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In vivo and *in vitro* effects of glucocorticosteroids on arachidonic acid metabolism and monocyte function in nonasthmatic humans

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ABSTRACT: Glucocorticosteroids are used as anti-inflammatory agents in a range of diseases, however, their mechanism of action is unknown. Recently, inhibition of arachidonic acid metabolism has been suggested as one possible mechanism of action.

A series of experiments were undertaken in nonasthmatic humans to examine the effects of oral prednisolone and dexamethasone and inhaled budesonide on the excretion of the urinary leukotriene E_4 (LTE_4), an established marker of total body leukotriene generation *in vivo*. In addition, the effect of the drugs on the *in vitro* and *ex-vivo* function of monocytes was examined.

***In vitro* dexamethasone $>10^{-8}$ M inhibited the thromboxane A_2 (TxA_2) release from human monocytes, an effect which recovered within 24 h. *In vivo*, neither inhaled budesonide (1.6 mg·day^{-1} for 7 days), nor a standard therapeutic dose of oral prednisolone (30 mg·day^{-1} for 3 days), nor high doses of oral dexamethasone (8 mg·day^{-1} for 2 days) altered the excretion of urinary LTE_4, despite the latter completely suppressing endogenous cortisol production. The *ex-vivo* zymosan stimulated release of TxA_2 release from monocytes was not altered by the standard dose prednisolone, but was reduced by high dose dexamethasone and inhaled budesonide.**

This study shows that high doses of systemic steroids have little effect on arachidonic acid metabolism in normal nonasthmatic humans. Inhaled budesonide, however, does reduce arachidonic acid metabolism in circulating monocytes, presumably by affecting these cells during their passage through the lung.
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Glucocorticosteroids are an effective treatment for inflammatory diseases, but their mechanism of action is still not known. In *in vivo* and *in vitro* animal models, glucocorticosteroids can inhibit arachidonic acid metabolism through the induction of the synthesis of "lipocortin" [1], which acts as an antagonist of phospholipase A_2 [2]. The action of lipocortin could, therefore, be the basis of the anti-inflammatory action of glucocorticosteroids as products of phospholipase A_2 can induce many of the components of inflammation [3].

Much emphasis has been placed on the use of glucocorticosteroids in the treatment of asthma [4]. It is assumed that the glucocorticosteroids are acting as anti-inflammatory agents in this condition. To date, much investigation of the action in man has relied on indirect measurements such as measurements of airway reactivity [4]. Bronchoscopy with biopsy or bronchoalveolar lavage may give additional information. However, only small samples are obtained in the former, and the latter can be associated with *ex-vivo* activation of inflammatory cells. Results of lavage will also be sensitive to change in the cell population in the lung, which is known to occur with glucocorticosteroids therapy [5].

To investigate in man the action of glucocorticosteroids on arachidonic acid metabolism, we have performed a series of studies in which the effect of oral and inhaled glucocorticosteroids were assessed on total body leukotriene (LT) production, which has been clearly shown to change with asthma [6]. In addition, we have examined the action of these drugs on the *ex-vivo* thromboxane (Tx) production from monocytes, which could act as an accessible marker of glucocorticosteroids action in the disease state. These studies were performed in normal volunteers to eliminate the natural variability in the state of activation and the number of inflammatory cells, which will occur in patients with inflammatory diseases.

Methods

Purification of human peripheral blood monocytes

We collected 100 ml of blood into tubes containing 3.8% sodium citrate. Following dextran sedimentation, mononuclear cells were separated on discontinuous plasma/percoll density gradients [7]. The mononuclear cells were washed three times and finally the cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM). Monocytes were separated from the lymphocytes by adhesion culture of 2×10^6 cells·well^{-1} for 1 h at 37°C in 12 mm diameter plastic wells (Becton Dickenson UK Ltd) yielding a >95% pure monocyte population.

Biochemical analysis

Protein was measured by the method of LOWRY *et al.* [8]. TxB_2 release was measured by radioimmunoassay as described previously [9]. Urinary LTE_4 was measured by one step reverse phase high performance liquid chromatography (HPLC) coupled with radioimmunoassay (Amersham International, Amersham, UK) as described previously [6], and the LTE_4 concentrations were corrected for urinary creatinine. Urinary cortisol was measured by standard radioimmunoassay utilizing ^{125}I cortisol (Baxter Healthcare Corporation, Cambridge, Mass., USA).

