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Bactericidal/permeability-increasing protein inhibits endotoxin-induced vascular nitric oxide synthesis

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Background: Endotoxin (lipopolysaccharide, LPS) up-regulates inducible nitric oxide synthase (iNOS) in blood vessels during septic shock. This promotes the production of nitric oxide (NO), leading to dilation of the vessels. The aim of the study was to investigate the effects of the LPS-binding endogenous antibiotic bactericidal/permeability-increasing protein (BPI) on the action of LPS on the blood vessels wall and to identify possible influence on underlying NO-related mechanisms.

Methods: Isolated segments of rat thoracic aorta and cultured primary smooth muscle cells were incubated for 5–48 h in the presence of the following combinations of compounds: (a) LPS; (b) interleukin-1β (IL-1β); (c) BPI; (d) BPI + LPS; (e) BPI + IL-1β or (f) neither BPI, LPS nor IL-1β (control). After incubation of intact segments, we measured smooth muscle contraction in response to phenylephrine and accumulation of the NO end products nitrate and nitrite in surrounding medium. Western blot was used to assess the levels of inducible nitric oxide synthase (iNOS) in cultured cells.

Results: Both LPS and IL-1β decreased contractility and increased NO production, as well as iNOS. Co-incubation with BPI attenuated all the effects of LPS but only the effects of prolonged exposure to IL-1β in cultured cells.

Conclusion: We conclude that BPI attenuates the LPS-induced changes in vascular reactivity by inhibiting the expression of iNOS resulting in decreased NO formation and restored responsiveness to vasoconstrictors. The data suggest that BPI can prevent circulatory disturbances during Gram-negative sepsis.

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Endotoxin (lipopolysaccharide, LPS), a major cell wall component of Gram-negative bacteria, activates the patient's host response during Gram-negative sepsis (1). The response to LPS may result in circulatory failure leading to regional hypoperfusion, thus contributing to multiorgan dysfunction and death (2, 3).

In rat models, injection of LPS results in increased expression and activity of inducible nitric oxide synthase (iNOS) in locations including vascular endothelial cells, smooth muscle and adventitia (4–7). iNOS contributes to the formation of nitric oxide (NO) from L-arginine (8). Locally produced NO relaxes vascular smooth muscle and expression of iNOS and excessive production of nitric oxide in the blood vessel wall mediates vasodilation resulting in hypotension during septic shock (3, 9).

Vascular preparations incubated with LPS *in vitro* within hours become hyporesponsive to constrictors such as the α1-adrenoceptor agonist, phenylephrine (10). The contractility is restored in the presence of

iNOS inhibitors indicating that it, at least in part, is dependent on enhanced NO formation (11). Thus, isolated blood vessel preparations are useful important models to study effects of LPS on blood vessel NO function *in vitro*.

Bactericidal/permeability-increasing protein (BPI) was initially isolated from granules of human polymorphonuclear leukocytes (12). It is present in the primary granules of neutrophils (13) and specific granules of eosinophils (14) but can also be detected on the surface of polymorphonuclear leukocytes (15) and monocytes (16). BPI is an endogenous antibiotic and as such part of the innate defence against infection. It rapidly kills Gram-negative bacteria via high affinity binding to the bacterial cell wall LPS (17). BPI is released from strongly stimulated neutrophils (18) and it probably also contributes to the killing of ingested bacteria (19).

Bactericidal/permeability-increasing protein could be important for binding and neutralizing LPS released after bacterial death, e.g. during treatment of

the infection with antibiotics (18). Thus, intact BPI or a recombinant N-terminal fragment attenuates LPS-induced neutrophil and endothelial cell activation *in vitro* (20, 21), as well as secretion of inflammatory mediators, neutrophil activation and circulatory changes *in vivo* (18, 22, 23). BPI, or BPI analogs, may therefore be beneficial as adjunctive treatment of severe infections (24, 25).

The aim of the present study was to investigate if BPI could modulate the effect of LPS on the reactivity of the smooth muscle in the blood vessel wall and to identify underlying NO-related mechanisms. The results may have bearing on the treatment of circulatory failure during Gram-negative sepsis.

Materials and methods

Material

The study was approved by the Institutional Review Board for the care of animal subjects and the care and handling of the animals were in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (250 gram body weight) were anaesthetized with halothane (Astra, Södertälje, Sweden) and bled to death. The thoracic aorta was removed, cleaned of adherent fat and cut into 3 mm long cylindrical segments.

