Murine Macrophages Produce Endothelin-1 After Microbial Stimulation

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Endothelin-1 (ET-1) was originally characterized as a potent vasoconstrictor secreted by the endothelium and participating in the regulation of vascular tone. Subsequent analysis has revealed ET-1 to be a multifunctional peptide produced by a wide variety of cells and tissues under normal and pathologic conditions. The importance of macrophages as a source of ET-1 during infection and inflammation is supported by clinical observations in humans and in animal models of inflammation. We hypothesize that the production of ET-1 is part of the characteristic macrophage response to infection, and have begun to investigate the ability of various classes of microbes or microbial products to induce macrophage ET-1 production. We report the production of ET-1 by murine macrophages in response to stimulation with both gram-positive and gram-negative bacteria. Stimulation of macrophages with yeast (*Candida albicans* or *Saccharomyces cerevisiae*) or the protozoan parasite *Leishmania major*, elicited no significant release of ET-1. The production of ET-1 in response to lipopolysaccharide (LPS) was dose and time dependent, and required the expression of a functional toll-like receptor 4 (TLR4). Pharmacologic inhibition of the transcription factor, nuclear factor-κB (NF-κB) suppressed LPS-induced ET-1 production. Our findings complement the growing body of literature implicating a role for macrophage-derived ET-1 in inflammatory pathologies. The production of ET-1 by macrophages during infection and inflammation has the potential to affect tissue perfusion, leukocyte extravasation, and immune cell function. Exp Biol Med 230:652–658, 2005

Key words: macrophage; endothelin-1; lipopolysaccharide; toll-like receptor 4

Introduction

Endothelin-1 (ET-1) is a member of the endothelin family of peptides that were initially described as potent vascular smooth muscle constrictors released from the endothelium by ischemia, injury, or inflammation (1). The majority of studies examining ET-1 expression and function have analyzed the production of ET-1 by the vascular endothelium and its ability to induce vasoconstriction and regulate vascular tone by interacting with receptors on vascular smooth muscle cells. However, ET-1 is now known to be produced by a wide variety of cells and tissues under normal and pathologic conditions. In addition to its vasoconstrictive properties, ET-1 acts as a mitogen for vascular smooth muscle cells, modulates the expression of adhesion molecules on endothelial cells, and is a chemoattractant and modulator of immune cell function (1–3).

Endothelin-1 has been postulated to participate in the pathogenesis of a number of diseases, such as myocardial infarction, bronchial asthma, pulmonary hypertension, renal failure, atherosclerosis, and sepsis. Among these pathophysiologic conditions, sepsis presents with the highest plasma levels of ET-1 (2, 3). Although it has been theorized that the elevated levels of ET-1 initially encountered in sepsis may be attributed to the normal homeostatic response to sepsis-associated hypotension, its prolonged and excessive rise may lead to excessive vasoconstriction in some vascular beds, resulting in decreased organ perfusion and increased organ damage (4). Several studies have demonstrated a strong correlation between ET-1 plasma levels and morbidity and mortality in septic patients (5, 6).

The potential importance of macrophages and monocytes as a source of ET-1 during sepsis or endotoxemia is supported by observations in humans and in animal models. Peripheral blood monocytes from septic patients express significantly higher levels of ET-1 mRNA than those of healthy control subjects (7). Kupffer cells (liver macrophages), in addition to hepatic vascular endothelial cells, seem to synthesize and release ET-1 in experimental models of endotoxemia (8). Increased concentrations of ET-1 have also been noted in the inflammatory infiltrate present after the induction of lethal peritonitis in rats (9). The results of

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these findings and others have led some to suggest that the dramatic increase in plasma ET-1 levels seen in patients suffering from sepsis or endotoxemia is caused primarily by monocyte-derived ET-1 and not from the increased production of ET-1 by endothelial cells (10).

