properties of ß-adrenoceptor agonists
-Concepts for therapy of acute inflammatory disorders-

C.A. Izeboud

Immunomodulatory properties of β-adrenoceptor agonists
- Concepts for therapy of acute inflammatory disorders -

Immuunmodulatie door β-adrenoceptor agonisten
- Concepten voor de behandeling van acute ontstekingsreacties -
(met een samenvatting in het Nederlands)

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The clinical definition of inflammation was written down for the first time by the Roman Cornelius Celsus, during the first century A.D. The four major signs of inflammation according to him, are: *"Rubor et tumor cum calore et dolore"* (redness and swelling, with heat and pain). The fifth cardinal sign was added by the German cellular pathologist Rudolph Virchow (1821-1902) in his book Cellular Pathology (1858) and was named *"functio leasa"* (disturbed function), meaning that inflammation induces organ dysfunction (1). To date, these five cardinal signs are regarded as the basic symptoms of an inflammatory response.
Diseases that are characterised by inflammation are of major importance in modern society. Especially in those cases where a systemic inflammatory response develops, such as in sepsis and systemic inflammatory response syndrome (SIRS), a high mortality rate is observed. These severe disorders are an increasing problem for intensive care units. Therefore, the processes involved in inflammation are subject of many studies. Several therapeutic strategies have been designed to intervene in the inflammatory cascade. Especially when the immune system itself is the cause of the disease, there is an obvious need for (drug-)therapy that controls the (excessive) immune reaction.
During the past two decades, evidence has accumulated that the sympathetic nervous system plays an important role in the regulation of the immune system. The principal neurotransmitter norepinephrine and the adrenal hormone epinephrine (noradrenaline and adrenaline) were both shown to modulate immune reactions (e.g. (2-4)). It is a challenge, both from a fundamental and an applied (clinical) scientific viewpoint, to elucidate mechanisms involved in the modulation of the immune reaction via the adrenoceptor system, and to derive potential therapeutical applications from this knowledge.

THE INFLAMMATORY RESPONSE

Inflammation is the body's reaction to an injury, such as an invasion by an infectious agent (bacteria, protozoa, viruses, fungi), physical injury or trauma. This response can be subdivided into three major events:

I. An increased blood supply to the inflamed area.
II. Increased capillary permeability caused by retraction of the endothelial cells. This permits larger molecules to traverse the endothelium and

thus allows the soluble mediators of immunity to reach the site of infection.

III. Leukocytes (particularly polymorphonuclear granulocytes) migrate out of the capillaries into the surrounding tissue, towards the site of inflammation by a process known as chemotaxis (5).

In summary, the sequence of events during an inflammatory response are characterised by vasodilatation and capillary permeability, cellular activation, release of cell-derived mediators, chemotaxis (cellular migration), and phagocytosis. The major distinction between local- and systemic- inflammatory reactions is the fact that, although the original infection or trauma might have been initiated locally, the response to the injury spreads throughout the whole organism which leads to a systemic (over)reaction. Several features of inflammation are important both in localised inflammatory reactions and during a systemic inflammatory response. These characteristics include cellular activation (especially activation of macrophages) and release of mediators (like eicosanoids, cytokines, reactive oxygen and -nitrogen intermediates) and will be discussed in more detail below. Ultimately, the outcome of the immune reactions is determined by the effectiveness of the response (as depicted in figure 1).

In general, inflammation can be separated into acute and chronic processes. In practice, the clinical course of inflammation is termed acute if it lasts for hours or days, and called chronic if it lasts for months or years. Examples of acute (systemic) inflammatory disorders are systemic immune response syndrome (SIRS) and sepsis. Chronic inflammatory diseases include asthma and rheumatoid arthritis.

