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Evaluation of the anti-inflammatory effects of β -adrenoceptor agonists on human lung macrophages

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Abstract

The principal mechanism by which bronchodilator β -adrenoceptor agonists act is to relax airways smooth muscle although they may also be anti-inflammatory. However, the extent of anti-inflammatory activity and the cell types affected by these agonists are uncertain. The purpose of this study was to evaluate whether β -adrenoceptor agonists prevent pro-inflammatory cytokine generation from activated human lung macrophages. Macrophages were isolated and purified from human lung. The cells were pre-treated with both short-acting (isoprenaline, salbutamol, terbutaline) and long-acting (formoterol, salmeterol, indacaterol) β -agonists before activation with lipopolysaccharide (LPS) to induce cytokine (TNF α , IL-6, IL-8 and IL-10) generation. The experiments showed that short-acting β -agonists were poor inhibitors of cytokine generation. Of the long-acting β -agonists studied, formoterol was also a weak inhibitor of cytokine generation whereas only indacaterol and salmeterol showed moderate inhibitory activity. Further experiments using the β_2 -adrenoceptor antagonist ICI-118,551 suggested that the effects of indacaterol were likely to be mediated by β_2 -adrenoceptors whereas those of salmeterol were not. These findings were corroborated by functional desensitization studies in which the inhibitory effects of indacaterol appeared to be receptor-mediated whereas those of salmeterol were not. Taken together, the data indicate that the anti-inflammatory effects of β -adrenoceptor agonists on human lung macrophages are modest.

Keywords

Alveolar macrophages, β -agonists, TNF α , indacaterol, salmeterol

1. Introduction

β -adrenoceptor agonists are used as bronchodilators in the treatment of respiratory diseases (Waldeck, 2002; Cazzola et al., 2012). In addition to bronchodilation, β -agonists may also possess anti-inflammatory activity (Barnes, 2006; Theron et al., 2013). Both in vitro and in vivo studies suggest that β -agonists may dampen inflammation although there are inconsistencies (Schild, 1937; O'Connor et al., 1994; Maris et al., 2004, Maris et al., 2005; Bosmann et al., 2012; Wex et al., 2015). It is noteworthy that in many of these studies animal models or cell lines have been employed. How representative these models are of human systems is open to interpretation.

The human lung macrophage plays an important role in innate and adaptive immune responses in the lung (Gordon, 2007). However, increased numbers of macrophages and excessive activation of these cells have been associated with respiratory diseases especially COPD (Peters-Golden, 2004; Barnes, 2008; Yang et al., 2012). Attenuating excessive macrophage activation would be expected to be beneficial in the treatment of respiratory diseases. Studies investigating the effects of β -agonists on monocytes, monocyte-derived macrophages (MDM) and macrophage-like cell lines suggest that these drugs may modulate a number of macrophage functions including the prevention of pro-inflammatory cytokine generation (Yoshimura et al., 1997; Izeboud et al., 1999; Donnelly et al., 2010; Shirato et al., 2013). By contrast, other studies show limited effects of β -agonists in these systems (Linden, 1992; Zetturlund et al., 1998; Ezeamuzie and Shihab, 2010). As a consequence, no consistent evidence has emerged to determine whether β -agonists effectively attenuate the pro-inflammatory activity of macrophages. This is

particularly true for human alveolar macrophages in which very few studies have been performed to investigate the effects of β -agonists on these cells.

The aim of the present study, therefore, was to determine whether β -agonists are effective inhibitors of pro-inflammatory cytokine release from macrophages isolated from human lung. To this end, a range of both short- and long-acting β -agonists were evaluated.

2. Materials and methods

2.1 Buffers

Phosphate buffered saline (PBS) contained (mM): NaCl 137; Na₂HPO₄·12H₂O 8; KCl 2.7; KH₂PO₄ 1.5. PIPES buffer contained (mM): PIPES (22), NaCl (110), KCl (5) and the pH was titrated to 7.4 with NaOH.

2.2 Preparation of compounds

Stock solutions (10 mM) of salmeterol, formoterol and indacaterol were prepared in dimethyl sulphoxide and stored at 4 °C. Salbutamol, terbutaline and ICI-118,551 were prepared as stock solutions (10 mM) dissolved in distilled water and stored at 4 °C. Stock solutions (10 mM) of (-)-isoprenaline bitartrate were prepared in 0.05% sodium metabisulphite (dissolved in 0.9% saline) and stored at 4 °C. LPS from *E. coli* serotype R515 (Re) was provided as a 1 mg/ml stock solution and stored at 4 °C. Poly(I:C)-LMW stock solution (10 mg/ml) was prepared in distilled water and stored at -20 °C.

2.3 Lung tissue

Lung tissue was obtained from surgical resections. Thirty-two lung preparations were used in this study, 21 preparations were derived from males and 11 from females. The age range of participants was 29 to 88 years with a median age of 68. Informed written consent was obtained in order to use the lung tissue for the experiments described in this study. The use of lung tissue was approved by the National Research Ethics' Service, UK (REC reference: 15/NW/0657).

2.4 Macrophage isolation

Macrophages were isolated from lung tissue using a modification of the protocol described by Liu and colleagues (1984). In brief, lung tissue was chopped extensively in RPMI-1640 buffer (containing L-glutamine) and the tissue filtered over 100 µm nylon mesh (Incamesh, Warrington, UK). The filtrate (~200 ml) was centrifuged (300 g, 10 min, room temperature) and the pellets resuspended in ~40 ml of RPMI-1640 supplemented with 10% heat inactivated FBS (foetal bovine serum), penicillin (25 U/mL), streptomycin (25 µg/ml), gentamicin (50 µg/ml) and fungizone (1 µg/ml). The cell suspensions were left to sediment at 4 °C for 1 h. After sedimentation, the supernatant was aspirated and the sedimentation step at 4 °C was repeated. The sedimented material was resuspended in 30 ml PIPES buffer and centrifuged (300 g, 10 min, room temperature). The resulting pellet was resuspended in PIPES buffer. The suspension was then filtered through nylon mesh before being reconstituted in PIPES buffer (20 ml for every 5 g of tissue).