Cell culture

Rat vascular smooth muscle cells were isolated from thoracic aorta by the explant method (26). In short, aorta segments were aseptically removed, trimmed and longitudinally cut open. The endothelium was removed by gently rubbing the intimal surface with a sterile cotton pad. Approximately 3×3-mm sections were placed with the intimal side down into 6-cm^3 Petri dishes. Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (10%), penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and amphotericin-B (250 ng ml^{-1}, all from Life Technologies, Täby, Sweden) was added to cover the tissue without disturbing the orientation of the explants. The dishes were incubated in a humidified incubator at 37 °C with 5% CO$_2$ in air.

Vascular cells were allowed to grow out from the aorta sections for 8 days, after which the sections were removed. When confluence was reached, the cells were harvested using 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA, both from Sigma-Aldrich, St. Louis, MO), rinsed in Hanks' Balanced Salt Solution (Life Technologies) and transferred to 25-cm^2 flasks (first passage). The cells were cultured to confluence in DMEM with 5% fetal bovine serum and

antibiotics as above. At subsequent passages the cells were seeded at a ratio of 1:4. Subcultured cells were found to have a mean doubling time of approximately 30 h. The cells were identified as smooth muscle cells by their characteristic hill and valley appearance in culture and by their expression of an approximately 40-kDa protein with immunoreactivity corresponding to smooth muscle α-actin as determined by Western blot (see below) using a monoclonal anti-smooth muscle α-actin antibody (Cat No. A-2547, Sigma-Aldrich).

Incubations

Intact aorta segments intended for contraction experiments were incubated at 37 °C for 5 h in Krebs-Ringer solution with penicillin (2000 U ml^{-1}) and streptomycin (0.2 mg ml^{-1}, both from Sigma-Aldrich) continuously gassed with 5% CO$_2$ in O$_2$. The Krebs-Ringer solution contained (mmol. l^{-1}): Na$^+$ 143, K$^+$ 5.9, Cl$^-$ 128, Ca^{2+} 2.5, HCO^{3-} 25.0, Mg^{2+} 1.2, SO$_4{}^{2-}$ 1.2, H$_2$PO$_4{}^-$ 1.2, Glucose 5.5 and EDTA 0.024 in LPS-free distilled water (Sigma-Aldrich). Intact aorta segments intended for measurement of nitrate/nitrite accumulation were incubated 24 h in DMEM without phenol red (ICN, Costa Mesa, CA) containing L-arginine (1 mmol l^{-1}, substrate for nitric oxide synthesis) and penicillin (2000 U ml^{-1}) and streptomycin (0.2 mg ml^{-1}, all from Sigma-Aldrich) in an atmosphere of 8% CO$_2$ in air. Cells cultured to confluence on 6-cm^2 Petri dishes (for Western blot) or 2-cm^2 wells in a 24 well plate (for DNA fragmentation and cytotoxicity experiments) were incubated for 16 or 48 h in DMEM with penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and amphotericin-B (250 ng ml^{-1}, all from Life Technologies) but without fetal bovine serum in an atmosphere of 5% CO$_2$ in air.

The segments and cells were incubated in the presence of the following combinations of compounds: (a) LPS (from *E. coli*, strain 0111:B4, Difco, Detroit, MI); (b) interleukin-1β (IL-1β, Alexis, San Diego, CA); (c) BPI (prepared from human neutrophils, Wieslab, Lund, Sweden); (d) BPI + LPS; (e) BPI + IL-1β or (f) neither BPI, LPS nor IL-1β (control). The concentrations of LPS and IL-1β used were determined from initial pilot experiments and chosen as they were found to be the lowest concentrations giving a clear response compared to control.

Measurement of smooth muscle contraction

The first set of experiments aimed to investigate the effects of incubation with LPS (100 ng ml^{-1}), IL-1β (0.2 ng ml^{-1}) and/or BPI (1 µg ml^{-1}) on circular smooth muscle contraction. After incubation, the segments