Protocols

In vivo *experiments*. In the first study, 6 normal nonasthmatic volunteers (5 male, aged 28–39 yrs) were studied on three occasions at least 1 week apart in a randomized, double-blind, double placebo, crossover design. The protocol was approved by the Ethics Committee of the Royal Postgraduate Medical School and The Hammersmith Hospital Special Health Authority. Subjects were treated with therapeutic doses of either inhaled budesonide (0.8 mg *b.i.d.*) for 7 days and placebo tablets for 3 days, or placebo inhaler for 7 days with prednisolone 30 mg daily (for 3 days), or double placebo. The subjects, therefore, received either two placebo therapies, or active budesonide and placebo prednisolone, or placebo budesonide and active prednisolone. Following the last dose of oral medication, a 24 h urine collection was commenced for measurement of LTE_4 and cortisol. The urine was collected *ad libitum* into preservative-free containers at home and kept at room temperature as in previous studies [6]. Immediately after the completion of the urine collection (*i.e.* on the morning of day 8), 100 ml of blood was taken and the monocytes isolated. Monocytes were then incubated with or without increasing concentrations of opsonised zymosan (0.1–1.0 mg·ml^{-1}) for 1 h. At the end of this incubation the supernatant was analysed for TxB_2 concentration.

In a second study a different group of 6 normal nonasthmatic volunteers (5 male, aged 25–39 yrs) were treated with either 2 mg dexamethasone *q.d.s.* for 48 h, or matched placebo, in a randomized, double-blind, crossover fashion separated by at least 1 week. The first dose of medication was taken at 12.00 h on day 1, and thereafter at 6 hourly intervals, the final dose being taken at 06.00 h on day 3. A urine collection for measurement of LTE_4 and cortisol was commenced immediately after the ingestion of the penultimate dose and closed at 09.00 h on day 3, when blood was drawn for the monocyte studies and in addition a differential leucocyte count.

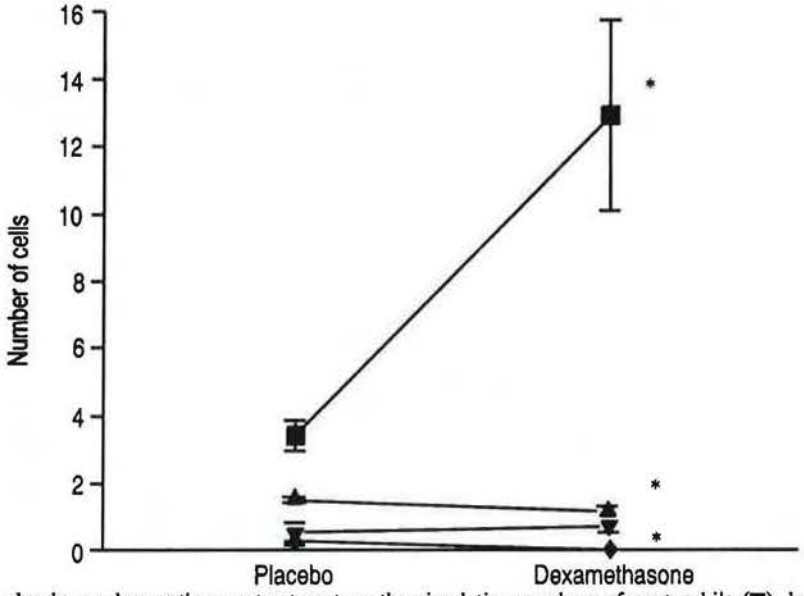

Fig. 1. – The effect of placebo or dexamethasone treatment on the circulating numbers of neutrophils (■), lymphocytes (▲), monocytes (▼) and eosinophils (♦). Mean±SEM. *: $p<0.05$ compared with placebo.

In vitro *experiments*. Following isolation (from non-treated volunteers, n=6) the monocytes were incubated for 16 h in the presence or absence of dexamethasone (10^{-9} to 10^{-5} M). Following this incubation the cells were washed and challenged with opsonized zymosan (1 mg·ml^{-1} DMEM) for 1 h. Following this challenge

the supernatant was harvested for later analysis of TxB_2. The cells were then removed for protein analysis in order to standardize the TxB_2 release.