The resuspended cells (20 ml) were layered over an 80% Percoll gradient (20 ml) and centrifuged (400 g, 20 min, room temperature). The interface was harvested and two washes were performed with PIPES buffer (50 ml). After centrifugation, (488 g, 10 min at room temperature) the pellet was resuspended in supplemented RPMI-1640 (~10 ml). The cells were then counted using a haemocytometer. Macrophages were seeded at 2×10^5 per well in 24-well cell culture plates in supplemented RPMI-1640 (1ml per well) and incubated overnight (37 °C, 5% CO₂).

Cytospins were prepared (Thermo Shandon Cytospin 3) and from these the purity of cell suspensions was determined by morphology. Cytospins were stained with Quick-Diff and processed according to the manufacturer's instructions. Cell

viability was assessed by erythrosin-B exclusion. Macrophage purity was $86\pm 2\%$ and cell viability was $92\pm 1\%$.

2.5 Preparation of monocyte-derived macrophages (MDM)

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient centrifugation from whole blood obtained from healthy volunteers, who gave informed written consent. Approval was granted by the South Sheffield Research Ethics' Committee (REC reference: 07/Q2305/7). PBMC were seeded at 2×10^6 cells/ml in 24 well plates in RPMI-1640 supplemented with 10% heat inactivated newborn calf serum. After 24 h, non-adherent cells were removed and adherent cells were cultured in RPMI-1640 supplemented with 10% heat inactivated FBS to give a final concentration of approximately 2×10^5 MDM/well at 14 days (Dockrell et al., 2001).

2.6 Macrophage activation protocol

After overnight incubation, medium from the wells was replaced with supplemented RPMI-1640 buffer (1 ml) and incubated ($37\text{ }^{\circ}\text{C}$, 5% CO_2) for 2 h before the start of the experiment. Where agonists and antagonists were used, cells were pre-incubated for 30 to 60 min with these ligands before addition of stimulus for 22 h. The cell culture supernatants were harvested and centrifuged (488 g, 4 min, room temperature) and the resulting supernatants stored at $-80\text{ }^{\circ}\text{C}$ for analysis of cytokine content. TNF α , IL-6, IL-8 and IL-10 were analysed using commercially-available ELISA kits (RSG kits, eBioscience, Hatfield, UK).

2.7 Materials

The following were purchased from the sources indicated: dimethyl sulphoxide, fungizone, indomethacin, isoprenaline, penicillin/streptomycin, Percoll, PIPES, salbutamol, terbutaline, (all Sigma, Poole, UK); gentamicin, heat inactivated FBS, heat inactivated newborn calf serum (Gibco, Paisley, UK); RPMI-1640 containing L-glutamine (Lonza, Slough, UK); salmeterol, ICI-118,551 (Tocris Bioscience, Bristol, UK); Quick-Diff (Reagen, Toivala, Finland); Ficoll-Paque (GE Healthcare Life Sciences, Little Chalfont, UK); LPS (Enzo Life Sciences, Exeter, UK); Poly(I:C)-LMW (InvivoGen, CA, USA); indacaterol maleate and formoterol fumarate were a kind gift of Novartis (Horsham, UK).

2.8 Data analysis

Values are expressed as means \pm S.E.M. Statistical significance was performed utilising either ANOVA followed by post hoc tests (Dunnett's or Tukey's tests) or paired and unpaired Student's t-tests as appropriate.

3. Results

3.1 Long-acting β -agonists are more effective than short-acting β -agonists as inhibitors of cytokine generation

The effects of short-acting and long-acting β -adrenoceptor agonists on LPS-induced cytokine generation from human lung macrophages were investigated (Fig. 1).

Neither isoprenaline, terbutaline nor salbutamol had any significant ($P>0.05$) inhibitory effects on cytokine generation from macrophages. By contrast, the long-acting β -agonists, indacaterol and salmeterol were effective ($P<0.05$) inhibitors of TNF α (Fig. 1A) and IL-6 (Fig. 1B), reducing cytokine generation from activated macrophages by ~40%. However, indacaterol and salmeterol were ineffective as inhibitors of IL-8 generation (Fig. 1C). The long-acting β -agonist, formoterol, was ineffective as an inhibitor of the stimulated release of all the cytokines investigated. The steroid dexamethasone was included as a positive control in these experiments. Dexamethasone was a highly effective inhibitor of TNF α , IL-6 and IL-8 generation reducing the stimulated release of all three cytokines by ~80%.

As well as studies investigating pro-inflammatory cytokine generation from macrophages, the potential effects of β -adrenoceptor agonists on the anti-inflammatory cytokine, IL-10, were determined (Fig. 1D). None of the β -adrenoceptor agonists studied induced IL-10 generation when used alone (data not shown). LPS activation of macrophages led to modest release of IL-10 generation. None of the β -adrenoceptor agonists studied, with the exception of salmeterol, had any effect on LPS-induced IL-10 generation whereas dexamethasone was a highly effective inhibitor.

The effects of β -agonists on the generation of TNF α from macrophages induced by either LPS or the viral mimic poly(I:C) were investigated (Fig. 2).