Human, rat, and guinea pig mononuclear phagocytes (monocytes and macrophages) have been demonstrated to produce ET-1 in response to a variety of stimuli, including lipopolysaccharide (LPS), phorbol esters (phorbol myristate acetate) and recombinant human immunodeficiency virus gp120 (11–14). Interferon-γ has been reported to potentiate ET-1 secretion by human monocytes (15). Currently, there are no reports in the literature describing the production of ET-1 by macrophages after their interaction with intact whole microbes or structural components of gram-positive or fungal pathogens. There are also no reports analyzing ET-1 production by murine macrophages.

We initiated a series of investigations to examine the production of ET-1 by murine macrophages in response to stimulation with various classes of microbes or their structural components. These studies demonstrate that macrophages produce ET-1 in response to a variety of microbial challenges, most notably gram-positive and gram-negative bacteria. In addition, we defined a specific receptor-ligand interaction (LPS to toll-like receptor 4 [TLR4]) that is sufficient to induce macrophage ET-1 production. The identification of cellular and molecular events that regulate macrophage ET-1 production may help us to elucidate the source and function of ET-1 observed during numerous pathologic conditions.

Materials and Methods

Mice. Female BALB/c, C3H/HeOuJ, and C3H/HeJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed in the Des Moines University animal care facility. The care and treatment of mice was in accordance with guidelines established by the Des Moines University Institutional Animal Care and Use Committee. All protocols and procedures involving animals were reviewed and approved by this committee.

Macrophage Isolation and Cultivation. Murine bone marrow–derived macrophages (BMDM) were established as previously described, with minor modifications (16). Briefly, femurs were flushed with cation-free Dulbecco's phosphate-buffered saline (DPBS; Mediatech, Herndon, VA) using a 23-gauge needle. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech) containing 20% L929 cell–conditioned medium, 10% heat-inactivated fetal calf serum, 2 m*M* L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. L929 cells were grown under identical conditions, with their cell growth conditioned media serving as a source of macrophage colony stimulating factor.

Cells were grown at 37°C in 5% CO_2 for 7–10 days until uniform monolayers of macrophages were established. After the initial 5 days of cultivation, cells were washed once to remove nonadherent cells and cell debris, and fresh media was added. Twenty-four hours before use, monolayers were washed to remove nonadherent cells, and macrophages removed from the original plastic petri dishes by treatment with 5 m*M* EDTA in DPBS. The BMDM were washed once and resuspended in DMEM containing 10% heat-inactivated fetal calf serum, L-glutamine, penicillin G, and streptomycin. Cells were plated at a density of 1×10^6 cells/ml into 24-well plates (0.5 ml/well) or 25 mm^2 tissue culture flasks (3 ml/flask).

Microorganisms. *Escherichia coli* (ATCC#25922), *Staphylococcus aureus* (ATCC#29213), *Enterococcus faecalis* (ATCC#19443), and *Saccharomyces cerevisiae* (ATCC#9763) were purchased from the American Type Culture Collection (Manassas, VA). *Rhodococcus equi* (equine clinical isolate, "238") was provided by David Mosser, Ph.D. (University of Maryland, College Park, MD). *Candida albicans* (clinical isolate, "328") was provided by Bryan Larsen, Ph.D. (Des Moines University, Des Moines, IA). *Leishmania major* (MHOM/IL/Friedlin) was provided by David Sacks, Ph.D. (National Institute of Allergy and Infectious Disease, Bethesda, MD).

Bacteria were grown overnight at 37°C in Brain Heart Infusion Broth (Difco, Detroit, MI). *C. albicans* and *S. cerevisiae* were grown in Yeast Nitrogen Base media (Difco). The protozoan parasite *L. major* was maintained in Schneider's *Drosophila* media (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated fetal calf serum, L-glutamine, penicillin G, and streptomycin. Parasite cultures were maintained at 26°C.

Before being used in macrophage stimulation assays, all microorganisms were washed extensively in Hank's balanced salt solution (Mediatech).

Biochemicals and Reagents. Bacterial LPS (*E. coli* 0127:B8), polymyxin B, paclitaxel (Taxol), and BAY 11–7082 were all purchased from Sigma.

