Systems Biology based studies on anti-inflammatory compounds

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Chapter 5

Inhibitory effects of the β_2-adrenergic receptor agonist Zilpaterol on the LPS-induced production of TNF-α *in vitro* and *in vivo*.

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Inhibitory effects of the β_2-adrenergic receptor agonist Zilpaterol on the LPS-induced production of TNF-α *in vitro* and *in vivo*.

In this study the immuno-modulating properties of zilpaterol, a β_2-adrenergic receptor (β_2-AR) agonist specifically developed as a growth promoter in cattle were investigated. Although zilpaterol has a different structure compared to the β_2-adrenergic receptor agonists known to date, it was noted that it was able to bind to both the β_2-adrenergic receptor ($Ki = 1.1x10^{-6}$ M) and the β_1-adrenergic receptor ($Ki = 1.0x10^{-5}$ M). Using lipopolysaccharide (LPS) exposed U937 macrophages, the production of cyclic adenosine-3',5'-cyclic monophosphate (cAMP) and tumor necrosis factor alpha (TNF-α) were investigated. Zilpaterol inhibited TNF-α release and induced intracellular cAMP level in a dose-dependent manner. The inhibition of TNF-α release and the induction of cAMP production was mainly mediated via the β_2-AR, as indicated by addition of β_1- and β_2-selective antagonists. The effects of zilpaterol were investigated in LPS-treated male Wistar rats. Zilpaterol dosed at 500 µg/kg bodyweight reduced TNF-α plasma levels. In conclusion, zilpaterol is a β_2-adrenergic agonists and an inhibitor of TNF-α production induced by LPS both in vivo and in vitro.

Introduction

Beta-adrenergic agonists can be used as so-called repartitioning agents. In muscle tissue, β-agonists promote protein synthesis and cell hypertrophy by inhibition of proteolysis, whereas in adipose tissue they promote lipolysis. On the basis of these properties, β-agonists can be used in cattle industry to increase feeding efficiency, increase carcass leanness, and promote animal growth [1, 2]. In most countries this is illegal. In addition, β-agonists have been shown to be abused to enhance athletic performance [3, 4]. In clinical practice, β_2-adrenergic agonists are used as bronchodilators in both humans and horses [5-9]. The predominant effect of β_2-agonists on the airways is relaxation of airway smooth muscle. Although some authors have suggested that β_2-agonists possess anti-inflammatory characteristics, these anti-inflammatory effects have mainly been observed *in vitro* [10, 11]. However previous studies demonstrated that clenbuterol suppressed the release of TNF-α by LPS activated U937 macrophages and in rats treated with LPS. Evidence for the involvement of the β_2-AR in this effect was also presented [12, 13]. Zilpaterol was originally designed as a growth promoter in cattle [14]. The use of zilpaterol has been approved in South Africa and Mexico, but is forbidden in the EU, USA and Asia [15, 16]. Little is known about the mechanism of action of zilpaterol. Although it has been claimed

by the manufacturer that zilpaterol acts via the β_2-adrenergic receptor (β_2-AR) [14], there are as far as we know no data in the open literature that support this basic property.