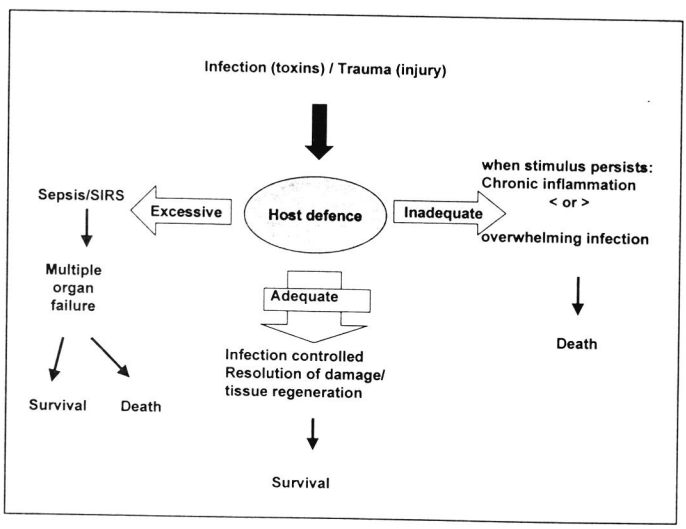

Figure 1. The balance for an appropriate host response to infection or trauma. Both an excessive and an insufficient response may lead to disability/ organ failure or death.

Typical pathogenic sequence of events during systemic inflammation

A frequent and serious problem confronting clinicians is the management of a serious infection including the systemic response to infection; syndromes that are termed sepsis and systemic inflammatory response syndrome (SIRS) (6). A systemic inflammatory response can be triggered not only by infections but also by non-infectious disorders, such as injury and trauma. Manifestations of sepsis and SIRS include those related to the systemic response to infection (tachycardia, tachypnea or hyperventilation, alterations in temperature, and marked increases or decreases in the number of circulating white blood cells) and those related to organ-system dysfunction (gastrointestinal, cardiovascular, respiratory, renal, hepatic, and haematology abnormalities) (6,7). Basically, the systemic inflammatory response starts with the nidus of infection (abscess, peritonitis, pneumonia, pyelonephritis) or trauma. From there, micro-organisms or inflammatory mediators may invade the bloodstream directly, or micro-organism may proliferate locally and release various substances (like toxins) into the bloodstream. This in turn leads to activation of monocytes and macrophages, neutrophils and other immune cells, which start to produce endogenous mediators of inflammation (see "inflammatory mediators") that are released into the plasma (8,9). These inflammatory mediators (like cytokines and NO) spread via the plasma through the whole organism and trigger other cells, tissues, and organs, which also produce inflammatory mediators (like lipid mediators, PAF, kinins, reactive oxygen intermediates, and proteolytic enzymes). In this way, the inflammatory cascade grows and branches like a tree. The production of the inflammatory mediators initiates pathophysiological effects like fever, complement activation, disseminated intravascular coagulation, vascular dysfunction, myocardial depression, tissue damage, and multiple organ failure (10). The systemic inflammatory response is visualised in a simplified scheme in figure 2. Endotoxin (including lipopolysaccharide, LPS) may be regarded as a prototype initiator of a systemic inflammatory response.

CELLULAR ACTIVATION

The cell types present in different inflammatory foci vary widely depending on the nature of the antigenic stimulus, its persistence, and the type of immune reaction it elicits. Acute inflammatory foci contain mostly neutrophils, whereas sites of chronic inflammation usually contain higher proportions of mononuclear cells (11). Macrophages play an important role during a systemic inflammatory response. The crucial role of macrophages is exemplified by their ability to bind, phagocytize and subsequently neutralise infectious agents (5,12). In addition, macrophages are a major source of inflammatory mediators like cytokines and prostaglandins (13-16).

