induced gastric damage in rats (Jia et al., 2007b). TLR4 protein is a positive regulator of inflammation (Szumilas et al., 2013). Signaling pathways activated by Toll-like receptors (TLRs) in the innate immune system are linked to the induction of pro-inflammatory cytokines. NF-κB, p38, and the MAPK family member c-Jun N-terminal kinase are the major factors activated by TLR4 (O'Neill, 2006) which, along with its co-receptor MD-2 and some inflammatory mediators, is upregulated by chronic mild stress (Garate et al., 2011). Hence it is a plausible hypothesis that TLR4 may increase gastric injury through the activation of inflammatory pathways in stress ulcer. However, we found just the opposite. In this study, it was observed that water immersion restraint stress-induced gastric ulceration was more severe in $TLR4^{-/-}$ than in WT mice. Hence, in addition to promoting inflammation, TLR4 signaling provides a gastroprotective function under stress conditions.

Many studies have found that glucocorticoid (corticosterone in rodents) release in response to acute stress protects the gastric mucosa against stress-induced ulceration (Filaretova et al., 1998, 2014; Hernandez et al., 1984; Yigiter et al., 2010). In the present study, corticosterone release was not stimulated during acute stress in $TLR4^{-/-}$ mice, which accorded with some other reports (Vakharia & Hinson, 2005; Zacharowski et al., 2006). Serum cortisol concentration showed a similar trend to that of corticosterone during the acute water immersion restraint stress, while other research reported that although plasma corticosterone is considered the main glucocorticoid involved in the regulation of stress responses in rodents, the presence of plasma cortisol and whether its level can be used as an indicator of stress has not been determined in rodents before (Gong et al., 2015). In the present study, furthermore,

WT and TLR4$^{-/-}$ mice. This indicates that the release of glucocorticoid during acute stress plays an important role in indicating injury and protecting the gastric mucosa during acute stress in mice.

Previous research showed that TLR4 signaling is sufficient to cause glucocorticoid release from adrenal cells (Vakharia & Hinson, 2005). LPS from Gram-negative bacteria, which act via TLR4, stimulates glucocorticoid release (Zacharowski et al., 2006). In addition, translocation from the intestinal flora of bacterial LPS occurs during chronic mild stress (Garate et al., 2011). LBP is essential for the recognition of LPS by TLR4 (Palsson-McDermott & O'Neill, 2004), and it has been found that plasma LPS and LBP expression is upregulated after cold restraint stress, which has been linked to changes in intestinal permeability and bacterial translocation (Garate et al., 2011). In this study, we also found that serum concentrations of LPS and of LBP were upregulated after 4-h water immersion restraint stress. Also, the serum cortisol and corticosterone concentrations both increased 4 h after LPS treatment in this study in WT, but importantly not in the TLR4$^{-/-}$ mice. In contrast, the corticotropin test showed increased circulating glucocorticoid (corticosterone and cortisol) concentrations in both types of mice, although the response was less in TLR4$^{-/-}$ mice. Hence, TLR4 in the adrenal gland is not essential for a response to ACTH, but has an important role in regulating the extent of glucocorticoid release during acute stress.

Conversion of cholesterol to pregnenolone in mitochondria is the first, rate-limiting, and hormonally regulated step in the synthesis of all steroid hormones (Halkerston et al., 1961; Koritz & Kumar, 1970; Stone & Hechter, 1955). Adrenal steroidogenesis is controlled by ACTH which initiates several enzymatic processes starting with conversion of cholesterol to pregnenolone mediated by the key side chain cleavage enzyme (CYP11A1), and then synthesis of particular steroids which takes place in the endoplasmic reticulum and mitochondria (Miller & Auchus, 2011). Forms of CYP11A1 engineered to lack the mitochondrial leader or that are targeted to the endoplasmic reticulum are inactive, demonstrating that the mitochondrial environment is required for activity (Black et al., 1994). In the present study, mitochondria in adrenocortical cells of TLR4$^{-/-}$ mice showed ultrastructural defects as compared to those of WT mice. Furthermore, the expression of CYP11A1 protein increased after 4 h stress and by LPS treatment in WT mice, but was decreased in TLR4$^{-/-}$ mice. Moreover, lipid-storing droplets constituting the substrates for steroidogenesis were reduced in TLR4$^{-/-}$ mice in Oil red O stained cryosections of whole adrenal gland. These changes may underlie

the differences in the synthesis and secretion of corticosterone and cortisol by adrenocortical cells between WT and TLR4$^{-/-}$ mice.

Exposure to low concentrations of endotoxin produces long-term changes in HPA axis activity (Shanks et al., 2000). In particular, early-life exposure to Gram-negative LPS increases corticosterone secretory pulse frequency and amplitude (Vakharia & Hinson, 2005). Moreover, preconditioning with mild stress provides gastroprotection to rats during acute stress (Filaretova et al., 2008). In another study, increasing glucocorticoid receptor expression induced a weaker response to restraint stress (Reichardt et al., 2000). Thus, it can be conjectured that long-term exposure to stressful environmental stimuli may promote the neuroendocrine/neuroimmune response during acute stress events, and that this form of adaptation is lost in the absence of TLR4, thereby decreasing protection against the adverse consequences of acute stress.

In summary, TLR4 deficiency resulted in aggravated gastric ulceration that was associated with a reduction in glucocorticoid release during acute stress, an effect that was alleviated by treatment with exogenous cortisol. TLR4 thus plays a gastroprotective role in stress-induced gastric ulceration that is exerted via modulation of glucocorticoid secretion by impacting the ultrastructure and the synthetic function of the adrenal gland through LPS/TLR4 signaling. In addition, as well as corticosterone, cortisol also plays an important role during acute stress in mice.

Disclosure statement

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

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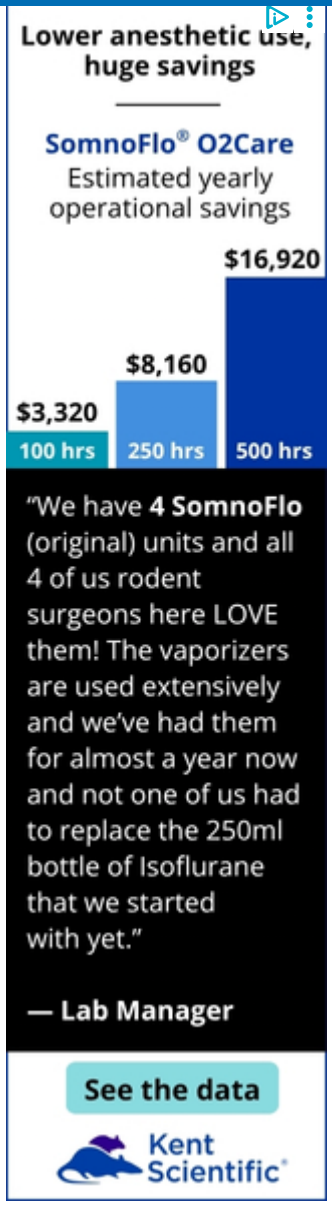

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