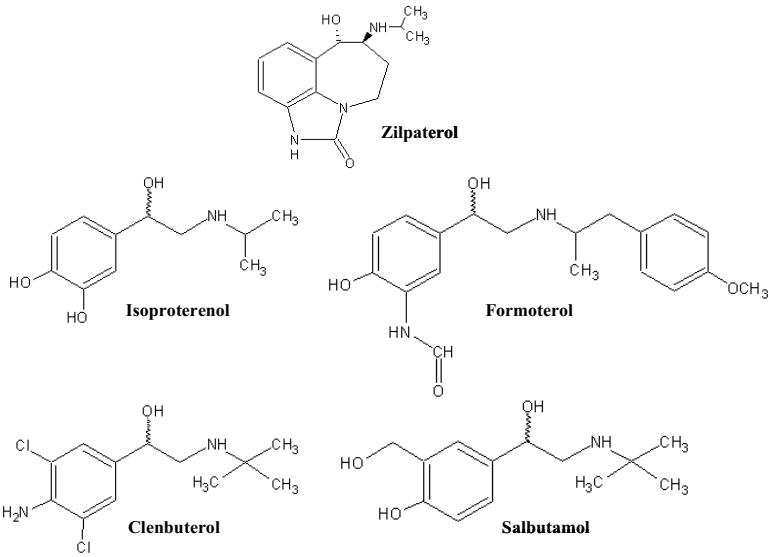

Figure 1 Comparison of the chemical structure of zilpaterol with several other β_2-AR agonists.

In this study it was investigated whether zilpaterol, which shows little structural resemblance to other β_2-agonists (e.g. clenbuterol, formoterol, salbutamol, and isoproterenol) (Fig. 1), has affinity for the β_1-AR and/or β_2-AR. Beta-ARs belong to the family of GTP-binding protein-coupled receptors (GPCR). Beta$_1$-AR and β_2-AR augment the activity of adenylate cyclase via Gs regulatory proteins. Binding to the β_1-AR or β_2-AR results in the production of the second messenger cyclic adenosine-3',5'-cyclic monophosphate (cAMP) [17-22].

This study also investigates the potential immuno-modulating effects of zilpaterol. Although results from previous studies from our laboratory [12, 13] and others [10, 11] suggest that this might be a general property of all β_2-agonists, it would be interesting to establish if such an effect is also induced by this compound of differing structure. It has been suggested that cAMP is involved in the inhibition of tumor necrosis factor-alpha (TNF-α) synthesis and release [23, 24]. Therefore, the study investigated the anti-inflammatory properties of zilpaterol by examining its effects on the production of cAMP and TNF-α. For this purpose the human monocyte-like histiocytic lymphoma cell line U937 was used as a model system. Furthermore, the roles of the β1- and β2-adrenergic receptors on the effects of zilpaterol on cAMP and TNF-α were examined. Finally, an endotoxemic rat model system was used to investigate the inhibitory effect of zilpaterol on the LPS-induced TNF-α plasma levels *in vivo*.

Material en Methods

Chemicals

Lipopolysaccharide (LPS, *E.coli* 0111:B4), propranolol, ICI 118551 and atenolol were obtained from Sigma Aldrich (St. Louis, MO, USA) and zilpaterol from Intervet Inc. (Millsboro, US).

Receptor radioligand binding

Cell membrane homogenates from β_2-adrenergic receptor-transfected Sf9 cells or β_1-adrenergic receptor transfected HEK-293 cells (15-20 µg protein) were incubated for 60 min at 22 ºC with 0.15 nM CGP 12177 in the absence or presence of varying concentrations of zilpaterol ($1x10^{-9}$ - $1x10^{-4}$ M) and clenbuterol ($1x10^{-10}$ - $1x10^{-5}$ M) in a buffer containing 50 mM Tris-HCl (pH 7.4), 10mM $MgCl_2$, and 2 mM EDTA. Non-specific binding was determined in the presence of 50 µM alprenolol. Following incubation, the samples were filtered rapidly under vacuum through GF/B glass fibre filters (Packard, Rungis, France) using a Unifilter 96-sample cell harvester (Packard). The filters were dried and subsequently counted for radioactivity in a Topcount scintillation counter (Packard) using Microscint scintillation cocktail (Packard). ICI 118551 was used as standard reference compound for the β_2 adrenergic receptor and atenolol for the β_1-adrenergic receptor.

The specific binding of ligands to the receptors was defined as the difference between the total binding and the non-specific binding determined in the presence of an excess of unlabelled ligand. The results are expressed as a percentage of control specific binding obtained in the presence of zilpaterol inhibition of the control radioligand specific binding. Binding studies were performed in triplicate. Using Graphpad Prism Software (San Diego, USA), the inhibition constants (Ki) were calculated from the Cheng Prushoff equation ($Ki = IC_{50}/(1 + (L/K_D))$), in which L = the concentration radioligand in the assay, and K_D = the affinity of the radioligand for the receptor.