In a further series of *in vitro* experiments cells from 6 normal volunteers were incubated for 16 h with 10^{-6} M dexamethasone. The cells were then washed and incubated in fresh DMEM for a further period of up to 32 h. At the end of each incubation time the cells were challenged with opsonized zymosan for 1 h as before.

Statistical analysis

In the text results are expressed as mean±SEM and statistical analysis was performed by analysis of variance and least significant difference tests.

Results

In vivo *studies*

Circulating leucocyte counts. Some of the blood taken at the end of the second *in vivo* study was analysed for leucocyte cell numbers. Figure 1 shows that compared to placebo there was a significant increase in neutrophil count ($p<0.05$) and a significant decrease in both the lymphocyte and eosinophil count ($p<0.05$), whilst the count of monocytes was unchanged.

Urinary cortisol and LTE_4. There was no difference between the urinary cortisols during treatment with double placebo or budesonide. However, as expected the values during prednisolone therapy were elevated because of the crossreactivity between prednisolone and cortisol in the urinary assay (table 1). There was, however, a significant decrease in the urinary cortisol excretion during dexamethasone treatment compared to placebo (table 1). In both studies there was no difference in the urinary excretion of LTE_4 (table 1) between any active therapy and the placebo period. The difference in excretion of LTE_4 in the two studies reflects the difference in subjects.

Table 1. – Level of urinary cortisol: and LTE_4 excretion 1) during treatment with double placebo (7 days), budesonide 0.8 mg *b.i.d.* (7 days) or prednisolone 30 mg·day^{-1} (3 days); 2) during treatment with placebo (2 days) or dexamethasone 8 mg·day^{-1} (2 days)

Study		Cortisol nM	LTE_4 ng·mmol^{-1} urinary creatinine
1	Double placebo	149±16	14.2±4.2
	Budesonide	121±15	14.7±4.1
	Prednisolone	>500	22.1±3.0
2	Placebo	99.5±11	35.5±10.9
	Dexamethasone	<25	33.1±13.0

LTE_4: leukotriene E_4.

Monocyte activation. Figure 2 shows the effect of treatment with budesonide, prednisolone or placebo on TxB_2 release from zymosan challenged cells compared to placebo. Budesonide, but not prednisolone, significantly ($p<0.05$) reduced the amount of TxB_2 released at each concentration of zymosan below 1.0 mg·ml^{-1}. In the second study compared to placebo, dexamethasone also reduced ($p<0.05$) the amount of TxB_2 released (fig. 3).

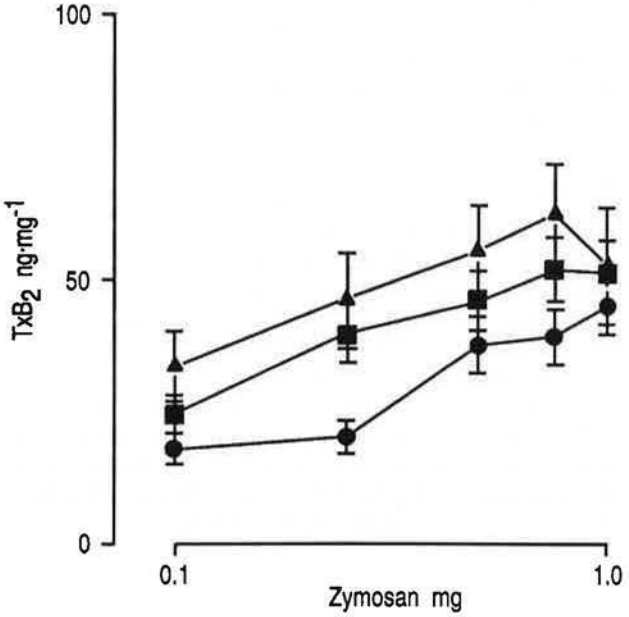

Fig. 2. – The effect of treatment of volunteers with prednisolone (▲), budesonide (●) and placebo (■) on the *ex-vivo* release of TxB_2 following zymosan challenge of human monocytes. The data is the mean±SEM. TxB_2: thromboxane B_2.

