

**THE effect of prostaglandin E<sub>2</sub>, iloprost and cAMP on both nitric oxide and tumour necrosis factor- $\alpha$  release in J774 macrophages has been studied. Both prostaglandin E<sub>2</sub> and iloprost inhibited, in a concentration-dependent fashion, the lipopolysaccharide-induced generation of nitric oxide and tumour necrosis factor- $\alpha$ . The inhibitory effect of these prostanoids seems to be mediated by an increase of the second messenger cAMP since it was mimicked by dibutyryl cAMP and potentiated by the selective type IV phosphodiesterase inhibitor RO-20-1724. Our results suggest that the inhibition of nitric oxide release by prostaglandin E<sub>2</sub> and iloprost in lipopolysaccharide-activated J774 macrophages may be secondary to the inhibition of tumour necrosis factor- $\alpha$  generation, which in turn is likely to be mediated by cAMP.**

**Key words:** cAMP, Macrophages, Nitric oxide, Phosphodiesterase inhibitor, Prostaglandins, Tumour necrosis factor

## Modulation of macrophage activation by prostaglandins

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### Introduction

Macrophages activated with bacterial lipopolysaccharide release a variety of mediators including nitric oxide (NO), tumour necrosis factor (TNF- $\alpha$ ) and prostaglandins, namely prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prostacyclin (PGI<sub>2</sub>).<sup>1–3</sup> The cytotoxic properties of activated macrophages depend, at least in part, on the biological activities of these mediators. Thus the synthesis of NO from the amino acid L-arginine has been shown to be a major cytotoxic mechanism of activated macrophages.<sup>1</sup> Moreover NO has been identified as an effector molecule of the cytotoxic effects produced by TNF- $\alpha$  in bovine endothelial cells.<sup>4</sup> Conversely, PGE<sub>2</sub> and PGI<sub>2</sub> inhibit the cytotoxic properties of activated macrophages.<sup>3</sup> Furthermore, increasing evidence suggests that the interaction existing within the biological actions of NO, prostaglandins and TNF- $\alpha$  may result in a mutual regulation of their synthesis and/or release. In fact TNF- $\alpha$  has been reported to induce NO synthase in murine peritoneal macrophages<sup>5</sup> and bovine endothelial cells.<sup>4</sup> TNF- $\alpha$  increases PGE<sub>2</sub> production in human synovial cells and human dermal fibroblasts,<sup>6</sup> mouse peritoneal macrophages<sup>7</sup> and rat Kupffer cells,<sup>8</sup> as well as PGI<sub>2</sub> generation in human endothelial cells.<sup>9</sup> Conversely, PGE<sub>2</sub> has been shown to down-regulate the generation of TNF- $\alpha$  in macrophages of different origin<sup>2,10</sup> and HL-60 cells.<sup>11</sup> Moreover it has been reported that the release of TNF- $\alpha$  by macrophages is inhibited by cAMP, a second messenger for both PGE<sub>2</sub> and PGI<sub>2</sub>.<sup>10–12</sup>

We have shown that PGE<sub>2</sub> and the stable analogue of PGI<sub>2</sub>, iloprost, inhibited the induction of NO synthase in lipopolysaccharide-activated J774 murine macrophages.<sup>13</sup> We hypothesized that the inhibition of NO synthase induction could be secondary to an increase in cAMP levels in the activated macrophages. This hypothesis was also supported by the inability of both prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ) and the stable analogue of thromboxane A<sub>2</sub> (TXA<sub>2</sub>), U46619, which do not enhance cAMP levels, to modify NO generation. However, since TNF- $\alpha$  induces NO synthase in murine macrophages<sup>7</sup> and PGE<sub>2</sub> has been shown to inhibit TNF- $\alpha$  release from these cells,<sup>2</sup> the inhibition of NO synthase induction by PGE<sub>2</sub> could also be secondary to the down-regulation of TNF- $\alpha$  in J774 cells.

In the light of the above considerations we have studied the effect of PGE<sub>2</sub>, PGI<sub>2</sub> and cAMP on the production of NO and TNF- $\alpha$  by lipopolysaccharide-stimulated J774 macrophages.