were placed on two L-shaped hooks in 2-ml tissue baths. The baths contained Krebs-Ringer solution gassed with 11.5% CO_2 in O_2 at a rate giving PCO_2 5.0 kPa, PO_2 approximately 40 kPa and pH 7.4. The temperature was thermostatically maintained at 37 °C. One of the hooks was connected to a Grass FTO3C force-displacement transducer (Grass Medical Instruments, Quincy, MA) for isometric measurement of tension. The vessel tension was recorded on a Grass polygraph model 7b. The segments were stretched to a pretension of 20 mN and allowed to equilibrate for 1 h. Thereafter, potassium chloride (90 mmol l^{-1}) was added and the resulting smooth muscle contraction was used to check the viability of the segments. After wash out, the α_1-adrenoceptor agonist, phenylephrine (10^{-9}-10^{-4} mol l^{-1}, Sigma-Aldrich), was added cumulatively in $_{10}$log units. The resulting contraction was registered and concentration-response curves drawn. The experiments were performed first in the absence and then, after wash out, during which the vessel tension returned to base-line, in the presence of the iNOS inhibitor L-nitro arginine monomethyl ester (L-NAME, 0.3 mmol l^{-1}, added 10 min prior to phenylephrine, Sigma-Aldrich).

A second set of experiments was performed in order to assess any direct effect of BPI on smooth muscle. Segments of rat aorta were mounted in organ baths, allowed to equilibrate and tested with potassium chloride as above. To some segments, BPI was added cumulatively in $_{10}$log units (10^{-12}-10^{-6} g ml^{-1}) and smooth muscle contraction was measured. Other segments were first precontracted with endothelin-1 (Sigma-Aldrich) to a stable submaximal contraction. BPI was then added cumulatively in $_{10}$log units (10^{-12}-10^{-6} g ml^{-1}) and smooth muscle relaxation was measured.

Measurement of nitrate/nitrite accumulation

Nitric oxide is rapidly oxidized to nitrite and nitrate (27). The NO release from the segments is therefore reflected in the accumulation of nitrate and nitrite in the incubation medium. The segments were incubated with LPS (1 ng ml^{-1}) or IL-1β (0.2 ng ml^{-1}) for 24 h. BPI (0.1, 1 or 3 µg ml^{-1}) was added at the beginning of the incubation ('Pre-incubation') or immediately before analysis of the nitrate/nitrite content ('Post-incubation'). This protocol was used in order to exclude any interaction of BPI with the nitrate/nitrite assay.

After incubation, the incubation medium was removed from the vessel segments and centrifuged at 11 000 × g for 5 min at room temperature. All nitrate was reduced to nitrite with nitrate reductase, which was subsequently detected by Griess reagent and measured in a spectrophotometer at 550 nm (Total Nitrate/Nitrite Assay Kit, Assay Designs Inc., Ann Arbor, MI). The detection limit of this assay was 0.75 µmol l^{-1}. Standard curves were constructed by analysing DMEM with different concentrations of sodium nitrate (Assay Designs).

Western blot

After incubation with LPS (0, 0.1, 1 or 10 ng ml^{-1}), IL-1β (0, 0.01, 0.1 or 1 ng ml^{-1}) and/or BPI (0, 1 or 3 µg ml^{-1}), the incubation medium was replaced by a lysis buffer containing phosphate buffered saline (PBS) with Triton X-100 (1%), a protease inhibitor cocktail (20 µ ml^{-1}) and benzamidine hydrochloride (10 mmol l^{-1}, all from Sigma-Aldrich). The cells were removed from the Petri dishes with a cell scraper. The cell suspension was frozen and thawed two times to enhance cytolysis and was then centrifuged for 5 min at 11 000 × g at room temperature.

Total protein concentration was determined on the basis of the Biuret reaction combined with colorimetric detection of the cuprous cation using a reagent containing bicinchonic acid (Pierce, Rockford, IL). Twenty µg protein was electrophoretically separated in a 7.5% SDS polyacrylamide gel, stacking 4% (28), together with protein size standards (Kaleidoscope, Bio-Rad, Hercules, CA).

The separated proteins were transferred to a PVDF membrane (Hybond ECL, Amersham Pharmacia, Freiburg, Germany) using a Bio-Rad Mini protean tank blot equipment. The membranes were blocked with 5%-non-fat milk (ICN) in 2 mol l^{-1} Tris buffered saline (TBS) with 0.1% Tween-20 (ICN) for 1 h at room temperature and subsequently exposed to a polyclonal rabbit anti-iNOS antibody 1/1000 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at room temperature for 1 h in blocking solution. After repetitive washings, membranes were incubated with a biotinylated goat antirabbit antibody 1/5000 (Santa Cruz) for another h in TBS. Detection was performed by using ECL Plus reagents and Hyper Film^{TM} (both from Amersham Pharmacia).

