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European Journal of Pharmacology 287 (1995) 257–261

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Endotoxin-induced changes of endothelial cell viability and permeability: protective effect of a 21-aminosteroid

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Received 27 April 1995; revised 31 July 1995; accepted 8 August 1995

Abstract

Endotoxic shock results in endothelial cell dysfunction and oedema formation. Endotoxin decreased in a concentration-dependent fashion endothelial cell viability with maximum effects by adding 10 μg/ml lipopolysaccharide for 48 h (47 $\pm$ 15% and 22 $\pm$ 2.6% of control cells in the presence or absence of foetal calf serum, respectively). Furthermore, incubation (10 h) with lower concentrations of lipopolysaccharide (1 μg/ml) significantly increased endothelial cell permeability to 250% compared to control values. The 21-aminosteroid U-74389G (10 μM) prevented the cytotoxic effect of lipopolysaccharide as well as the lipopolysaccharide-induced increase in endothelial cell permeability. By contrast, the glucocorticoid methylprednisolone was less effective even at higher concentrations (100 μM). The effect of lipopolysaccharide is possibly due to oxidative stress and/or membrane destabilization rather than to the induction of inflammatory mediators, because of the reduced efficacy of the glucocorticoid.

Keywords: Lipopolysaccharide; Antioxidant; Vitamin E; Vitamin C; Allopurinol; Glucocorticoid

1. Introduction

Endothelial injury can seriously damage the function of the tissue and may result in the loss of barrier permeability and oedema formation. Septic/endotoxic shock, ischaemia/reperfusion injury or trauma lead to the formation or release of several inflammatory mediators such as cytokines (tumour necrosis factor-α, interleukin-1 and -8), kinins or oxygen free radicals mainly by neutrophils and monocytes. These mediators can act directly on the endothelium causing activation (Westlin and Gimbrone, 1993). Furthermore, activated neutrophils adhere to the endothelial cells through specific receptor interactions, consequently causing either direct cytolysis or detachment of endothelial cells (Westlin and Gimbrone, 1993; Schneeberger et al., 1994).

Beside the neutrophil-mediated effects, endotoxin, the active component from the wall of Gram-negative bacteria, is also directly involved in oedema formation in vivo (Brigham, 1992). The pathophysiological effect on endothelial cells without interaction with neutrophils, however, has still not been adequately researched. We therefore were interested in the influence of endotoxin on the viability and permeability of endothelial cells.

Glucocorticoids prevent production of various cytokines as well as induction of inducible enzymes (cyclooxygenase, nitric oxide synthase) in vitro and in vivo, by binding to a specific intracellular receptor (Amano et al., 1992; Rees et al., 1990; Barnes and Adcock, 1993). The variety of side effects from glucocorticoids led to the pursuit of non-glucocorticoid steroid analogues, the 21-aminosteroids, which are strong antioxidants but do not effect translation or transcription (Hall, 1992; Thomas et al., 1993). Thus, we wanted to determine the effect of the 21-aminosteroid U-74389G on the endotoxin-induced endothelial cell dysfunction in comparison to methylprednisolone.

2. Materials and methods

2.1. Materials

Cell culture materials were from Gibco, Berlin, Germany, and Dispase II from Boehringer Mannheim,

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SSDI 0014-2999(95)00499-8

Mannheim, Germany. Transwell collagen-treated filters (pore size, 3.0 μm) were purchased from Costar Corporation, Bodenheim, Germany and microtiter plates from Sarstedt, Germany. The 21-aminosteroid, U-74389G (21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione, (*z*)-2-butenedioate) was kindly donated by Upjohn Company, Kalamazoo, USA. Methylprednisolone hemisuccinate was from Hoechst, Frankfurt, Germany. Lipopolysaccharide from *Escherichia coli* (serotype 055:B5) and all chemicals used were from Sigma Chemical Company, St. Louis, USA.

2.2. Cell culture

Pig aortic endothelial cells were isolated, identified (Gryglewski et al., 1986) and grown in 75 cm^2 fibronectin-treated tissue culture flasks with Dulbecco's modified Eagle medium containing 20% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. Culture flasks as well as microtiter and Transwell membrane plates were preincubated with 2.5 μg/ml human fibronectin for at least 60 min. After 6 days this primary culture reached confluence and the cells were detached with Dispase (1.2 U/ml) and spread either on fibronectin-coated 96-well microtiter plates or on fibronectin-coated 24-Transwell collagen-treated filters.