Cell cultures

Human monocyte-like histiocytic lymphoma cells U937 [25] obtained from the ATCC (CRL-1593.2) were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (Life technologies, Breda, The Netherlands) at 37 °C and 5% CO_2 in a humidified atmosphere. U937 monocytic cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA, 10 ng/ml, overnight, Omnilabo, Breda, The Netherlands) as described previously [26].

The PMA-differentiated macrophages were allowed to recover from the PMA treatment for 48 h, during which the culture medium was replaced daily.

Macrophage activation and TNF-α assay

U937 macrophages were cultured at a concentration of $1x10^6$ cells per well. Cells were incubated for 4 h with LPS (*E.Coli* 0111:B4, 1μg/ml), LPS in the presence of a dilution series of zilpaterol ($1x10^{-8}$ - $1x10^{-4}$ M), LPS in the presence of a dilution series of clenbuterol ($1x10^{-10}$ - $1x10^{-6}$ M) or LPS plus zilpaterol ($1x10^{-6}$ M) in combination with a dilution series of β-AR antagonists ($1x10^{-8}$ – $1x10^{-5}$ M), respectively. The following β-AR antagonists were used; ICI 118551 (β_2-AR), atenolol (β_1-AR), and propranolol (non-selective antagonist for β_1-AR and β_2-AR). The incubations were performed in 6-fold.

The concentration of TNF-α in the culture supernatants was determined by ELISA using the cytoset antibody pair kit for TNF-α from Biosource (Etten-Leur, The Netherlands) according to the manufacturer's protocol. After removal of culture medium the cells were lysed in 0.1 M NaOH and used for protein determination by the modified method of Bradford (Bio-Rad, Veenendaal, The Netherlands). The toxicity of the zilpaterol concentration used was determined by measuring the enzyme activity of lactate dehydrogenase (LDH). LDH activity was quantitatively determined using the *in vitro* assay for LDH activity from Roche Diagnostics (Mannheim, Germany) for automated clinical chemistry analyzer Hitachi 911 (Hitachi, Japan).

Measurements of intracellular cAMP formation

U937 macrophages were cultured in 24 well cell culture plates at a concentration of $1x10^6$ cells per well. Cells were incubated for 10 minutes at 37 ºC with 1 μg/ml LPS in the absence or presence of a dilution series of zilpaterol ($1x10^{-8}$ - $1x10^{-4}$ M). In addition, cells that were used for antagonism studies were incubated with 1 μg/ml LPS and $1x10^{-5}$ M zilpaterol in combination with a dilution series of β-AR antagonists ($1x10^{-8}$ - $1x10^{-4}$ M). The following β-AR antagonists were used; ICI 118551, atenolol, and propranolol. The incubations were performed in 6-fold. The culture medium was aspirated and the cells were placed directly on ice. The cells were lysed in 0.25 ml 0.1 M HCl and after centrifugation the samples were assayed directly using the cyclic AMP EIA kit from Cayman Chemical (Ann Arbor, USA).

In Vivo effect of zilpaterol on TNF-α release

Twenty four male Wistar rats (200-250 g) were purchased from Charles River Inc. (Sulzfeld, Germany). The animals were randomized into four groups of 6 animals and received food and water *ad libitum*. Room temperature was kept constant and light was maintained at a 12 h cycle. The rats were fasted the night before the experiment (only water available, *ad libitum*). Saline (Eurovet animal health BV, Bladel, The Netherlands) was used for administration either as control or as vehicle for the following doses: LPS-solution (2 mg/kg bodyweight) and zilpaterol (500 µg/kg bodyweight). The doses and observation period were based on previously performed *in vivo* experiments [12, 27] and for zilpaterol a dose was estimated based on the data obtained in the *in vitro* data presented. The control group and LPS group were administered saline orally, one hour before an intra-peritoneal injection of saline or LPS respectively. The LZ (LPS + zilpaterol) group and Zil (zilpaterol) group received both an oral dose of zilpaterol followed after 1 hour by an intra-peritoneal injection of LPS or saline respectively. Blood samples from all rats were drawn at 2 h after LPS challenge. Blood was collected in EDTA containing tubes and centrifuged for 15 min (3000g, 4 ºC). Plasma was stored at -80 ºC until analysis. The concentration of TNF-α in rat plasma was determined by ELISA using the Rat TNF-α Ultrasensitive kit from Biosource (Etten-Leur, The Netherlands) according to the manufacturer's protocol. The study protocol was approved by the Ethical Committee for experiments on Animals of our Institute.