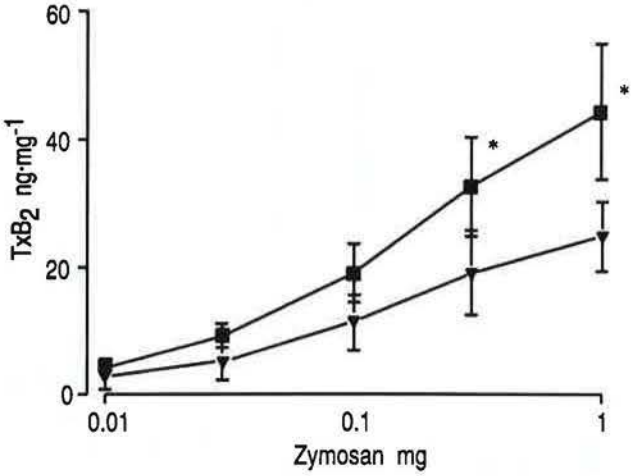

Fig 3. – The effect of treatment of volunteers with placebo (■) or dexamethasone (▼) on the *ex-vivo* release of TxB_2 following zymosan challenge of human monocytes. The data is the mean ±SEM. *: $p<0.05$ compared with the placebo. TxB_2: thromboxane B_2.

In vitro *studies*

Figure 4 shows that 16 h incubation with dexamethasone at concentrations above 10^{-8} M significantly ($p<0.05$) inhibits TxB_2 release from the monocytes

from each volunteer. Figure 5 shows that the amount of TxB_2 released per mg cell protein was reduced with prolonged incubation and the additional inhibitory effect of dexamethasone was lost by 24 h incubation without dexamethosone.

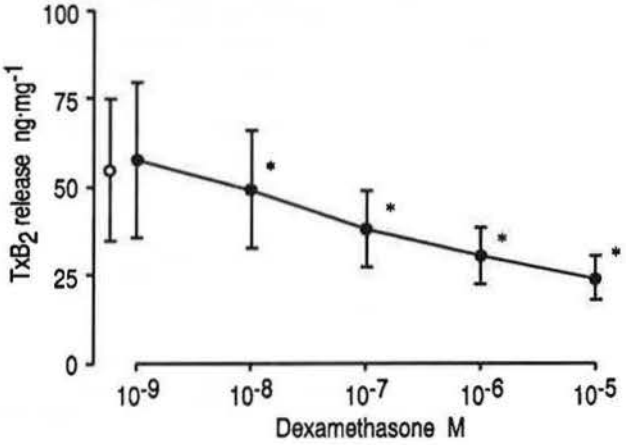

Fig. 4. – The effect on monocytes of 16 h *in vitro* incubation with various concentrations of dexamethasone on zymosan stimulated release of TxB_2 from human monocytes. The data is the mean±SEM. *: $p<0.05$ compared with the control (open circles). TxB_2: thromboxane B_2.

The lack of effect of high and conventional doses of oral glucocorticosteroids on urinary excretion of LTE_4 was surprising in view of the proposed mechanism of action of these drugs [2, 4, 9, 10] and, in view of the urinary cortisol measurements, cannot be due to lack of compliance to the therapy. It is consistent with the previous observations of the lack of effect of glucocorticosteroids therapy on urinary prostaglandin excretion [11–13], and nasal leukotriene release following challenge [14]. These observations could be explained if the proposed inhibitory effect on phospholipase A_2 (PLA_2) [1, 2, 9, 10] does not occur *in vivo* in humans at these concentrations of glucocorticosteroids or that the enzyme inhibition is too short-lived to result in a measurable change in the urinary metabolite excretion. In addition, lipocortin may only be active at higher levels of stimulation of PLA_2. The effect of oral glucocorticosteroids on monocyte function in the *ex-vivo* protocols is compatible with the latter hypothesis. In these experiments only high dose dexamethasone therapy reduced TxB_2 release and this was only significant at the high levels of zymosan stimulation. To explore the possibility that the action of the oral glucocorticosteroids might be transient, recovery of function of the