Macrophage Stimulation. Intact and viable microorganisms were added to macrophage monolayers to achieve a multiplicity of infection of approximately 50 to 1. Lipopolysaccharide and Taxol were added to achieve the final concentrations indicated. In some assays, cells were preincubated with 15 μg/ml of the LPS-neutralizing compound, polymyxin B, or an inhibitor of nuclear factor-κB (NF-κB) activation, BAY 11–7082, for 15 mins before stimulation. At the indicated times, culture supernatants were collected, centrifuged to remove microbes and cellular debris, and frozen and stored at –80°C for later analysis. Monolayers were washed with DPBS, and total cellular RNA was isolated for analysis of ET-1 transcript abundance.

Analysis of ET-1 mRNA Expression. RNA was isolated from washed macrophage monolayers using the Purescript RNA Isolation Kit (Gentra Systems, Minneapolis, MN). cDNA was synthesized from 1 μg of total RNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Relative ET-1 and β-actin mRNA

levels were determined using a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The PCR primers and amplification parameters are presented in Table 1. Primers were designed to produce relatively small PCR products and to span introns (thereby generating different size products when amplifying cDNA vs. potentially contaminating genomic DNA as a template). The number of amplification cycles used was determined empirically, such that amplification occurred in a linear fashion (25 cycles for β-actin, and 30 cycles for ET-1).

Analysis of ET-1 Peptide Levels. The amount of ET-1 peptide present in culture supernatants was determined using the ET-1 enzyme immunoassay (EIA) kit from Assay Designs, Inc. (Ann Arbor, MI).

Statistical Analysis. Data analysis was performed using the GraphPad InStat program (GraphPad Software, Inc, San Diego, CA), with statistical significance ($P < 0.05$) determined using analysis of variance (ANOVA) and Student's t tests.

Results

LPS-Induced ET-1 Production Is Time and Dose Dependent. Stimulation with LPS has been previously reported to trigger the release of ET-1 from human mononuclear phagocytes (11). To determine the effect of LPS stimulation on ET-1 production by murine macrophages, BMDM from BALB/c mice were stimulated with 100 ng/ml of LPS for periods of time ranging from 2–12 hrs. At each time point, culture supernatants and total cellular RNA were collected and analyzed for the presence of ET-1 peptide and mRNA. As previously reported for macrophages from human and other animal models, LPS stimulates the production of ET-1 by murine BMDM (Fig. 1). This induction was rapid, with abundant message detected as early as 2 hrs after stimulation (Fig. 1A), and accumulated peptide detected in the culture supernatant as early as 4 hrs after the addition of LPS (Fig. 1B). Peak message and peptide levels appeared approximately 6–8 hrs after initial LPS stimulation. Further analysis demonstrates that macrophages rapidly and dramatically increase transcription of the ET-1 gene and secrete ET-1 peptide when stimulated with considerably lower concentrations of LPS (Fig. 2). Transcription of the ET-1 gene and secretion of ET-1 peptide by murine BMDM was detected after the stimulation of macrophages with concentrations of LPS as low as 0.1 ng/ml, with maximum production occurring after stimulation with 100 ng/ml or greater.

These data demonstrate that the production of ET-1 by murine BMDM in response to LPS is time and dose dependant, with maximum production occurring approximately 6 hrs after initial stimulation, and with LPS concentrations of 100 ng/ml or greater.

ET-1 Is Produced by Macrophages in Response to Microbial Challenge. To determine the effect of microbial stimulation on macrophage ET-1 production, BMDM from BALB/c mice were incubated with the following microorganisms at a multiplicity of infection (MOI) of 50 to 1: gram-negative bacteria, *E. coli*; gram-positive bacteria, *S. aureus*, *E. faecalis*, and *R. equi*; yeast, *S. cerevisiae* and *C. albicans*; and protozoa, *L. major*. Six hours after stimulation, culture supernatants and total cellular RNA were isolated. The array of organisms used in these assays was selected to include bacteria frequently associated with sepsis (*E. coli*, *S. aureus*, *E. faecalis*), fungal agents causing sepsis (*C. albicans*), and intracellular pathogens of macrophages (*R. equi* and *L. major*). *S. cerevisiae* was included because of its historical use as a macrophage stimulant. Each of these organisms varies in surface structure and presumably interacts with a diverse array of macrophage receptors.