### Materials and Methods

The murine monocyte macrophage cell line J774 (American Tissue Culture Catalogue TIB 67, page 231) was grown in Dulbecco's modified Eagle's medium (Gibco) at 37°C as described previously.<sup>13</sup> The cells were plated in 24-well culture plates (Falcon) at a density of  $2.5 \times 10^5$  cells/ml and allowed to adhere at 37°C in 5% CO<sub>2</sub>/95% air for 2 h. Thereafter the medium was replaced with fresh medium, cells were activated with lipopolysaccharide (0.1  $\mu$ g/ml) from *Salmo-*

*nella thyphosa* (Difco) and incubated in the presence of various concentrations (see Results) of test compounds: PGE<sub>2</sub>, PGF<sub>2α</sub> and dibutyryl cAMP (Sigma), iloprost (Schering), RO-201724 (Biomol).

NO was measured as nitrites (NO<sub>2</sub><sup>-</sup>, nmol per 10<sup>6</sup> cells) accumulated in the incubation media 24 h after lipopolysaccharide challenge. A spectrophotometric assay based on the Griess reaction was used.<sup>14</sup>

The level of TNF-α (U/ml) in the cell medium, 3 h after lipopolysaccharide challenge, was assessed in WEHI-164 cells by a biological assay using recombinant human TNF-α (Sigma) as reference standard and rabbit antimurine TNF-α (Genzyme) antiserum which cross-reacts with rat TNF-α in order to assess the specificity of TNF-α-dependent cytolytic activity.<sup>15</sup>

Data are expressed as percent of control release (mean ± standard error of the mean of *n* observations). Comparisons were made by the unpaired two-tailed Student's *t*-test. The level of statistically significant difference was defined as *p* < 0.05.

## Results

The production of NO<sub>2</sub><sup>-</sup> by unstimulated J774 macrophages was undetectable (< 1 nmol per 10<sup>6</sup> cells in 24 h, *n* = 6). The cells stimulated with lipopolysaccharide (0.1 μg/ml) released, as previously reported (Marotta *et al.*<sup>13</sup>), substantial amounts of NO, measured as NO<sub>2</sub><sup>-</sup> (64.5 ± 2.3 nmol per 10<sup>6</sup> cells in 24 h, *n* = 8). When the cells were stimulated in the presence of dibutyryl cAMP (10<sup>-4</sup> – 10<sup>-6</sup> M), PGE<sub>2</sub> (10<sup>-6</sup> – 10<sup>-8</sup> M) or iloprost (10<sup>-7</sup> – 10<sup>-9</sup> M), added concomitantly with LPS, a concentration-dependent decrease of NO<sub>2</sub><sup>-</sup> accumulation was observed (Fig. 1). PGE<sub>2</sub>, iloprost and cAMP, at any of the concentrations tested, did not affect NO<sub>2</sub><sup>-</sup> generation when added to the cells 6 h after lipopolysaccharide challenge (data not shown). Conversely, PGF<sub>2α</sub> (10<sup>-6</sup> – 10<sup>-8</sup> M) did not significantly affect NO<sub>2</sub><sup>-</sup> accumulation when added concomitantly with lipopolysaccharide or 6 h later (data not shown). The inhibition produced by 10<sup>-4</sup> M dibutyryl cAMP was about 50% (*p* < 0.01) and was virtually superimposable on the inhibition produced by 10<sup>-6</sup> M PGE<sub>2</sub> and 10<sup>-7</sup> M iloprost. The selective inhibitor of cAMP-specific phosphodiesterase type IV, RO-201724,<sup>16</sup> significantly (*p* < 0.01) increased the inhibition induced by dibutyryl cAMP. In fact, in the presence of 10<sup>-4</sup> M RO-201724, the inhibition of NO<sub>2</sub><sup>-</sup> accumulation by 10<sup>-4</sup> M dibutyryl cAMP was significantly increased from about 50% to about 70% (Fig. 1). RO-201724 also potentiated the inhibitory action of

