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Effect of endotoxin on the angiotensin II receptor in cultured vascular smooth muscle cells

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1 In some tissues, a decrease in the number of cell surface receptors and alterations of the receptor coupling have been proposed as possible mechanisms mediating the deleterious effects of bacterial endotoxin in septic shock.

2 The effects of bacterial lipopolysaccharide (*Escherichia coli* 0111-B4; LPS) on vascular angiotensin II and vasopressin receptors have been examined in cultured aortic smooth muscle cells (SMC) of the rat by use of radioligand binding techniques.

3 In vascular SMC exposed to 1 $\mu g\ ml^{-1}$ endotoxin for 24 h, a significant increase in angiotensin II binding was found. The change in [^{125}I]-angiotensin II binding corresponded to an increase in the number of receptors whereas the affinity of the receptors was not affected by LPS. In contrast, no change in [^{3}H]-vasopressin binding was observed.

4 The pharmacological characterization of angiotensin II binding sites in control and LPS-exposed cells demonstrated that LPS induced an increase in the AT_1 subtype of the angiotensin II receptors. Receptor coupling as evaluated by measuring total inositol phosphates was not impaired by LPS.

5 The effect of LPS on the angiotensin II receptor was dose-, time- and protein-synthesis dependent and was associated with an increased expression of the receptor gene.

6 The ability of LPS to increase angiotensin II binding in cultured vascular SMC was independent of the endotoxin induction of NO-synthase.

7 These results suggest that, besides inducing factors such as cytokines and NO-synthase, endotoxin may enhance the expression of cell surface receptors. The surprising increase in angiotensin II binding in LPS exposed VSM cells may represent an attempt by the cells to compensate for the decreased vascular responsiveness. It may also result from a non-specific LPS-related induction of genes.

Keywords: Angiotensin II; vasopressin; endotoxin; receptor; smooth muscle cells; nitric oxide

Introduction

Septic shock in man is characterized by a non-specific loss of vascular reactivity to vasoconstrictor agents (Groenenweld & Thijs, 1986). This phenomenon which contributes to the high mortality of septic patients can be reproduced in animals by the infusion of endotoxin. Thus, rats infused with *Escherichia coli* lipopolysaccharide (LPS) exhibit a significantly blunted blood pressure response to catecholamines, angiotensin II and vasopressin (Schaller *et al.*, 1985; Evequoz *et al.*, 1987: Burnier *et al.*, 1988). Similarly, hyporeactivity to various vasoconstrictors can be demonstrated in arteries of endotoxaemic animals studied *ex vivo* or in isolated vessels studied *in vitro* after incubation with LPS (Wakabayashi *et al.*, 1987; Beasley, 1990; Beasley *et al.*, 1990; Julou-Schaeffer *et al.*, 1990; McKenna, 1990; Schneider *et al.*, 1992). *In vitro*, endotoxin has also been shown to reduce the contractility of isolated cardiac myocytes (Brady *et al.*, 1992).

Several pathogenic mechanisms have been proposed to explain the diminished responsiveness to vasoconstrictor agents in endotoxaemia. In recent years, increasing evidence suggests that the L-arginine-nitric oxide (NO) pathway plays an important role in mediating the haemodynamic effects of endotoxin. Indeed, treatment, with endotoxin induces expression of a calcium-independent NO-synthase in the endothelium and in vascular smooth muscle cells (Knowles *et al.*, 1990; Radomski *et al.*, 1990; Rees *et al.*, 1990). The expression of this enzyme leads to the vasodilatation and hyporesponsiveness to vasoconstrictors characteristic of endotoxin shock (Rees *et al.*, 1990; Moncada *et al.*, 1991). *In vitro*, the depressed contractility of arteries taken from endotoxaemic animals can be restored by L-arginine analogues such as N^{ω}-nitro-L-arginine methyl-ester (L-NAME) and N^{ω}-monomethyl-L-arginine (Fleming *et al.*, 1990; Julou-Schaeffer *et al.*, 1990; Kilbourn *et al.*, 1990; Hollenberg *et al.*, 1993; Schott *et al.*, 1993).

Other mechanisms may also contribute to the vascular effects of endotoxin. Cytokines such as interleukin-1 or tumour necrosis factor have been shown to mimic the properties of endotoxin in inducing NO-synthase and causing vascular hyporeactivity (Beasley *et al.*, 1989; Gulick *et al.*, 1989; Beasley, 1990; Schultz *et al.*, 1992). Alterations in vasoactive hormone receptors might also underlie some of the metabolic and haemodynamic consequences of sepsis and endotoxaemic shock. Indeed, impairments in receptor-stimulated phosphoinositide hydrolysis have been reported in chronically endotoxin-treated rats (Spitzer *et al.*, 1989). Moreover, significant decreases in the total number of hepatic vasopressin and α_1-adrenoceptors have been found after chronic endotoxin infusion in the rat (Roth & Spitzer, 1987). In addition, a modification in the β-adrenoceptors-adenylate cyclase coupling has been described in myocardial membranes of endotoxaemic rats (Romano & Jones, 1986).