As illustrated in Figure 3, the exposure of macrophages to gram-positive or gram-negative bacteria resulted in the rapid induction of ET-1 transcripts and secretion of ET-1 peptide. Interestingly, the incubation of macrophages with eukaryotic microbes (yeast; *S. cerevisiae*, *C. albicans*; protozoa; *L. major*) had little or no effect on the production of ET-1 by macrophages. Although stimulation with *S. cerevisiae* resulted in increased levels of ET-1 mRNA (see representative assay in Fig. 3A) the amount of ET-1 peptide secreted was not significantly greater than the amount produced by unstimulated cells.

ET-1 Is Produced in Response to TLR4 Ligation. The predominant receptor on macrophages responsible for recognizing LPS from gram-negative organisms is known as TLR4 (17). To determine the contribution of TLR4 ligation to macrophage ET-1 production, macrophages from C3H/HeJ (TLR4-signaling deficient) and C3H/HeOuJ (wild type) mice were stimulated for 6 hrs with either media alone, LPS, or intact and viable *E. coli* or *S. aureus*. Stimulation was performed in the absence or presence of the lipid A (LPS binding)–neutralizing compound, polymyxin B (18). In each of three independent experiments, summarized in Figure 4, macrophages from TLR4-sufficient C3H/HeOuJ mice secreted significant levels of ET-1 peptide in response to all stimuli (LPS, *E. coli*, *S. aureus*). Although the response to LPS was abrogated by the inclusion of polymyxin B, this treatment had only a modest effect ($P < 0.05$) on the C3H/HeOuJ macrophage response to *E. coli*, and no effect on the response to *S. aureus*. In contrast to macrophages from C3H/HeOuJ mice, cells from TLR4-deficient C3H/HeJ mice produced no ET-1 in response to LPS and a diminished response to *E. coli*.

In addition to binding LPS, murine TLR4 has been demonstrated to recognize the chemotherapeutic agent, Taxol (19). The stimulation of murine macrophages with Taxol has been demonstrated to induce the expression of a number of genes and the secretion of many inflammatory cytokines via a signal transduction pathway indistinguishable from that triggered by LPS (20). To determine whether TLR4 ligands other than LPS were cable of triggering the

Table 1. PCR Primers and Conditions

Target	Forward primer	Reverse primer	Cycles	Annealing temp (°C)	Product size (bp)
ET-1	AAGCGCTGTTCCTGTTCTTC	CTTGATGCTATTGCTGATGG	30	60	383
β-actin	GTGGGCCGCTCTAGGCACCA	CTCAGCTGTGGTGGTGAAGC	25	55	510

release of ET-1, macrophages were stimulated with Taxol in the absence or presence of polymyxin B. As demonstrated in Figure 5, macrophages release ET-1 in response to both of the TLR4 ligands, LPS and Taxol.

These data demonstrate that the ligation of TLR4 by LPS or Taxol is sufficient to induce ET-1 production by murine macrophages, and that the ligation of TLR4 on macrophages by LPS in addition to other unknown ligands is responsible for much of the ET-1 secretion induced by *E. coli*. Interestingly, *S. aureus* (a gram-positive organism containing no LPS) is a potent inducer of ET-1, and this induction is independent of TLR4. These data suggest that the ligation of receptors other than TLR4 can induce the production of ET-1 by macrophages.