2.3. Determination of cell viability

In microtiter plates the cells (5×10^5 cells/ml) were grown for one additional day. Then, the cells were incubated with lipopolysaccharide and U-74389G or methylprednisolone for 48 h as indicated. The experiments were carried out with and without foetal calf serum. Cell viability was determined with the viability assay of Mosmann (1983). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) was added, and after 4 h incubation the supernatants were discharged. Adherent cells were resolved in 100 μl/well 0.04 NHCl in isopropanol. The absorption was detected photometrically by means of a multiwell scanning spectrometer (MR5000; Dynatech) in dual-wavelength mode (570 nm and 630 nm as reference).

2.4. Determination of permeability

Endothelial cells (3.5×10^6 cells/ml) were grown on Transwell membranes and maintained for 3–4 days until they reached confluence. The cells were incubated with lipopolysaccharide and/or U-74389G, methylprednisolone, vitamin C, vitamin E or allopurinol with and without preincubation for 10 h. After preincubation the medium in the lower chamber was replaced by Medium 199 (1 ml). The upper compartment was refilled with trypan blue-bovine serum albumin (BSA) solution (100 μl), and substances as indicated. Transwell plates were incubated at 37°C with mild, continuous agitation using an orbital mixer. We determined permeability changes by measuring trypan blue-labelled albumin diffusion photometrically (590 nm) at 30 min intervals.

3. Results

3.1. Effect on endothelial cell viability

Incubation of endotoxin (lipopolysaccharide) showed a time-and concentration-dependent cytotoxic effect on endothelial cells. Maximum cytotoxic effects were determined with 10 μg/ml lipopolysaccharide after incubation for 48 h by means of the MTT assay (Mosmann, 1983). Viability was reduced to 47 ± 15% and 22 ± 2.6% of control values in the presence or absence of foetal calf serum, respectively ($n = 4$; means ± S.D.). Although under optimal cell growth conditions in the presence of foetal calf serum the cytotoxicity of lipopolysaccharide appeared less impressive, we demonstrated a significant reduction of cell viability in comparison to control cells ($P < 0.05$). The cytotoxic effects of lipopolysaccharide were ensured by measuring cell viability with the crystal violet dye and they were not only due to inhibition of proliferation. Methylprednisolone (100 μM) addition did not prevent the lipopolysaccharide effects, as shown in Fig. 1. By contrast, endothelial cell destruction caused by lipopolysaccha-

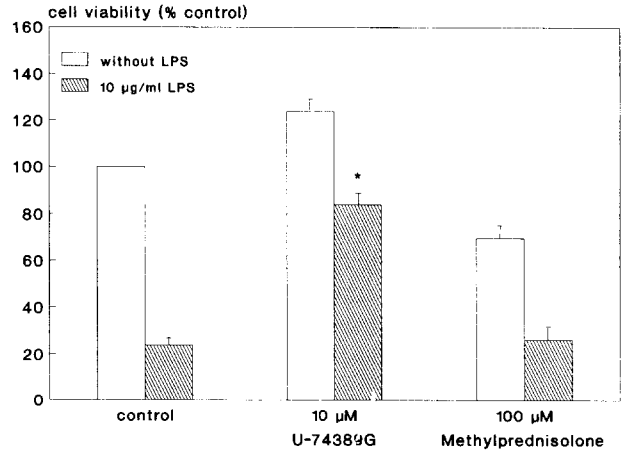

Fig. 1. Effect of U-74389 or methylprednisolone on endothelial cell viability. Porcine endothelial cells (5×10^4 cells/well) were incubated with 10 μg/ml lipopolysaccharide (LPS) for 48 h in the absence of foetal calf serum and cell viability was determined with the MTT assay as described in Materials and methods. U-74389G (10 μM) or methylprednisolone (100 μM) were added 30 min before lipopolysaccharide addition. Values are means ± S.E.M. from four different preparations with $n = 3$ per experiment. * $P < 0.05$ compared to lipopolysaccharide-treated cells.

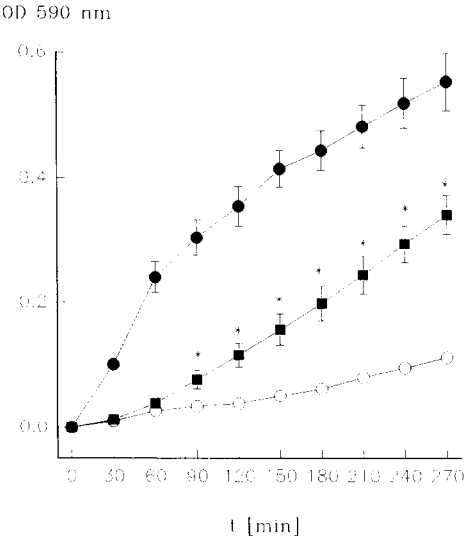

Fig. 2. Effect of lipopolysaccharide on endothelial cell permeability. Endothelial cells were grown on Transwell plates as described in Materials and methods. Adding trypan blue-BSA solution to the upper chamber, optical density was detected in the lower chamber at 30-min intervals. (●) Transwell membranes without endothelial cells; (○) endothelial cells without addition of lipopolysaccharide; (■) endothelial cells which were preincubated with 1 μg/ml lipopolysaccharide for 10 h. Values are expressed as means ± S.E.M., $n = 6$. * $P < 0.05$ versus control cells.