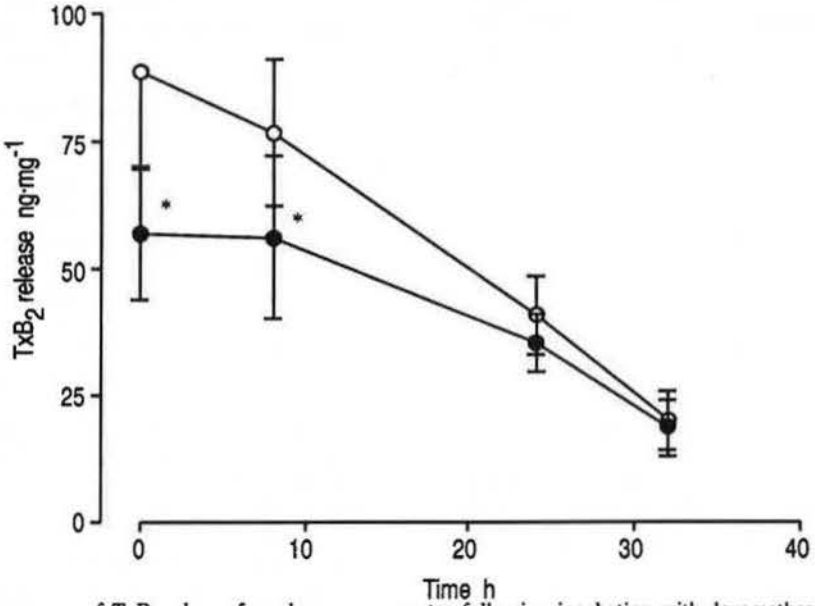

Fig 5. – The *in vitro* recovery of TxB_2 release from human monocytes following incubation with dexamethasone (10^{-6} M) for 16 h prior to washing. The time points refer to hours after the washing step. The data is the mean±SEM. *: $p<0.05$ compared with the control (open circles). TxB_2: thromboxane B_2.

Discussion

The effects of glucocorticosteroids on arachidonic acid metabolism in normal humans was studied. The results have show that there was no detectable reduction in the whole body production of LTE_4 despite profound effects on cortisol production and circulating cell numbers with oral dexamethasone. In addition, high doses of oral and inhaled glucocorticosteroids were required to reduce the ability of peripheral blood monocytes to produce TxB_2 following stimulation with zymosan *ex-vivo*.

monocyte following incubation with dexamethasone *in vivo* was studied. During the recovery both the control and treated cells showed a reduction in TxB_2 release, which is a characteristic of the maturing monocyte *in vitro*. However, the inhibitory effect of dexamethasone was lost at 24 h. It is therefore conceivable that the lack of effect of prednisolone on the circulating monocyte could be due to the time of the last dosing. However, the duration of clinical effect of the therapy would be expected to be in excess of this time as alternate day therapy is effective [15]. This suggests that inhibition of arachidonic acid

metabolism is unlikely to be a major action of the drug. The action of dexamethasone on the zymosan stimulation of the monocyte is unlikely to be due to alteration in the cell number and maturity of the cell, as could be the case with the neutrophil as this did not change in the study.

The inhibitory effect of budesonide on both sensitivity and amount of release of TxB_2 *ex-vivo* from monocytes is intriguing, as it occurs in the absence of any systemic effect of the drug on cortisol, which at the dose used would have been minimal [15]. The budesonide may be having its action on the cells as they pass through the lungs, or by reducing the release of cytokines from the lung which control the recruitment and perhaps state of activity of the circulating monocytes. This is consistent with the data from bronchitics, where inhaled budesonide in similar doses has been shown to inhibit the responses of circulating neutrophils [16], and in healthy males, where alteration in the clinical variation of circulating leucocyte number follows treatment with high dose budesonide [17].

These studies in nonasthmatics suggest that at low, but clinically effective, doses of glucocorticosteroids, there is little evidence of a sufficient inhibitory action on PLA_2 activity to reduce LTE_4 generation, suggesting that the anti-inflammatory action of the drugs may be upon the release of cytokines and, therefore, exerting an indirect effect on the activity of inflammatory cells. Further work is required to study the effect of glucocorticosteroids in the diseases in which they are effective.

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