NF-κB Activation Is Necessary for Macrophage ET-1 Production. After ligation, all TLRs activate common signaling pathways that culminate in the activation of the transcription factor, NF-κB. To investigate the role for NF-κB in the regulation of ET-1 production by macrophages, cells were stimulated with LPS in the presence of increasing concentrations of a pharmacologic inhibitor of NF-κB activation, BAY 11–7082. As demonstrated in Figure 6, LPS-induced ET-1 production was significantly inhibited (88.5 ± 4.5%) by micromolar concentration of BAY 11–7082. A putative NF-κB binding site identified in the promoter region of the human ET-1 gene has recently been implicated in regulating the expression of ET-1 by endothelial cells in response to stress (21). The region of the ET-1 promoter involved in regulating macrophage ET-1 production is a current area of investigation.

Discussion

The production of ET-1 by endothelial cells under physiologic and pathologic conditions has received a considerable amount of attention. Here, we report the production of ET-1 by murine macrophages after microbial challenge and in response to ligation of TLR4.

Our observations that murine macrophages produce ET-1 in response to both gram-positive and gram-negative bacteria (Fig. 3), as well as in response to LPS stimulation (Figs. 1 and 2), support the notion that ET-1 production is part of the characteristic macrophage response to microbial challenge. Furthermore, our observations suggest that

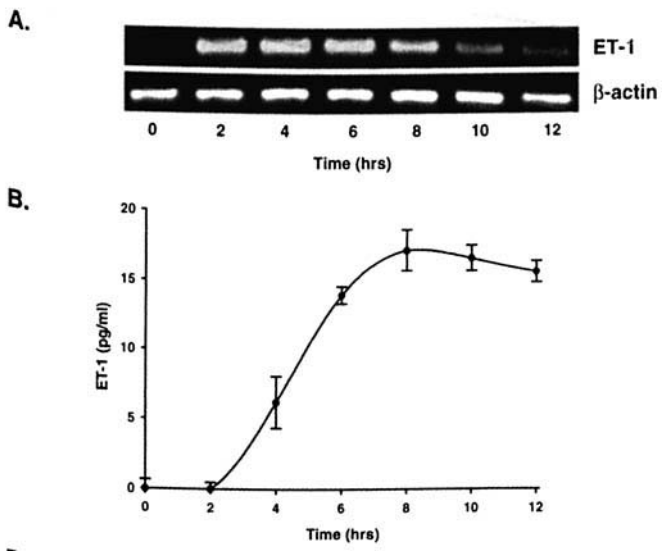

Figure 1. The kinetics of ET-1 production by macrophages in response to LPS stimulation. BMDM were stimulated with 100 ng/ml of LPS for increasing periods of time. Supernatants were collected and analyzed for ET-1 peptide concentration using a commercially available EIA kit (B). Monolayers were washed and RNA was collected for RT-PCR analysis of ET-1 and β-actin transcript levels (A). Peptide data represent the mean ± SD of triplicate determinations.

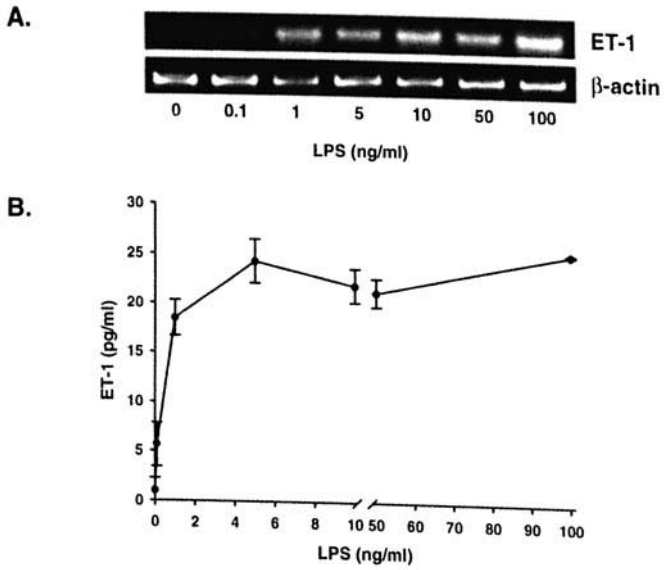

Figure 2. The effect of different concentrations of LPS on macrophage ET-1 production. BMDM were stimulated for 6 hrs with increasing concentrations of LPS. Supernatants were collected and analyzed for ET-1 peptide concentration using a commercially available EIA kit (B). Monolayers were washed and RNA was collected for RT-PCR analysis of ET-1 and β-actin transcript levels (A). Peptide data represent the mean ± SD of triplicate determinations.

