Interleukin-1β, but not interleukin-6, enhances renal and systemic endothelin production in vivo

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Boesen EI, Sasser JM, Saleh MA, Potter WA, Woods M, Warner TD, Pollock JS, Pollock DM. Interleukin-1β, but not interleukin-6, enhances renal and systemic endothelin production in vivo. Am J Physiol Renal Physiol 295: F446–F453, 2008. First published June 4, 2008; doi:10.1152/ajprenal.00095.2008.—The inflammatory cytokines IL-1β and IL-6 have been shown to stimulate production of endothelin-1 (ET-1) by several cell types in vitro, but their effects on renal ET-1 production in vivo are not known. To test whether IL-1β and IL-6 stimulate renal ET-1 production and release in vivo, urine was collected from male C57BL/6 mice over 24-h periods at baseline and on days 7 and 14 of a 14-day subcutaneous infusion of IL-1β (10 ng/h), IL-6 (16 ng/h), or vehicle. By day 14, plasma ET-1 was significantly increased by IL-1β infusion (1.7 ± 0.1 vs. 0.8 ± 0.1 pg/ml for vehicle, P < 0.001). Compared with vehicle infusion, IL-1β infusion induced significant increases in urinary ET-1 excretion rate and urine flow but did not affect conscious mean arterial pressure (telemetry). IL-1β infusion significantly increased renal cortical and medullary IL-1β content (ELISA) and prepro-ET-1 mRNA expression (quantitative real-time PCR). In contrast, 14 days of IL-6 infusion had no significant effect on plasma ET-1 or urinary ET-1 excretion rate. To determine whether IL-1β stimulates ET-1 release via activation of NF-κB, inner medullary collecting duct (IMCD-3) cells were incubated for 24 h with IL-1β, and ET-1 release and NF-κB activation were measured (ELISA). IL-1β activated NF-κB and increased ET-1 release in a concentration-dependent manner. The effect of IL-1β on ET-1 release could be partially inhibited by pretreatment of IMCD-3 cells with an inhibitor of NF-κB activation (BAY 11-7082). These results indicate that IL-1β stimulates renal and systemic ET-1 production in vivo, providing further evidence that ET-1 participates in inflammatory responses.

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these cytokines could stimulate renal ET-1 production in vivo. Having found that IL-1β, but not IL-6, stimulated renal ET-1 production, we also performed experiments using a mouse inner medullary collecting duct (IMCD) cell line (mIMCD-3 cells) (42) to determine whether NF-κB might be an intermediate between IL-1β and renal ET-1 production.

METHODS

Effect of chronic IL-6 and IL-1β infusions on renal ET-1 production. All procedures were approved in advance by the Medical College of Georgia Institutional Animal Care and Use Committee. Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed individually and maintained on a diet of regular rodent chow (Harlan Teklad, Madison, WI) and tap water throughout the study. After a 1-wk baseline period, mice were placed in metabolic cages (Lab Products, Seaford, DE) for 24 h to facilitate urine collection. On the following day, microosmotic pumps (Alzet 1002, DURECT, Cupertino, CA) were implanted subcutaneously in the interscapular region under anesthesia with 2% isoflurane (Baxter Healthcare, Deerfield, IL) for delivery of mouse recombinant IL-6 (16 ng/h; R & D Systems, Minneapolis, MN), mouse recombinant IL-1β (10 ng/h; R & D Systems), or their respective vehicles (0.9% NaCl or PBS, both containing 0.1% bovine serum albumin) for 14 days (n = 6–8 in each group). At the end of each week, mice were again placed in metabolic cages for 24-h urine collection periods. Inasmuch as all intact endothelin found in the urine is of renal origin (1), urinary ET-1 excretion rate was used as an index of renal endothelin production. After the 2-wk IL-6, IL-1β, or vehicle infusion, the mice were anesthetized with pentobarbital sodium (60 mg/kg; Abbott Laboratories, North Chicago, IL), a terminal blood sample was taken by cardiac puncture and centrifuged at 4°C, and the plasma was collected. Kidneys from IL-1β- and vehicle-infused mice were dissected into cortex and medulla. Plasma, renal tissue, and urine samples were snap frozen in liquid nitrogen and stored at −80°C.