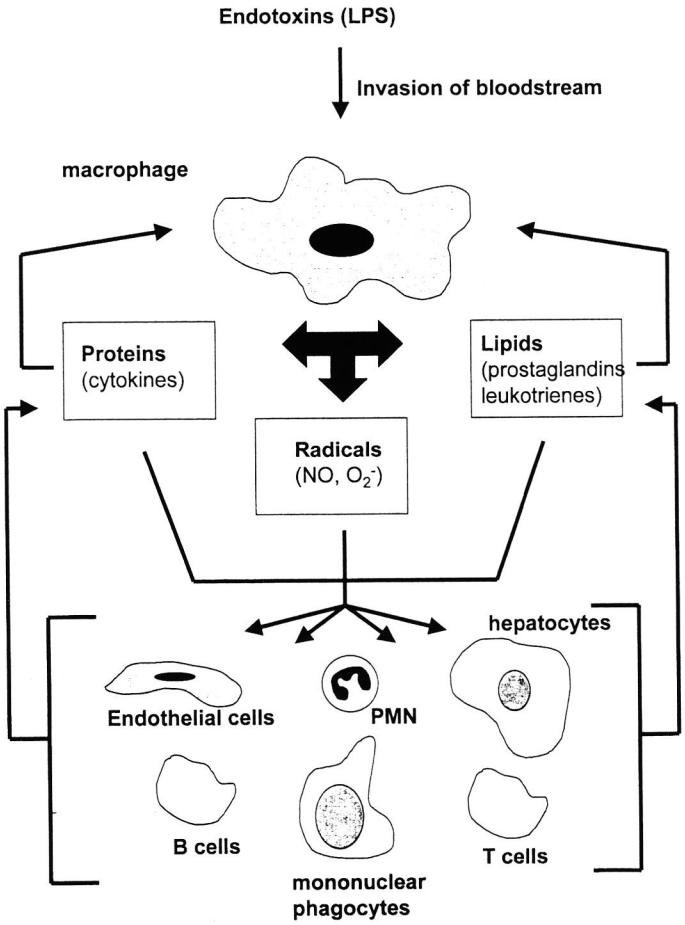

Figure 2. *Cascade of events after endotoxin enters the circulatory system of a organism*

Macrophages exist as resident (tissue bound) and (immature) circulating macrophages (monocytes). Resident macrophages are present throughout the organism, e.g. liver-macrophages (Kupffer cells), lung macrophages (alveolar macrophages), brain macrophages (glial cells), and peritoneal macrophages. The various macrophages possess special characteristics due to their specific locations. During the onset of a systemic inflammatory response the Kupffer cells are very important. Kupffer cells are located in a strategic position within the capillaries (sinusoids) of the liver where they monitor all the incoming (portal) blood. These liver- macrophages represent 80% of the tissue-fixed macrophages and are part of the reticulo endothelial system (RES), they bind the majority of invading toxins and foreign particulate material (17). This characteristic was clearly shown in studies where (radiolabelled) endotoxin was injected: most of the injected lipopolysaccharide (LPS) ended up in the liver and within the liver it was bound to the Kupffer cells (18,19). In other studies, Kupffer cells were neutralised (e.g. by gadolinium chloride) which completely altered the systemic response to infection (20-24), and emphasised the importance of macrophages during an inflammatory response.

Macrophages are in a resting state during homeostasis, but can change from a resting into an activated state. Activation is achieved after triggering by different kinds of extracellular stimuli like (remnants) of micro-organisms (such as zymosan and endotoxins, including LPS (8,25-28)), peptide-mediators (29,30), lipid mediators (31,32), or radicals that are secreted by other cells (33-35). Peptide mediators are for example cytokines and chemokines; an important group of lipid mediators are the prostaglandins.

The first step after binding of the extracellular stimuli to their subsequent receptors on the cellular surface, is the initiation of an intracellular signalling cascade that leads to the actual cellular activation. A thoroughly studied (intracellular) signalling cascade is the initiation of cell activation by LPS. Although the exact mechanisms of LPS binding and subsequent induction of intracellular signal transduction and cellular activation are still not fully characterised, the current idea of the cascade of events after invasion of the system is as follows. LPS binds (potentially with the help of LPS-binding protein LBP) to CD14 on the cell surface of immune cells. Since CD14 has no membrane spanning domain it can not directly initiate intracellular signal transduction. However, after binding of LPS the interaction of CD14 with Toll-like receptors (Tlr) leads to intracellular signal transduction (36-40). Recent findings suggest a central role for Tlr4 in recognition of LPS (41-43). Recently, a possible explanation for the often observed species-differences in sensitivity to LPS was given by Smirnova *et al.*, who demonstrated highly variable regions in both the extracellular and cytoplasmic domain of the polymorphic protein Tlr4 (44).