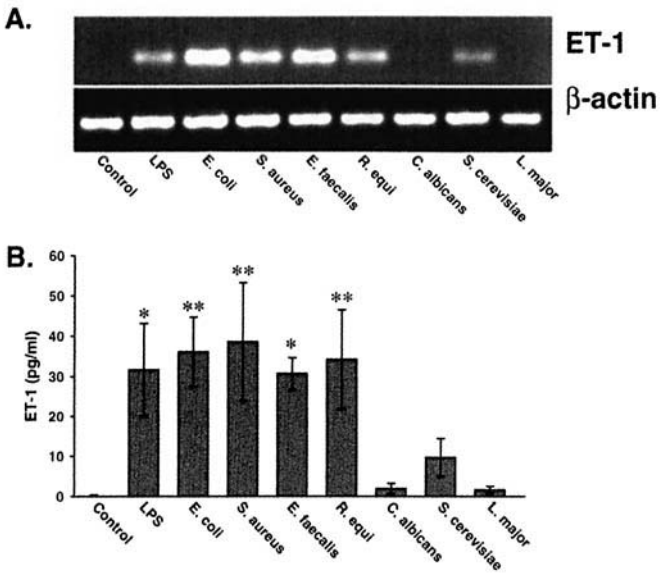

Figure 3. The effect of microbial challenge on macrophage ET-1 production. BMDM were stimulated for 6 hrs with either 100 ng/ml of LPS, or the indicated microorganisms at a multiplicity of infection of 50 to 1. Supernatants were collected and analyzed for ET-1 peptide concentration using a commercially available EIA kit (B). Monolayers were washed and RNA was collected for RT-PCR analysis of ET-1 and β-actin transcript levels (A). The RT-PCR data are representative of three independent assays. The peptide data represent the mean ± SEM of three independent assays, each performed in triplicate. Asterisks indicate a significant difference (* $P < 0.05$; ** $P < 0.01$) from control cells as determined using Student's *t* test.

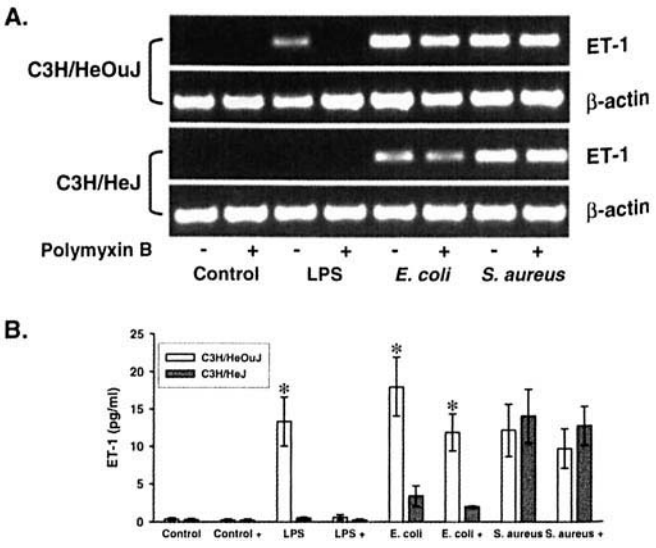

Figure 4. The importance of TLR4 signaling in macrophage ET-1 production. BMDM from wild-type C3H/HeOuJ or TLR4-signaling deficient C3H/HeJ mice were stimulated with LPS, *E. coli*, or *S. aureus* for 6 hrs in the absence (–) or presence (+) of 15 μg/ml of the LPS-neutralizing compound, polymyxin B. Culture supernatants were collected and analyzed for the presence of the ET-1 peptide (B). Monolayers were washed and RNA was extracted for RT-PCR analysis (A). The ET-1 peptide data represent the mean ± SEM of four independent experiments, each performed in triplicate. The RT-PCR data are representative of one of these assays. Asterisks indicate significant differences (* $P < 0.001$) in the response of cells from C3H/HeOuJ mice compared with those from C3H/HeJ mice, as determined using Student's *t* test.

macrophages may be a key source of ET-1 during infection or inflammatory conditions that develop in response to microbial challenge.

