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Evidence that mast cell degranulation, histamine and tumour necrosis factor α release occur in LPS-induced plasma leakage in rat skin

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1 In the present study we investigated the role of mast cells during inflammation in rat skin. As the release of several pro-inflammatory mediators, such as histamine and tumour necrosis factor α (TNFα), occurs following mast cell activation we studied whether mast cell degranulation and the release of both histamine (H) and TNFα occurred in a model of lipopolysaccharide (LPS)-induced plasma leakage in rat skin.

2 Plasma leakage in the rat skin was measured over a period of 2 h as the local accumulation of intravenous injection of ^{125}I-human serum albumin (^{125}I-HSA) in response to intradermal injection of LPS. LPS (10 μg site^{-1}) produced an increase of plasma leakage (50.1 ± 2.3 μl site^{-1}) as compared to saline (9.0 ± 3.2 μl site^{-1}). Histological analysis of rat tissue showed that LPS induced a remarkable mast cell degranulation (59.8 ± 2.1%) as compared to saline (13.5 ± 2.2%).

3 Ketotifen (10^{-9}–10^{-7} mol site^{-1}), a well-known mast cell-membrane stabilizer, produced a dose-related inhibition of LPS-induced plasma leakage by 36 ± 3.5%, 47 ± 4.0%, 60 ± 3.3% respectively. In addition, ketotifen (10^{-7} mol site^{-1}) inhibited mast cell degranulation by 59.2 ± 2.7%.

4 Chlorpheniramine maleate (CPM) (10^{-9}–10^{-7} mol site^{-1}), an H_1 histamine receptor antagonist only partially inhibited LPS-induced plasma leakage in rat skin (38 ± 1.1% at the highest dose). Furthermore, CPM (10^{-7} mol site^{-1}) did not prevent mast cell degranulation.

5 A polyclonal antibody against TNFα (1 : 500, 1 : 100, 1 : 50 v v^{-1} dilution), locally injected, decreased LPS-induced plasma leakage in the skin by 15 ± 2.0%, 24 ± 2.1% and 50 ± 3.0% respectively.

6 Taken together these results suggest that LPS-induced plasma leakage in rat skin is mediated, at least in part, by mast cell degranulation and by the release of histamine and TNFα from these cells.

Keywords: LPS; plasma leakage; mast cell degranulation; TNFα; ketotifen; histamine

Abbreviations: CPM, chlorpheniramine maleate; H, histamine; IgE, immunoglobulin E; ^{125}I-HSA, ^{125}I-human serum albumin; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; TNFα, tumour necrosis factor α

Introduction

Mast cells are present mainly in the airways, gastrointestinal tract and skin, i.e. sites that directly interface with the external environment. The anatomical arrangement of mast cells place them in the first line of defence against injury and infections. Mast cells have been traditionally associated with immediate hypersensitivity, an immune reaction resulting from the release of chemical mediators, such as H, following IgE mediated activation (Ishizaka & Ishizaka, 1989). H is involved in numerous pathophysiological processes including inflammation, since it induces vasodilatation and increased microvascular permeability. The effects of H are mediated by three different classes of receptors, H_1, H_2, H_3 (Falus & Mèretey, 1992). However, increased vascular permeability is mediated solely by H_1 receptors (Owen *et al*., 1980; Woodward *et al*., 1985). Recently, the direct involvement of mast cells in the inflammatory and immune process has been clearly demonstrated, since mast cells are a potential source of cytokines, such as TNFα, which are released upon IgE-dependent stimulation (Plaut *et al*., 1989; Burd *et al*., 1989). TNFα is a multifunctional cytokine and a key mediator of immune and inflammatory response (Tracey & Cerami, 1994) and it has been found pre-formed and stored in granules of mast cells or newly synthesized following mast cell activation (Gordon & Galli, 1990, 1991).

Both H and cytokine release by mast cells is not merely a response to IgE dependent activation but may occur in response to a variety of stimuli including bacterial LPS (Church *et al*., 1987; Brzezinska-Blasczyk *et al*., 1988; Leal-Berumen *et al*., 1994).

We have previously demonstrated that intradermal injection of LPS in rat skin causes an inflammatory reaction, characterized by increased plasma leakage at the injured site reaching a maximum at 2 h after endotoxin challenge (Iuvone *et al*., 1998). To the best of our knowledge, no data are available on the involvement of mast cells in this model of acute inflammation. The aim of our study was to investigate whether mast cell degranulation occurred at the inflamed site and whether mediators such as H and TNFα, released by these cells, may contribute to the increase in plasma exudation.