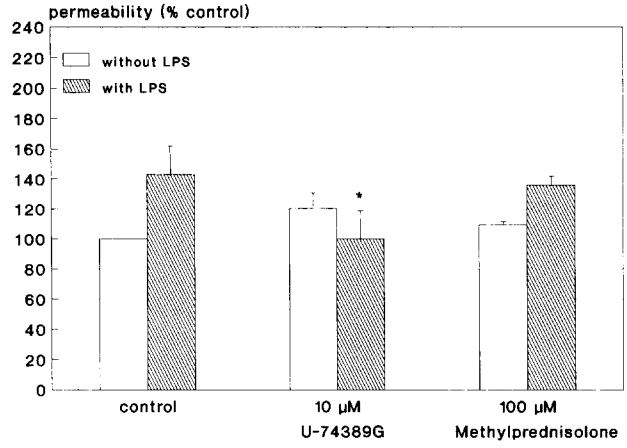

Fig. 3. Effect of U-74389G or methylprednisolone on lipopolysaccharide (LPS)-induced increase in endothelial cell permeability. Lipopolysaccharide (1 μg/ml) and U-74389G or methylprednisolone (as indicated) were preincubated for 10 h. Then, endothelial cell permeability was detected 120 min after adding trypan blue-labelled albumin. Values are calculated as percentage of control values (control = 100%) and expressed as means ± S.E.M., $n = 6$. * $P < 0.05$ versus lipopolysaccharide-treated cells.

ride was prevented by U-74389G in the presence as well as in the absence of foetal calf serum. This effect was dose-dependent with a maximal effective dose of 10 μM, whereas lower concentrations were less effective. Even in the absence of lipopolysaccharide, U-74389G significantly improved the viability of endothelial cells.

3.2. Effect on endothelial cell permeability

The functional integrity of the endothelial cell monolayer was determined by measuring the transfer of trypan blue-labelled albumin across confluent monolayers of pig aortic endothelial cells grown on polycarbonate membranes (Gudgeon and Martin, 1989). Without cells (control) the trypan blue-labelled albumin crossed the membrane in a time-dependent way (Fig. 2). This led to an increase in optical density, whereas in

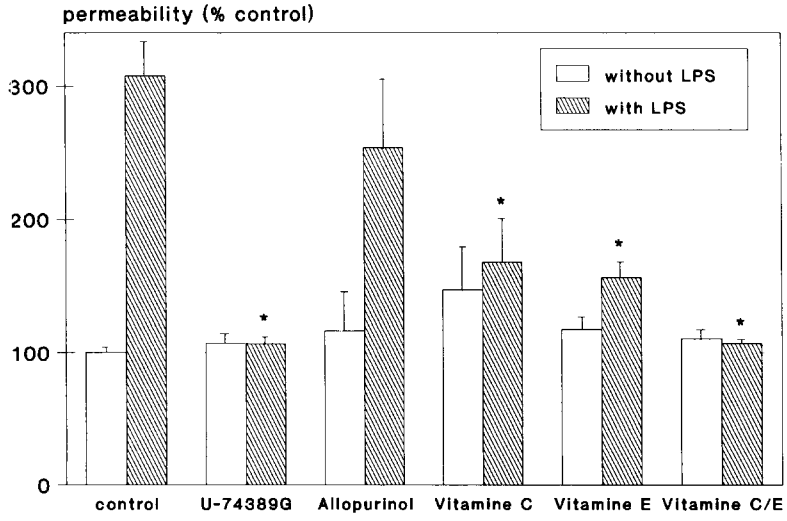

Fig. 4. Effect of antioxidants on lipopolysaccharide (LPS)-induced endothelial permeability. Lipopolysaccharide (1 μg/ml) and the different antioxidants (U-74389G, 10 μM; allopurinol, 10 μM; ascorbate/vitamin C, 10 μM; α-tocopherol/vitamin E, 10 μM) were preincubated for 10 h. Endothelial permeability was detected 270 min after addition of trypan blue-labelled albumin. Values are percentages of control values (means ± S.E.M.; $n = 6$). * $P < 0.05$ versus lipopolysaccharide-treated cells.

experiments with an intact endothelial cell layer only a minor amount of albumin crossed the membrane. The lipopolysaccharide effect depended on the preincubation time. Addition of 1 μg/ml lipopolysaccharide without preincubation at the beginning of the permeability assay did not affect endothelial cell permeability (112 ± 28.9% compared to control values, means ± S.D., $n = 4$). Ten hours of preincubation with lipopolysaccharide (1 μg/ml), however, resulted in an enhanced endothelial cell permeability of about 250% in comparison to control values (Fig. 2). Incubation for 10 h with 1 μg/ml lipopolysaccharide did not change endothelial cell viability, as demonstrated with the MTT assay or crystal violet dye (data not shown).

