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$P2X_7$ Receptor Activation Amplifies Lipopolysaccharide-Induced Vascular Hyporeactivity via Interleukin-1β Release

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ABSTRACT

Lipopolysaccharide (LPS) stimulates cytoplasmic accumulation of pro-interleukin (IL)-1β. Activation of $P2X_7$ receptors stimulates conversion of pro-IL-1β into mature IL-1β, which is then secreted. Because both LPS (in vivo) and IL-1β (in vitro) decrease vascular reactivity to contractile agents, we hypothesized the following: 1) $P2X_7$ receptor activation contributes to LPS-induced vascular hyporeactivity, and 2) IL-1β mediates this change. Thoracic aortas were obtained from 12-week-old male C57BL/6 mice. The aortic rings were incubated for 24 h in Dulbecco's modified Eagle's medium, LPS, benzoylbenzoyl-ATP (BzATP; $P2X_7$ receptor agonist), LPS plus BzATP, oxidized ATP (oATP; $P2X_7$ receptor antagonist), or oATP plus LPS plus BzATP. After the treatment, the rings were either mounted in a myograph for evaluation of contractile activity or homogenized for IL-1β and inducible nitric-oxide synthase (iNOS) protein measurement. In endothelium-intact aortic rings, phenylephrine (PE)-induced contractions were not altered by incubation with LPS or BzATP, but they significantly decreased in aortic rings incubated with LPS plus BzATP. Treatment with oATP or IL-1ra (IL-1β receptor antagonist) reversed LPS plus BzATP-induced hyporeactivity to PE. In the presence of N^G-nitro-L-arginine methyl ester or *N*-([3-(aminomethyl)phenyl]methyl)ethanimidamide (selective iNOS inhibitor), the vascular hyporeactivity induced by LPS plus BzATP on PE responses was not observed. BzATP augmented LPS-induced IL-1β release and iNOS protein expression, and these effects were also inhibited by oATP. Moreover, incubation of endothelium-intact aortic rings with IL-1β induced iNOS protein expression. Thus, activation of $P2X_7$ receptor amplifies LPS-induced hyporeactivity in mouse endothelium-intact aorta, which is associated with IL-1β-mediated release of nitric oxide by iNOS.

Invasion of the bloodstream by bacteria, viruses, or parasites induces activation of the immune system and triggers systemic defense mechanisms (Pinsky, 1996). Extreme triggering of defense mechanisms leads to severe inflammation [sepsis/septic shock (Morrison and Ulevitch, 1978; McCabe et al., 1983). In 1988, Cerami and Beutler showed that endotoxic shock is not caused directly by toxins secreted from microorganisms, but it is the result of a strong, incontrollable immune response in the host (Cerami and Beutler, 1988). Lipopolysaccharide (LPS), a major component of Gram-negative bacteria, once released into bloodstream induces multiple and excessive endogenous proinflammatory mediators, including cytokines [such as tumor necrosis factor-alpha and interleukin (IL)-1, -6, and -8], leukotrienes, and thromboxane A_2, all playing a role in the responses associated with sepsis/septic shock. For example, i.v. injection of IL-1 induces lowering of blood pressure (Dinarello and Wolff, 1993). Prolonged exposure to IL-1β results in decreased vascular tone (Beasley et al., 1989; Takizawa et al., 1999), and it also increases nitric-oxide synthase (NOS) protein expression and activity (French et al., 1991; Gui et al., 2000). Many studies have indicated that a subtype of purinergic receptors, the $P2X_7$ receptor, plays a key role in IL-1 processing and release in inflammatory cells. The P2 family of purinergic receptors includes, as of now, 15 members that have been cloned and classified into subfamilies: P2Y receptors and P2X receptors, which are G protein-coupled and cation-selective channels, respectively (Burnstock and Knight, 2004). The $P2X_7$ receptor, a member of the P2X purinergic superfamily of receptors, functions as an ATP-gated ion channel (Di Virgilio, 1995; Falzoni et al., 1995). Activation of Toll-like receptors by LPS in monocytes, macrophages, and microglial cells induces large-quantity synthesis of pro-IL-1β (molecular mass, 31 kDa, a form of biologically inactive procytokine), which accumulates and aggregates with the inflammasome component

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ABBREVIATIONS: LPS, lipopolysaccharide; IL, interleukin; NOS, nitric-oxide synthase; BzATP, benzoylbenzoyl-ATP; PE, phenylephrine; L-NAME, N^G-nitro-L-arginine methyl ester; 1400W, *N*-([3-(aminomethyl)phenyl]methyl)ethanimidamide; oATP, oxidized ATP; IL-1ra, IL-1β receptor antagonist; DMEM, Dulbecco's modified Eagle's medium; iNOS, inducible NOS; ELISA, enzyme-linked immunoadsorbent assay.

in the cell cytoplasm. Activation of $P2X_7$ receptors by ATP triggers potassium efflux, procaspase-1 cleavage, conversion of pro-IL-1β into mature IL-1β (molecular mass, 17 kDa, a biologically active form of cytokine), and mature IL-1β release to the extracellular environment (Perregaux and Gabel, 1994; Sanz and Di, 2000; Ferrari et al., 2006). Experiments performed in vitro and in vivo in $P2X_7$ receptor knockout mice conclusively identified the receptor responsible for ATP-dependent IL-1β release, upon LPS challenge, as the $P2X_7$ receptor (Ferrari et al., 1997; Solle et al., 2001; Labasi et al., 2002).