DNA fragmentation and cytotoxicity experiments

Confluent cells were incubated in serum free DMEM for 16 h with IL-1β (1 ng ml^{-1}), IL-1β + BPI (1 or 3 µg ml^{-1}) or H_2O_2 (10 µmol l^{-1}). H_2O_2 has previously been found to induce apoptosis in cultured smooth muscle cells and was used as a positive control in these experiments (29). After incubation, the cells were lyzed and DNA-fragmentation was assessed using an ELISA kit according to the manufacturer's instructions (Cell Death Detection ELISA, Roche Molecular Bio-
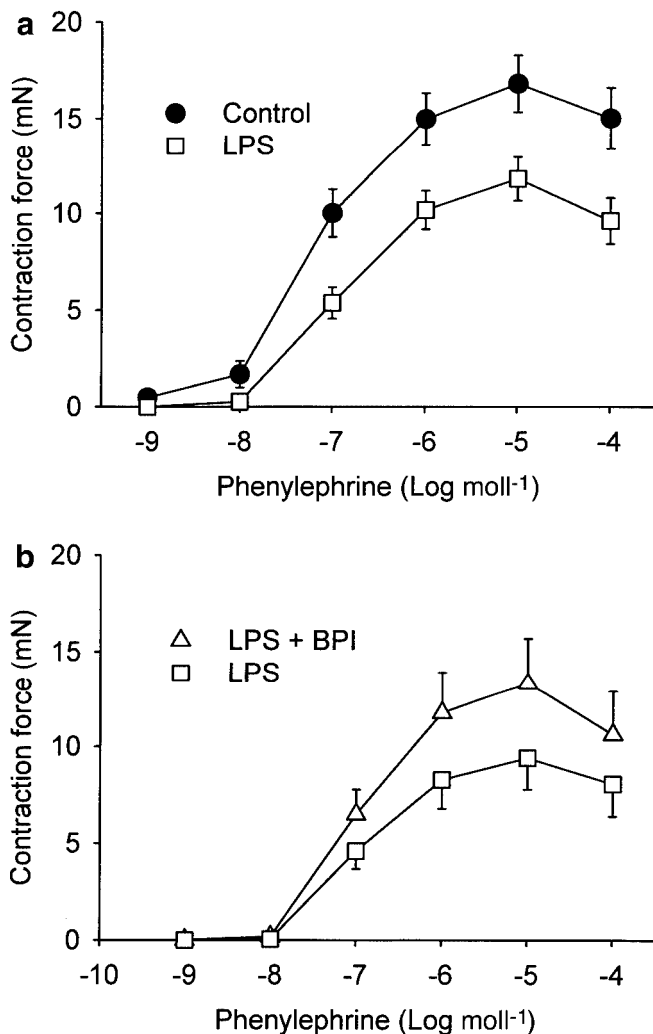
a

b

Fig. 1. Phenylephrine-induced smooth muscle contraction in rat aorta. Phenylephrine induced a concentration-dependent smooth muscle contraction. (a) The contraction was significantly weaker after 5 hours' incubation with LPS (100 ng ml^{-1}, open square) compared to control (filled circle, n = 13, two way repeated measurements ANOVA, P = 0.016). (b) The contraction induced by phenylephrine was significantly stronger in segments incubated for 5 h in the presence of both BPI(1 µg ml^{-1}) and LPS (100 ng ml^{-1}, open triangle) compared to segments incubated in the presence of LPS alone (open square, n = 6, two-way repeated measurements ANOVA, P = 0.029). This indicates that BPI can neutralize the effects of LPS on the aorta segments. Values are means ± SEM.

chemicals, Germany). Cell viability after 48 h was determined using trypan blue (Sigma–Aldrich) on trypsinized cells by counting in a hemocytometer. Only dead cells are permeable to trypan blue.

Statistics
Concentration-response curves were compared using two-way repeated measurement ANOVA on logarithmically transformed data in order to eliminate inequality of the variances. Student's paired t-test was

used to compare the levels of nitrate/nitrite. Significance was accepted at $P < 0.05$. Values are means ± SEM and 'n' equals number of rats.