Whether similar alterations of cell surface receptors occur in the smooth muscle cells of the vascular wall during endotoxaemia is not known. The purpose of the present experiments was therefore to examine the effects of LPS on the angiotensin II and vasopressin receptors *in vitro* on cultured vascular smooth muscle cells.

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Methods

Cell culture

Experiments were carried out on cultures of rat aortic smooth muscle cells (SMC). SMC were obtained as described previously (Burnier *et al.*, 1991). Cells were used after 2 to 15 passages.

Angiotensin II binding studies

Binding studies were performed as described previously (Burnier *et al.*, 1991). The cells were grown on 24 well plates and studied subconfluent 5 days later; at that time each well contained about 400,000 cells or 175 μg protein/well ($n=24$). Displacement curves were performed with increasing concentrations of unlabelled angiotensin II. Each point of the curve was measured in duplicate or triplicate. The displacement curves were obtained by incubating the cells for 1 h at 37°C with 0.01 nM [^{125}I]-angiotensin II (specific activity 2200 Ci mmol^{-1}, New England Nuclear). The specific binding of angiotensin II was defined as the total binding of 0.01 nM [^{125}I]-angiotensin II in the absence of unlabelled angiotensin II minus the nonspecific binding i.e. the binding of 0.01 nM [^{125}I]-angiotensin II in the presence of 1 μM unlabelled angiotensin II.

At 37°C, angiotensin II is internalized after binding to its receptor (Ullian & Linas, 1989). Therefore, binding studies were also conducted at 4°C to prevent internalization. In these studies, cells were incubated with the radiolabelled angiotensin II for 6 h to ensure equilibrium in angiotensin II binding. The amount of angiotensin internalized was also evaluated. For this purpose, the binding was performed at 37°C. After incubation with radiolabelled angiotensin II, the receptor-bound angiotensin II was removed by an acid wash (glycine 50 mM and NaCl 150 nM at pH 3.0). The intracellular radioactivity was then counted in a gamma-counter.

[^{3}H]-vasopressin binding

Vasopressin binding was carried out in DMEM as binding buffer. After being washed with the buffer, cells were incubated with 3 nM [^{3}H]-arginine-vasopressin (New England Nuclear, specific activity 20 Ci mmol^{-1}) for 15 min at 37°C. The binding was stopped with a phosphate buffer solution (Dulbecco) supplemented with 1 mM $MgCl_2$, 0.8 mM $CaCl_2$ and bovine serum albumin (0.1% weight/vol) was added at pH 7.4. Cells were removed from the wells with a lysis buffer containing 0.1% sodium dodecyl sulphate, 2% Na_2CO_3 and 0.1 N NaOH. Non-specific binding was measured in the presence of 10 μM unlabelled vasopressin and total binding in the absence of radiolabelled vasopressin. Cells were incubated with 1 μg ml^{-1} LPS or its vehicle for 24 h before being studied.

Endotoxin exposure

All studies were performed simultaneously in control cells and cells exposed to endotoxin 0111 : B4 LPS isolated from *Escherichia coli* (Difco, Detroit, MI, U.S.A.). In all experiments, the LPS incubation was done in presence of 15% 'endotoxin free' foetal calf serum. Twenty four hours incubation with LPS had no effect on cell growth as the number of cells counted in control and LPS-treated wells was comparable (367'395 $\pm$ 77'000 cells/well in controls and 371'770 $\pm$ 29'170 cells/well in LPS-treated plates or 176.2 $\pm$ 7.1 μg protein/well in controls and 171.3 $\pm$ 13.4 μg protein/well in LPS-treated dishes, mean $\pm$ s.d., $n=24$ in each group). The protein content was determined with the Lowry method using bovine serum albumin as a standard (Lowry *et al.*, 1951). The dose-dependency of the endotoxin effects was evaluated by incubating vascular SMC with increasing doses of LPS (0.01 ng ml^{-1} to 1000 ng ml^{-1}). The time-dependency was assessed by incubating the cells with LPS for 1, 3, 6 or 24 h. To exclude non-specific effects, angiotensin II binding studies were also performed in the presence or absence of LPS on cell lines which do not express angiotensin II receptors such as (COS cells) or SK-NMC cells.