In the present study, we addressed the hypothesis that $P2X_7$ receptor activation contributes to LPS-induced changes in vascular reactivity through IL-1β release. To test our hypothesis, we determined, in mouse thoracic aortas, the effects of an agonist of $P2X_7$ receptors, benzoylbenzoyl-ATP (BzATP), on vascular responses and IL-1β release induced by LPS. In addition, we elucidated the downstream mediators of this signaling pathway.

Materials and Methods

Materials. Bacterial LPS (*Escherichia coli* serotype 0.127:B8), phenylephrine (PE), N^G-nitro-L-arginine methyl ester (L-NAME), *N*-([3-(aminomethyl)phenyl]methyl)ethanimidamide (1400W), BzATP, and oxidized ATP (oATP) were all obtained from Sigma-Aldrich (St. Louis, MO). Recombinant mouse IL-1β receptor antagonist (IL-1ra) was obtained from R&D Systems (Minneapolis, MN). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT).

Isolated Artery Preparation and Vascular Incubation Protocol. Thoracic aortas were obtained from 12-week-old male C57BL/6 mice. The thoracic aortas were cleaned of adhering periadventitial fat, cut into 3-mm length rings, and then incubated for 24 h with DMEM (supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, and 1% L-glutamine), LPS (100 μg/ml), BzATP ($P2X_7$ receptor agonist, 150 μM), LPS plus BzATP, oATP ($P2X_7$ receptor antagonist, 50 μM), oATP plus LPS plus BzATP (oATP, 50 μM; incubated 1 h before LPS and BzATP), or IL-1β (20 ng/ml). In some experiments, the endothelium was mechanically removed from the aortic rings by rubbing the intimal surface with a stainless steel wire.

Vascular Function Studies. After the incubation, the rings were mounted in a myograph (Danish Myo Technology A/S, Aarhus, Denmark) containing warmed (37°C), oxygenated (95% O_2/5% CO_2) physiological salt solution consisting of the following: 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4 \cdot 7H_2O$, 1.56 mM $CaCl_2 \cdot 2H_2O$, 14.9 mM $NaHCO_3$, 5.6 mM glucose, and 0.03 mM EDTA. The preparations were equilibrated for at least 60 min under a passive tension of 5 mN. After the equilibration period, the aortic rings were stimulated with PE (0.1 μM) followed by relaxation with acetylcholine (1 μM), which was used as evidence of an intact endothelium. Cumulative concentration-response curves to PE (10^{-10}–10^{-5} M) were performed. Functional experiments were also performed in the presence of IL-1ra (100 ng/ml; 4 h), L-NAME (NOS inhibitor, 100 μM; 40 min), or 1400W [selective inducible NOS (iNOS) inhibitor, 1 μM; 40 min].

The contractile response to 120 mM KCl was also tested at the end of each time functional study to rule out the possibility of vascular damage due to the 24-h incubation with the different reagents.

Measurement of Vascular IL-1β by Enzyme-Linked Immunoadsorbent Assay. After 24 h of incubation (as described above), aortas were homogenized in a T-PER tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL) and proteinase inhibitor mixture (Sigma-Aldrich). After a 30-min centrifugation at 14,000*g*, the supernatant was aliquoted and used for protein assay (with BCA protein assay reagent A and B; Pierce Biotechnology), IL-1β measurement with an enzyme-linked immunoadsorbent assay (ELISA) kit (Pierce Biotechnology), or Western blots analysis of iNOS expression.

iNOS Protein Expression in Aorta by Western Blot. Forty micrograms of protein were eluted from the supernatant and loaded directly into the SDS sample buffer for 10% SDS-polyacrylamide gel electrophoresis. After transfer onto a 0.45-μm pure nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad, Hercules, CA), the membranes were blocked with 5% defatted milk in Tris buffer solution containing 0.1% Tween 20 for 1 h and then incubated with 1:100 dilution of specific polyclonal antibody against iNOS (BD Biosciences Transduction Laboratories, Lexington, KY) in Tris buffer solution containing 0.1% Tween 20 for 24 h at 4°C. The membranes were washed and finally incubated with a 1:1000 dilution of sheep anti-mouse IgG-horseradish peroxidase antibody (GE Healthcare, Chalfont St. Giles, UK) for 1 h at room temperature. After successive washes, the immunocomplexes were developed using an enhanced peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents; Pierce Biotechnology) and exposed to X-ray film (Carestream Health, Rochester, NY).