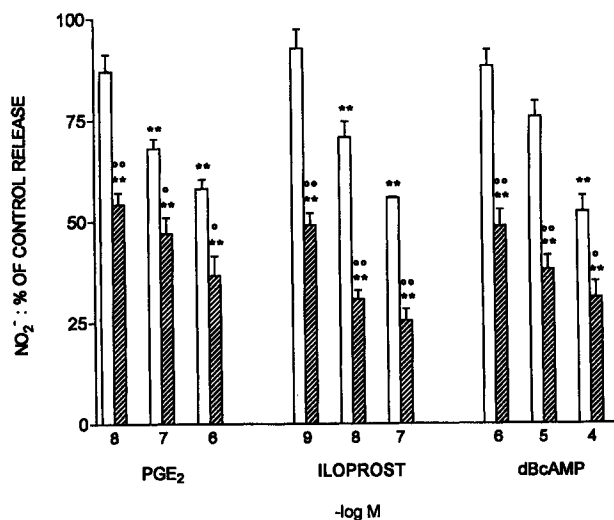


FIG. 1. Effect of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), iloprost and dibutyryl cAMP (dBcAMP) on NO<sub>2</sub><sup>-</sup> generation by J774 murine macrophages 24 h after lipopolysaccharide challenge (0.1 μg/ml) in the absence (open bars) or presence (hatched bars) of RO-201724 10<sup>-4</sup> M. Data are expressed as mean ± standard error of the mean of five to six experiments. \*\**p* < 0.01 vs. control (lipopolysaccharide alone = 100% release); °*p* < 0.05, °°*p* < 0.01 vs. the corresponding concentration of the inhibitor in the absence of RO-201724.

PGE<sub>2</sub> and iloprost on NO<sub>2</sub><sup>-</sup> generation (Fig. 1). RO-201724 alone (10<sup>-4</sup> M) did not produce any effect (data not shown).

We have also studied the effect of PGE<sub>2</sub>, PGI<sub>2</sub> (iloprost) and cAMP (dibutyryl cAMP) on TNF-α release from lipopolysaccharide-activated J774 cells. Three h after lipopolysaccharide-challenge these cells released 297 ± 20.5 U/ml of TNF-α (*n* = 8), compared to the undetectable release by unstimulated cells (< 1 U/ml in 3 h, *n* = 8). Both PGE<sub>2</sub> (10<sup>-6</sup> – 10<sup>-8</sup> M) and iloprost (10<sup>-7</sup> – 10<sup>-12</sup> M) produced a concentration-dependent inhibition of TNF-α release (Fig. 2). PGF<sub>2α</sub> (10<sup>-6</sup> – 10<sup>-8</sup> M) did not produce any effect (data not shown). It is interesting to note that iloprost, as observed for NO<sub>2</sub><sup>-</sup> generation, was more potent than PGE<sub>2</sub>, also an inhibitor of TNF-α release. Moreover, both prostanoids significantly inhibited (*p* < 0.01) TNF-α release at concentrations which were virtually ineffective on NO<sub>2</sub><sup>-</sup> production (10<sup>-8</sup> M PGE<sub>2</sub> or 10<sup>-9</sup> M iloprost). The phosphodiesterase inhibitor RO-201724 (10<sup>-4</sup> M) potentiated the effect of iloprost for significant inhibition (*p* < 0.01) of TNF-α release was observed at concentrations of 10<sup>-11</sup> and 10<sup>-12</sup> M, (10.5 ± 1.1% and 41.1 ± 3.7% of control release, respectively; *n* = 4). Dibutyryl cAMP (10<sup>-4</sup> – 10<sup>-6</sup> M) inhibited lipopolysaccharide-induced TNF-α release in a concentration-dependent fashion (Fig. 2). Moreover, as observed for PGE<sub>2</sub> and iloprost, dibutyryl cAMP was more potent as an inhibitor of TNF-α release