LPS induces the expression of a large number of genes involved in inflammatory reactions by activating several types of transcription factors. One of the transcription factors that plays a key role in the activation of genes induced by LPS is nuclear factor-kappaB (NF-κB), which induces the expression of several important inflammatory mediators including TNFα (26,36,41,45-48). An important characteristic of macrophage activation is the

production and release of pro- and anti-inflammatory cytokines (8,28,47,49) . Cytokines are important in the onset and progression of the inflammatory cascade (50-52). Pro-inflammatory cytokines are held responsible for detrimental side-effects like tissue damage and organ failure (8,53,54). Other indications of macrophage activation during inflammation are the induction of enzymes, like nitric-oxide synthase (iNOS)(55-58) and cycloxygenase-2 (prostaglandin-H-synthase-2, COX-2) (48,55,59,60). Inherent to this up regulation of enzymatic activity is the production and release of agents that play a pivotal role during inflammation: (nitric) oxide-intermediates (55,61,62), leukotrienes and prostaglandins (63,64). Other characteristics of activated macrophages are the upregulation of cellular adhesion molecules like VCAM and ICAM (65). These molecules are able to bind (infiltrating) leucocytes and facilitate (trans)migration of polymorphonuclear granulocytes and mononuclear phagocytes.

INFLAMMATORY MEDIATORS

Histamine, serotonin, bradykinin, and lipid mediators (e.g. prostaglandins, leukotrienes) are generally recognised for several decades as important mediators of inflammation. More recently, peptide mediators (e.g. cytokines) and NO have also been acknowledged. In acute inflammatory reactions the peptide- and lipid- mediators and reactive nitrogen intermediates play a pivotal role; these mediators will therefore be discussed in more detail.

Peptide mediators

Peptide mediators are produced by a wide variety of (immune) cells during the inflammatory response. Cytokines play a critical role during an inflammatory response, since they have an important regulatory function. The concentrations of these agents determine for a major part whether they are beneficial or detrimental to the host.

Cytokines are relatively small proteins with an intercellular signalling function, and can be subdivided in pro- and anti-inflammatory cytokines. However, it must be noted that this division is not very strict, since several cytokines have been reported to possess both pro- and anti-inflammatory characteristics (e.g. IL-6 (66,67)).

Pro-inflammatory cytokines are mediators that initiate and increase the inflammatory response, and are often reported to exert detrimental side-effects when produced in excessive amounts (50-52). Tumour Necrosis Factor-α (TNFα) (68), interleukin-1β (IL-1β) (69), interleukin-6 (IL-6) (70), interleukin-12 (IL-12) (71), and interleukin-18 (IL-18)(72), are regarded as important pro-inflammatory cytokines. Anti-inflammatory cytokines decrease the (effects of the) inflammatory response, e.g. by down-regulating the production of pro-inflammatory cytokines, lipid mediators and NO (49,73-75). Examples of anti-inflammatory cytokines are interleukin-10 (IL-10), interleukin-4 (IL-4) and interleukin-13 (IL-13). The following cytokines were subject to a more detailed

study in this thesis: TNFα, IL-1β, IL-6 and IL-10, and will therefore be discussed in more detail below.

TNFα

TNFα, a polypeptide cytokine, is one of the first mediators to be released during the onset of the inflammatory cascade in response to infection or trauma (68,76,77). Beneficial effects of TNFα are its micobicidal activity and its tumoricidal action. TNFα has cytotoxic properties, it can initiate both cellular- or tissue-necrosis (organ damage) and apoptosis (54,68,78). When TNFα is injected into humans or animals, it initiates almost the same inflammatory response as can be seen after infection by micro-organisms or after the injection of endotoxins (50,52,68). This illustrates the important role of TNFα in the onset of the inflammatory cascade. TNFα is produced by many immune cells, but the major producers of TNFα during a systemic inflammatory response are the macrophages, in particular Kupffer cells (13). TNFα is also able to regulate the production of many inflammatory mediators, like prostaglandins (29) and NO (57,79). Neutralisation of TNFα, either by neutralising antibodies or by soluble receptors, greatly reduces the severity of the inflammatory response and associated organ failure (53,80). Therefore, this cytokine is subject of many studies and therapeutical strategies to prevent the production or release in order to reduce the detrimental effects of the inflammatory reaction (68,81).

