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Regulation of tumour necrosis factor production by adrenal hormones *in vivo*: insights into the antiinflammatory activity of rolipram

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1 The role of adrenal hormones in the regulation of the systemic and local production of tumour necrosis factor (TNFα) was examined in male Balb/c mice.

2 Intraperitoneal injection of 0.3 mg *E. coli* lipopolysaccharide (LPS, 0111:B4) led to high levels of circulating TNFα without stimulating TNFα production in the peritoneal cavity. Systemic production of TNFα in response to LPS was increased in adrenalectomized animals and in normal animals treated with the β-adrenoceptor antagonist, propranolol. The glucocortoid antagonist, RU 486, did not modify systemic TNFα production. These results indicate that systemic TNFα production is regulated by adrenaline but not by corticosterone.

3 When mice were primed with thioglycollate, TNFα was produced in the peritoneal cavity in response to low dose LPS (1 μg). The levels of TNFα in the peritoneal cavity were not enhanced by adrenalectomy or by treatment with either propranolol or RU 486, indicating local production of TNFα in the peritoneal cavity is not regulated by adrenaline or corticosterone.

4 The phosphodiesterase type IV (PDE-IV) inhibitor, rolipram, inhibited both the systemic production of TNFα in response to high dose endotoxin (ED_{50} = 1.3 mg kg^{-1}) and the local production of TNFα in the peritoneal cavity in response to low dose endotoxin (ED_{50} = 9.1 mg kg^{-1}). In adrenalectomized mice there was a slight reduction in the ability of rolipram to inhibit the systemic production of TNFα (ED_{50} = 3.3 mg kg^{-1}) while the ability of rolipram to inhibit the local production of TNFα in the peritoneal cavity was virtually abolished (24% inhibition at 30 mg kg^{-1}). The glucocorticoid antagonist, RU 486, also reduced the ability of rolipram to inhibit local TNFα production while propranolol was without effect.

5 Systemic treatment with rolipram increased the plasma concentrations of corticosterone in normal mice but not in adrenalectomized mice indicating that rolipram can cause adrenal stimulation *in vivo*.

6 In summary, these data indicate that systemic production of TNFα in response to high dose endotoxin is controlled differently from the local production of TNFα in response to low dose endotoxin. The systemic production of TNFα is regulated by catecholamines, but not by corticosterone, while the local production of TNFα in the peritoneal cavity is not regulated by basal levels of either catecholamines or corticosterone.

7 These data also show that the ability of rolipram to inhibit the local production of TNFα is dependent on the release of corticosterone from the adrenal glands.

Keywords: Tumour necrosis factor; rolipram; inflammation; endotoxin

Introduction

Cytokines such as tumour necrosis factor (TNFα) are produced from host tissues in response to bacterial endotoxins during infection, and systemic overproduction of these molecules can precipitate shock. It has been appreciated for many years that removal of the adrenal glands can exacerbate many aspects of acute inflammation and increase susceptibility to endotoxic shock. It is now realized that adrenal hormones regulate the production of TNFα and interleukin-1 (Perretti *et al.*, 1989; 1993; Butler *et al.*, 1989; Parant *et al.*, 1991) and can modulate the sensitivity of animals to the toxic action of these cytokines (Bertini *et al.*, 1988). The effects of adrenalectomy have been largely attributed to a reduction in the levels of circulating corticosterone but since β-adrenoceptor agonists are also potent inhibitors of TNFα production (Severn *et al.*, 1992), circulating catecholamines may also regulate cytokine production in response to endotoxin.

The inhibition of TNFα production by β-adrenoceptor agonists is a transcriptional effect mediated through elevation of the intracellular levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP). Elevation of cyclic AMP leads to inhibition of a diverse range of leukocyte functions and the effect of β-adrenoceptor agonists can be mimicked and enhanced by phosphodiesterase inhibitors which inhibit the hydrolysis of cyclic AMP. Phosphodiesterase type-IV is the predominant phosphodiesterase isoenzyme present in inflammatory cells and is selectively inhibited by rolipram, a drug originally developed as an antidepressant (Beavo & Reifsnyder, 1990). There are ample reports that rolipram can inhibit leukocyte function. These effects include inhibition of superoxide production from neutrophils (Nielson *et al.*, 1989; Simpson *et al.*, 1992) and esoinophils (Dent *et al.*, 1991), inhibition of histamine release and leukotriene production from basophils (Peachell *et al.*, 1992) and mast cells (Griswold *et al.*, 1993) and inhibition of TNFα production by macrophages (Semmler *et al.*, 1993).