Salbutamol was ineffective as an inhibitor of TNF α generation induced by both stimuli whereas salmeterol and indacaterol were effective against LPS-induced TNF α generation but not that driven by poly(I:C). Dexamethasone was an effective inhibitor of TNF α generation driven by either stimulus.

The possibility that the relative ineffectiveness of short-acting β -agonists to inhibit cytokine generation might be related to the strength of the stimulus was investigated (Fig. 3). The effects of salbutamol on TNF α generation following activation of macrophages with a range of concentrations of LPS (0.1, 1 and 10 ng/ml) were studied. Salbutamol inhibited TNF α generation to a modest (~30%) but statistically significant ($P < 0.05$) extent when macrophages were activated with lower (< 10 ng/ml) concentrations of LPS.

Macrophages have been shown to release prostaglandin E₂ (PGE₂) following activation with LPS and the addition of exogenous PGE₂ is known to inhibit macrophage responses (Rowe et al., 1997; Ratcliffe et al., 2007; Buenestado et al., 2012). This LPS-induced PGE₂ may act in a paracrine fashion to inhibit macrophage responses and this might interfere with the inhibitory activity of β -agonists. To establish whether this might be the case, the inhibitory activity of salbutamol was investigated in the absence and presence of the cyclooxygenase inhibitor indomethacin (Fig. 4). Preliminary studies of ours showed that indomethacin (1 μ M) completely blocked the generation of PGE₂ from macrophages activated with LPS (data not shown). The extent to which salbutamol inhibited LPS-induced TNF α generation was very similar whether indomethacin was present or not.

3.2 β -adrenoceptor agonists are more effective in MDM than in lung macrophages

Previous studies have demonstrated that β -adrenoceptor agonists are effective inhibitors of cytokine generation from MDM (Donnelly et al., 2010). The effects of both short-acting and long-acting β -agonists on cytokine generation from LPS activated MDM were investigated (Fig. 5). In contrast to the situation seen with human lung macrophages, the data show that both short-acting and long-acting β -agonists were effective inhibitors of TNF α generation from MDM.

3.3 The effects of indacaterol, but not salmeterol, are β -adrenoceptor-mediated

The effects of the β_2 -adrenoceptor antagonist, ICI-118,551, on the inhibitory effects of indacaterol and salmeterol were evaluated (Fig. 6). Pretreatment of macrophages with ICI-118,551 partly reversed the inhibitory effects of indacaterol against LPS-induced TNF α generation. By contrast, the inhibitory effects of salmeterol were not reversed by ICI-118,551.

To investigate further the mechanism by which indacaterol and salmeterol inhibit cytokine generation from macrophages, functional desensitization experiments were performed. Overnight incubation of macrophages with indacaterol abolished the subsequent ability of indacaterol to inhibit LPS-induced TNF α generation (Fig. 7A). By contrast, overnight treatment with salmeterol did not affect the inhibitory activity of salmeterol (Fig. 7B).

Cross-desensitization experiments were also performed. Long-term treatment with indacaterol had no effect on the subsequent salmeterol inhibition of TNF α generation (data not shown). Interpretation of the converse experiment where macrophages were treated with salmeterol overnight and then the inhibitory effects of indacaterol investigated were difficult to interpret because of a persisting residual inhibitory effect of salmeterol (data not shown).

4. Discussion

In the present study we have investigated the effects of short-acting and long-acting β -agonists on pro-inflammatory cytokine release from human lung macrophages. Our data demonstrate that long-acting β -agonists display superior anti-inflammatory activity than short-acting β -agonists although overall the inhibitory effects of this class of drugs are modest.

The effects of a number of short-acting β -agonists on LPS-induced cytokine generation from macrophages were investigated. Neither salbutamol nor terbutaline was particularly effective as an inhibitor of cytokine generation from macrophages. As salbutamol and terbutaline are generally recognised as partial agonists at the β_2 -adrenoceptor (Cazzola et al., 2012), the effects of the full agonist, isoprenaline, were also studied. Isoprenaline was essentially ineffective as an inhibitor of cytokine generation. However, it should be noted that isoprenaline as well as being short-acting is known to be susceptible to metabolism (Cazzola et al., 2012). These factors may have limited the potential inhibitory effects of isoprenaline.

The possibility that aspects of the experimental system might have influenced the inhibitory effects of short-acting β -agonists were considered. For example, the strength of the activating stimulus, LPS, was evaluated and when sub-maximal concentrations of LPS were used to activate macrophages, salbutamol was moderately more effective as an inhibitor. In further studies we wondered whether the endogenous production of PGE₂ might mask responses to β -agonists. It is known that macrophages produce PGE₂ in response to LPS and PGE₂ is inhibitory in macrophages (Rowe et al., 1997; Ratcliffe et al., 2007; Buenestado et al., 2012; Birrel et al., 2015). When experiments were performed in the presence of the cyclooxygenase inhibitor, indomethacin, at a concentration known to block PGE₂

production by macrophages completely, there was no improvement in the inhibitory activity of salbutamol. Altogether these studies demonstrate that even when the experimental system is geared to potentially facilitate increased inhibitory activity, the extent of inhibition seen with salbutamol was still relatively modest.

In addition to short-acting β -agonists, the effects of the long-acting β -agonists salmeterol, formoterol and indacaterol were also studied in this system. Salmeterol and indacaterol were reasonably effective inhibitors of cytokine generation from lung macrophages whereas formoterol was ineffective. These findings differ with those reported for MDM in which both formoterol and salmeterol were capable of reducing the extent of LPS-induced cytokine generation (Donnelly et al., 2010). Indeed, our own studies indicate that all the short-acting β -agonists tested (isoprenaline, salbutamol and terbutaline) as well as the long-acting β -agonist formoterol are considerably more effective inhibitors of cytokine generation in MDM than in lung macrophages (Fig. 5). These findings highlight some obvious functional differences between MDM and lung macrophages that may reflect influences related to site (lung cf blood), extent of macrophage differentiation or exposure to environmental factors.