Results

Smooth muscle contraction
Phenylephrine induced a concentration-dependent smooth muscle contraction that was significantly weaker after incubation with LPS compared to control (Fig. 1a, $P = 0.016$). In the presence of the nitric oxide synthase inhibitor, L-NAME, the contraction did not
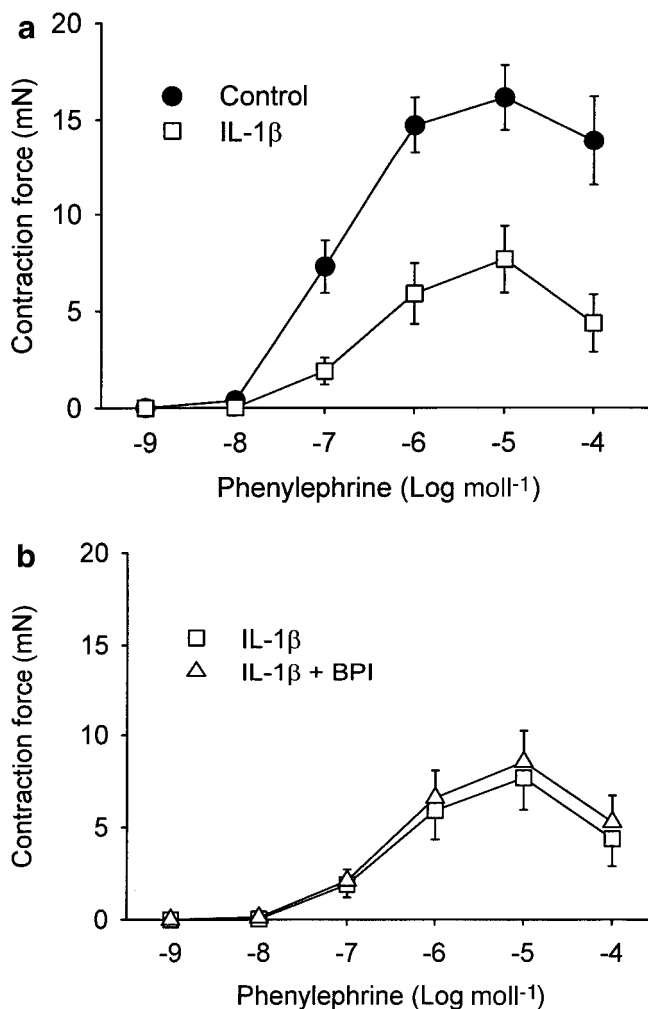
a

b

Fig. 2. Phenylephrine-induced smooth muscle contraction in rat aorta. (a) The contraction was significantly weaker after 5 hours' incubation with IL-1β (0.2 ng ml^{-1}, open square) compared to control (filled circle, n = 6, two-way repeated measurements ANOVA, P = 0.034). (b) The contraction induced by phenylephrine was not significantly different in segments incubated for 5 h in the presence of both BPI (1 µg ml^{-1}) and IL-1β (0.2 ng ml^{-1}, open triangle) compared to segments incubated in the presence of IL-1β alone (open square, n = 6). This suggests that the neutralizing effect of BPI can be specific for LPS. Values are means ± SEM.

differ from control, indicating that the reducing effect of LPS incubation on the contraction is dependent on NO synthesis (not shown, $n = 13$). Incubation with BPI alone did not affect the contraction induced by phenylephrine (not shown, $n = 6$). However, the contraction was significantly stronger in segments incubated with both LPS and BPI compared to segments incubated with LPS alone (Fig. 1b, $P = 0.029$). The effect of BPI was not seen in the presence of L-NAME (not shown, $n = 6$). This indicates that BPI can neutralize the NO-mediated effects of LPS on the aorta segments.

Similar to after incubation with LPS, the phenylephrine-induced contraction was significantly weaker after incubation with IL-1β compared to control (Fig. 2a, $P = 0.034$). In the presence of L-NAME, the contraction after IL-1β incubation did not differ from control indicating that the effect of IL-1β is NO mediated

(not shown, $n = 6$). Co-incubation with BPI did not affect the contraction after IL-1β incubation either in the presence or in the absence of L-NAME (Fig. 2b). This suggests that the action of BPI is LPS specific.

Bactericidal/permeability-increasing protein added to the organ baths neither induced contraction of resting aorta segments nor relaxed aorta segments precontracted by endothelin-1 (not shown, $n = 2$).