Effect of chronic IL-1β infusion on core body temperature and arterial pressure. Male C57BL/6 mice (n = 5 in each group) were instrumented with telemetry transmitters for recording of core body temperature (model TA-F20, Data Sciences International, St. Paul, MN) or arterial pressure (model PA-C20, Data Sciences). With the animals under 2% isoflurane anesthesia, the TA-F20 transmitter was implanted into the abdominal cavity via a small midline incision, and the wound was closed using 5-0 chromic sutures. The wound was infiltrated with 0.125% bupivacaine (Abbott), and the mice were given acetylsalicyclic acid (Sigma Chemical, St. Louis, MO; 2 g/l in drinking water) for the following 2 days. The PA-C20 transmitters were implanted as previously described (28). After 1 wk of recovery from surgery, baseline data were recorded for 4 days before implantation of microosmotic pumps for infusion of IL-1β or vehicle as described above. Data were recorded throughout the 14-day infusions, and mice were then killed and plasma was collected and stored as described above.

Effect of IL-1β on ET-1 production by mIMCD-3 cells. mIMCD-3 cells (American Type Culture Collection, Rockville, MD) were cultured in 1:1 DMEM-Ham’s F-12 medium containing 10% fetal calf serum, 1% penicillin-streptomycin, and 2 mM L-glutamine. Cells were incubated at 37°C in 5% CO2-95% air. At passages 4–8, cells were grown to confluence on 96-well plates (Costar, Corning International, Corning, NY). In initial experiments, cells were treated with mouse recombinant IL-1β (10 ng/ml; R & D Systems) for 0.5–48 h. Incubation of cells with IL-1β for 24 h increased the rate of ET-1 release into culture medium to ~2.5 times that of cells incubated for the same duration without IL-1β (n = 2); therefore, concentration-response curves were constructed after incubation of cells with IL-1β (0.01–10 ng/ml) for 24 h. Medium was removed and stored at −20°C until the immunoreactive ET-1 content was analyzed. To determine whether IL-1β stimulates ET-1 release via activation of NF-κB, cells were grown to confluence in 100-mm plates and then incubated with IL-1β (0.0001–1 ng/ml) for 24 h. Cells were rinsed twice with ice-cold PBS, harvested, and subjected to analysis of NF-κB activation. In additional experiments, cells were pretreated for 1 h with 0 or 10 μM BAY 11-7082 (Biomol, Plymouth Meeting, PA), an irreversible inhibitor of IkBα phosphorylation and degradation. Cells were then incubated with IL-1β (10 ng/ml) for 24 h, medium was removed and stored for later analysis of immunoreactive ET-1 content, and cell viability was determined spectrophotometrically as the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical) to formazan. Cells were incubated with MTT (0.5 mg/ml) at 37°C for 45 min at the end of each experiment. MTT was then removed by aspiration, and cells were solubilized in 100 μl of dimethyl sulfoxide. The reduction of MTT to formazan was quantified by measurement of the optical density at 550 nm. Values are means ± SE and represent results of cells from three to seven experiments, each assayed in triplicate.

Assays. Plasma IL-6 and IL-1β concentrations were measured by ELISA (R & D Systems). For measurement of renal tissue IL-1β concentration, tissue was first homogenized in a hypotonic buffer containing protease inhibitors as described previously (38), the homogenate was centrifuged at 15,000 g at 4°C for 20 min, and the supernatant IL-1β concentration was measured by ELISA (R & D Systems), with IL-1β content expressed as picograms per milligram of total protein, determined by standard Bradford assay (Bio-Rad, Hercules, CA). Urine electrolyte concentrations were determined by ion-sensitive electrodes (Synchron ELISE, Beckman Instruments, Brea, CA). Urinary immunoreactive endothelin concentrations were measured by radioimmunoassay (Amersham Pharmaica Biotech, Arlington Heights, IL). Plasma and cell culture media immunoreactive endothelin concentrations were measured by chemiluminescent immunomunoassay (R & D Systems). Urinary protein excretion was measured by standard Bradford assay (Bio-Rad) and albumin excretion by ELISA (Albwell M ELISA, Exocell, Philadelphia, PA). NF-κB activation in mIMCD-3 cell lysates was measured by an ELISA (TransAM NF-κB, Active Motif, Carlsbad, CA) utilizing a primary antibody that recognizes an epitope on the p65 subunit of NF-κB that is only accessible when NF-κB has been activated.