It was noteworthy that indacaterol and salmeterol showed some effectiveness as inhibitors of LPS-induced TNF α generation but were unable to prevent TNF α release induced by poly(I:C). LPS is a bacterial product that signals through Toll-like receptor (TLR)4 whereas poly(I:C) is a viral mimic that acts through TLR3 (Akira et al., 2006). The disparate mechanisms of action of these two stimuli could underpin the relative effectiveness of β -agonists against TNF α release induced by the two stimuli. These data could also suggest that β -agonists might provide greater anti-inflammatory potential when macrophages are activated by bacteria rather than viruses.

It was of interest that although indacaterol and salmeterol had some effect as inhibitors of LPS-induced TNF α and IL-6 generation, neither agonist was effective against IL-8. The reasons for this outcome are difficult to reconcile since there is a strong likelihood that LPS-induced cytokine release is mediated through a common pathway involving activation of nuclear factor- κ B (Blackwell and Christman, 1997). One possible explanation might relate to the prodigious quantities of IL-8 generated by macrophages following LPS activation which seem far greater than the quantities of either TNF α or IL-6 released. These quantities of IL-8 might be difficult for a β -adrenoceptor agonist to counter. The reason why macrophages release a great deal of IL-8 may be connected to the kinetics of cytokine generation. Whereas the generation of TNF α and IL-6 appear to plateau by 16 h after LPS challenge, IL-8 generation appears to be continuing even after 22 h (see Supplemental Information, Fig. S1). These findings suggest that there may be differences in the mechanism by which individual cytokines are generated by macrophages following LPS challenge.

As well as considering effects on pro-inflammatory cytokine generation, we also explored the possibility that β -adrenoceptor agonists might influence the generation of the anti-inflammatory cytokine, IL-10. A previous study in murine macrophages demonstrated that β_2 -adrenoceptor activation led to increased generation of IL-10 and a switch in phenotype of macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) (Grailer et al., 2014). β -adrenoceptor agonists had no such effect on IL-10 generation from human lung macrophages. Indeed, macrophages activated with LPS were comparatively weak releasers of IL-10 compared to the pro-inflammatory cytokines TNF α , IL-6 and IL-8. These findings highlight the fact that human lung macrophages display a prominently pro-inflammatory phenotype which is in keeping with the finding of others (Tomlinson et al., 2012).

We performed some experiments to determine whether the inhibitory effects of indacaterol and salmeterol were receptor-mediated. The β_2 -adrenoceptor antagonist, ICI-118,551, antagonized the inhibitory effects of indacaterol but not those of salmeterol. These findings suggest that indacaterol acts at β_2 -adrenoceptors to stabilise macrophages but that salmeterol does not.

Functional desensitization experiments were performed in order to evaluate further the mechanisms by which indacaterol and salmeterol act in macrophages. A long-term desensitizing treatment of macrophages with indacaterol led to a loss in the subsequent ability of indacaterol to inhibit TNF α generation from macrophages. These findings support the idea that the effects of indacaterol are receptor-mediated. This same desensitizing treatment with indacaterol had no effect on the subsequent inhibitory effects of salmeterol suggesting that indacaterol and salmeterol act at different targets. A long-term desensitizing treatment of macrophages with salmeterol did not desensitize the subsequent response to salmeterol. These findings suggest that the inhibitory effects of salmeterol are probably not receptor-mediated.

In summary, the present study demonstrates that while β -adrenoceptor agonists may inhibit cytokine generation from human lung macrophages these anti-inflammatory effects are modest.

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Conflict of interest statement

None

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Figure legends

Fig. 1 Effects of short-acting and long-acting β -agonists on cytokine generation.

Macrophages were incubated (30 min) with or without isoprenaline (iso), terbutaline (ter), salbutamol (salb), formoterol (form), salmeterol (salm), indacaterol (inda) all at 10^{-5} M or dexamethasone (dex; 10^{-7} M) before challenge with LPS (10 ng/ml). After 22 h, cell supernatants were harvested and the amount of (A) TNF α , (B) IL-6, (C) IL-8 and (D) IL-10 generated was assessed. Values are means \pm S.E.M., n=6 (A) or n=5 (B, C, D). Statistically significant ($P < 0.05$ at least) levels of inhibition, compared to the unblocked control, are indicated by an asterisk.

Fig. 2 Effects of β -agonists on TNF α generation induced by poly(I:C). Macrophages were incubated (30 min) with or without salbutamol (salb), salmeterol (salm), indacaterol (inda) all at 10^{-5} M or dexamethasone (dex; 10^{-7} M) before challenge with either (A) LPS (10 ng/ml) or poly(I:C) (10 μ g/ml). After 22 h, cell supernatants were harvested and the amount of TNF α generated was assessed. Values are means \pm S.E.M., n=4. Statistically significant ($P < 0.05$ at least) levels of inhibition, compared to the unblocked control, are indicated by an asterisk.

Fig. 3 Effects of stimulus strength on the inhibitory effects of salbutamol.

Macrophages were incubated (30 min) with or without salbutamol (10^{-6} M) before challenge with a range of LPS concentrations (0.1, 1 or 10 ng/ml) for 22 h. Cell supernatants were recovered and TNF α content assessed. Values are means \pm S.E.M., n=3. Statistically significant ($P < 0.05$) levels of inhibition by salbutamol, compared to the corresponding unblocked control, are indicated by an asterisk.