Interleukin-1

Currently, three isoforms of IL-1 have been identified: IL-1α, IL-1β, and IL-1-receptor antagonist (IL-1ra). The biological properties of IL-1α and IL-β are in most studies indistinguishable, although IL-1β has been studied more extensively. IL-1β is mainly produced by macrophages, dendritic cells, endothelium, fibroblasts and B cells. TNFα and IL-1β share many properties, these cytokines resemble eachother in size, in the early release during the onset of the inflammatory cascade and in the effects provoked, such as fever, tachycardia and gastric dysfunction (50,52,69). Characteristics, which are thought to be typical for IL-1β compared to TNFα, are the induction of enzyme synthesis and prostaglandin synthesis in osteoclasts, chondrocytes and fibroblasts (5). Additionally, IL-1β has an important role in the induction of iNOS and NO production (82,83). The pro-inflammatory characteristics of IL-1β have been studied extensively, but recently also attention is paid to IL-1ra and its anti-inflammatory characteristics (84). The anti-inflammatory effect of IL-1ra is due to the fact that this protein blocks the IL-1 receptor, thereby preventing IL-1α and IL-1β to initiate pro-inflammatory effects.

Interleukin-6

IL-6 is produced a little later than TNFα and IL-1β during the onset of the inflammatory cascade. IL-6 is released by activated macrophages, fibroblasts and T-cells. This agent is an endogenous pyrogen and evokes fever (67). An important characteristic of IL-6 is that it initiates the production of acute phase

proteins by hepatocytes. Additionally, other features that have been reported include its role in the induction of arthritis (85), and its role in cell-growth and differentiation (86). Some investigators claim that IL-6 is an anti-inflammatory cytokine (66) since it is needed to control the inflammation. The beneficial and detrimental effects of IL-6 were reviewed recently by Gadient and Otten (67). IL-6 levels have often been reported to be very high in patients with severe systemic inflammation (like in septic shock), and high levels of IL-6 are prognostic for increased risk of mortality (6,7,87). However, the precise role of IL-6 in systemic inflammatory disorders is yet not fully understood.

Interleukin-10

IL-10 is an anti-inflammatory cytokine that is also released relatively early during the onset of an inflammatory response. Its main characteristic is the ability to reduce the inflammatory reaction via negative feedback mechanisms. IL-10 suppresses the release of pro-inflammatory mediators by inhibiting the activation of the transcription factor NF-κB (49,88,89). The neutralisation of this protein leads to increased lethality during endotoxemia (90), which emphasises the importance of IL-10 in controlling the inflammatory reaction.
IL-10 is produced and released mainly by activated macrophages and T-cells. Because of its anti-inflammatory effects this cytokine is subject of extensive study, and is also tested for its therapeutical potential (91).