Although not readily apparent in figures demonstrating the mean amount of ET-1 peptide secreted in multiple experiments (Figs. 3 and 4), within an individual replicate, the amount of ET-1 message detected was linearly correlated with the amount of ET-1 peptide present in the culture supernatant (data not shown). Stringent regulation of ET-1 transcription and the relatively short half-life of ET-1 mRNA suggest that ET-1 production is primarily regulated at the level of transcription in endothelial cells (22, 23). Recently, some reports have implicated post-transcriptional and post-translational mechanisms that may contribute to the secretion of ET-1 by endothelial cells (24, 25). Transcriptional and post-transcriptional mechanisms regulating macrophage ET-1 production are a current area of investigation in our laboratory.

The most extensively studied member of the TLR family is TLR4. These receptors vary in ligand specificity, but collectively recognize a myriad of pathogen-associated molecular patterns, including bacterial endotoxin, and cell wall and membrane components of bacteria, fungi, and protozoa. Toll-like receptor 4 is an essential component in the recognition of LPS from gram-negative bacteria by macrophages (17). Ligation of TLR4 has been demonstrated to result in the production of numerous inflammatory mediators, including tumor necrosis factor TNF-α, interleukin IL-1β, IL-6, IL-12, and nitric oxide (26). C3H/HeJ mice, which are LPS-hyporesponsive, possess a point mutation in their *tlr4* gene, resulting in a lack of LPS-induced signal transduction, and, therefore, a diminished inflammatory response when stimulated with LPS (17).

Using these TLR4-signaling deficient animals, we demonstrate that TLR4 ligation is sufficient to induce macrophage ET-1 production (Fig. 4). Furthermore, in addition to LPS, other TLR4 ligands, such as Taxol, are capable of triggering ET-1 production (Fig. 5). The observations that the gram-positive (LPS negative) bacterium, *S. aureus*; and the gram-negative (LPS positive) bacterium, *E. coli* are capable of stimulating ET-1 production in the absence of TLR4 signaling suggest that other macrophage receptors, in addition to TLR4, can trigger macrophage ET-1 production. A role for TLR2, a macrophage receptor for bacterial lipoproteins and lipoteichoic acids, in triggering macrophage ET-1 production in response to both gram-positive and gram-negative bacteria is a current area of investigation in our laboratory.

After ligation, all TLRs activate similar signaling pathways that culminate in the activation of the transcription factor, NF-κB (27). Activation of NF-κB in macrophages results in the production of numerous proinflammatory cytokines, chemokines, and growth factors

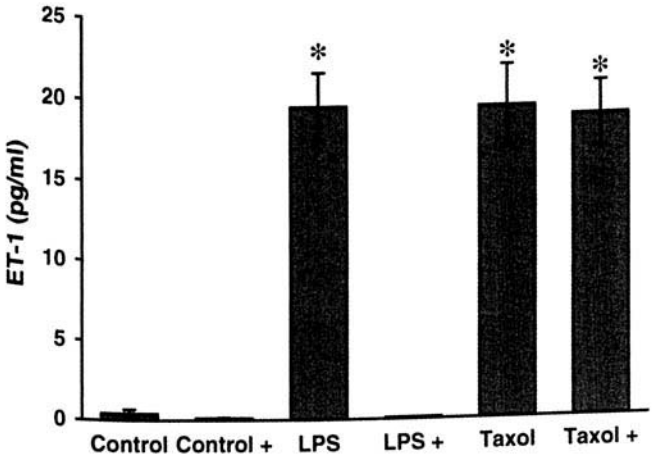

Figure 5. Endothelin-1 production in response to TLR4 ligation. BMDM were stimulated for 6 hrs in the presence of media alone (Control), 100 ng/ml of LPS, or 25 μ*M* Taxol. Stimulation was performed in the absence or presence (+) of 15 μg/ml of the LPS-neutralizing compound, polymyxin B. Supernatants were collected and analyzed for the presence of ET-1 peptide. Data represent the mean ± SEM of three independent experiments, each performed in triplicate. Asterisks indicate a significant differences (* $P < 0.001$) from the control, as determined using Student's *t* test.