Statistical Analysis. All values in the figures and text are expressed as mean ± S.E.M. of *n* observations, where *n* represents the number of animals studied. For measurement of IL-1β and iNOS, three aortas from the same group were pooled, and each pool was considered as one number. Statistical evaluation was performed by using analysis of variance followed by a Bonferroni's multiple comparison test in the vascular functional studies. Differences in IL-1β production and iNOS expression were analyzed by a Dunnett's multiple comparison test. A P value less than 0.05 was considered to be statistically significant.

Results

Effects of LPS and $P2X_7$ Receptor Activation on PE Vascular Reactivity. Incubation with LPS (100 μg/ml) plus BzATP (150 μM) for 24 h, but not with BzATP or LPS alone, significantly attenuated PE-induced contractile responses in endothelium-intact aortic rings, compared with control (DMEM) ($LogEC_{50}$ of control: −7.36 ± 0.05; BzATP: −7.29 ± 0.06; LPS: −7.16 ± 0.05; LPS plus BzATP: −6.80 ± 0.06) (Fig. 1). No changes in PE-induced contractile responses were observed in endothelium-denuded aortic rings incubated with DMEM and LPS plus BzATP ($LogEC_{50}$ of control: −7.62 ± 0.06; LPS plus BzATP: −7.49 ± 0.06).

Effect of IL-1ra and NOS Inhibitors on LPS Plus BzATP-Induced Hyporeactivity to PE. Incubation of endothelium-intact aortic rings with IL-1ra (100 ng/ml for 4 h) completely prevented the attenuated contractile response to PE in aortas treated with LPS plus BzATP ($LogEC_{50}$ of control: −7.36 ± 0.05; LPS plus BzATP: −6.80 ± 0.06; LPS plus BzATP plus IL-1ra: −7.16 ± 0.08) (Fig. 2). Incubation with IL-1ra alone did not modify PE responses ($LogEC_{50}$ of IL-1ra: −7.43 ± 0.09). To determine the role of nitric oxide in BzATP-amplified LPS-induced hyporeactivity, we tested the effects of a nonselective NOS inhibitor (L-NAME, 100 μM) and a selective iNOS inhibitor (1400W, 1 μM) on vascular function. Incubation with L-NAME for 40 min resulted in a leftward shift of the PE concentration-response curve in control and LPS plus BzATP arteries (Fig. 3A). Incubation with 1400W for 40 min significantly reversed the decreased contractile response to PE only in LPS plus BzATP group (Fig. 3B).

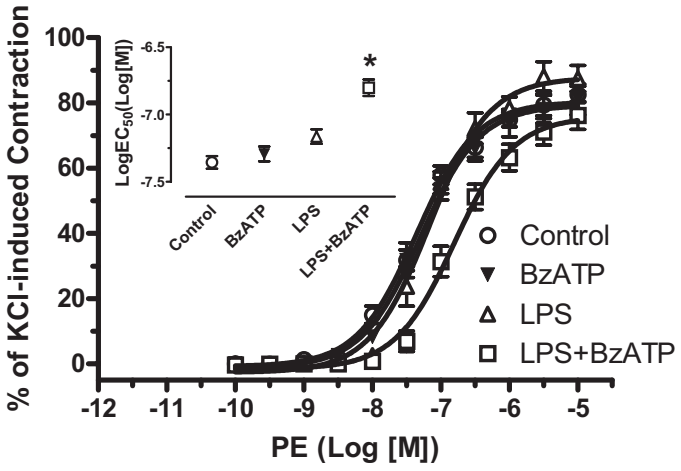

Fig. 1. PE-induced vascular contractile responses are attenuated by LPS plus BzATP. Endothelium-intact aortic rings were incubated with DMEM (control; $n = 8$), LPS (100 μg/ml; $n = 9$), BzATP (150 μM; $n = 5$), and LPS plus BzATP [LPS (100 μg/ml) plus BzATP (150 μM); LPS+BzATP; $n = 7$]. Data are plotted as the percentage of maximal contractile response to 120 mM KCl. $LogEC_{50}$ to PE are represented. Data are expressed as mean ± S.E.M. of n animals. *, $P < 0.001$ versus control.