Lipid Mediators

Changes in the plasma membrane that are associated with cellular activation allow phospholipase A_2 to release arachidonic acid, an essential fatty acid. Arachidonic acid can be metabolised by lipoxygenase or cyclo-oxygenase (COX) enzymes into products that are called lipid mediators (5) or eicosanoids (prostaglandins, PG's, and leukotrienes, LT's). Eicosanoids are important mediators of metabolism and play a regulatory role in several pathological conditions (63,64,92-94). The enzymes lypoxygenase and COX-1 are constitutively expressed in various immune and non-immune cells (5). COX-2 is an inducible isoform of the cyclo-oxygenase enzyme which is normally absent but can be upregulated during an inflammatory response (55). Responsible for this upregulation are LPS, TNFα, and IL-1β (59,60,95,96). Additionally, COX-2 is upregulated in colon of patients with colorectal cancer (97) and in liver during alcoholic liver disease (98). In turn, eicosanoids are able to regulate cytokine and NO-production (99-102). It is generally believed that eicosanoids derived from lipoxygenases and COX-1 are essentially 'good' mediators involved in homeostasis. The products of COX-2, however, are thought to have potentially negative effects during inflammatory reactions (60,64). Therefore, much effort has been put into the development of specific COX-2 inhibitors (103,104). Moreover, the classic non-specific COX-inhibitors like aspirin were reported to have side-effects like gastric ulcering, renal tubular necrosis and increased bleeding times, which are assumed to be evoked by the inhibition of both COX-1 and COX-2. The inhibition of COX-1 is assumed to lead to a decrease of essential prostaglandins that are needed for vital physiological functions (60).

Reactive nitrogen intermediates

Another important group of inflammatory mediators are the reactive nitrogen intermediates, also called reactive nitrogen species. These mediators are produced by the enzyme nitric oxide synthase of which three isoforms are currently known: NOS-1 (cNOS), a constitutive isoform originally isolated from neuronal sources, NOS-2 (iNOS), an inducible isoform that may generate large quantities of NO, and NOS-3 (eNOS), a constitutive isoform identified as an endothelial cell-specific NOS. Another basis for differentiation between the constitutive and inducible enzymes is the requirement for calcium binding to calmodulin in the constitutive isoforms (16,105). The inducible isoform iNOS is of particular interest because this enzyme is not expressed under non-pathological circumstances, but is upregulated during an inflammatory response, similar to COX-2. Upregulation of iNOS is initiated by microbial products, inflammatory cytokines and during reperfusion ischemia (16,57,83,105,106).

NOS converts the amino acid L-arginine into L-citrulline, and an additional product of this reaction is NO (107). NO is vulnerable to a plethora of biologic reactions, the most important being those involving higher nitrogen oxides (NO2-), nitrosothiol, and nitrosyl iron-cysteine complexes, the products of which (e.g. peroxynitrite) are believed to be highly cytotoxic (105). These intermediates react and bind to all sorts of targets inside and outside cells, thereby causing damage to membranes, tissues, and enzymes (105,108-110). Furthermore, NO has been demonstrated to induce apoptosis in the liver (106). By contrast, release of NO in small quantities may be beneficial because it has been shown to decrease tumour cell growth and levels of prostaglandins and to increase protein synthesis and DNA-repair enzymes in isolated hepatocytes. Additionally, NO has a physiological role as a vasodilator (105). In summary, NO possesses both cytoprotective and cytotoxic properties depending on the amount and the isoform of NOS by which it is produced, therefore therapeutic 'fine regulation' of (excessive) NO-release during inflammation might be beneficial and reduce the negative side-effects it evokes.

WHEN THE IMMUNE SYSTEM ITSELF IS CAUSE OF DISEASE

Undesirable consequences of immunity or an excessive immune response are multiple organ failure during a (systemic) inflammatory response, allergy, autoimmunity, and transplantation (graft-) rejection. Referring to the fifth symptom of inflammation (disturbed function of organs), which is exemplified by multiple organ failure often observed in the later phase of sepsis and SIRS (6,7), the control of an excessive immune response might greatly improve the recovery of patients. Inflammatory mediators determine for a major part the progress of the disease and negatively influence the recovery of patients (6,7,10).The detrimental effects like organ-damage and shock, induced by the release of excessive amounts of proinflammatory cytokines, has often been described (7,8,53), with special emphasis on TNFα (76,111) and reactive nitrogen intermediates (62,108).

Therefore, immunomodulatory treatments (immunosuppressive treatment) might aid to prevent the detrimental effects of an (over)active immunesystem.