Nitrate/nitrite production

Incubation with LPS significantly increased the production of nitrate/nitrite in the incubation medium compared to control (Fig. 3). Co-incubation with BPI concentration-dependently diminished the nitrate/nitrite production induced by LPS to control values. The production of nitrate/nitrite during incubation with BPI alone did not differ from the control values (not
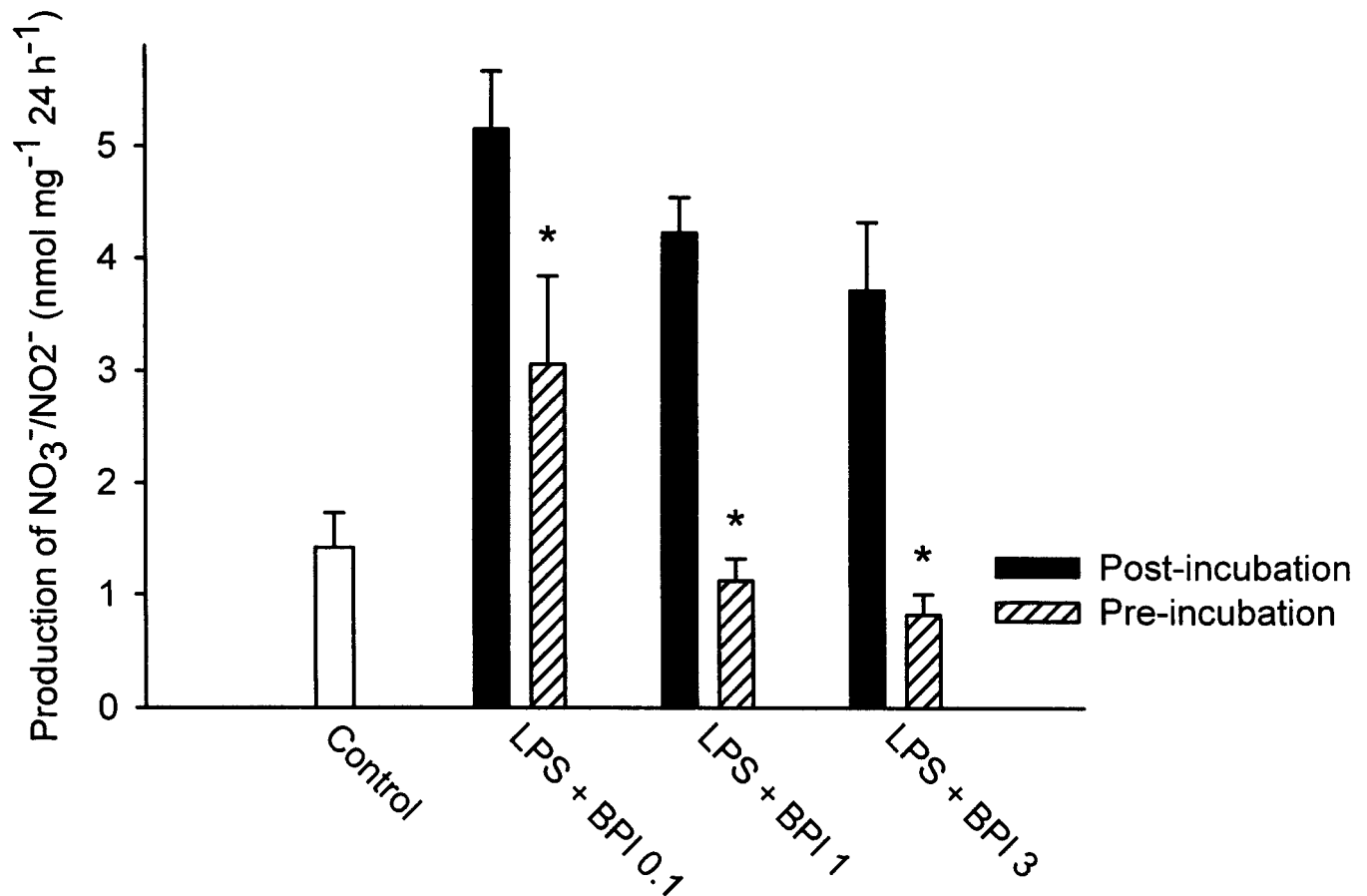

Fig. 3. Production of the nitric oxide end products nitrate and nitrite from segments of rat aorta during 24 hours' incubation as measured with Griess reagent. Compared to control (open bar), the production of nitrate/nitrite was higher after incubation with LPS ($1 \, ng \, ml^{-1}$) followed by addition of BPI (0.1, 1 or $3 \, \mu g \, ml^{-1}$) immediately before the analysis ('Post-incubation'). When BPI (0.1, 1 or $3 \, \mu g \, ml^{-1}$) was added at the start of the incubation together with LPS ('Pre-incubation', hatched bars) the nitrate/nitrite production was lower compared to when BPI was added at the end of the incubation ('Post-incubation', filled bars, Student's paired t-test, $P < 0.05$, *). This effect of BPI was concentration-dependent. It confirms that BPI can inhibit the LPS-induced nitric oxide production in rat aorta. The addition of BPI at the end of the incubation was used to exclude any interaction of BPI with the nitrate/nitrite assay. Values are means + SEM. ($n = 6$).

shown, $n = 6$). This confirms that BPI can inhibit the LPS-induced NO production in rat aorta.