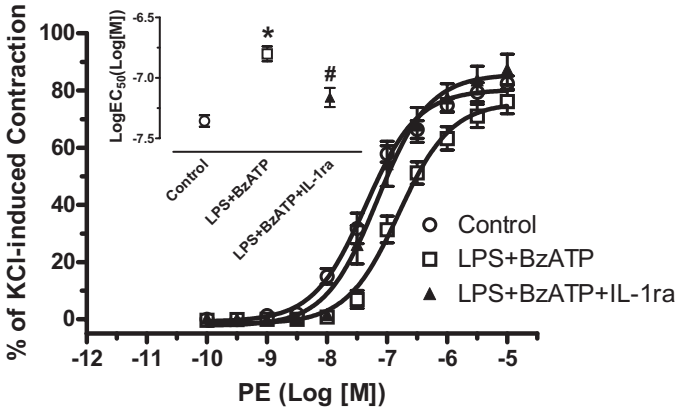

Fig. 2. IL-1ra reverses the attenuated vascular contractile response to PE after incubation with LPS plus BzATP. Endothelium-intact aortic rings were incubated with DMEM (control; $n = 8$), LPS plus BzATP [LPS (100 μg/ml) plus BzATP (150 μM); LPS+BzATP; $n = 7$], and LPS plus BzATP plus IL-1ra [LPS (100 μg/ml) plus BzATP (150 μM) plus IL-1ra (100 ng/ml); LPS+BzATP+IL-1ra; $n = 7$]. Data are plotted as the percentage of maximal contractile response to 120 mM KCl. $LogEC_{50}$ to PE are represented. Data are expressed as mean ± S.E.M. of n animals. *, $P < 0.001$, LPS+BzATP versus control; #, $P < 0.01$, LPS+BzATP+IL-1ra versus LPS+BzATP.

Effect of $P2X_7$ Receptor Antagonist on LPS Plus BzATP-Induced Hyporeactivity to PE. Incubation of endothelium-intact aortic rings with oATP ($P2X_7$ receptor antagonist, 50 μM for 1 h before the 24-h incubation with LPS) completely abolished the attenuated contractile response to PE induced by LPS plus BzATP. Incubation with oATP alone did not modify the contractile responses to PE. ($LogEC_{50}$ of control: −7.36 ± 0.05; LPS plus BzATP: −6.80 ± 0.06; oATP+LPS+BzATP: −7.18 ± 0.06; oATP: −7.37 ± 0.05) (Fig. 4).

Effects of LPS and $P2X_7$ Receptor Activation on Vascular IL-1β Levels and iNOS Protein Expression. All groups were normalized by the respective total protein. In endothelium-intact aortas, incubation with LPS (100 μg/ml, 24 h) increased IL-1β levels (Fig. 5, left). Incubation with BzATP (150 μM, 24 h) augmented LPS-induced IL-1β release, and this effect was inhibited by the pretreatment with oATP (50 μM, 1 h) (Fig. 5, left). However, in endothelium-denuded aortic rings, neither LPS nor LPS plus BzATP increased IL-1β release (Fig. 5, right).

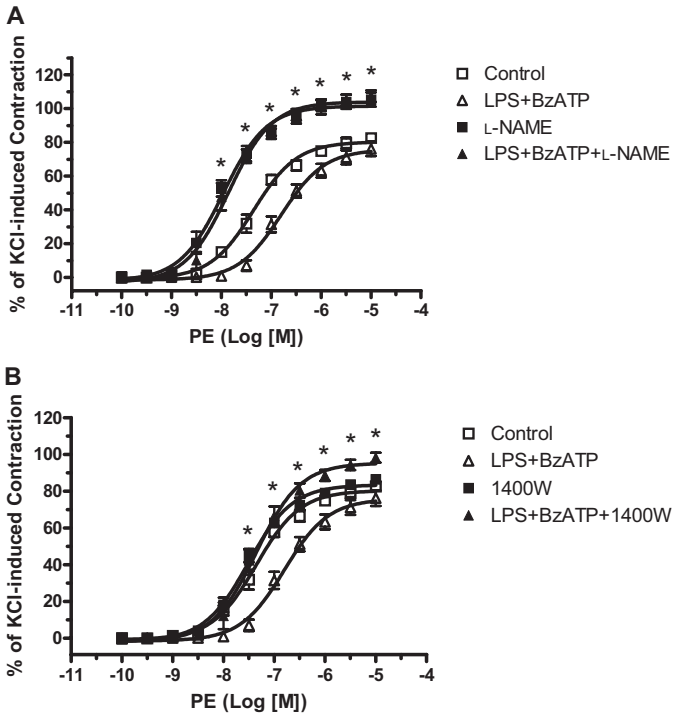

Fig. 3. NOS inhibitors abolish the effects of LPS plus BzATP on PE-induced vascular reactivity. A, endothelium-intact aortic rings were incubated with DMEM (control; $n = 8$), LPS plus BzATP [LPS (100 μg/ml) plus BzATP (150 μM); LPS+BzATP; $n = 7$], L-NAME (L-NAME 100 μM for 40 min in the organ bath after 24-h incubation with DMEM; $n = 5$), and LPS plus BzATP plus L-NAME (L-NAME 100 μM for 40 min in the organ bath after 24-h incubation with LPS plus BzATP; LPS+BzATP+L-NAME; $n = 5$). B, the same curves were represented as (A) in DMEM and LPS plus BzATP. Endothelium-intact aortic rings were incubated with 1400W (1400W 1 μM for 40 min in the organ bath after 24-h incubation with DMEM; $n = 5$) and LPS plus BzATP plus 1400W (1400W 1 μM for 40 min in the organ bath after 24-h incubation with LPS plus BzATP; LPS+BzATP+1400W; $n = 5$). Data are plotted as the percentage of maximal contractile response to 120 mM KCl. Data are expressed as mean ± S.E.M. of n animals. *, $P < 0.001$, LPS+BzATP versus LPS+BzATP+NOS inhibitor (L-NAME or 1400W).