CURRENT ANTI-INFLAMMATORY THERAPIES

Immunopharmacology is the study of the regulation of the immune system and of therapeutic strategies that selectively modify immune function in human and animal diseases.
The aim of immunomodulatory therapy is either to increase the activity of the immune system, which is outside the scope of this thesis, or decrease the intensity of an immune reaction. To date, immunosuppressive therapy is mostly used to inhibit graft rejection in transplantation and to reduce autoimmunity. For these purposes often used immunosuppressive agents include: corticosteroids, cyclophosphamide , methotrexate, cyclosporin A, and tacrolimus.
With regard to the treatment of severe (systemic) inflammatory disorders, time has passed when the management of sepsis and SIRS could be summarised as supportive care (e.g. to control hemodynamic abnormalities and to perform vasopressor therapy) and the administration of antibiotics (112,113). Many new strategies for the control of acute and systemic inflammatory diseases have been developed. These strategies vary widely in their targets and sites of action. However, the overall-aim is to prevent the detrimental side-effects, like multiple organ failure and tissue damage, and to decrease mortality of patients suffering from an excessive systemic immune response initiated by infection, injury, or trauma.
Immunomodulatory therapy for the treatment of acute (systemic) inflammatory disorders can be applied at several levels, and during various periods during the inflammatory response.
First target in the pathological sequence of events are the remnants of micro-organisms, the endotoxins including LPS. Treatments have been designed to prevent the release of LPS from the micro-organisms, the detoxification or removal of endotoxins (e.g. by polymixin B, or apoE), the neutralisation of circulating LPS (e.g. using anti-LPS antibodies), and the prevention of the presentation and binding of LPS to its receptors (e.g. anti-LBP and anti-CD14 antibodies) (10,114-116). Next step in the inflammatory cascade is the cellular signal transduction initiated by endotoxins. At this level, inhibition can be obtained using inhibitors of protein kinases or NF-κB (10). However, during the past two decades most effort has been put into anti-inflammatory strategies aiming to affect the inflammatory mediators that are released after the inflammatory cascade has already been initiated. Although the cascade of events is already ongoing, the rationale behind these anti-inflammatory strategies is to prevent tissue damage which is associated with excessive release of inflammatory mediators and to prevent shock, and eventually decrease mortality of patients. Different therapeutics have been designed to either prevent the release of inflammatory mediators or to bind and neutralise these agents. Anti-inflammatory compounds that proved to be successful in laboratories in *in vitro* experiments and in simplified animal experiments, which

have been tested in clinical experiments are corticosteroids, Non-Steroidal Anti-Inflammatory Drugs (NSAIDS), and phoshodiesterase inhibitors. Cortocosteroids are anti-inflammatory and antipyretic, and are generally used to suppress various (local or topical) inflammatory responses. Corticosteroid therapy can resolve some (clinical) symptoms of inflammation within hours to days (113,117-119). The best known group of NSAIDS are the cox-inhibitors. These drugs prevent the release of lipid mediators, prostaglandins in particular. Although NSAIDs are frequently used to relieve pain or reduce fever, they also attenuate granulocyte functions, such as chemotaxis, phagocytosis, and bacterial killing. For this reason NSAIDs have been tested in several clinical trials of human endotoxemia (115,120). In addition, findings in recent studies involving animals and human volunteers, injected with endotoxin suggest that pretreatment with NSAIDs enhances production of TNFα and Il-1β. The increase in blood levels of these cytokines is most likely due to the prevention of feedback-inhibition by prostaglandin E2. Thus, NSAIDs may contribute to the sudden onset of shock, organ failure, and aggressive infection by inhibiting neutrophil function, augmenting cytokine production, and attenuating the cardinal manifestations of inflammation (120). Phosphodiesterase (mainly PDE-IV) inhibitors are compounds that inhibit the enzymatic breakdown of cAMP, thereby increasing the intracellular levels of this second messenger, which has been described to be a potent anti-inflammatory mechanism (121-123). Phosphodiesterase inhibitors have been shown to exert beneficial anti-inflammatory effects in laboratory and clinical experiments (124-128). Other strategies that aim to neutralise pivotal cytokines are neutralising antibodies, receptor antagonists and soluble receptors (81,84,125,129), or the removal of mediators from the system by use of hemofiltration (130,131).