Rolipram has also been shown to inhibit TNFα production *in vivo* (Turner *et al.*, 1993; Sekut *et al.*, 1995) and since TNFα plays a central role in leukocyte recruitment and tissue injury in inflammatory diseases (Derkx *et al.*, 1993; Elliott *et al.*, 1994), reduction in the concentrations of this cytokine in inflamed tissues may represent an important therapeutic property of PDE-IV inhibitors. Consequently, in addition to evaluating the role of endogenous adrenal hormones in the regulation of TNFα production, we have examined the ability of rolipram to inhibit TNFα production in different tissues in

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mice injected with bacterial endotoxin. Since it has been reported that the PDE-IV inhibitor, denbufylline, can cause release of corticosterone from rodent adrenal glands *in vivo* (Hadley *et al.*, 1991), we have also examined the ability of rolipram to inhibit TNFα production in adrenalectomized mice.

Methods

Animals

Normal male Balb/c mice, adrenalectomized mice and their sham controls were purchased from Charles River Laboratories (Raleigh, NC, U.S.A.).

The drinking water of the adrenalectomized mice was supplemented with 0.9% sodium chloride.

Determination of the effect of rolipram on the production of TNFα in vivo

Male Balb/c mice (20–25 g) were dosed with vehicle or rolipram (1–30 mg kg^{-1}) by oral gavage 0.5 h before injection of LPS (0.3 mg, i.p.). After 1 h, blood was collected in serum separator tubes and centrifuged at 10,000 *g*. The serum samples were stored at −20°C before assay. Adrenalectomized male Balb/c mice (20–25 g) and sham controls were treated in the same way. In some experiments control mice were pretreated with propranolol (5 mg kg^{-1}, i.p.) or RU 486 (25 mg kg^{-1}, i.p.) 15 min before injection of LPS.

To determine the effect of rolipram on the local production of TNFα, male Balb/c mice (20–25 g) were primed with an i.p. injection of Brewer thioglycollate (0.5 ml of a 10% solution) followed 4 days later by an i.p. injection of LPS (1 μg). One hour later, the peritoneal cavities were lavaged with 3 ml heparinized saline containing the protease inhibitors, phenylmethylsulphonyl fluoride (0.25 mM), leupeptin (1 μg ml^{-1}) and EDTA (1 mM) and the lavage fluids centrifuged at 10,000 *g*. The supernatants were stored at −20°C before assay. Control male Balb/c mice were pretreated with propranolol (5 mg kg^{-1}, i.p.) or RU 486 (25 mg kg^{-1}, p.o.) 15 min before dosing with rolipram.

TNFα assay

Samples were diluted 1 to 10 and assayed for TNFα in duplicate by ELISA (Genzyme, Boston, MA, U.S.A.). Standard curves were performed in the appropriate concentration of normal mouse serum or plasma. Under these conditions the level of detection was 0.1 ng per ml.

Corticosterone assay

In some experiments plasma levels of corticosterone were determined with a [^{3}H]-corticosterone radioimmunoassay kit (ICN, Costa, Mesa, CA, U.S.A.). All samples were diluted 1 in 1000 prior to assay and under these conditions the lower limit of detection was 50 ng ml^{-1}.

Materials

Rolipram (4-[3-cyclopentyloxy-4-methoxyphenyl]-2-[1H]-pyrrolidone) and RU 486 (11β-(4-dimethyl aminophenyl) 17 β-hydroxy, 17 α (prop-1-ynyl) estra 4,9-dien-3-one) were synthesized in the department of Medicinal Chemistry, Pfizer Inc. Brewer thioglycollate medium was purchased from Difco (Detroit, MI, U.S.A.). All other reagents were purchased from the Sigma Chemical Company (St Louis, MO, U.S.A.).