Statistical evaluation

All values presented are means ± SD of 6 individual incubations unless stated otherwise. For the comparison of receptor-binding data and the dose response curves (logEC-50) the F-test was performed. The levels of TNF-α and cAMP were compared using the Student's *t*-test assuming normal distributions and equal variance. TNF-α results from the *in vivo* study were compared using one-way ANOVA followed by the Bonferroni's multiple comparison test. The effect on bodyweight of animals treated with zilpaterol and the control group were analysed using the Mann-Whitney test (nonparametric). The results were considered significantly different if $p < 0.05$. All analyses were performed using Graphpad prism 4.0 software package (Graph Pad Software Inc., San Diego, USA).

Results

Receptor-binding

Specific receptor-binding assays for the β_1-AR and β_2-AR were used to measure the affinity of zilpaterol to these receptors. The binding curves in Figure 2 show that zilpaterol did bind to the β_2-AR (Ki = 1.0 x10^{-6} M), although with a lower affinity compared to ICI 118551 (Ki = 1.3 x10^{-9} M) and clenbuterol (Ki = 4.2 x 10^{-8} M). The Ki of the three compounds were significantly different ($p < 0.05$, F-test). The binding of zilpaterol to the β_1-AR (Ki = 1.0 x 10^{-5} M) was found to be weaker than to the β_2-AR in this assay. The difference in Ki was significantly different ($p < 0.05$, F-test).

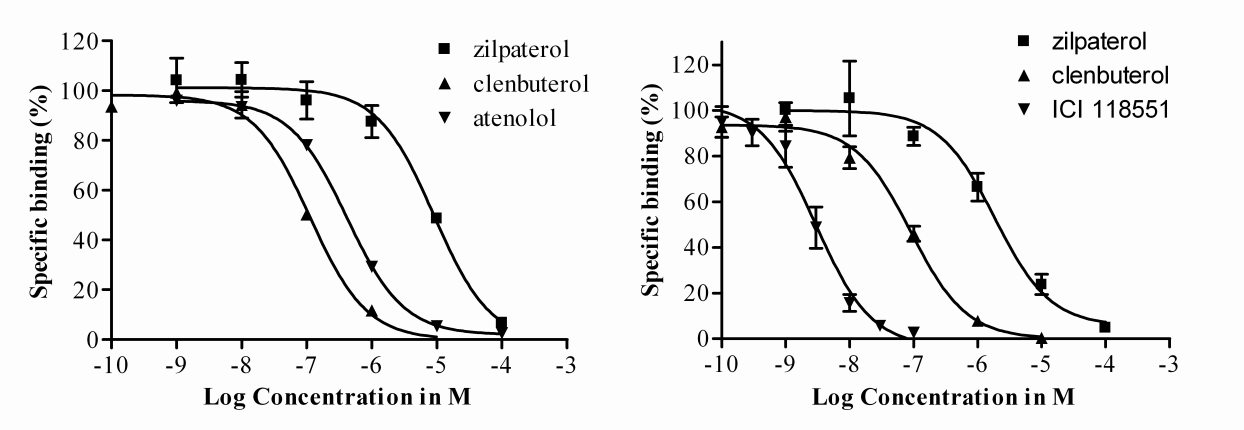

Figure 2 Receptor binding curves of zilpaterol to the β_1-AR (left panel) and to the β_2-AR (right panel). Cell membrane homogenates of HEK293 cells transfected with β_1-AR or Sf9 cells transfected with β_2-AR were incubated with [^{3}H] CGP 12177 (control radioligand) in the absence or presence of zilpaterol (■), clenbuterol (▲) or reference compound (▼). ICI 118551 was used as reference compound for binding to the β_2-AR and atenolol for β_1-AR binding. The results are expressed as percentage inhibition of control radioligand-specific binding (mean ± SD of a triplicate measurement).