Renal preproendothelin mRNA expression. Total RNA was isolated from mouse renal tissue using ultrapure TRIzol reagent according to the procedure described by the manufacturer (Invitrogen, Carlsbad, CA). Total RNA concentration and purity were determined spectrophotometrically using absorbance at 260 nm (A260) and the ratio of A260 to A280, respectively. RNA (1 μg) was reverse transcribed using the QuantiTect RT kit (Qiagen, Valencia, CA). A dilution of the resulting cDNA was used to quantify the relative content of mRNA by real-time PCR (iCycler IQ real-time PCR detection system, Bio-Rad) using commercially available QuantiTect primer assays (Qiagen) to detect mouse GAPDH and prepro-ET-1 (catalog nos. QT03090999 and QT00253512 respectively), with SYBR green as the fluorescent probe. Fluorescence data were acquired at the end of extension. A melt analysis run was performed for all products to determine the specificity of the amplification. The cycle threshold (CT) values for each gene were measured and calculated by computer software (iCycler IQ OSS, version 3.0a, Bio-Rad). Expression of prepro-ET-1 mRNA relative to GAPDH was calculated on the basis of the change in CT, in which ΔCT = CTTarget – CTGAPDH, and normalized between the control group and corresponding treatment group and expressed as −ΔΔCT.

With use of this method, an mRNA that is expressed at a greater level in the experimental than in the control group will have a negative ΔΔCT value and a positive (−ΔΔCT) value. The relative fold expression was calculated as 2−ΔΔCT.

Statistical analyses. Values are means ± SE, except for plasma cytokine concentrations, which are presented as median (range). Plasma cytokine concentrations were compared between two groups by Mann-Whitney’s U-test. Plasma ET-1 concentrations, renal tissue IL-1β concentrations, and renal prepro-ET-1 mRNA expression were
compared between two groups by Student’s unpaired t-test. Data from the three weekly metabolic cage collection periods and from body temperature recordings were analyzed by repeated-measures ANOVA with (Bonferroni’s) post hoc contrasts. Blood pressure and heart rate before and after IL-1β infusion were compared within the same mice by paired t-test. Data from cell culture experiments were analyzed by one-factor ANOVA, and Dunn’s post hoc test was performed when $P < 0.05$. $P \leq 0.05$ was considered statistically significant.

RESULTS

**Chronic IL-6 infusion in mice.** Infusion of IL-6 subcutaneously for 14 days produced a median plasma IL-6 concentration of 57 pg/ml (range 32–109 pg/ml), whereas IL-6 was below detection in four vehicle-infused mice and 7 pg/ml in the other two vehicle-infused mice ($P < 0.01$). Plasma ET-1 concentration was not significantly affected by IL-6 infusion ($0.44 \pm 0.05$ and $0.44 \pm 0.03$ pg/ml in vehicle- and IL-6-infused mice, respectively). There were no significant differences in urinary ET-1 excretion rate, urine flow, Na+ excretion, or food and water intake between vehicle- and IL-6-infused mice over the course of the experiment (Fig. 1). Body weight of mice in both groups increased over the 3-wk period ($P$ for time effect ($P_{time}$) = 0.0001), although IL-6-infused mice gained weight at a significantly slower rate ($P$ for time-dependent difference between groups ($P_{group \times \ time}$) $< 0.05$; Fig. 1F).

**Chronic IL-1β infusion in mice.** Infusion of IL-1β subcutaneously for 14 days produced a median plasma IL-1β concentration of 179 pg/ml (range 120–268 pg/ml), whereas IL-1β was below detection in all eight vehicle-infused mice ($P < 0.001$). At day 14, plasma ET-1 concentration of IL-1β-infused mice was approximately double that of vehicle-infused mice ($1.7 \pm 0.1$ vs. $0.8 \pm 0.1$ pg/ml, $P < 0.001$). Infusion of IL-1β significantly increased renal cortical and medullary concentrations of IL-1β ($P < 0.01$ in both cases; Fig. 2A). Expression of prepro-ET-1 mRNA was significantly increased in the renal cortex and medulla of IL-1β-infused mice compared with vehicle-infused mice ($P < 0.05$; Fig. 2B).