Fig. 4 Effect of indomethacin on the inhibitory effects of salbutamol. Macrophages were incubated (30 min) with or without indomethacin (10^{-6} M) and then incubated together with or without salbutamol before challenge with LPS (1 ng/ml). After 22 h, cell supernatants were harvested and the amount of TNF α generated was assessed. Values are expressed as the % inhibition of the unblocked releases of TNF α which were 395 ± 123 ng/ml (- indomethacin) and 454 ± 233 ng/ml (+ indomethacin). Values are means \pm **S.E.M.**, n=4 (-indomethacin) and n=6 (+ indomethacin).

Fig. 5 Comparison of the effects of β -agonists on monocyte-derived macrophages (MDM) and human lung macrophages (HLM). Cells were incubated (30 min) with or without isoprenaline (iso), terbutaline (ter), salbutamol (salb), formoterol (form), salmeterol (salm), indacaterol (inda) all at 10^{-5} M or dexamethasone (dex; 10^{-7} M) before challenge with LPS (10 ng/ml). After 22 h, cell supernatants were harvested and the amount of TNF α generated was assessed. Values are expressed as the % inhibition of the control unblocked TNF α generation which was 1145 ± 383 and 1108 ± 271 pg/ml for MDM and HLM, respectively. Values are means \pm **S.E.M.**, n=4 (MDM) or n=6 (HLM). The effectiveness of a given β -agonist to inhibit TNF α generation between cell types was compared statistically. Significant ($P < 0.05$ at least) differences are indicated by asterisks.

Fig. 6 Effects of ICI-118,551 on the inhibitory effects of formoterol and salmeterol. Macrophages were incubated (1 h) with (+) or without (-) ICI-118,551 (10^{-6} M) and then with or without (A) indacaterol (10^{-5} M) or (B) salmeterol (10^{-5} M) for a further 30 min. The cells were then challenged with LPS (10 ng/ml) for 22 h after which the amount of TNF α generated in the supernatants was assessed. Values are means \pm

S.E.M., n=4. Statistically significant differences compared to control (grey bar) are indicated by asterisks ($P < 0.05$ at least).

Fig. 7 Desensitization of the inhibitory effects of indacaterol but not those of salmeterol. Macrophages were incubated (24 h) with (+) or without (-) a desensitizing treatment (1st incubation) of beta agonist (10^{-5} M). After this incubation, the cells were washed three times. The cells were then incubated (30 min) with (+) or without (-) a beta agonist (10^{-5} M) for a second time (2nd incubation) to determine inhibitory effects after the desensitizing treatment. The cells were then challenged with LPS (10 ng/ml) for 22 h after which the amount of TNF α generated in the supernatants was assessed. The figure shows (A) the effects of indacaterol pre-treatment on the subsequent inhibition by indacaterol and (B) the effects of salmeterol pre-treatment on the subsequent inhibition by salmeterol. Values are means \pm **S.E.M.**, n=5 (A) or n=4 (B). Statistically significant comparisons are indicated by asterisks ($P < 0.05$ at least) and non-significant comparisons by 'ns'.