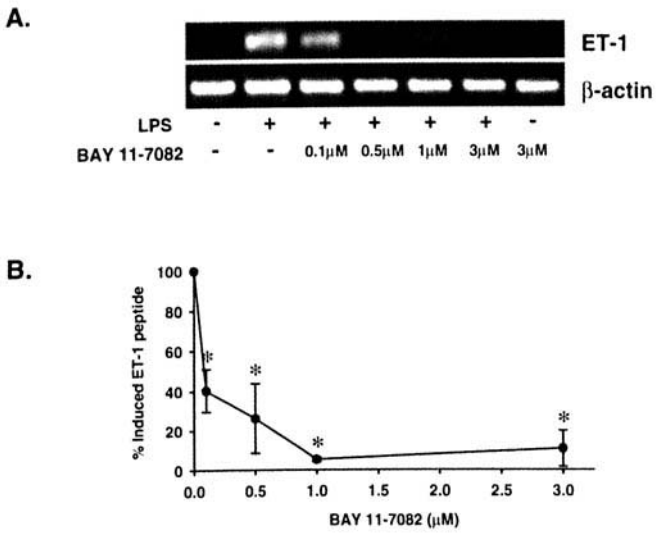

Figure 6. The effect of an inhibitor of NF-κB activation on macrophage ET-1 production. BMDM were stimulated for 6 hrs in the presence of media alone (–) or 100 ng/ml of LPS (+). Stimulation was performed in the presence of increasing concentrations of BAY 11–7082, an irreversible inhibitor of IκB phosphorylation, and subsequent NF-κB nuclear translocation. Supernatants were collected and analyzed for the presence of ET-1 peptide. Monolayers were washed and RNA was extracted for RT-PCR analysis. Induced peptide values represent the mean ± SD of triplicate determinations. This figure is representative of three independent assays, each performed in triplicate. Asterisks indicate significant difference (* $P < 0.001$) from control (no inhibitor), as determined using Student's *t* test.

(28). Our ability to abrogate macrophage ET-1 production through the pharmacologic inhibition of NF-κB activity (Fig. 6) supports the inclusion of ET-1 as a macrophage-derived mediator of inflammation.

Recent studies in transgenic mice reveal that the over expression of ET-1 results in pulmonary fibrosis, chronic lung inflammation, and chronic kidney inflammation with increased tissue density of lymphocytes and macrophages (29, 30). Studies in endothelin receptor knockout mice suggest a role for the endothelin system in cutaneous inflammation and inflammatory pain (31). Macrophages found in fatty streaks and fibrous plaque areas from human atherosclerotic lesions express high levels of ET-1 (32), and native and oxidized low-density lipoprotein have been reported to increase ET-1 expression by both vascular smooth muscle cells and monocyte-derived macrophages. These studies, in addition to the reported contribution of ET-1 to the pathophysiology of sepsis, support the hypothesis that ET-1 can act as a potent proinflammatory mediator in a diverse spectrum of pathologic conditions.

Our findings complement this growing body of literature, implicating a role for ET-1 in inflammatory pathologies. Our studies appear to be the first to examine ET-1 production by murine macrophages, the first to demonstrate ET-1 production by macrophages after their exposure to intact microorganisms (gram-positive and gram-negative bacteria), and the first to identify a specific receptor-ligand interaction responsible for inducing the release of ET-1 by macrophages. Current investigations are aimed at the identification of additional receptor-ligand interactions that trigger macrophage ET-1 production, and the cellular and molecular mechanisms that regulate this response.

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