Angiotensin II receptor characterization

To define the type of angiotensin II receptor expressed in the presence or absence of endotoxin, displacement curves were done in the presence of increasing concentrations of the selective angiotensin II AT_1 receptor antagonist, losartan (DuP 753, MK 954) or the angiotensin II AT_2 receptor antagonist, (1 - 4 (4 - amino - 3 - methyl - phenyl)methyl-5-diphenylisoethyl-4, 5, 6,7-tetrahydro - 1H - imidazo [4,5-C] pyridine-6-carboxylic acid-2HC) (PD 123177, Bumpus *et al.*, 1991).

Angiotensin II receptor: RNA isolation and Northern blot analysis

Cells were grown to 80% confluency and total RNA was isolated after homogenization of cells in 4 M guanidine isothiocyanate, followed by sedimentation of the RNA through a caesium chloride cushion. RNA was quantitated by determination of the 260/280 nm absorbance ratio and staining with ethidium bromide after gel electrophoresis (Waeber *et al.*, 1993). For Northern blot analysis, 15 μg of RNA was size-fractionated on a 1.2% agarose gel containing formaldehyde and transferred by diffusion to Gene Screen membrane (Du Pont, Boston, MA, U.S.A.). The blots were then hybridized with random primed (Boehringer, Mannheim, Germany) β-actin and angiotensin II receptor AT_1 cDNA probes in 5 x SSC (standard saline citrate; 1 x SSC = 0.15 M NaCl and 0.015 M Na citrate), 100 mM sodium phosphate pH 6.5, 5 x Denhardt's solution, 50% formamide, 10 mM EDTA, 1% sodium dodecyl sulphate and 100 μg ml^{-1} yeast tRNA overnight at 42°C. The blots were washed in 2 x SSC followed by 0.2 x SSC, 0.1% sodium dodecyl sulphate at 50°C. The autoradiograms were then exposed to Hyperfilm-MP (Amersham-Rahn, Zurich, Switzerland). The level of AT_1 receptor mRNA was determined in control cells and cells incubated with LPS (1 μg ml^{-1}) for 1, 3, 6 and 24 h.

Determination of inositol phosphates and cyclic GMP

Inositol phosphates were determined in control and angiotensin II-stimulated cells with or without exposure to LPS. For the determination of total inositol phosphates, confluent cells were incubated with [*myo*-^{3}H]-inositol (Amersham, 10 μCi 5 ml^{-1} culture medium) for 24 h in inositol-containing DMEM. Then, cells were washed with PBS and stimulated with angiotensin II for 15 min at 37°C in the presence of 20 mM $LiCl_2$. Incubation was stopped by placing the dish on ice, discarding the medium and recovering the cells with a rubber policeman. Inositol phosphates were extracted with methanol/chloroform/0.3 M formic acid (2 : 1 : 1), in the presence of 2 mM EGTA. The aqueous phase was recovered, lyophylized, and resuspended in 1 ml 0.1 M formic acid. Inositol phosphates were separated on 1 ml Dowex AG 1-X8 (formate form, Biorad) columns. Columns were conditioned with 2 ml of ammonium formate buffer (2 M ammonium formate acidified to pH 5 with concentrated formic acid), two washes with 5 ml water and one with 2 ml formic acid 0.1 M. Samples of 800 μl were applied to the columns and free inositol and glycerophosphoinositol were eluted with 8 ml formic acid 0.1 M. Inositol phosphates were then eluted as a pool with 7 ml ammonium formate buffer; 1 ml fractions were collected, mixed with 10 ml OptiPhase HiSafe 3 (LKB) and radioactivity measured in a beta counter. Eluted counts per minute (c.p.m.) were normalized with total c.p.m. in the extract prior to chromatography.

Binding studies were also done on cells incubated for 24 h with cycloheximide (5 μM) to inhibit protein synthesis. To evaluate the role of NO-synthase activation in mediating the effects of LPS, cells were incubated with N$^{\omega}$-nitro-L-arginine

methyl-ester (L-NAME, 300 μM). The ability of L-NAME to block NO-synthase activity was evaluated by measuring guanosine 3′:5′-cyclic monophosphate (cyclic GMP). The intracellular cyclic GMP concentration and the cyclic GMP released by the cells in the supernatant were measured by radioimmunoassay using a commercially available kit (Amersham cGMP kit). For this purpose, SMC were incubated with 1 μg ml^{-1} LPS for 1, 3, 6 and 24 h in the presence of 3-isobutyl-1-methylxanthine (IBMX, Sigma, 10^{-5} M). After incubation, the culture medium was collected and lyophylized. Intracellular cyclic GMP was extracted by addition of ice-cold 65% ethanol. The supernatant was then drawn into test tubes. The remaining precipitate was washed again with ice cold 65% ethanol and added to the appropriate tubes. The extracts were centrifuged at 2000 *g* for 15 min at 4°C and the supernatant transferred to fresh tubes. The combined extracts were then evaporated and kept at −20°C. Before analysis, the dried extracts were dissolved in the assay buffer. An aliquot of the cell pellet was kept for protein determination.