We used two structurally unrelated drugs, CPM and ketotifen, which exhibit a different mechanism of action. CPM was tested because it inhibits the effects of H mediated by H_1 receptors, such as the H-dependent oedema formation

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in the skin (Maling *et al*., 1974). Ketotifen, despite of its anti-H_1 action, was used to prevent mast cell degranulation since it has been demonstrated to have a stabilizing effect on mast cell membranes (Martin & Baggiolini, 1981; Grant *et al*.,1990) dissociated from its anti-H_1 action (Martin & Romer, 1978). Ketotifen has been used as selective stabilizer of connective mast cells by several investigators (Brown *et al*., 1998). In addition, the involvement of TNFα in LPS-induced plasma leakage was also investigated by using an anti-TNFα antibody.

Methods

Plasma leakage measurement in rat skin

The formation of local plasma leakage in the skin was measured in male Wistar rats (250–300 g) (Iffa Credo, Belgium). The rats were anaesthetized with Nembutal (60 mg kg^{-1} i.p.) and the back of the rats was shaved. ^{125}I-HSA (2 μCi kg^{-1} of body weight in 1% of Evans blue solution) was injected *via* the tail vein. Plasma leakage was induced by intradermal injection of 100 μl of LPS (10 μg $site^{-1}$) and compared to saline. Ketotifen (10^{-9}–10^{-7} mol $site^{-1}$), CPM (10^{-9}–10^{-7} mol $site^{-1}$) and rabbit anti-mouse TNFα polyclonal antibody (1 : 500, 1 : 100, 1 : 50 v v^{-1} dilutions) cross reacting with rat TNFα (Genzyme, Cambridge, MA, USA) were injected intradermally (100 μl) 10 min before LPS, according to a balanced site injection plan, in duplicate immediately before ^{125}I-HSA administration. In some experiments plasma leakage was induced by local administration of H (10^{-8} mol $site^{-1}$). After 2 h blood samples were taken by cardiac puncture and the animals were killed. The injection sites were punched out and samples were counted in an automatic gamma-counter (Cobra5005, Packard). Plasma leakage at each site was expressed as μl of plasma by dividing skin sample ^{125}I counts by ^{125}I counts in 1 μl of plasma (Williams, 1979).

Histology

Frozen sections (6–8 mm) were made from rat skin samples and fixed in methanol for 15 min. Tissues were stained for 10 min in 0.05% w v^{-1} toluidine blue solution (50 mg toluidine blue, 39 ml saline, 1 ml acetic acid, 10 ml 40% formol, 50 ml ethanol) then washed and counterstained for 1 min with 0.1% w v^{-1} nuclear fast red solution (0.1 g nuclear fast red in 100 ml 5% $(NH_4)_2$ SO_4 in distilled water). In order to evaluate the percentage of degranulation we counted mast cells present in 20 fields (262144 μm^2 each field area; magnification 60×) distinguishing between deep blue (not degranulated) and light blue (degranulated) mast cells.

Chemicals

All compounds, unless otherwise stated, were purchased from Sigma Aldrich (Bornem, Belgium). ^{125}I-HSA was obtained from Amersham (Brussels, Belgium).

Statistical

Results are expressed as the mean±s.e.mean of *n* animals where each value is the average of responses in duplicate sites. Statistical comparisons were made by one way-ANOVA followed by Bonferroni's test for multiple comparisons or by a non parametric test (Mann-Whitney-*U* test).

Results

Effect of ketotifen and CPM on LPS-induced plasma leakage

LPS (10 μg $site^{-1}$) injected intradermally in rat skin caused an increase in plasma leakage after 2 h (51.0±2.3 μl $site^{-1}$) as compared to rats injected with saline (9.0±3.2 μl $site^{-1}$) (Figure 1). Ketotifen (10^{-9}–10^{-7} mol $site^{-1}$) injected 10 min before LPS, dose-dependently inhibited plasma leakage by 36.0±3.5%, 47.0±4.0% and 60.4±3.3%, respectively (Figure 1). In contrast, treatment with CPM (10^{-9}–10^{-7} mol $site^{-1}$), injected 10 min before LPS (10 μg $site^{-1}$), resulted in slight inhibition of plasma leakage by 4.0±0.5%, 13.0±0.6% and 38.0±1.1%, respectively (Figure 1).