Urinary excretion of ET-1 was similar in both groups at baseline but increased during the 14-day IL-1β infusion to levels that were significantly elevated compared with vehicle-infused mice at day 14 and compared with baseline and day 7 of the IL-1β infusion ($P < 0.05$; Fig. 3A). Similarly, urine flow was similar in both groups at baseline but increased markedly over the course of the experiment in IL-1β-infused mice, with a significant elevation in IL-1β-infused mice at day 14 compared with vehicle-infused mice and compared with earlier time points within the same group ($P < 0.05$; Fig. 3B). The rate of Na+ excretion also increased significantly over the course of the experiment ($P_{time} < 0.05$; Fig. 3C) but was not significantly different between the two groups. Water intake increased significantly over the course of the experiment in both groups and was significantly greater in the IL-1β-infused mice ($P$ for treatment group effect ($P_{group}$) = 0.05; Fig. 3D), whereas food intake did not change significantly in either group (Fig. 3E). Body weight of vehicle-infused mice increased over the course of the experiment, whereas body weight of IL-1β-infused mice did not, with the weights of the two groups diverging significantly from day 7 of the 14-day infusions ($P < 0.05$; Fig. 3F). Urinary protein excretion rate was not significantly different between vehicle- and IL-1β-infused mice, nor did it change over the course of the study in either group ($1.16 \pm 0.1$ and $1.10 \pm 0.13$ mg/day at day 14 in vehicle and IL-1β groups, respectively). Urinary albumin excretion, which might be a more sensitive marker of renal injury, was not significantly different between vehicle- and IL-1β-infused groups at baseline (25 ± 2 and 22 ± 2 μg/day, respectively) or after 1 wk of IL-1β infusion. However, there was a small, but significant ($P < 0.05$), increase in albumin excretion in IL-1β-infused mice at day 14 (32 ± 4 μg/day) compared with vehicle-treated mice (19 ± 2 μg/day) and with day 7 in IL-1β-infused mice (19 ± 4 μg/day).

Because of the known critical role of IL-1β in fever, core body temperature was measured in separate groups of mice before and during IL-1β or vehicle infusion. Core body tem-
per temperature was similar in both groups of mice before microosmotic pump implantation (Fig. 4A). After implantation of microosmotic pumps, temperature rose transiently in both groups, with this effect being significantly greater in IL-1β-infused mice on days 2 and 3 of the infusion (P < 0.05; Fig. 4A). However, by day 4 of the subcutaneous infusions, temperatures were similar to preinfusion levels and not significantly different between the two groups. Chronic infusion of IL-1β did not significantly affect conscious mean arterial pressure, which averaged 108 ± 3 mmHg on the 2 days before commencement of IL-1β infusion and 103 ± 8 mmHg on the final 2 days of the 14-day IL-1β infusion (Fig. 4B). Heart rate rose transiently on commencement of IL-1β infusion, following a pattern similar to that observed for body temperature, but was not significantly elevated by the end of the 14-day IL-1β infusion (552 ± 27 and 534 ± 27 beats/min on the 2 days before and the final 2 days of IL-1β infusion, respectively; Fig. 4C).

IL-1β-induced ET-1 production in IMCD-3 cells. Since the IMCD is a major site of renal ET-1 production and appears to contribute much of the ET-1 found in the urine (2), the molecular mechanisms underlying the effect of IL-1β on ET-1 production were further investigated using the mIMCD-3 cell line. IL-1β increased immunoreactive ET-1 concentration in the culture medium of mIMCD-3 cells in a concentration-dependent manner (P = 0.0001; Fig. 5A). To determine whether IL-1β stimulated ET-1 production via NF-κB, we first examined whether IL-1β activated NF-κB in mIMCD-3 cells. IL-1β caused a concentration-dependent increase in the content of the activated p65 subunit of NF-κB in mIMCD-3 cells (P = 0.0001), with this effect reaching statistical significance at IL-1β concentrations of ≥0.03 ng/ml (Fig. 5B). We then tested whether inhibition of NF-κB would attenuate the IL-1β-induced increase in ET-1 release from mIMCD-3 cells. Cells were treated with inhibitors of NF-κB before incubation with IL-1β. BAY 11-7082, an irreversible inhibitor of IκBα phosphorylation and degradation that results in inactivation of NF-κB, significantly reduced the IL-1β-induced stimulation of ET-1 release at 10 μM to 71 ± 12% of IL-1β alone (P < 0.05), with no decrease in cell viability. Higher concentrations of BAY 11-7082, as well as other inhibitors of NF-κB activation, parthenolide and MG-132, reduced cell viability (data not shown) and, therefore, could not be used to assess the involvement of NF-κB activation in the response of mIMCD-3 cells to IL-1β.

DISCUSSION

The major finding of the present study is that chronic IL-1β infusion increased renal plasma and urinary ET-1 levels and increased renal prepro-ET-1 mRNA, indicating that IL-1β stimulates ET-1 production in vivo. These findings agree well with data from cultured cells in this and previous studies (6, 21, 35, 48, 55). Furthermore, the use of telemetry has enabled us to confirm that these effects of IL-1β on the endothelin system were not secondary to an ongoing febrile response, nor are they associated with induction of hypertension.
In contrast to IL-1β, chronic infusion of IL-6 had no effect on plasma or urinary ET-1. Although it is not possible to exclude some localized effect of IL-6 on tissue ET-1 concentrations, our results do not support the idea that IL-6 stimulates ET-1 production in vivo, at least not at the dose used. We chose to infuse IL-6 at a rate of 16 ng/h on the basis of a previous study that employed this dose to mimic levels seen in obesity (25) and infused IL-1β at an approximately equimolar rate.