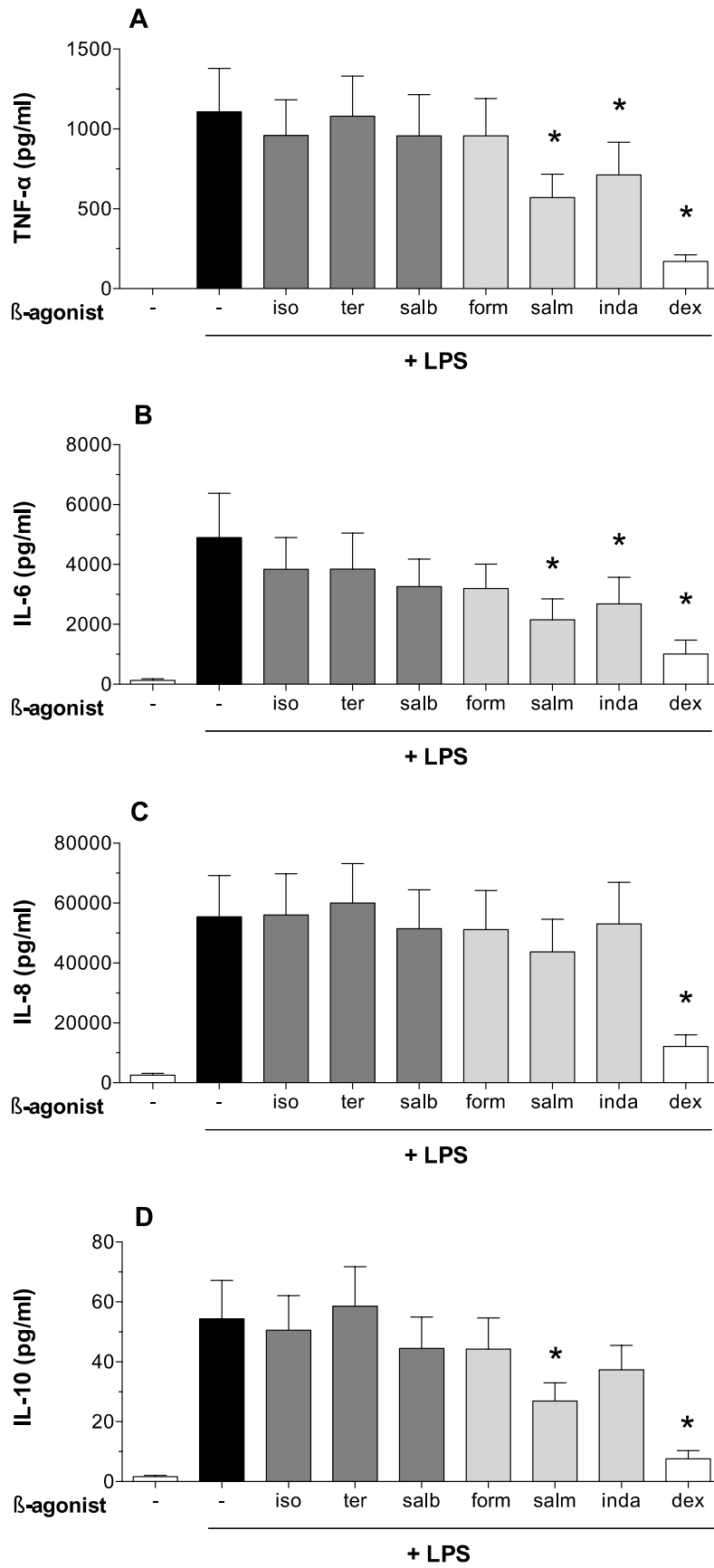


Fig. 1

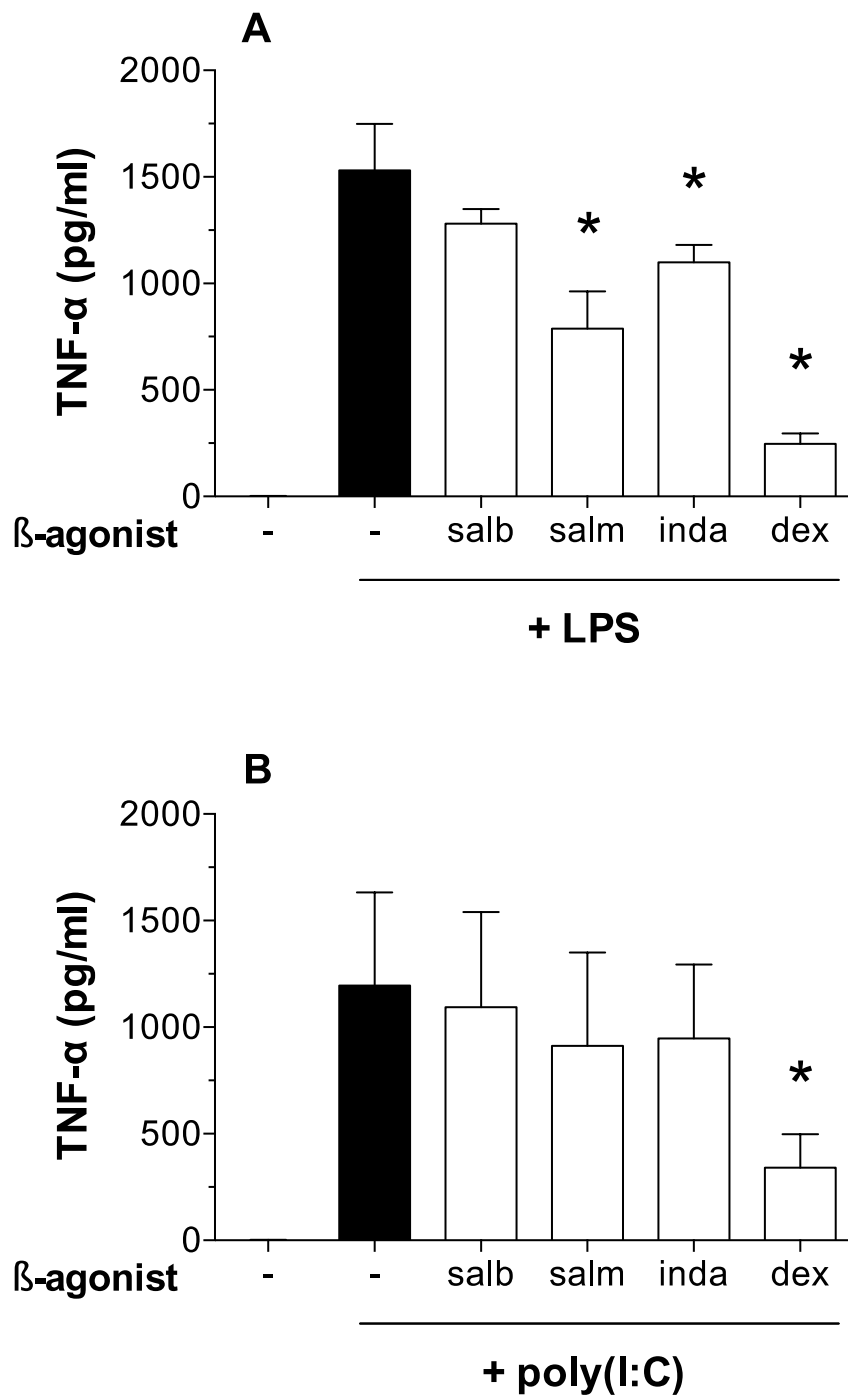


Fig. 2