Incubation with IL-1β also significantly increased the accumulation of nitrate/nitrite (4.7 ± 0.68 nmol mg^{-1} tissue 24 h^{-1}, $n = 5$) compared to control (1.4 ± 0.31 nmol mg^{-1} tissue 24 h^{-1}, $n = 5$, $P = 0.003$, not shown). Co-incubation with BPI (0.1–3 μg ml^{-1}) did not affect the accumulation induced by IL-1β (not shown, $n = 5$). This confirms that the action of BPI on vascular NO synthesis is LPS specific.

Western blot
The anti-iNOS antibodies detected a band on the Western blot corresponding to the molecular weight of iNOS (approximately 135 kDa). The band was visible after incubation with LPS at 10 ng ml^{-1} and IL-1β at 1 ng ml^{-1} but not at lower concentrations indicating up-regulation of iNOS (Fig. 4). BPI at both 1 and 3 μg ml^{-1} inhibited the LPS-induced up-regulation of iNOS. BPI also concentration-dependently inhibited the IL-1β-induced up-regulation. These results support the view that BPI inhibits the LPS-induced NO production in rat aorta via inhibition of iNOS expression. However, during our experimental conditions, BPI also seems to be able to inhibit the expression induced by IL-1β.
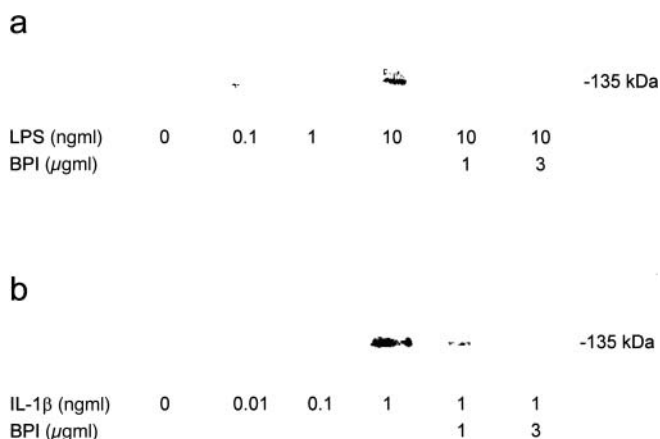

Fig. 4. iNOS in cultured rat aorta smooth muscle cells after 48 hours' incubation with (a) LPS (0, 0.1, 1 and 10 ng ml^{-1}) or LPS (10 ng ml^{-1}) and BPI (1 and 3 μg ml^{-1}) or (b) IL-1β (0, 0.01, 0.1 and 1 ng ml^{-1}) or IL-1β (1 ng ml^{-1}) and BPI (1 and 3 μg ml^{-1}). The proteins of cell lysates were electrophoretically separated according to the molecular weight and transferred to a PVDF membrane. Immunoreactive iNOS was detected by an anti-iNOS antibody. A band corresponding to the molecular weight of iNOS (approximately 135 kDa) was detected. The band was visible after incubation with LPS at 10 ng ml^{-1} and IL-1β at 1 ng ml^{-1}, indicating up-regulation of iNOS. Co-incubation with BPI (1 and 3 μg ml^{-1}) inhibited both the LPS- and the IL-1β-induced up-regulation.

Cytotoxicity and DNA fragmentation
H_2O_2 caused DNA fragmentation in the cultured smooth muscle cells after 16 h of exposure ($504 \pm 85\%$ of control) and reduced the number of attached trypan blue impermeable cells after 48 h ($51 \pm 4.7\%$ of control) indicating cytotoxicity via an apoptosis pathway. Neither IL-1β nor IL-1β + BPI induced DNA fragmentation or affected the number of attached trypan blue impermeable cells (three independent experiments). This indicates that IL-1β and BPI do not induce apoptosis and are not cytotoxic in our model.