Statistical analysis

Binding parameters (K_d and B_{max}) were determined using a Scatchard analysis of the displacement curves. All values are presented as mean ± s.e.mean. Standard differences were analysed using a standard Student's *t* test for paired or unpaired data or analysis of variance and a least significant difference test when appropriate.

Results

Effect of LPS on angiotensin II and vasopressin binding

Displacement curves of [^{125}I]-angiotensin II by unlabelled angiotensin II in control and LPS-treated vascular SMC at 37° and 4°C are shown in Figure 1. When cells were incubated for 24 h with LPS (1 μg ml^{-1}), a significant increase in specific binding was observed. Non-specific binding was always below 5%. At 37°C (Figure 1a), maximal binding increased up to 140% ($P<0.001$) depending on the dose of LPS. To avoid internalization, the binding studies were repeated at low temperature. At 4°C (Figure 1b), maximal binding with 1 μg ml^{-1} LPS increased by 27.0 ± 5.3% ($P=0.009$). The concentration of unlabelled angiotensin II inducing 50% binding inhibition (IC_{50}) at 37° and 4°C was comparable in control cells (0.5 nM) and in LPS-treated cells (0.6 nM). Scatchard analysis of the binding data obtained at 4°C revealed a linear plot demonstrating a single class of angiotensin II binding sites in control as well as in LPS-treated SMC (Figure 1). The dissociation constant (K_d) was 0.58 ± 0.3 nM ($n=4$) in control conditions and 0.58 ± 0.5 nM ($n=4$) after LPS exposure. However, the maximum number of binding sites (B_{max}) was significantly increased after LPS (14′433 ± 480 sites/cell, $P<0.05$) when compared to control cells (9′886 ± 560 sites/cell). In contrast to the angiotensin II receptor, vasopressin binding was not different in control cells and cells exposed to 1 μg ml^{-1} LPS. At 37°C, total binding measured in the absence of unlabelled vasopressin was 395 ± 24 c.p.m./well in control and 334 ± 11 c.p.m./well in LPS-exposed cells ($n=2$ in triplicates). Non-specific binding measured in the presence of 10 μM unlabelled vasopressin was 108 ± 20 c.p.m./well in control and 121 ± 18 c.p.m./well in LPS-exposed cells ($n=2$ in triplicates).

Figure 2 demonstrates that the effect of LPS on angiotensin II receptors is both time- and dose-dependent. Indeed, no effect of LPS was observed after 1 h of incubation whereas significant increases in binding were found at 6 and 24 h (Figure 2a). The dose-response relationship was obtained between 0.1 ng ml^{-1} and 1 μg ml^{-1} LPS (Figure 2a). The response to LPS was attenuated in cells with a higher number of passages. Thus, the LPS-induced increase in maximal binding was 58 ± 10% (mean ± s.e.mean, $n=12$) in cells studied between the first and the sixth passage. When cells were studied between the 6th and 10th passage, the increase in maximal binding was 35 ± 16% ($n=5$). No effect of LPS was obtained in cells studied after more than 15 passages (2 ± 2%, $n=6$).

In control cells incubated for 1 h with [^{125}I]-angiotensin II, 69% of the bound angiotensin II was actually internalized