Macrophage activation and TNF-α assay

Although binding to the β_1- and β_2-AR was found to be relatively weak, the role of the two receptors in the biological response of the cell to zilpaterol was investigated. Because other β_2-adrenergic agonists are known to inhibit pro-inflammatory responses of cells in *in vitro* experiments, we investigated if zilpaterol was able to inhibit the TNF-α production of U937 macrophages exposed to LPS. Figure 3 indicates that zilpaterol inhibited the TNF-α release of U937 cells exposed to LPS in a dose-dependent manner. The inhibition was not due to a toxic effect induced by zilpaterol, as indicated by LDH activity in the culture medium of exposed cells. Untreated cells had an LDH activity of 50.6 ± 3.4 U/l, whereas U937 macrophages treated with LPS in combination with 1x10^{-4} M zilpaterol (highest dose) had an LDH activity of 55.0 ± 3.1 U/l. The LDH activities induced by the two treatments were not significantly different ($p > 0.05$). However the inhibition of the LPS-induced TNF-α production by

zilpaterol (EC50 = $5.8x10^{-9}$ M) was less pronounced than the inhibition by clenbuterol (EC50 = $7.1x10^{-10}$ M).

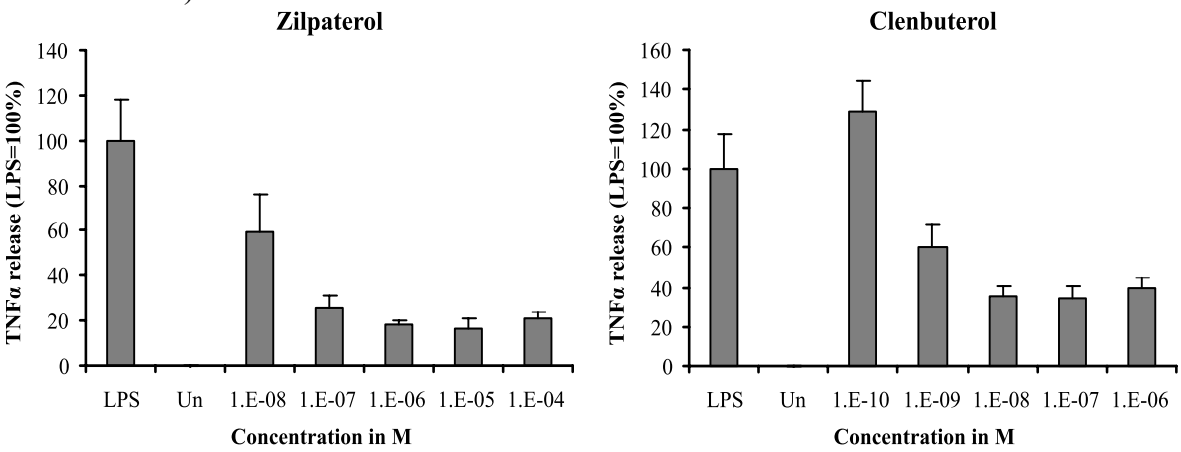

Figure 3 Inhibition of TNF-α release from untreated U937 macrophages (Un) and macrophages incubated for 4 h with 1 µg/ml LPS in the absence (LPS) or presence of a dilution series of zilpaterol ($1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, and $1x10^{-4}$ M, respectively (left panel) or a dilution series of clenbuterol ($1x10^{-10}$, $1x10^{-9}$, $1x10^{-8}$, $1x10^{-7}$, and $1x10^{-6}$ M, respectively (right panel). The release of TNF-α is corrected for protein content and calculated with respect to the TNF-α release from U937 cells exposed to LPS alone (17.3 ± 5.5 ng/ml). The results are presented as means ± SD (n=6).