Discussion

These results show that BPI specifically attenuates the LPS-induced changes in vascular reactivity. This seems to be due to inhibition of the LPS-induced expression of iNOS, resulting in decreased NO formation and restored responsiveness to vasoconstrictors. This could be explained by binding of BPI to LPS (18), inhibiting the LPS interaction with cell surface receptors in the blood vessel wall. It suggests that BPI may prevent circulatory disturbances during Gram-negative sepsis.

It has previously been shown that IL-1β up-regulates NO synthesis in vascular tissue (30). Incubation with IL-1β or with both IL-1β and BPI reduced the contraction in response to phenylephrine and increased NO production to the same extent. This demonstrates that BPI, in a concentration sufficient to neutralize the effect of LPS, does not affect IL-1β-induced NO stimulation. It indicates that the NO-reducing BPI effect is LPS-specific and therefore supports the hypothesis of LPS binding to BPI.

We were therefore surprised to find that BPI reduced not only the LPS-induced iNOS expression but also the iNOS expression induced by IL-1β as assessed by Western blot. In these experiments we had to use cultured smooth muscle cells and a prolonged incubation time in order to achieve detectable amounts of iNOS. This suggests that the mechanisms for iNOS induction by LPS might be different in cultured cells compared to intact vessel segments and/or that the Western blot method used has a high detection threshold. The inhibitory action of BPI on IL-1β-induced iNOS expression cannot be explained by BPI being solely a LPS scavenger. Van der Schaft and colleagues (31) have recently shown that BPI during 72 h of exposure induces apoptosis in endothelial cells in concentrations about one order of magnitude higher than the concentrations used in the present study. They were not able to demonstrate any effects on human fibroblasts or a human adenocarcinoma cell-line

and concluded that these effects of BPI are endothelial cell specific. Contrary to the conclusions by Van der Schaft and colleagues, the results from the Western blot experiments suggest that BPI could have a cytotoxic effect also on cultured smooth muscle cells and/or after prolonged exposure. However, we did not find any evidence for apoptosis or cytotoxicity induced by IL-1β or BPI in our model. Taken together, it cannot be excluded that BPI has anti-inflammatory properties acting directly on the *cultured smooth muscle cells*. This remains to be further tested. BPI did not alter the NO-mediated action of IL-1β on contractility and NO-production in intact aorta rings. Thus it seems unlikely that a cytotoxic or non-specific anti-inflammatory action was responsible for the inhibition by BPI on the LPS-effects in the *intact blood vessel wall*.

One should be careful to compare the potency of BPI found to reduce the effects of LPS in different models. We found a significant reduction by BPI at an *in vitro* concentration of $1 \mu g \ ml^{-1}$. However, on a molar basis, this is similar to the potency of intact BPI to inhibit LPS-induced expression of the surface antigens CR1 and CR3 on human neutrophils (20). It is also similar to the potency of rBPI$_{23}$, a recombinant modified N-terminal fragment of human BPI with preserved LPS binding activity, to inhibit tumour necrosis factor secretion and nitrite production in mouse macrophages (32).

Earlier efforts to modulate the extreme inflammatory response during sepsis have focused on reducing the levels or effects of secondary inflammatory mediators such as proinflammatory cytokines. However, the results in the clinical setting have been disappointing (33). The present results suggest a way to interfere earlier in the cascade of events during Gram-negative sepsis by directly reducing the inflammatory action of the bacteria and their products.

Recently the results of a large scale prospective randomized multicentre study evaluating a recombinant BPI analog (rBPI$_{21}$) as adjunctive treatment for meningococcal sepsis in children was published (25). The study included 393 patients who received either rBPI$_{21}$ or placebo. The study could not demonstrate any significant reduction of mortality. This could be due to problems with the study design, which suffered from a time-consuming inclusion procedure. Thus, most deaths occurred in the interval between identification of the patients and study drug administration and, of those who finally got the drug, the majority would have survived anyway (34). However, among the surviving children, fewer had multiple severe amputations, suggesting that BPI protects the circulation.

The present results suggest that the protection could be, at least partly, mediated via effects on the vascular nitric oxide system.

The present study shows that BPI attenuates the LPS-induced changes in vascular reactivity by inhibiting the expression of iNOS, resulting in decreased NO formation and restored responsiveness to vasoconstrictors. They suggest that BPI can prevent circulatory disturbances during Gram-negative sepsis. Hopefully, the present results will stimulate further clinical studies with BPI and its analogs in septic patients since they emphasize the dual beneficial action of BPI: being both an antibiotic and an anti-inflammatory compound.

Acknowledgements

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