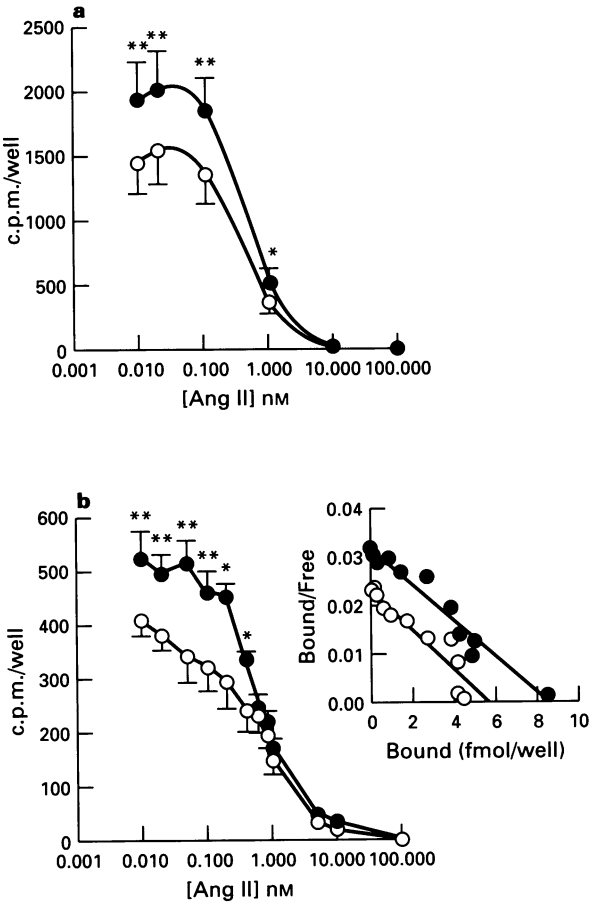

Figure 1 [^{125}I]-angiotensin II binding in control (○) and endotoxin-exposed (●) rat vascular smooth muscle cells (passages 2 to 7). Cells were incubated with endotoxin (1 μg ml^{-1}) for 24 h. Binding experiments were performed at 37°C (a, $n=11$) and at 4°C (b, $n=4$). The Scatchard plot of the results obtained at 4°C is indicated in (b). Data represent mean ± s.e.mean of the total binding in c.p.m./well including uptake for the experiments performed at 37°C. *$P<0.05$, **$P<0.01$ vs control (paired *t* test).

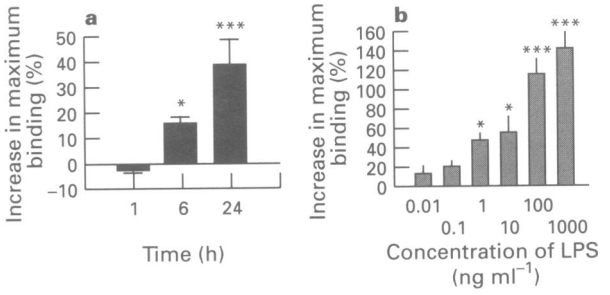

Figure 2 (a) Time-dependency of the effect of LPS (1 μg ml^{-1}) on angiotensin II binding; (b) dose-dependency of the effect of LPS on angiotensin II binding. Values represent the percentage change (LPS exposed − control) in maximal binding of [^{125}I]-angiotensin II in the absence of unlabelled angiotensin II after subtraction of the non-specific binding. Experiments were carried out at 37°C (thus including uptake of radiolabelled angiotensin II) on cell passages 3 to 7. Data are mean ± s.e.mean of 3 experiments in triplicate. *$P<0.05$, ***$P<0.001$ LPS versus control by a paired *t* test.

(total binding in intact cells: 1398±193 c.p.m.; total binding after acid wash: 969±130 c.p.m./well, $n=2$ in triplicate). In the LPS-exposed cells, the internalisation of angiotensin II was proportionally comparable at 69.9% (total binding in intact LPS-exposed cells: 1864±263 c.p.m.; total binding after acid wash: 1303±184 c.p.m./well, $n=2$ in triplicate). In the two cell lines (COS and SK-NMC) which do not express angiotensin II binding sites, no specific angiotensin II binding was found after LPS exposure.

Angiotensin II receptor characterization and expression

As shown in Figure 3, LPS enhanced the number of angiotensin II AT_1 receptor subtype only. Indeed, in control as well as in LPS-treated cells, all the [^{125}I]-angiotensin II was displaced by the AT_1 antagonist, losartan with an IC_{50} of 10 nM whereas the AT_2 receptor antagonist, PD 123177 had no effect on angiotensin II binding in control and LPS-treated cells.

Northern blot analysis of vascular SMC RNA hybridized with the AT_1 angiotensin II receptor cDNA probe confirmed the presence of the AT_1 receptor subtype in these cells. In cells pretreated with LPS (1 μg ml^{-1} for 1, 3, 6 and 24 h), the AT_1 receptor/β-actin mRNA ratio was increased as compared to control cells suggesting that the expression of the angiotensin II receptor gene is enhanced under the influence of endotoxin (Figure 4).

The increase in angiotensin II binding sites induced by LPS was also dependent on protein synthesis as it was inhibited by cycloheximide. In normal conditions, 24 h incubation with LPS (10 ng ml^{-1}) increased the maximal binding from 1735 c.p.m./well to 2455 c.p.m./well ($n=2$ in duplicate). In the presence of cycloheximide, no difference in total binding was observed between control (1154 c.p.m./well) and LPS-exposed cells (1105 c.p.m./well).

To evaluate the receptor coupling in intact and LPS-exposed cells, total inositol phosphates were measured. In control cells, total inositol phosphates represented 5.5±0.5% of the total counts in the aqueous extract ($n=3$ in duplicate). In cells treated with LPS for 24 h, total inositol phosphates increased slightly to 7.85±0.9% (NS). Upon stimulation with 100 nM angiotensin II, total inositol phosphates accumulated significantly to 12.2±1.5% ($P<0.01$ vs control cells using a one-way analysis of variance and a least significance difference test). The response to angiotensin II was even more pronounced in LPS-treated cells (16.8±2.0%, $P<0.01$ vs control and LPS-exposed cells).