Comparing the relative potencies of IL-1β and IL-6 in stimulating ET-1 production in vivo is, however, problematic. Although the two cytokines were infused at a similar rate, a greater plasma concentration of IL-1β was reached by day 14 of the infusion. It is worth noting that the concentrations of IL-6 and IL-1β achieved by our infusion protocol are much lower than those seen in sepsis (30). It is also possible that IL-6 had a transient effect on ET-1 production, since the first measurement of ET-1 production (urinary ET-1 excretion rate) was not made until 1 wk after commencement of cytokine infusions.

Chronic IL-1β infusion had a marked effect on plasma ET-1 concentration, which was approximately double that of vehicle-infused mice. This effect is consistent with the previously
reported stimulatory effect of IL-1β on ET-1 release by endo-
thelial cells (48, 55). An additional possibility is that IL-1β may somehow promote downregulation or dysfunction of the endothelin ETB receptor. This receptor is thought to act as a “clearance” receptor for endothelins, and genetic disruption (11) or pharmacological blockade of the receptor (12) results in increased plasma ET-1 concentrations. One study reported that IL-1β reduces ETB receptor expression in cultured endothelial cells (34); however, IL-1β has been shown to increase (4) or decrease ETB receptor expression (57) in other cell types.

Since immunoreactive ET-1 found intact in the urine is of renal, rather than circulating, origin (1), the increase in urinary IL-1-infused mice. The cell types in which IL-1/sistent with the effect observed on renal prepro-ET-1 mRNA in the kidney. The data also showed that urine flow was markedly increased by chronic IL-1 infusion. Acute administration of IL-1β to rats has also been shown to produce natriuresis and diuresis (3). The proposed mechanisms for IL-1-induced natriuresis and diuresis include PGE2-dependent inhibitory effects on Na+ -K+ -ATPase activity (56) and a PGE2-independent increase in anion secretion (18) by the collecting duct. Since ET-1 is thought to exert a diuretic effect in the healthy kidney (45), the diuretic effect observed in our study may be due to a combination of the direct diuretic effect of IL-1β and IL-1β stimulation of renal ET-1 production. Determination of the relative contributions of these factors to the diuresis observed in this study was, however, beyond the scope of the present investigation. Water intake was also significantly higher in IL-1β-than in vehicle-infused mice. This was likely a compensatory response to the diuretic effect of IL-1β infusion, rather than a direct effect on thirst, since previous studies reported that acute or chronic central administration or acute peripheral administration of IL-1β has no effect or a dose-dependent inhibitory effect on water intake in rodents (31, 36, 37).

Interestingly, the marked elevation of plasma ET-1 concentra-
tion and renal production of ET-1 induced by chronic IL-1β infusion did not cause hypertension. It is possible that activation of endothelial or tubular ETB receptors offsets the pressor actions of ET-1. Indeed, in rats, chronic infusion of ET-1 only induces hypertension when the rats are maintained on a high salt diet (9, 32). In addition, the marked diuretic effect of IL-1β infusion may also have offset any prohypertensive effect of the elevated ET-1 levels. Although not examined in our study, infusion of IL-6 at 16 ng/h also has no effect on blood pressure of male C57BL/6 mice (M. W. Brands, unpublished observations). Chronic IL-1β infusion had no effect on urinary protein excretion rate but did slightly, but significantly, increase albumin excretion rate, which suggests induction of some mild renal injury. The albumin excretion rate observed in IL-1β-infused mice is, however, much lower than that observed in models of hypertension e.g., chronic angiotensin II infusion plus high salt diet (29) or streptozotocin-induced diabetes (22). C57BL/6 mice were used in these experiments, and this strain is known to be relatively resistant to renal injury (15, 40). It is therefore possible that chronic infusion of IL-1β would have more injurious effects in other strains of mice or in other species.

The present study provides evidence that IL-1β can increase renal and circulating ET-1 levels in vivo. ET-1 has been implicated in a number of renal inflammatory conditions, as evidenced by direct measurements of increased renal ET-1 expression (54) and/or beneficial effects of endothelin blockade on albuminuria and renal histology (17, 44). The data from the present study suggest that activation of the endothelin
IL-1β stimulates endothelin production in vivo

system in such inflammatory conditions may at least in part be driven by IL-1β released from infiltrating macrophages and other immune cells.

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