Effect of ketotifen and CPM on H-induced plasma leakage

In order to assess the efficacy of both ketotifen and CPM as H_1 antagonists, in some experiments plasma leakage was induced

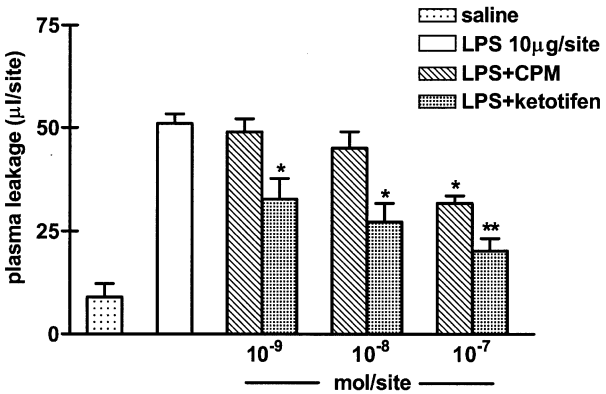

Figure 1 Dose-dependent effect of ketotifen and CPM on LPS-induced plasma leakage in rat skin. Ketotifen (10^{-9}–10^{-7} mol $site^{-1}$) and CPM (10^{-9}–10^{-7} mol $site^{-1}$) were injected i.d. 10 min before LPS (10 μg $site^{-1}$). Plasma leakage was measured over a period of 2 h as local accumulation of i.v. injected ^{125}I-HSA. Each column represents the mean±s.e.mean of $n=5$ experiments in duplicate. $^*P<0.05$, $^{**}P<0.01$ versus LPS.

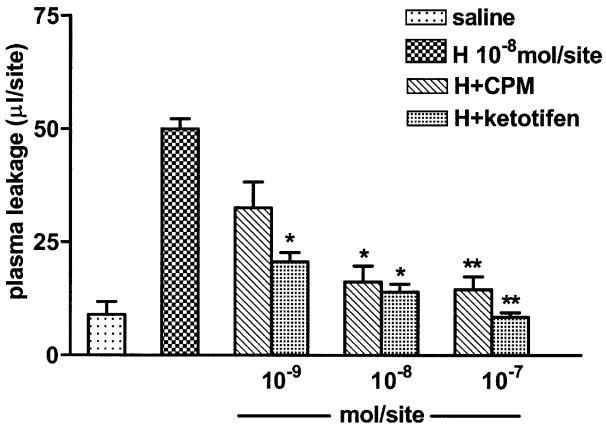

Figure 2 Dose-dependent effect of ketotifen and CPM on H-induced plasma leakage in rat skin. Ketotifen (10^{-9}–10^{-7} mol $site^{-1}$) and CPM (10^{-9}–10^{-7} mol $site^{-1}$) was injected 10 min before H (10^{-8} mol $site^{-1}$). Plasma leakage was measured over a period of 2 h as local accumulation of i.v. injected ^{125}I-HSA. Each column represents the mean±s.e.mean of $n=3$ experiments in duplicate. $^*P<0.05$, $^{**}P<0.01$ versus H.

by H (10^{-8} mol site^{-1}) resulting in an increased plasma exudation of 50.0 ± 2.2 μl site^{-1} as compared to control saline (13 ± 1.6 μl site^{-1}) (Figure 2). Both ketotifen (10^{-9}–10^{-7} mol site^{-1}) and CPM (10^{-9}–10^{-7} mol site^{-1}) dose-dependently and strongly inhibited H-induced plasma leakage (ketotifen by $59.0 \pm 5.2\%$, $72.0 \pm 6.0\%$ and $83.2 \pm 7.0\%$; CPM by $35 \pm 2.7\%$, $67 \pm 3.5\%$, $71 \pm 5.7\%$ respectively) (Figure 2).

Effect of ketotifen and CPM on mast cell degranulation

In order to evaluate LPS-induced mast cell degranulation in the skin, sections of rat skin were prepared and histologically stained for mast cells. Figure 3 shows a representative histological section. Histology showed that mast cells from saline-injected rats were deep blue stained demonstrating a slight degranulation of $13.3 \pm 3.2\%$. In contrast, mast cells from rats treated with LPS (10 μg site^{-1}) were light blue stained demonstrating a degranulation of $59.8 \pm 2.1\%$. Rats treated with ketotifen (10^{-7} mol site^{-1}) before LPS challenge, showed a mast cells degranulation of $24.4 \pm 2.5\%$ resulting in an inhibition of degranulation by $59.2 \pm 2.7\%$ as compared to LPS. In contrast, treatment with CPM (10^{-7} mol site^{-1}) before LPS challenge did not affect mast cell degranulation (Figure 4).