Statistical analysis

The data were statistically analysed by use of the Student's unpaired *t* test and considered significant when $P<0.05$.

Results

Systemic TNFα production in control and adrenalectomized mice in response to high dose endotoxin: effect of rolipram

High levels of TNFα were detected in the serum of control mice at 1 h after challenge with a lethal dose of LPS (0.3 mg, i.p.). The levels of TNFα were 3–4 fold higher in adrenalectomized animals in reponse to the same dose of LPS (Figure 1). Pretreatment with rolipram inhibited TNFα production in control mice ($ED_{50}=1.3$ mg kg^{-1}, p.o., $n=3$ independent experiments). Rolipram was also effective in inhibiting TNFα production in adrenalectomized mice ($ED_{50}=3.3$ mg kg^{-1}, p.o., $n=3$ independent experiments), albeit with a modest reduction in potency. It should be noted, however, that the levels of TNFα in adrenalectomized mice dosed with 10 mg kg^{-1} rolipram were equivalent to those levels in vehicle-treated control mice and, even at 30 mg kg^{-1}, the inhibition of TNFα was not complete.

Systemic production of TNFα in response to high dose endotoxin in mice treated with propranolol or RU 486

Systemic production of TNFα in response to LPS was enhanced by treatment with propranolol but not RU 486 (Figures 2 and 3). The inhibitory activity of rolipram on the systemic production of TNFα was not significantly modified by either propranolol or RU 486 (Figures 2 and 3).

Local production of TNFα in peritoneal cavities of control and adrenalectomized mice in response to low dose endotoxin: effect of rolipram

Although TNFα could not be detected in peritoneal lavage fluids of normal mice injected with LPS (despite elevation of this cytokine in serum), high levels of TNFα were detected in peritoneal lavage fluids from thioglycollate - primed mice when challenged with an intraperitoneal injection of a low dose of LPS (1 μg) (Figure 4). In contrast to the systemic production of TNFα, the local production of TNFα in response to LPS in primed mice was not elevated by removal of the adrenal glands (Figure 4), even though this dose of LPS did cause enhanced levels of circulating TNFα (data not shown) as described for the high dose of LPS in the previous section. In addition, rolipram inhibited TNFα production in the peritoneal cavities of normal mice ($ED_{50}=9.1\pm1.2$ mg kg^{-1}, p.o., $n=4$ in-

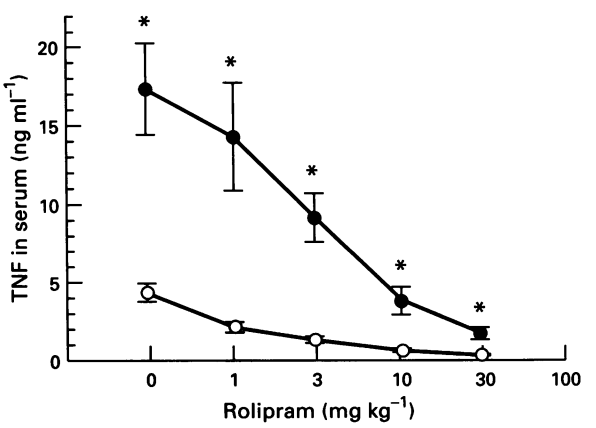

Figure 1 Effect of rolipram on the systemic production of TNFα in response to endotoxin in control male Balb/c mice (○) or adrenalectomized mice (●). Rolipram was administered 30 min prior to challenge with endotoxin and serum samples were collected at 1 h after injection of endotoxin for measurement of TNFα. Each point is the mean±s.e.mean of data from 3 independent experiments and each experiment was conducted with 5 mice per group.

dependent experiments) but was much less effective in inhibiting the production of TNFα at this site in adrenalectomized animals (24±10% inhibition at 30 mg kg^{-1} p.o., $n=4$ independent experiments).