In endothelium-intact aortas, iNOS protein expression was detected in LPS (100 μg/ml) treatment, but not in the control (DMEM) group (Fig. 6, left). Incubation with LPS plus BzATP (150 μM) further increased iNOS protein expression (Fig. 6, left), but no changes in endothelial NOS expression were observed after incubation with LPS or LPS plus BzATP (data not shown). Pretreatment of aortas with oATP for 1 h inhibited the augmented LPS plus BzATP-induced iNOS protein expression (Fig. 6, left). In endothelium-denuded aortas, iNOS protein expression did not change after incubation with LPS or LPS plus BzATP (Fig. 6, right).

Effects of IL-1β Treatment on Vascular iNOS Protein Expression. Incubation of endothelium-intact aortic rings with IL-1β (20 ng/ml) for 24 h increased iNOS protein expression, but not in endothelium-denuded aortic rings (Fig. 7).

Discussion

Previous studies have shown that endotoxin induces a variety of biological responses and disease. Hypotension,

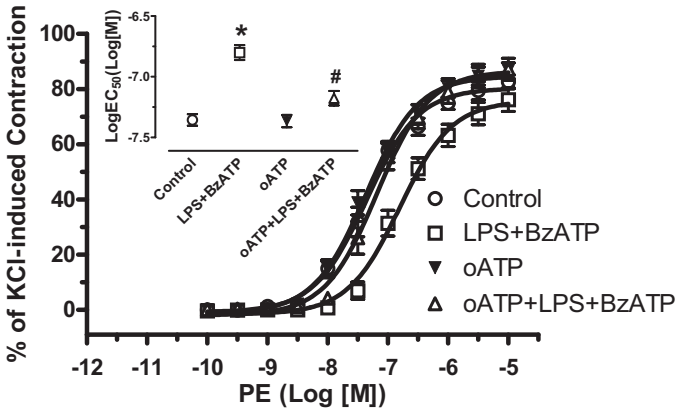

Fig. 4. Oxidized ATP (oATP, P2X$_7$ receptor antagonist) reverses the attenuated vascular contractile response to PE induced by LPS plus BzATP. Endothelium-intact aortic rings were incubated with DMEM (control; $n = 8$), LPS plus BzATP [LPS (100 μg/ml) plus BzATP (150 μM); LPS+BzATP; $n = 7$], oATP (50 μM for 1 h before incubation with DMEM; $n = 6$), and oATP plus LPS plus BzATP (oATP, 50 μM for 1 h before incubation with LPS plus BzATP; oATP+LPS+BzATP; $n = 6$). Data are plotted as the percentage of maximal contractile response to 120 mM KCl. LogEC$_{50}$ to PE are represented. Data are expressed as mean ± S.E.M. of n animals. *, $P < 0.001$, LPS+BzATP versus control or oATP; #, $P < 0.01$, oATP+LPS+BzATP versus LPS+BzATP.

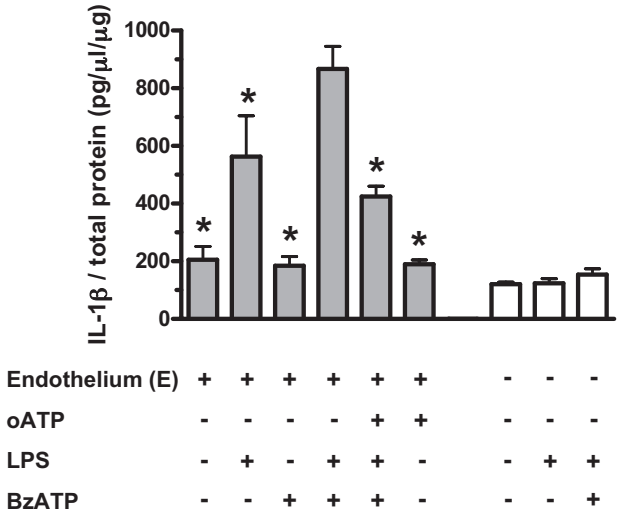

Fig. 5. BzATP augments IL-1β production induced by LPS in endothelium-intact vessels. The changes in endothelium-intact and -denuded aortic IL-1β levels were assessed by ELISA, after incubation with DMEM [control; E(+), $n = 6$; E(−), $n = 3$], LPS [100 μg/ml; E(+), $n = 3$; E(−), $n = 3$], BzATP [150 μM; E(+), $n = 4$], LPS plus BzATP [LPS (100 μg/ml) plus BzATP (150 μM); LPS+BzATP; E(+), $n = 4$; E(−), $n = 3$], oATP plus LPS plus BzATP [oATP (50 μM) for 1 h before incubation with LPS plus BzATP; oATP+LPS+BzATP; E(+), $n = 3$], and oATP [50 μM for 1 h before incubation with DMEM; E(+), $n = 3$]. Data are expressed as mean ± S.E.M. of n animals. *, $P < 0.05$ versus LPS+BzATP in endothelium-intact vessels.