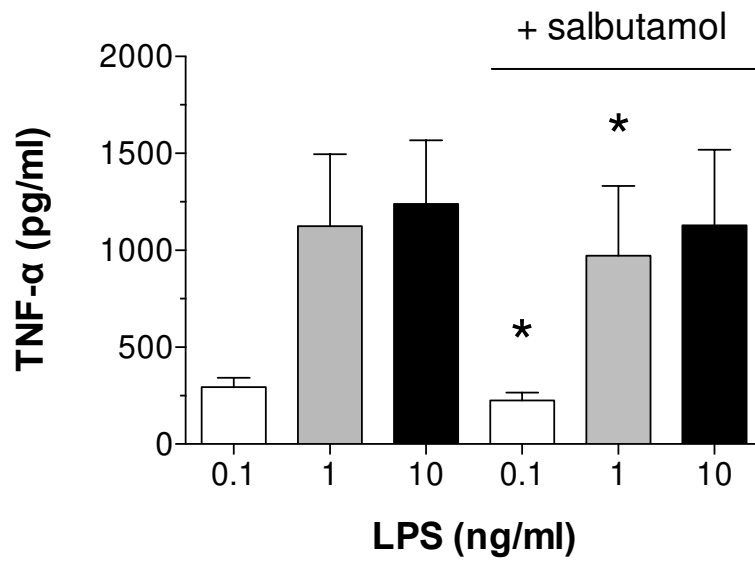


Fig. 3

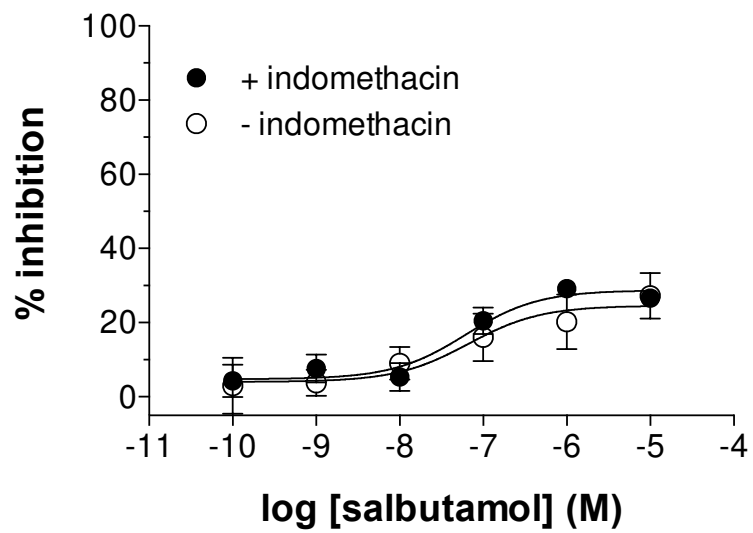


Fig. 4

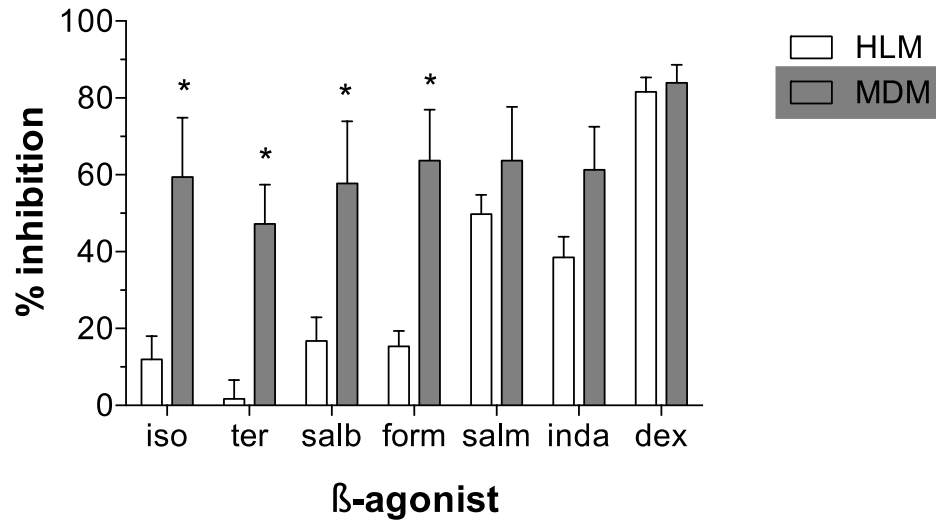