Local production of TNFα in response to low dose endotoxin in the peritoneal cavities of mice pretreated with RU 486 or propranolol: effects of dexamethasone and rolipram

Pretreatment with dexamethasone (0.1 mg kg^{-1}, p.o.) reduced TNFα production in the peritoneal cavity (1.8±0.2 ng ml^{-1} in dexamethasone-treated animals versus 4.2±0.2 ng ml^{-1} in controls, $n=6$ mice per group). This inhibition was reversed by coadministration of 25 mg kg^{-1} RU 486 (4.2±0.4 ng ml^{-1} in RU486/dexamethasone-treated animals, $n=6$ mice per group). However, this dose of RU 486 did not enhance local TNFα production but did diminish the inhibitory activity of rolipram (Figure 5). Propranolol at doses which elevated systemic TNFα production did not modify the local production of TNFα in the peritoneal cavity nor did it affect the inhibitory activity of rolipram (Figure 5).

Effects of endotoxin and rolipram on the levels of corticosterone in the plasma of normal and adrenalectomized mice

The plasma of vehicle-treated Balb/c mice contained 253.7±37.8 ng ml^{-1} ($n=5$) corticosterone. This was significantly increased to 398.2±24.9 ng ml^{-1} ($n=5$) by treatment with rolipram (30 mg kg^{-1}, p.o.) and to 571.7±32.7 ng ml^{-1} ($n=5$) by treatment with LPS (0.3 mg, i.p.). Combinations of rolipram and LPS were approximately additive (779.6±66.5 ng ml^{-1}, $n=5$). The levels of corticosterone in the plasma from adrenalectomized mice were below the lower limit of detection (<50 ng ml^{-1}) under these assay conditions, providing confirmation that the mice had been effectively adrenalectomized.

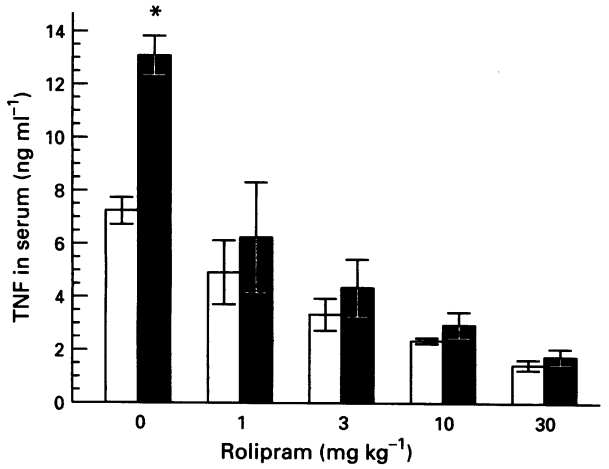

Figure 2 Effect of pretreatment with (±)-propranolol (5 mg kg^{-1}, i.p.) on the ability of rolipram to inhibit the systemic production of TNFα in response to LPS in male Balb/c mice. Mice pretreated with propranolol are depicted by solid columns and those pretreated with vehicle are shown by open columns. Each column is the mean± s.e.mean from 6 mice.

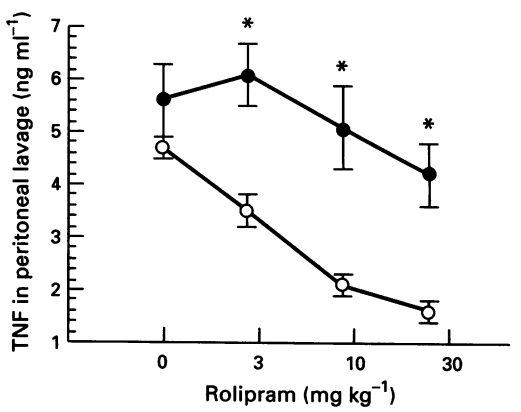

Figure 4 Effect of rolipram on the local production of TNFα in the peritoneal cavities of control thioglycollate - primed mice (○) and in primed mice which had been adrenalectomized (●). Peritoneal lavage fluids were collected at 1 h after injection of endotoxin. Each point is the mean±s.e.mean of data from 4 independent experiments and each experiment was conducted with 5 mice per group.