tachycardia, and vascular hyporeactivity to constrictor stimuli are common in animals with endotoxic shock (Szabó et al., 1993). In addition, proinflammatory cytokines are thought to be involved. Several studies have suggested that the P2X$_7$ receptor is an important regulator of inflammatory cell responsiveness to LPS (Falzoni et al., 1995; Solle et al., 2001; Guerra et al., 2003). LPS-activated isolated macrophages, as well as blood from the P2X$_7$ receptor-deficient mouse, are incapable of releasing mature IL-1β in response to ATP challenge (Solle et al., 2001; Labasi et al., 2002). Our current

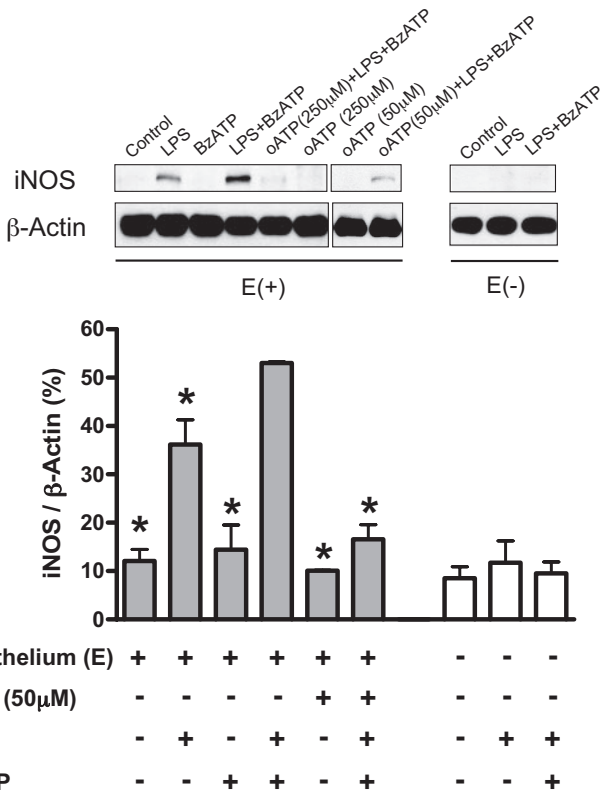

Fig. 6. BzATP augments iNOS protein expression induced by LPS in endothelium-intact vessels. The graphic depicts a typical Western blot image of iNOS protein expression (top) and the statistical analysis of changes of iNOS protein expression in mouse endothelium-intact and -denuded aortic rings, which were incubated with DMEM [control; E(+), $n = 3$; E(−), $n = 3$], LPS [100 μg/ml; E(+), $n = 3$; E(−), $n = 3$], BzATP [150 μM; E(+), $n = 3$], LPS plus BzATP [LPS (100 μg/ml) plus BzATP (150 μM); LPS+BzATP; E(+), $n = 3$; E(−), $n = 3$], oATP plus LPS plus BzATP [oATP (50 μM or 250 μM) for 1 h before incubation with LPS plus BzATP; oATP (50 μM) + BzATP+LPS; E(+), $n = 3$ or oATP (250 μM) + BzATP+LPS; E(+), $n = 3$], and oATP [50 μM or 250 μM for 1 h before incubation with DMEM; oATP (50 μM); E(+), $n = 3$ or oATP (250 μM); E(+), $n = 3$]. Data are expressed as mean ± S.E.M. of n animals. *, $P < 0.05$ versus LPS+BzATP in endothelium-intact vessels.

study extends these initial findings by showing that P2X$_7$ receptor activation facilitates LPS-induced vascular hyporeactivity. Incubation of endothelium-intact aortic rings with LPS plus BzATP significantly attenuated PE-induced contractile responses. oATP, a P2X$_7$ receptor inhibitor, effectively blocked the LPS plus BzATP-induced attenuation of PE contractile responses. It has been previously shown that incubation of rat aorta with IL-1β for 24 h attenuates PE-induced smooth muscle contraction (Takizawa et al., 1999). In this study, we also demonstrated that IL-1β and iNOS-derived nitric oxide are involved in this attenuated contractile response to PE induced by LPS plus BzATP. Thus, these studies implicated the P2X$_7$ receptor as a critical factor to regulate vascular reactivity in inflammation or inflammatory-related conditions.