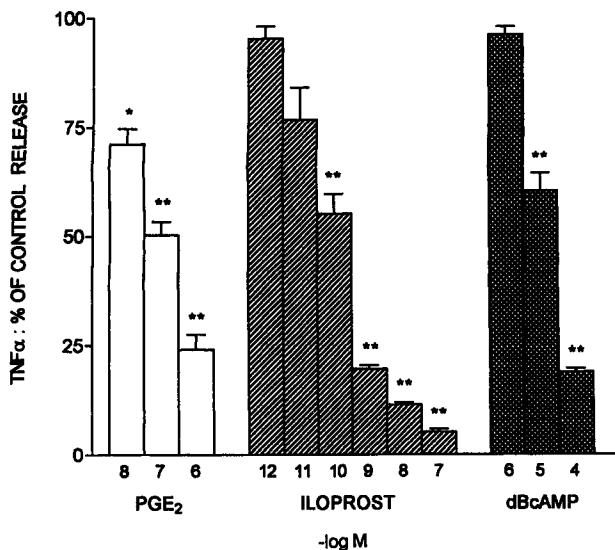


FIG. 2. Effect of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>, open bars) iloprost (hatched bars) and dibutyl cAMP (dBcAMP, cross-hatched bars) on TNF- $\alpha$  release by J774 murine macrophages 3 h after lipopolysaccharide challenge (0.1  $\mu$ g/ml). Data are expressed as mean  $\pm$  standard error of the mean of five to six experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 vs. control (lipopolysaccharide alone = 100% release).

than as an inhibitor of NO<sub>2</sub><sup>-</sup> generation, since at a concentration of 10<sup>-5</sup> M, which was poorly effective for NO<sub>2</sub><sup>-</sup> accumulation, a significant inhibition ( $p$  < 0.01) of TNF- $\alpha$  release was produced.

## Discussion

We have shown<sup>13</sup> that PGE<sub>2</sub> and PGI<sub>2</sub>, and its stable analogue iloprost, which are known to activate adenylate cyclase, inhibit the induction of NO synthase in lipopolysaccharide-activated J774 macrophages. We also demonstrated that this action was not shared by PGF<sub>2 $\alpha$</sub>  and the stable analogue of TXA<sub>2</sub>, U46619, which do not enhance cAMP levels. The results of the present study show that cAMP, as its permeable form dibutyl cAMP, inhibits lipopolysaccharide-induced NO release by J774 macrophages, and confirm that this release is inhibited by PGE<sub>2</sub> and iloprost but not by PGF<sub>2 $\alpha$</sub> . The selective type IV phosphodiesterase inhibitor, RO-201724,<sup>16</sup> potentiated not only the inhibitory effect of cAMP but also the effect of the two prostanoids. In the light of these findings our results strongly suggest that the inhibitory action of the two prostanoids on NO synthase induction in lipopolysaccharide-activated J774 macrophages is mediated by cAMP. This evidence is also supported by recent results showing that in murine peritoneal macrophages, cAMP is an intermediate in the down-regulation of NO synthase by prostaglandins.<sup>17</sup>

Our results also show that PGE<sub>2</sub> and iloprost, but not PGF<sub>2 $\alpha$</sub> , inhibit in a concentration-dependent fashion of lipopolysaccharide-induced TNF- $\alpha$  production by J774 macrophages. This effect was exhibited also by cAMP. The phosphodiesterase inhibitor RO-201724 potentiated this action. PGF<sub>2 $\alpha$</sub>  was unable to inhibit lipopolysaccharide-induced TNF- $\alpha$  release by J774 macrophages. These data suggest that, as observed for NO generation, the inhibitory action of the two prostanoids on TNF- $\alpha$  release is likely to be mediated by cAMP. In this respect it is interesting to note that all the tested compounds were more potent in inhibiting TNF- $\alpha$  than NO<sub>2</sub><sup>-</sup> production. Since NO seems to be the effector molecule of the cytotoxic activity of TNF- $\alpha$ ,<sup>5</sup> and PGE<sub>2</sub> and iloprost modulate the production of TNF- $\alpha$  and NO,<sup>17</sup> it could be hypothesized that the inhibitory action of the two prostanoids on NO synthase induction might be secondary to the inhibition of TNF- $\alpha$  generation which seems to be mediated by cAMP. This hypothesis appears conceivable considering that in macrophages lipopolysaccharide stimulates not only TNF- $\alpha$  and NO but also a sustained production of the prostaglandins, following the expression of inducible cyclooxygenase.<sup>18</sup> In this light the down-regulation of TNF- $\alpha$  and consequently of NO production by endogenous prostaglandins may represent a relevant feed-back mechanism in modulating the cytotoxic effects of a sustained NO production following immunological stimulation.

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