Inhibition of NO synthase activity by L-NAME (300 μM) did not modifiy the ability of LPS to enhance angiotensin II binding sites (Figure 5) although L-NAME markedly reduced intra- and extracellular cyclic GMP in LPS-exposed cells.

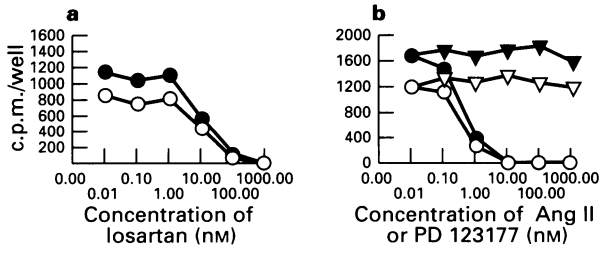

Figure 3 (a) [^{125}I]-angiotensin II binding in presence of increasing concentrations of the AT_1 receptor antagonist losartan in control (○) or LPS-exposed cells (1 μg ml^{-1} for 24 h, ●). (b) [^{125}I]-angiotensin II binding in presence of increasing concentrations of angiotensin II (circles) or the AT_2 receptor antagonist PD 123177 (triangles). Experiments ($n=2$ in duplicate) were conducted in the absence (open symbols) or presence of endotoxin (closed symbols, LPS 1 μg ml^{-1} for 24 h). Data represent the mean of the total binding in c.p.m./well including uptake (experiments were carried out at 37°C).

Vascular SMC exposed to endotoxin for 24 h exhibited significantly higher intracellular cyclic GMP concentrations as compared to control cells (Figure 6). The difference between LPS-exposed and control cells was even greater when extracellular cyclic GMP was measured (32.2±5.4 after LPS vs 1.95±0.16 pmol mg^{-1} protein, $n=5$, $P<0.01$). The effect of LPS on cyclic GMP was found only in cells exposed to endotoxin for 6 h or more. L-NAME significantly reduced but did not completely normalize cyclic GMP production in LPS-exposed cells. In control cells, extracellular cyclic GMP concentration was 1.56±0.27 pmol mg^{-1} protein in the absence and 1.77±0.46 in the presence of 300 μM L-NAME. In contrast, in LPS-exposed cells (1 μg ml^{-1} for 6 h), extracellular cyclic GMP was 16.8±5.02 in the absence and 3.0±0.7 pmol mg^{-1} protein in the presence of L-NAME ($n=4$).

Discussion

These results demonstrate that vascular SMC chronically exposed to LPS exhibit an increased number of AT_1 angiotensin

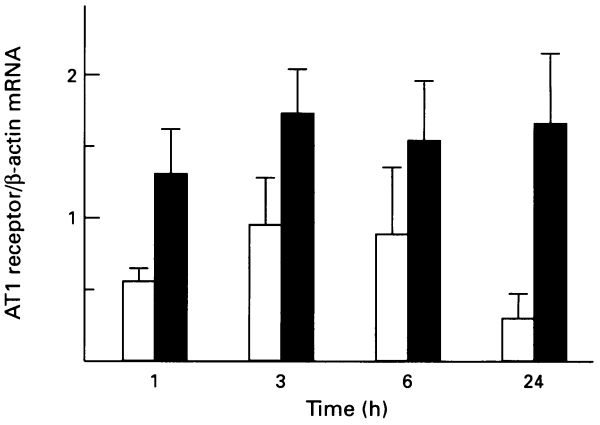

Figure 4 AT_1 receptor/β-actin mRNA ratio in control cells (open column) and cells exposed to LPS (1 μg ml^{-1}) for 1, 3, 6 and 24 h (closed columns). The figure shows the results of three separate experiments (mean±s.e.mean). Cells were studied after 2 to 4 passages. P values are not significant on times 3 and 6 h; $P=0.049$ at 1 h and 0.07 at 24 h (t test).

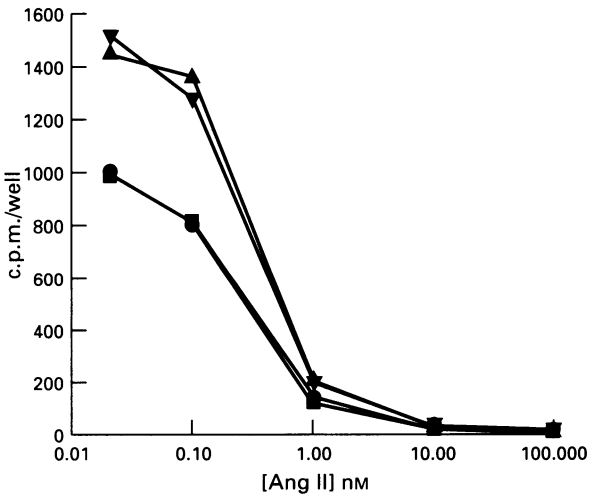

Figure 5 Effect of L-NAME (300 μM) on [^{125}I]-angiotensin II binding in control and LPS-exposed cells. Symbols (●) control; (■) control + L-NAME; (▲) LPS (1 μg ml^{-1}); (▼) LPS + L-NAME. Data represent the mean of the total binding in c.p.m./well including uptake (experiments were carried out at 37°C, $n=2$ in triplicate).