ATP, the natural agonist for P2X$_7$ receptor activation, was initially considered an intracellular energy source or enzyme cofactor involved in biosynthetic function. Later, ATP was recognized as a neurotransmitter involved on nonadrenergic, noncholinergic nerve responses (Burnstock et al., 1970; Burnstock et al., 1972). P1 receptors and P2 receptors constitute the two major families of purinergic receptors. Aden-

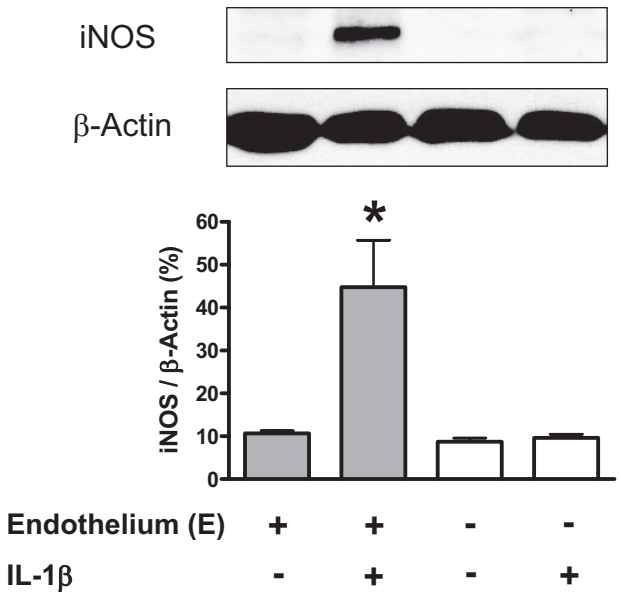

Fig. 7. IL-1β induces iNOS protein expression in endothelium-intact vessels. The graphic depicts a typical Western blot image of iNOS protein expression (top) and the statistical analysis of changes of iNOS protein expression in mouse endothelium-intact and -denuded aortic rings, which were incubated with DMEM [control; E(+), $n = 3$; E(−), $n = 3$] and IL-1β [20 ng/ml; E(+), $n = 3$; E(−), $n = 3$]. Data are expressed as mean ± S.E.M. of n animals. *, $P < 0.01$, IL-1β versus control in endothelium-intact vessels.

osine, which is formed by breakdown of ATP by ubiquitous intracellular or extracellular ectonucleotidases, exerts its effects by activating P1 receptors. ATP achieves its effects by activating the P2 receptor family, which based on structural and mechanistic differences was divided into two subsets—P2X and P2Y. The P2X receptors are ligand-gated ion channels classified in seven subtypes, $P2X_{1-7}$, some of which seem to modulate vascular tone, contraction of muscle cells, and signal transduction in cardiac cells (Burnstock and Knight, 2004). However, an important role in inflammation was inferred from the finding that ATP also elicited the processing and release of proinflammatory cytokines from LPS-primed macrophages by acting on a receptor (North, 2002). Until gene cloning, this receptor was recognized to have similar properties to the $P2X_7$ receptor (Surprenant et al., 1996). The putative LPS-binding region is also present in the $P2X_7$ receptor cytosolic domain (Khakh and North, 2006). Expression of $P2X_7$ receptor message and protein have been reported in different cell types, such as macrophages (Falzoni et al., 1995; Solle et al., 2001), leukocytes (Labasi et al., 2002), microglia (Bianco et al., 2006), epithelial cells (Gröschel-Stewart et al., 1999), endothelial cells (Ramirez and Kunze, 2002), and fibroblasts (Solini et al., 1999).

In the present study, we tested the hypothesis that $P2X_7$ receptor activation plays a role in the functional vascular changes induced by LPS. Our results showed that simultaneous incubation of mouse endothelium-intact aortic rings with LPS plus the $P2X_7$ receptor agonist BzATP, but not LPS or BzATP alone, significantly attenuated PE-induced vessel constriction (Fig. 1), indicating that $P2X_7$ receptor activation does play an important role in LPS-mediated vascular effects. Our results are in accordance with previous work showing that rat aortic rings after 20 to 24-h incubation with LPS and media alone displayed similar response to PE (Wylam et al., 2001). It is important to mention that incubation with media alone may elicit vascular synthesis of cytokines (Newman et al., 1996), and it is possible to prolong exposure to include very small amounts of LPS in nominally sterile media (McKenna, 1990). We were able to detect IL-1β in aortas incubated with DMEM, although in a smaller concentration than observed after LPS incubation (Fig. 5).

In a previous study, Takizawa et al. (1999) showed that incubation of rat aortas with IL-1β for 24 h decreases contractile responses to PE. In addition, IL-1β has been shown to increase in plasma 3 h after LPS was injected into rats (Izeboud et al., 2004). Accordingly, in our current study, addition of IL-1ra reversed vascular hyporeactivity induced by LPS plus BzATP incubation (Fig. 2), and we suggested that vascular hyporeactivity from LPS plus BzATP was dependent on the IL-1β release. The IL-1ra is a competitive inhibitor of IL-1α and IL-1β binding to the IL-1 receptor, and it blocks IL-1-dependent signal transduction, thus functioning as an endogenous, IL-1-selective inhibitor of inflammation (Dinarello, 2000). In contrast, it can be noted that in human vascular endothelial cells, LPS treatment followed by BzATP stimulation results in a maximal release of IL-1ra into the extracellular medium (Wilson et al., 2004). The balance of proinflammatory IL-1β and anti-inflammatory IL-1ra may determine the overall inflammatory response.