This difference in inhibitory effect was statistically significant ($p < 0.05$, F-test). The involvement of β-ARs in the inhibitory effect of zilpaterol on the TNF-α production was investigated by using a selective β_2-AR antagonist, ICI 118551, a selective β_1-AR antagonist, atenolol, and a non-selective β-AR antagonist, propranolol (β_1 and β_2). From Figure 4 it can be concluded that the inhibition of the TNF-α release was reversed in a dose-dependent manner by both ICI 118551 and propranolol, whereas atenolol did not affect the inhibitory effect of zilpaterol on the TNF-α production completely. The inhibitory effect was slightly reversed at a concentration of $1x10^{-5}$ M.

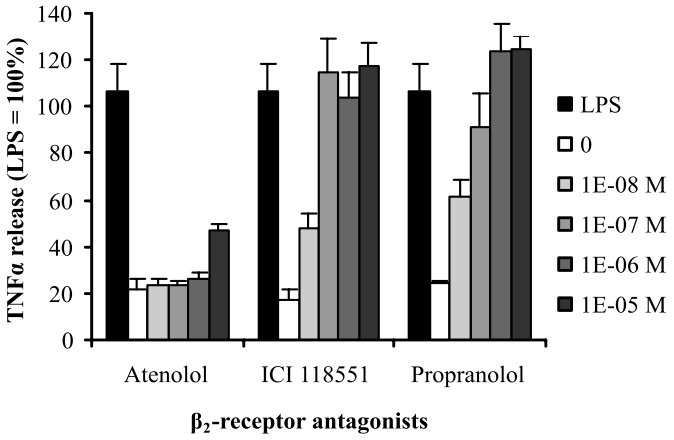

Figure 4 Effect of β_2-AR antagonists on the inhibition of TNF-α production by zilpaterol. U937 macrophages were exposed to 1 µg/ml LPS (black bars), LPS in combination with $1x10^{-6}$ M zilpaterol (white bars) or LPS in combination with zilpaterol in the presence of a dilution series of atenolol, ICI 188551 or propranolol (from left to right; $1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$, and $1x10^{-5}$ M respectively). The release of TNF-α is corrected for protein content and calculated with respect to the TNF-α release from U937 cells exposed to LPS alone (22.2 ± 6.8 ng/ml). The results are presented as means ± SD (n=6).

Measurements of intracellular cAMP formation

β-ARs belong to the GPCR family and are known to activate Gs proteins, which subsequently stimulate the production of cAMP. Therefore it was investigated if zilpaterol was able to induce intracellular cAMP levels. As shown in Figure 5 (left panel), zilpaterol induced the cAMP production in a dose-dependent manner. The addition of LPS alone had no effect on the cAMP production. Propranolol, ICI 118551, and atenolol inhibited the production of cAMP by zilpaterol in combination with LPS. However the effect of atenolol was less pronounced (Fig. 5). From these results can be concluded that the stimulation of cAMP production by zilpaterol was mainly mediated by the β_2-AR and weakly by the β_1-AR.