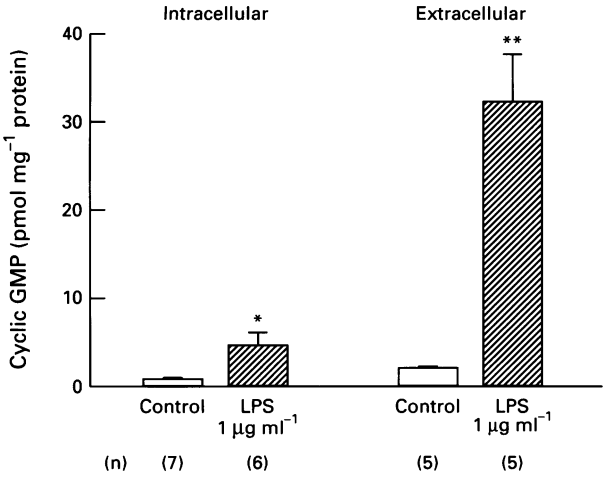

Figure 6 Effect of LPS (1 μg ml^{-1} for 24 h) on intracellular and extracellular cyclic GMP in rat vascular smooth muscle cells. *$P<0.05$, **$P<0.01$ vs control (unpaired t test). Values are mean ± s.e.mean of 5 to 7 experiments.

II binding sites. This effect of LPS is time-, dose- and protein synthesis-dependent. The LPS-induced increase in receptor number results from an increased expression of the receptor gene and induced AT_1 receptors appear to be normally coupled to their second messenger system. The LPS induction of angiotensin II receptors can be dissociated from the LPS induction of NO-synthase. LPS has no effect on vasopressin binding.

The Scatchard analysis of the angiotensin II displacement curves indicates that LPS essentially enhances the number of binding sites but the affinity of angiotensin II for its receptor is not affected. The lack of effect of LPS on K_d is in agreement with a previous report in hepatocytes in which LPS reduced the number but did not modify the K_d of vasopressin and prazosin to their specific receptors (Roth & Spitzer, 1987).

A priori, the LPS-induced increase in angiotensin II binding could result from several different mechanisms and might not necessarily represent an increase in angiotensin II receptor number. Indeed, angiotensin II like vasopressin and other peptides, is rapidly internalized upon binding to its receptor (Ullian & Linas, 1989; Briner *et al.*, 1992). Thus, when angiotensin II binding studies are performed at 37°C, intracellular radioactivity accounts for almost 70% of the total cell-associated counts. Endotoxin could have increased internalization and thereby produce an apparent increase in binding sites. This hypothesis can be excluded in several ways. First, the effect of LPS on angiotensin II binding sites was reproduced in intact cells studied at 4°C, a temperature that prevents internalization. Second, internalization was comparable in control- and LPS-exposed cells and LPS induced no disproportionated internalization of the radiolabelled angiotensin II. One could also postulate that endotoxin perturbs the lipid bilayer of the plasma membrane and hence produces non-specific binding sites for angiotensin II. This possibility was excluded by evaluation of the effect of LPS on angiotensin II binding in COS and SK-NMC cells that do not express angiotensin II receptors. A prolonged incubation of such cells with a high dose of LPS did not result in an increased angiotensin II binding.

The use of selective, nonpeptide angiotensin II receptor antagonists has demonstrated the existence of the AT_1 and the AT_2 subtypes (Bumpus *et al.*, 1991). The former can be antagonized by losartan and is coupled to calcium as intracellular second messenger (Burnier *et al.*, 1991). The latter can be blocked by PD 123177 and its function is not yet defined. Vascular smooth muscle cells normally express only the AT_1 receptor (Burnier *et al.*, 1991). The increase in angiotensin II binding induced by LPS could result from an increase in AT_1 receptors but also from an expression of the AT_2 subtype. All the angiotensin II binding was displaced by losartan whereas PD 123177 had no effect. These results clearly demonstrate the absence of AT_2 receptors in our vascular smooth muscle cells even after endotoxin exposure. Our finding of an increased production of inositol phosphates in LPS-exposed cells stimulated with angiotensin II as compared to control cells is also consistent with an increased synthesis of AT_1 receptors as these latter are coupled to the phosphoinositol-calcium pathway.