We observed significant attenuation of PE-induced contractile response in endothelium-intact aortic rings incubated with LPS plus BzATP (Fig. 1), but not in endothelium-denuded aortic rings. Thus, this implicates that the endothelium is necessary for $P2X_7$ receptor activation by BzATP to amplify the LPS-induced vascular hyporeactivity. In addition, $P2X_7$ receptor immunoreactivity has been detected in aorta, but a weak level of $P2X_7$ receptor immunoreactivity was associated with the smooth muscle layer (Lewis and Evans, 2001). In our results, we found that BzATP significantly enhanced iNOS protein expression induced by LPS in endothelium-intact, but not in endothelium-denuded aortic rings (Fig. 6). oATP not only reversed vascular hyporeactivity to PE (Fig. 4), but it also inhibited the enhanced IL-1β level and iNOS expression in aorta treated with LPS plus BzATP (Figs. 5 and 6). The endothelium is necessary for BzATP-induced amplification of LPS-induced hyporeactivity in mouse aorta. We also found that increased IL-1β level and iNOS protein expression are related to the presence of endothelium (Figs. 5 and 6). BzATP may activate $P2X_7$ receptor on the endothelium, stimulating the conversion of pro-IL-1β into mature IL-1β, which is then released from the endothelial cells, affecting adjacent cells. Accordingly, Gui et al. (2000) suggested that IL-1β plays an autocrine/paracrine role in the induction of iNOS in rat aorta tissue. We have detected increased IL-1β after 24-h incubation in endothelium-intact aortic tissue in LPS plus BzATP treatment (Fig. 5, left). However, IL-1β levels did not change in endothelium-denuded aortas treated with LPS plus BzATP (Fig. 5, right), and no IL-1β was detectable in the medium (data not shown). In addition, to understand the signaling relationship between IL-1β and iNOS, we directly incubated aortic rings with IL-1β for 24 h. iNOS protein expression was significantly increased in the endothelium-intact, but not in the endothelium-denuded aortic tissue (Fig. 7). Thus, these results demonstrate that IL-1β plays an au-

tocrine role acting on the adjacent vascular endothelial cells to induce iNOS production.

Another study has shown that during LPS-mediated acute inflammation, an increase in ATP release was measured from endothelial cells (Bodin and Burnstock, 1998). The endothelium may be exposed to high levels of extracellular ATP under inflammatory conditions, due to release from degranulating platelets, via sympathetic nerve stimulation, as well as damaged cells in atherosclerosis, hypertension, restenosis, and ischemia (Burnstock, 2002). Although we did not observe vascular functional changes in LPS alone-treated vessels (Fig. 1), we found an increase in IL-1β level and iNOS protein expression in endothelium-intact aorta (Figs. 5 and 6). LPS-induced endogenous ATP release from endothelium may not be high enough to enhance the vascular hyporeactivity by LPS until extra-additional ATP analog (BzATP) is added. Thus, it will be important to understand the main source of ATP in systemic LPS treatment, because systemic vascular hyporeactivity to norepinephrine is evident in LPS alone-treated animals (Chiao et al., 2005). Moreover, our results showed that a $P2X_7$ receptor antagonist, oATP, decreased the expression of iNOS induced by LPS plus BzATP to a level below that obtained with LPS alone in endothelium-intact aortic tissue (Figs. 5 and 6). Because $P2X_7$ receptor immunoreactivity associated with the smooth muscle layer of the arteries is weak (Lewis and Evans, 2001), no activity of the $P2X_7$ receptor was shown in endothelium-denuded vessels, even those treated with LPS or LPS plus BzATP, implicating that the action of LPS involves activation of $P2X_7$ receptors in endothelial cells. Accordingly, it had been demonstrated that oATP inhibits LPS-stimulated nitric oxide production and iNOS expression, and attenuates LPS-induced activation of nuclear factor-κB and extracellular signal-regulated kinase-1/2 in macrophages (Hu et al., 1998). $P2X_7$ receptors can cross-talk with the LPS-dependent pathways, because LPS signals through CD14/MD2/Toll-like receptor-dependent pathways as well as through CD14/$P2X_7$-dependent pathways (Guerra et al., 2003). In addition, the putative LPS-binding region is present in the $P2X_7$ receptor cytosolic domain (Khakh and North, 2006). Thus, this evidence implicates that the vascular action of LPS may involve the activation of $P2X_7$ receptor.

In summary, our results demonstrate for the first time that activation of $P2X_7$ receptor amplifies LPS-induced vascular hyporeactivity. $P2X_7$ receptor-mediated effects on vascular reactivity are due to IL-1β release from endothelial cells, which, in turn, induces downstream nitric oxide production by iNOS. Finally, we suggest that the $P2X_7$ receptor is an important regulator for vascular hypotensive responses in inflammation or inflammatory-related disease.

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