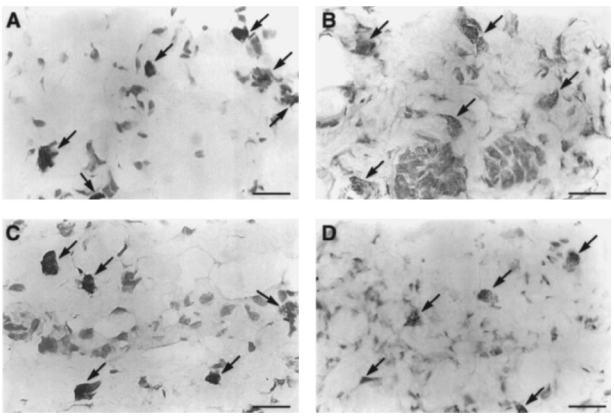

Figure 3 A representative histochemical analysis of rat skin injected with (A) saline; (B) LPS (10 μg site^{-1}); (C) LPS plus ketotifen (10^{-7} mol site^{-1}); (D) LPS plus CPM (10^{-7} mol site^{-1}) injected 10 min before LPS. Mast cell degranulation was evaluated on microscopically visible connective mast cells stained with 0.05% (w v^{-1}) toluidine blue and counterstained with 0.1% (w v^{-1}) nuclear fast red (magnification 60×). Arrows show mast cells in the skin.

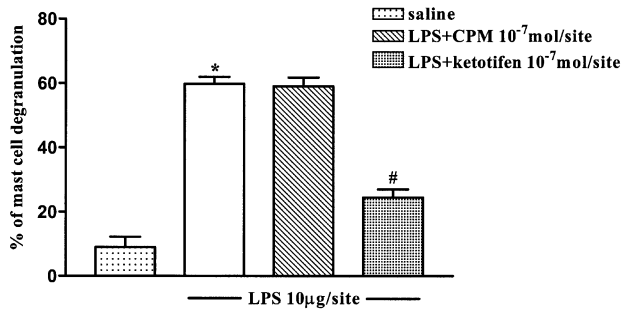

Figure 4 Effect of ketotifen on LPS-induced degranulation in rat skin. Ketotifen (10^{-7} mol site^{-1}) and CPM (10^{-7} mol site^{-1}) were injected 10 min before LPS (10 μg site^{-1}). A differentiation between not degranulated cells (deep blue) and degranulated cells (light blue) was performed. Results are expressed as percentage of degranulation (*$P<0.05$ versus saline and #$P<0.05$ versus LPS).

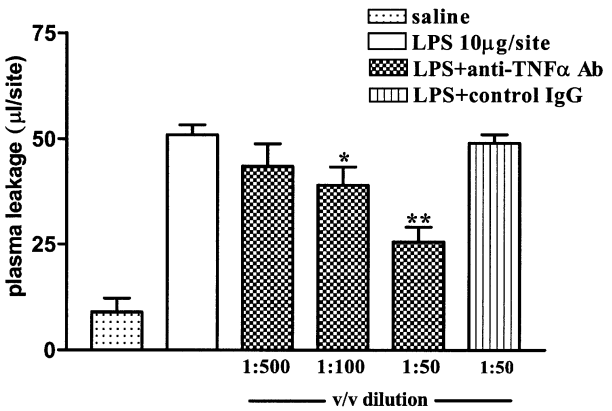

Figure 5 Dose-dependent effect of several dilution (1 : 500, 1 : 100, 1 : 50 v v^{-1}) of anti-TNFα antibody on LPS-induced plasma leakage in rat skin. Anti-TNFα and control rabbit IgG (1 : 50 v v^{-1} dilution) were injected i.d. 10 min before LPS (10 μg site^{-1}). Plasma leakage was measured over a period of 2 h as local accumulation of i.v. injected ^{125}I-HSA. Each column represents the mean ± s.e.mean of $n=5$ experiments in duplicate. *$P<0.05$, **$P<0.01$ versus LPS.

Effect of polyclonal anti-TNFα antibody on LPS-induced plasma leakage

The involvement of TNFα in LPS-induced plasma leakage was investigated by using an anti-TNFα antibody. Polyclonal rabbit anti-mouse TNFα antibody (dilutions 1 : 500, 1 : 100, 1 : 50 v v^{-1}), injected 10 min before LPS (10 μg site^{-1}) significantly reduced plasma leakage by $14.7 \pm 2.0\%$, 24.0 ± 2.1 and $50.0 \pm 3.0\%$ as compared to LPS alone (51.0 ± 2.3 μl site^{-1}). Rabbit anti-mouse IgG (dilution 1 : 50 v v^{-1}), injected into the skin, was used as control (49.0 ± 3.2 μl site^{-1}) (Figure 5).