The ability of endotoxin to increase angiotensin II binding in vascular SMC was time- and protein synthesis-dependent. Indeed, no effect of LPS was obtained when cells were incubated with endotoxin for 1 h whereas significant increases were found in cells exposed to LPS for 6 and 24 h. Moreover, the effect of LPS was abolished in the presence of cycloheximide, an inhibitor of protein synthesis. These observations evoke at least two potential mechanisms of action of LPS. First, endotoxin could directly enhance the expression of the angiotensin II receptor gene and hence the synthesis of new angiotensin II receptors. Alternatively, endotoxin could affect the turnover of the angiotensin II receptor either directly or indirectly via a third factor the synthesis of which is increased by LPS.

Whether endotoxin directly increases the angiotensin II receptor gene expression has been examined by Northern blot analysis. The LPS-treated cells indeed showed an increased AT_1 receptor gene expression in comparison to untreated cells and the enhanced expression was already measurable 1 h after LPS exposure. Therefore, the LPS-induced increase of angiotensin II binding sites seems to be transcriptionally mediated. The 5'-regulatory region of the rat AT_1 receptor has been recently cloned (Murasawa *et al.*, 1993). Interestingly, the promoter sequence of the gene contains three AP_1 sites which have not been functionally tested. As the activation of the protein kinase C pathway can enhance or repress gene transcription through AP_1 sites, one could speculate about such a mechanism to explain the LPS effect on gene expression since it has been suggested that endotoxin activates protein kinase C (Weightman & Raetz, 1984). In our results, there appears to be a dissociation between the time-course of the variations in AT_1 receptor gene expression and that of the LPS-induced increase in angiotensin II binding. Indeed, the effect of LPS on AT_1 receptor gene transcription and/or mRNA stabilization is evident at 1 h and is maintained up to 24 h whereas the effect on angiotensin II binding is observed only after 6 h of LPS exposure. A possible explanation for this apparent discrepancy is that the mechanisms of translation and translocation to a mature, functional membrane receptor is likely to take more time than gene expression. In addition, whether LPS interferes with the translation and translocation processes is not known.

The observation that the effect of LPS on angiotensin II binding needs several hours to develop and that it can be suppressed by cycloheximide could also support the hypothesis of a third factor mediating the effect of endotoxin. In macrophages, endothelial and vascular smooth muscle cells, endotoxin has been shown to induce the expression of several factors. These include cytokines, cytokine receptors, enzymes such as NO synthase or NADPH-dependent diaphorase and cofactors for NO synthase activity such as tetrahydrobiopterin (Stuehr & Marletta, 1985; Knowles *et al.*, 1990; McKenna, 1990; Radomski *et al.*, 1990; Rees *et al.*, 1990; Gross & Levi, 1992; Mitchell *et al.*, 1992). In our study, the respective role of these factors has not been exhaustively assessed. However, because induction of an inducible Ca^{2+}-independent NO synthase by LPS in endothelial and vascular smooth muscle cells appears to be a major pathway whereby endotoxin reduces vascular responsiveness and this induction requires *de novo* protein synthesis and a similar time course, we have examined the effects of LPS in cells incubated with the NO-synthase inhibitor L-NAME. Inhibition of NO-synthase activity with L-NAME did not blunt the effect of endotoxin on angiotensin II binding sites suggesting that an increased intracellular production of NO and hence a rise in intracellular cyclic GMP is

not involved in this specific receptor response to LPS. Thus, if the effect of endotoxin on angiotensin II receptors is indeed mediated by a third factor, this latter remains to be defined.

An increase in vascular angiotensin II receptors in LPS-exposed cells is rather surprising as vascular responsiveness is known to be decreased during endotoxaemia. The increase in angiotensin II binding induced by LPS may represent a failed attempt of the cells to compensate for the decreased vascular responsiveness. It may also result from an LPS-related induction of genes. In this respect, the specificity of the LPS effect on vascular receptors has not been evaluated extensively in the present study. Nevertheless, the observation that LPS does not increase vasopressin binding in SMC indicates the existence of some specificity. In accordance with our results, an increased number of endothelin receptors has recently been found in kidneys of endotoxaemic rats (Nambi *et al.*, 1991). The reason why some receptors are induced by LPS while others are not, may simply reflect the fact that different genes may be regulated in different ways by the same substance.

In summary, our results show that endotoxin increases the number of functional AT_1 angiotensin II receptors in cultured vascular SMC. The ability of LPS to increase angiotensin II receptors is related to an increased receptor gene expression independent of the induction of NO-synthase activity. The knowledge that LPS can induce the expression of some receptors in cultured cells may be particularly relevant for investigators studying receptors *in vitro* as the presence of LPS in serum or medium may represent an important confounding factor. The LPS content of serum given by quality control certificates can vary from 0.1 ng ml^{-1} in so called LPS-free serum to 70 ng ml^{-1} in normal serum.

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