An additional preincubation with the 21-aminosteroid U-74389G (10 μM) significantly reduced this lipopolysaccharide-induced increase in endothelial cell permeability to control values (Fig. 3), whereas the solvent (CS-4) did not influence endothelial cell permeability. The glucocorticoid methylprednisolone ameliorated the lipopolysaccharide effect at a concentration 10 times higher than the 21-aminosteroid.

These effects point to an involvement of oxidants or radicals in the lipopolysaccharide-induced increase of endothelial cell permeability. Therefore we investigated the influence of other antioxidants (Fig. 4). The xanthin oxidase inhibitor allopurinol (10 μM) only had a minor effect. α-Tocopherol, the principal antioxidant constituent of vitamin E, and ascorbate added solely ameliorated the permeability changes but did not completely reduce the lipopolysaccharide-induced increase in endothelial permeability to control values (Fig. 4). The combination of ascorbate and the lipid-soluble vitamin E completely prevented the lipopolysaccharide effect and was as effective as the 21-aminosteroid.

Control experiments ensured that neither U-74389G nor the solvent incubated solely affected cell permeability.

4. Discussion

In conclusion, the data presented confirmed that lipopolysaccharide has a direct effect on endothelial cells in the absence of neutrophils. At low concentrations (1 μg/ml), lipopolysaccharide increases endothelial cell permeability and at higher concentrations (10 μg/ml) lipopolysaccharide also destroys endothelial cells. The effect of low lipopolysaccharide concentrations incubated for 10 h on endothelial cell permeability was not due to cell destruction as demonstrated by our own results and other studies (Thomas et al., 1988).

Both effects are long-term and required incubation for several hours. The 21-aminosteroid prevented the lipopolysaccharide-induced decrease in endothelial cell viability. The cytotoxic effects, however, were not prevented by glucocorticoids, therefore protein synthesis is not likely to be necessary for this process. The membrane-stabilizing potential (Hall, 1992; Hall et al., 1994) or the capacity to interfere with the oxygen free radical metabolism (Thomas et al . 1993) may be two plausible explanations for the beneficial function of the 21-aminosteroid.

By contrast, the increase in endothelial cell permeability caused by lipopolysaccharide was ameliorated by methylprednisolone. However, U-74389, even if added at a lower concentration, was more effective and may therefore have a stronger antioxidative and membrane-stabilizing capacity compared to glucocorticoids. Furthermore, the hypothesis that the lipopolysaccharide effect is mediated by 'oxidative stress' leading to membrane destabilization was confirmed by the beneficial effects of other antioxidants and the induction of similar effects by oxidative mediators such as H_2O_2 and O_2^- at low concentrations (data not shown). Allopurinol, the inhibitor of xanthin oxidase (Gerdin and Haglund, 1994), as well as the water-soluble vitamin C were not as effective as the lipid-soluble vitamin E. The combination of vitamin E and C revealed comparable results with the 21-aminosteroid. This led us to assume that the prevention of lipid peroxidation by incorporation into the membrane is essential for the protective effect. As already proposed by Hall in 1994 (Hall et al., 1994), the 21-aminosteroid seems to combine both the membrane-stabilizing and the antioxidative property.

The novel non-glucocorticoid 21-aminosteroid prevented both endothelial destruction and increase in permeability, and may therefore be a therapeutic agent in Gram-negative septic shock or other diseases, such as acute central nervous system trauma, where endothelial destruction seems to be involved (Hall et al., 1994; Zuccarello and Anderson, 1988). The mechanisms of the effects of lipopolysaccharide on endothelial cells probably involve oxidative stress and/or lipid peroxidation rather than cytokine-mediated processes. This is supported by results from an experimental study demonstrating an attenuation of microvascular leakage in endotoxic shock by antioxidants (Matsuda et al., 1991) and experiments with pulmonary endothelial cells (Heflin and Brigham, 1981). Further studies should evaluate these mechanisms to give more insight into the functions of the 21-aminosteroid.

Acknowledgements

We thank Mrs. A. Elbers for her technical assistance and Mrs K. Nasskau for her help.

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