Discussion

We have previously demonstrated that intradermal injection of LPS in rat skin produced an increased plasma exudation at the inflamed site, reaching a maximum 2 h after LPS challenge (Iuvone *et al*., 1998). In this study we present evidence that treatment with ketotifen significantly reduced LPS-induced plasma leakage in rat skin. In contrast, CPM inhibited LPS-induced plasma leakage to a lesser extent as compared to ketotifen. Moreover, LPS injection into the skin results in mast cell degranulation. Ketotifen was able to prevent mast cell degranulation induced by LPS in the skin paralleling the effect on plasma leakage, as demonstrated by histochemical analysis. On the contrary, CPM did not protect mast cells from LPS-induced degranulation.

Ketotifen and CPM are both H_1 antagonists (Grant *et al*., 1990; Maling *et al*., 1974) and, in our experiments, ketotifen and CPM exibited the same potency in inhibiting H-induced plasma leakage.

In addition, ketotifen has been described to have a stabilizing effect on mast cell membrane (Martin & Romer, 1978; Grant *et al*., 1990). This effect is dissociated from its anti-histaminic effect (Martin & Baggiolini, 1981) and therefore ketotifen has been extensively used to protect mast cells from degranulation. Our results are in agreement with previous studies showing a protective effect of ketotifen on mast cells in bradykinin-induced plasma leakage in rat skin (Moroshita *et*

al., 1996) and in LPS-induced rat colon permeability (Brown *et al*., 1998).

In contrast, CPM as well as other classic anti-H_1 antagonists are less efficent in reducing the permeability response due to endogenously released H following mast cell degranulation. In fact, CPM inhibits only by one-third the oedema induced by compound 48/80, a well-known mast cell degranulator agent (Maling *et al*., 1974), while it strongly reduces plasma leakage in response to exogenous H (Weg *et al*., 1991).

Therefore, taken together our results suggest that LPS induces mast cell degranulation in the skin resulting in an increased plasma exudation. The increased plasma leakage seems to be due only in part to the release of H from mast cells, since CPM failed to inhibit LPS-induced plasma leakage to the same extent as ketotifen. Thus, other mechanisms and other non-histaminergic vasoactive mediators may be involved in the increased plasma exudation occurring in LPS-induced rat skin inflammation.

Since mast cells are a potential source of TNFα, we investigated whether TNFα could be involved, at least in part, in our model of inflammation. In our experiments, an anti-TNFα antibody strongly inhibited plasma leakage. This inhibition was comparable to that exerted by ketotifen but greater than that induced by CPM. Therefore these results suggest that TNFα together with H is responsible for the increased plasma exudation induced by LPS in rat skin. The exact mechanism by which TNFα induces plasma leakage is not known. TNFα has been shown to promote neutrophil-dependent oedema formation in rabbit skin (Rampart *et al*., 1989) and rat paw (Chen *et al*., 1994). Moreover, it has been recently demonstrated that TNFα activates the transcription factor nuclear factor-κB (NF-κB) (Legrands-Poels *et al*., 1997; Pan *et al*., 1998). NF-κB regulates the transcription of several genes involved in the inflammatory process, including genes coding for cytokines, such as TNFα, adhesion molecules and inducible nitric oxide (NO)-synthase (Xie *et al*., 1994; Wulczyn *et al*., 1996). In a previous study we demonstrated that NO, produced by the inducible NO-synthase isoform, increased LPS-induced plasma leakage in rat skin through the involvement of NF-κB (Iuvone *et al*., 1998). Therefore, we suggest that TNFα increases LPS-induced plasma leakage by activation of NF-κB which leads, in turn, to the release of NO and pro-inflammatory cytokines as well as to enhanced expression of adhesion molecules. Moreover, TNFα released following LPS challenge may enhance its own synthesis by activating NF-κB and may in turn amplify the inflammatory process.

Although mast cells probably do not represent the only source of TNFα in the skin, nevertheless these cells lying in close opposition to dermal blood vessels may play a crucial role in the development of oedema formation by the release of the pro-inflammatory mediators TNFα and H.

The mechanism by which LPS causes mast cell degranulation, at the present, is still unclear and remains to be investigated. However, the formation of reactive oxygen species, such as superoxide anions (Kanwar *et al*., 1994) and hydrogen peroxide (Kurose *et al*., 1994), following LPS challenge have been both involved in mast cell degranulation. This was recently confirmed by Brown *et al*. (1998) who found that administration of the antioxidant enzyme, superoxide dismutase, before LPS challenge, inhibited the LPS-associated increase in mucosal mast cell degranulation in rat neonatal colon. These results indeed suggest that reactive oxygen species may have an important role in LPS-induced mast cell degranulation although further studies are required to clarify the exact mechanism involved.

Part of this work has been presented to the British Pharmacological Society Meeting in Oporto, April 1999.

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