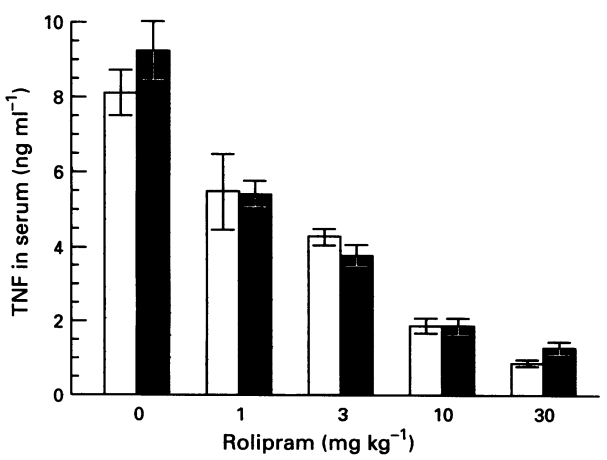

Figure 3 Effect of pretreatment with RU 486 (25 mg kg^{-1}, i.p.) on the ability of rolipram to inhibit the systemic production of TNFα in response to LPS in male Balb/c mice. Mice pretreated with RU 486 are depicted by solid columns and those pretreated with vehicle are depicted by open columns. Each column is the mean±s.e.mean from 6 mice.

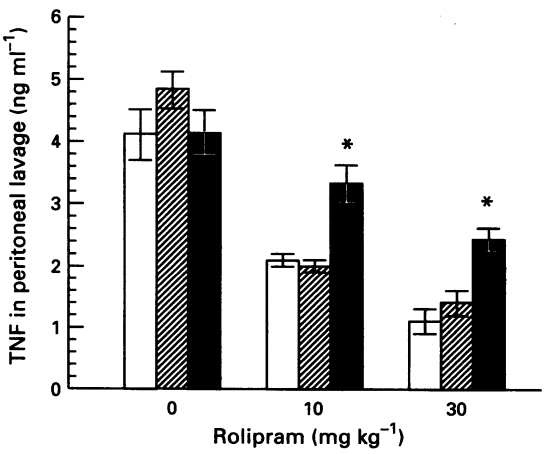

Figure 5 Comparison of the effect of propranolol or RU 486 pretreatment on the ability of rolipram to inhibit the local production of TNFα in the peritoneal cavities of thioglycollate-primed mice. Mice pretreated with vehicle are depicted by open columns, those pretreated with propranolol are depicted by hatched columns and those pretreated with RU 486 are depicted by solid columns. Each column is the mean±s.e.mean from 6 mice.

Discussion

We have confirmed previous observations that systemic TNFα production in endotoxic shock is enhanced by removal of the adrenal glands. This effect has been generally assumed to be due to a reduction in the levels of corticosterone but our data indicate that adrenaline plays a more important regulatory role than corticosterone since TNFα production was enhanced by the β-adrenoceptor antagonist, propranolol but not by the corticosterone antagonist, RU 486. This result is in agreement with previous studies which demonstrated that RU 486 does not enhance systemic TNFα production in response to LPS at doses which effectively block the action of glucocorticoids (Hawes *et al.*, 1992; Perretti *et al.*, 1993) but is surprising since it is well established that glucocorticoids can inhibit TNFα production *in vitro* and *in vivo*. The implication is that the tissues responsible for systemic production of TNFα are more sensitive to the inhibitory action of β-adrenoceptor agonists than to that of glucocorticoids. In this context, TNFα production by peripheral blood *in vitro* is exquisitively sensitive to inhibition by adrenaline but less sensitive to inhibition by dexamethasone (Pettipher, unpublished observations). Other tissues may be more sensitive to the inhibitory activity of glucocorticoids. However, these results do suggest that the contribution of catecholamines to the regulation of inflammatory responses may have been somewhat overlooked and, in some cases, the contribution of glucocorticoids overestimated. Indeed, in a model of carrageenin-induced pleurisy, adrenalectomy caused a two fold increase in oedema, leukocyte infiltration and lipid mediator production (Flower *et al.*, 1986) while RU 486 caused only a modest (< 50%) increase in inflammatory responses (Peers *et al.*, 1988). It would be of interest to determine the effect of propranolol in carrageenin-induced pleurisy. It should be noted, however, that corticosteroids may be more important than adrenaline in the regulation of processes not controlled by the cellular levels of cyclic AMP, such as interleukin-1 production (Bailly *et al.*, 1990; Molnar-Kimber *et al.*, 1992) which is enhanced in adrenalectomized animals (Perretti *et al.*, 1989). In addition to β-adrenoceptor agonists, systemic production of TNFα is also regulated by other agents which activate adenylyl cyclase since we have previously shown that inhibition of prostaglandin production by cyclo-oxygenase inhibitors enhances TNFα production in endotoxic shock (Pettipher & Wimberly, 1994).