Fig. 5

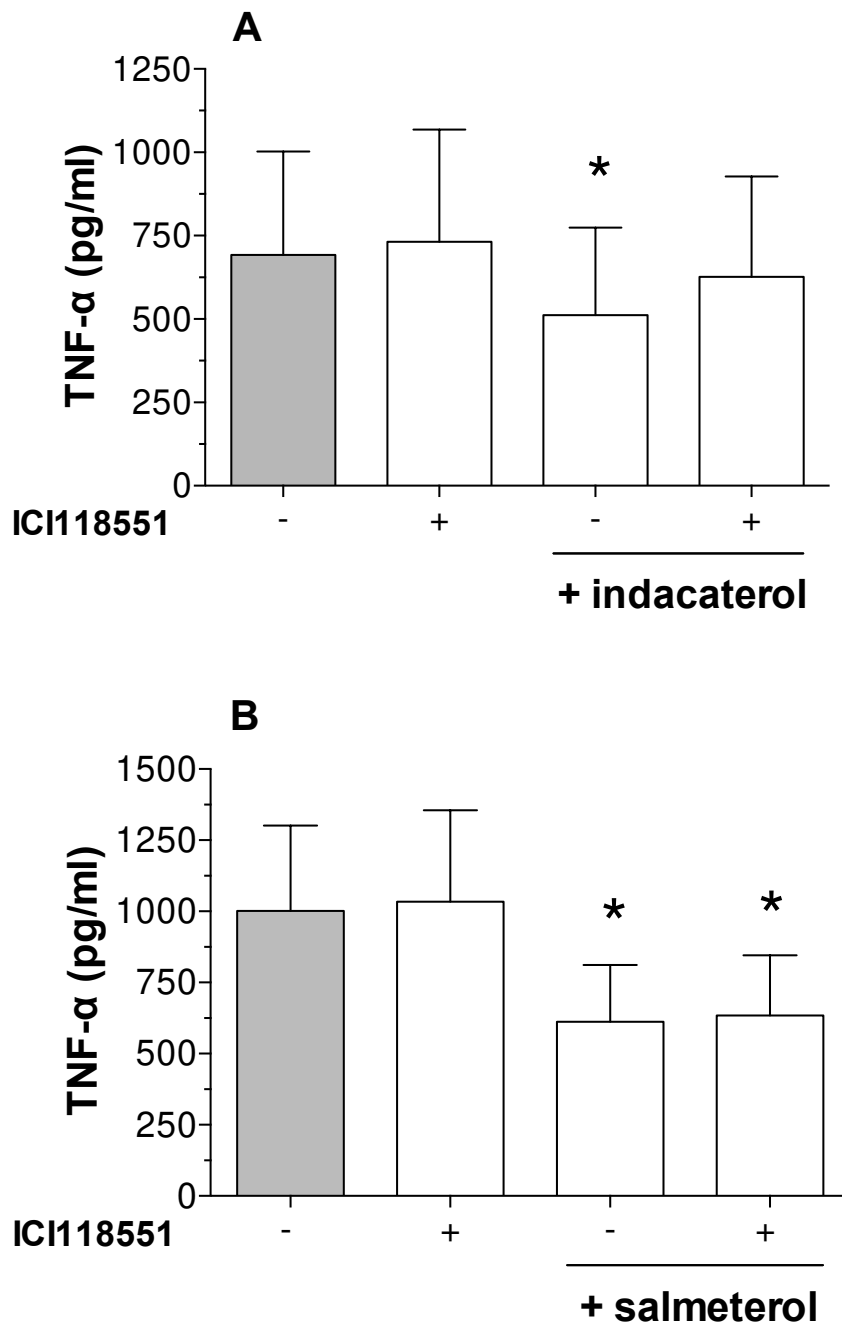


Fig. 6

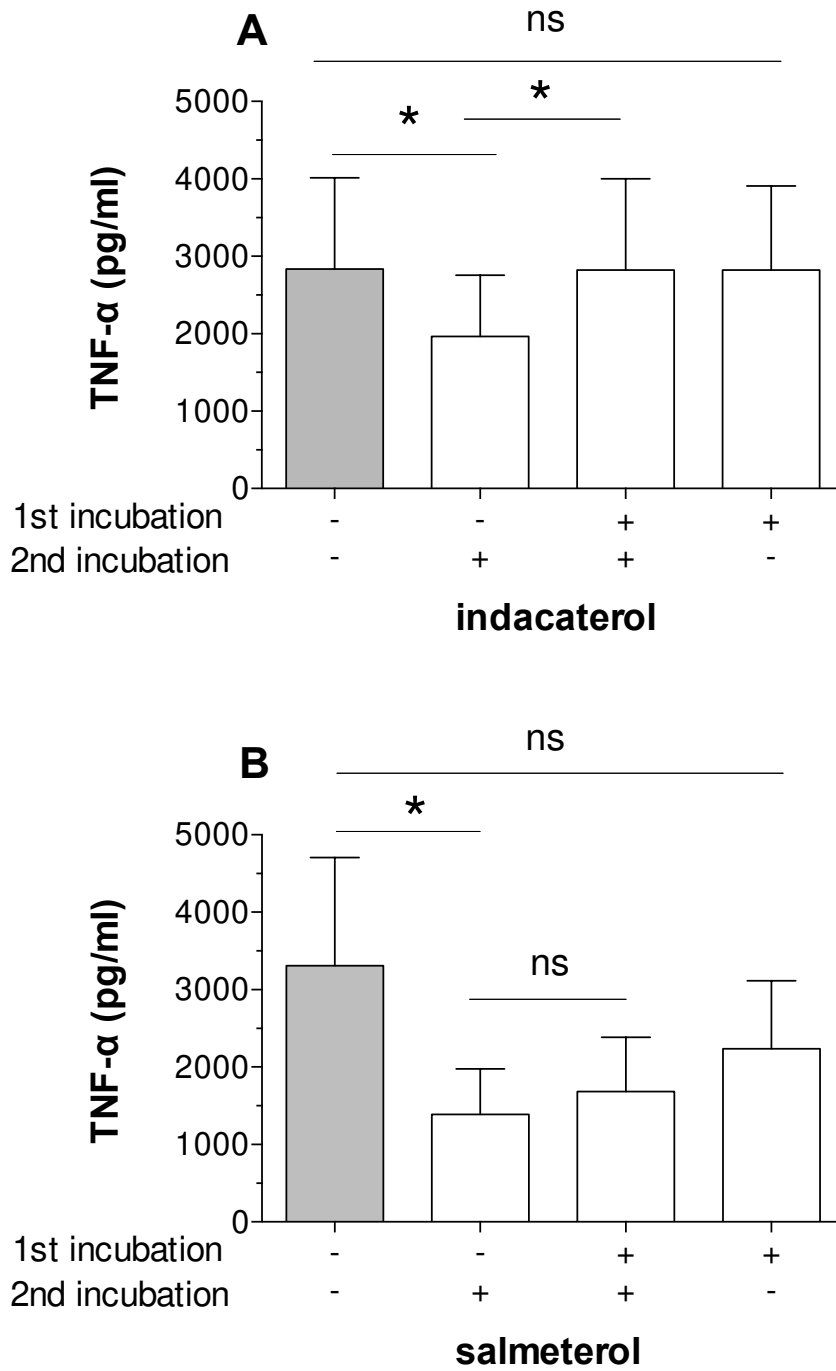


Fig. 7