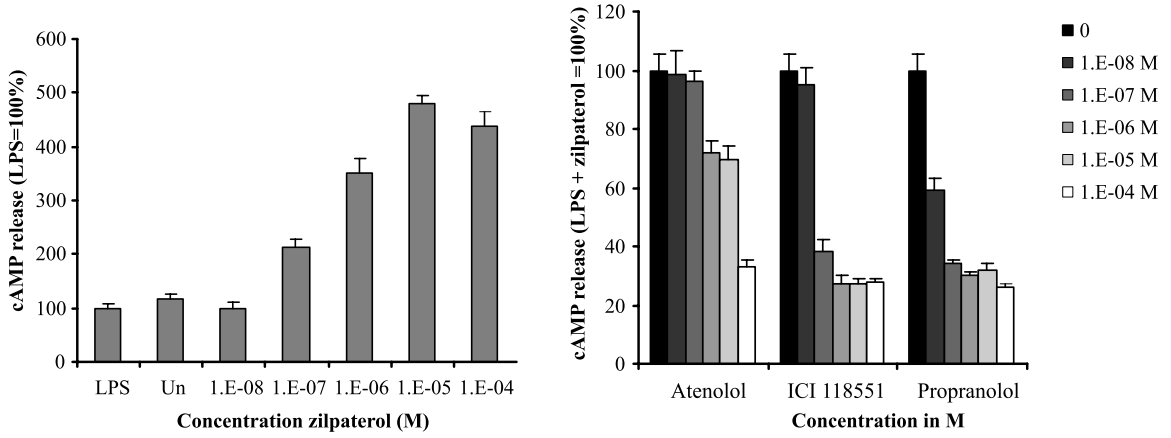

Figure 5 Left panel: induction of cAMP release by U937 macrophages untreated (Un) and treated with 1 µg/ml LPS in the absence (LPS) or presence of a dilution series of zilpaterol ($1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$ and $1x10^{-4}$ M, respectively. Right panel: the effect of β-AR antagonists, atenolol, ICI 118551 and propranolol on the inducing effect of zilpaterol on cAMP production. U937 macrophages were exposed to 1 µg/ml LPS in combination with $1x10^{-5}$ M zilpaterol (black bars) or LPS in combination with zilpaterol in the presence of a dilution series of the three β-AR antagonists mentioned above (from left to right; $1x10^{-8}$, $1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, and $1x10^{-4}$ M, respectively). The release of cAMP is calculated with respect to the cAMP release from U937 cells exposed to LPS alone (left panel) or LPS in combination with zilpaterol (right panel). The highest cAMP level measured was 85.4 ± 6.8 pmol/ml) for U937 macrophages incubated with LPS in combination with $1x10^{-4}$ M zilpaterol. The data are represented as means ± SD (n=6).

In vivo effect of zilpaterol on TNF-α release

The inhibitory effect of zilpaterol on the TNF-α production *in vitro* reflects the effects of other β_2-AR agonist, such as clenbuterol [12, 13]. In order to investigate whether zilpaterol could also inhibit the TNF-α production *in vivo*, the effects of zilpaterol on rats exposed to LPS was examined. Two hours after administration of LPS, rats showed high plasma levels of TNF-α (Fig. 6). TNF-α production was markedly inhibited by the treatment of rats with 500 µg/kg zilpaterol. The treatment of rats with saline or zilpaterol alone had no effect on the TNF-α production; however the administration of zilpaterol alone did have a clear effect on the

bodyweight of the animals. The increase in bodyweight (mean increase in bodyweight 12.7 ± 5.5 g) was significantly ($p = 0.026$, Mann-Whitney test, two tailed) enhanced with respect to the control group (mean increase in bodyweight: 4.9 ± 4.0 g). As zilpaterol was originally designed for this purpose, it is therefore of interest to note that this effect is already visible after one day of treatment of rats with only a single dose of 500 µg/kg zilpaterol.