In contrast to the systemic production of TNFα, the local production of TNFα in primed mice was not affected by adrenalectomy or treatment with propranolol or RU 486. This may reflect a lack of sensitivity of the peritoneal cells to inhibition by adrenal hormones or suggest that the levels of adrenal hormones in the peritoneal cavity are too low to inhibit TNFα production. Since we know that TNFα production in the peritoneal cavity is sensitive to low doses of dexamethasone, the latter possibility is a more likely explanation, although the sensitivity to β-adrenoceptor agonists is unknown.

The PDE-IV inhibitor, rolipram, is able to inhibit TNFα production *in vivo* (Turner *et al.*, 1993; Sekut *et al.*, 1995), an effect which has been presumed to be due to a direct effect on inflammatory cells. We found rolipram to be a potent inhibitor of systemic TNFα production in reponse to high dose endotoxin. Rolipram was also effective in reducing TNFα levels in the peritoneal cavities of primed mice but was less potent in this system. The reduced potency of rolipram in the peritoneal model compared to the sytemic model may reflect lower peripheral tissue concentrations of drug or a different pharmacological property of rolipram. Rolipram was also able to inhibit systemic TNFα in adrenalectomized mice but with a slight reduction in potency (2.5 fold). Although this reduction in potency is small it may be significant since rolipram, like all PDE-IV inhibitors, has a narrow therapeutic index. The inhibitory activity of rolipram was not modified by pretreatment with RU 486 or propranolol despite the ability of propranolol to enhance TNFα production. It is uncertain why propranolol did not reduce the potency of rolipram to the same extent as adrenalectomy but suggests that there may be additional factors derived from the adrenal glands which modulate the effects of rolipram on TNFα production. The effect of adrenalectomy on the ability of rolipram to inhibit TNFα production in the peritoneal cavity was much more marked. Only slight inhibition of TNFα was observed in adrenalectomized animals at the highest dose of rolipram and this effect did not reach statistical significance. The effect of adrenalectomy was mimicked by administration of the glucocorticoid antagonist, RU 486, which suggests that the inhibitory effect of rolipram on peritoneal TNFα is mediated through release of corticosterone from the adrenal glands. This is supported by our finding that rolipram increased the plasma concentrations of corticosterone which then presumably reached sufficient concentrations in the peritoneal cavity to inhibit TNFα production. We have also observed that the ability of rolipram to inhibit TNFα production in mouse blood *ex vivo* is largely due to the release of corticosterone (Pettipher, unpublished observations). It has been previously demonstrated that adrenalectomy can abrogate other anti-inflammatory activities of rolipram through removal of β-adrenergic tone (Underwood *et al.*, 1992; Griswold *et al.*, 1993). In these cases this may simply reflect synergy of the PDE-IV inhibitor with resting levels of catecholamines. Based on our present study, it appears that in addition to its direct effect on the PDE-IV enzymes present in inflammatory cells, rolipram has an anti-inflammatory property mediated by the release of corticosterone and that in some circumstances the latter property may predominate over its direct effects. The release of corticosterone is clearly an unacceptable mechanism of action for any anti-inflammatory agent but may only occur in rodents. If so, it suggests that experiments in rodents may overestimate the efficacy and potency of PDE-IV inhibitors.

In summary, we have shown that adrenalectomy can affect the ability of rolipram to inhibit TNFα production in some tissues *in vivo* and we suggest that recent reports (Sommer *et al.*, 1995; Sekut *et al.*, 1995) that rolipram and other PDE-IV inhibitors have dramatic anti-inflammatory efficacy in rodents should be re-evaluated in this light.

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