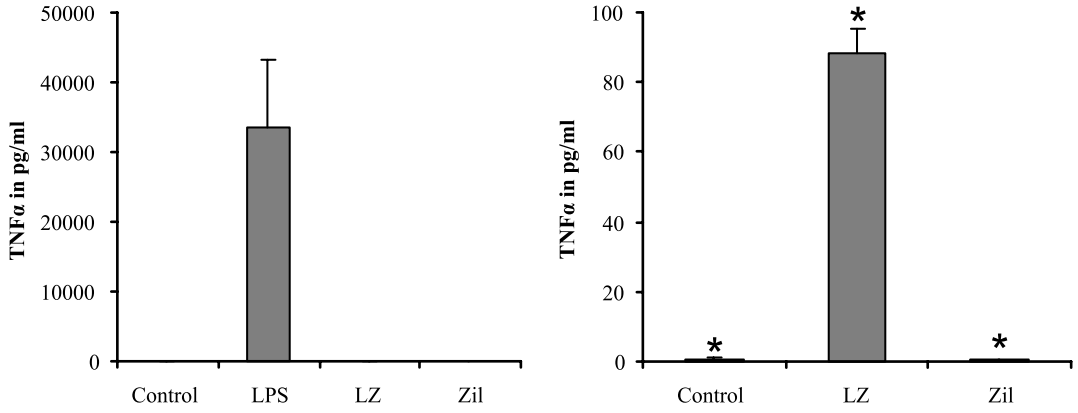

Figure 6 Effect of zilpaterol on TNF-α plasma levels, 2 hours after administration of LPS or saline. Rats were orally administered with saline one hour before intra-peritoneal injection of saline (Control) or before intra-peritoneal injection of 2 mg/kg bodyweight LPS (LPS), orally administered zilpaterol (500 µg/kg bodyweight) one hour before LPS-challenge (LZ) or orally administered zilpaterol (500 µg/kg bodyweight) one hour before intra-peritoneal injection of saline (Zil). The left panel shows the results of all treated groups and in the right panel the LPS group is omitted. The TNF-α plasma levels are presented as means of 6 animals ± SEM. * indicates significant difference with respect to LPS ($p < 0.05$) according to one way ANOVA followed by the Bonferroni's multiple Comparison Test.

Discussion

In this study, the affinity of zilpaterol for the β_1-AR and β_2-AR was investigated using specific receptor-binding assays. Like clenbuterol, zilpaterol binds to the β_1-AR as well as the β_2-AR, although clenbuterol binds to both receptors more potently.

Furthermore the U937 macrophage was used to investigate the immuno-modulating properties of zilpaterol and its mechanisms of action. The U937 cell line is a well established in vitro model system for human macrophages that express both the β_1-AR as well as the β_2-AR [13]. Zilpaterol inhibits TNF-α release from U937 macrophages exposed to LPS in a dose-dependent manner and this inhibitory effect is mainly mediated by the β_2-AR. To achieve the same inhibitory effect as clenbuterol on the TNF-α release, at least a ten times higher dose for zilpaterol is needed.

Activation of the β-ARs results in a Gs protein-mediated activation of adenylate cyclase, which subsequently catalyses the conversion of ATP in to cAMP [17-22]. Enhanced levels of

cAMP have been found to result in the inhibition of TNF-α production stimulated either *in vivo* or *in vitro* with LPS [23, 24]. This is in line with our findings that zilpaterol inhibits the TNF-α production and augments the cAMP production in a dose-dependent manner. The induction of cAMP can be mediated by both the β_1-AR and the β_2-AR, but our results demonstrate that the induction of cAMP by zilpaterol in U937 macrophages is mediated by the β_2-AR and to a lesser extend by the β_1-AR. This study demonstrates that the inhibitory effect on the TNF-α production is mainly mediated via the β_2-receptor. The effect of zilpaterol on TNF-α production and cAMP reflects the effects of other β_2-AR agonists *in vitro*. Salmeterol inhibits the release of TNF-α from LPS-stimulated THP-1 cells and inhibits the LPS-induced increase of murine serum TNF-α levels *in vivo* [28]. Moreover, isoproterenol which inhibits the TNF-α production in THP-1 cells, also increases intracellular cAMP levels [29]. Similar results have been observed for clenbuterol both *in vivo* and *in vitro* [12, 13]. Our results show that zilpaterol is a potent inhibitor of TNF-α plasma levels of rats exposed to LPS. Clinical observations were in line with the TNF-α data, as the animals receiving zilpaterol did not show the typical effects of LPS e.g. low body temperature, in-appetite and lower activity.

In conclusion, this study demonstrates that zilpaterol is a β_2-adrenergic receptor agonist and an inhibitor of TNF-α release, both *in vitro* and *in vivo*.

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