Unfortunately, these anti-inflammatory approaches and drugs have several drawbacks. Compounds that looked promising in preclinical experiments turned out to be not effective in clinical trials (e.g. (10,114,132,133), and references therein), or the drugs were found to evoke side effects (e.g. pentoxifylline(134)) or initiate toxicity (e.g. methotrexate in liver) (135). Immunosuppressive therapy may provoke opportune infections with viruses, fungi, protozoa, and intracellular bacterial pathogens, as was seen in patients that were treated with corticosteroids or NSAIDs (50,113). Another important factor appears to be the timing of drug-administration: systemic inflammation is characterised by waves of active and less active immune response (hyper and hypo-inflammatory phases (132,133)). This phenomenon might request for a need to continuous bed-side monitoring of blood-concentrations of inflammatory mediators to reveal the right time for drug-administration, or perhaps (more practical) drugs that could act as profylaxis against waves of inflammatory mediator release into the system. An interesting hypothesis was proposed by Cirino (55) who commented on the current trends in inflammation research. He argued that due to the great complexity of the biochemical, pharmacological, immunological, and pathological processes that are involved in inflammation, there is a tendency to investigate in depth the pathogenic role played by one single agonist at a time rather than to analyse the effects of multiple factors in the initiation of tissue damage. This approach is justified, since such complex models may be fraught

with many technical and interpretational difficulties. However, the dilemma is that many of the mediators produced operate in a network, and most of the time in an experimental system more than one enzyme or protein is induced. This might be one of the reasons why current anti-inflammatory therapies for the treatment of sepsis and SIRS have failed in clinical trials.
In conclusion, there is a firm ground for research into the mechanisms of the inflammatory response and to investigate potential therapies that prevents its detrimental side effects.

THE ß-ADRENOCEPTOR

Several *in vitro* and *in vivo* studies have provided evidence for the modulation of immune function by catecholamines via the adrenoceptor-system (reviewed by Madden *et al*, and Rosman and Brooks (2,136)). In addition, the concentrations of catecholamines in blood appear to alter during the course of an inflammatory response (137,138).
Adrenoceptors (AR) is the collective name for receptors that recognise the endogenous sympathetic neurotransmitter norepinephrine (noradrenaline, NE) and the adrenal medullar hormone epinephrine (adrenaline, EPI). In the classic study of Ahlquist (139) a series of sympathomimetic agents were investigated, providing the first evidence that these compounds could activate more than one type of adrenoceptor. Based on distinct potency orders of several ligands, it was proposed that two types of adrenoceptors exist, termed α- and β-adrenoceptors. Up to date the development of selective α- and β-AR agonists revealed the existence of several subtypes of α_1-, α_2 and β-adrenoceptors. With the use of molecular biological techniques, the existence of the three major families was confirmed and each of the families may be subdivided into three subtypes (135,140). The endogenous agonists for the adrenoceptors EPI and NE are important regulators of many physiological functions like the control of cardiovascular function, airway reactivity, energy metabolism, behaviour, and stress response.
The adrenoceptors have been studied extensively as targets for drugs (agonists and antagonists) including those for the treatment of cardiovascular diseases, asthma, and uterine relaxation.
Activation of the α_1-adrenoceptor has been reported to increase an inflammatory response (141), which therefore seems of less therapeutical value in the treatment of acute inflammatory disorders. Immunomodulatory action, like the suppression of release of inflammatory mediators, appeared to be operated particularly via the β-subtype of the adrenoceptor, and was studied extensively in many *in vitro* studies (142-146).
The β-AR possesses 7-transmembrane spanning domains and are coupled to G-proteins, and are usually referred to as: 7TM-GPCR. The mechanism, by which activation of β-AR leads to the ultimate generation of a pharmacological response, become to be well understood. Stimulation of β-AR leads to the activation of the membrane- bound enzyme adenylyl cyclase, which subsequently catalyses the conversion of adenosine triphosphosphate (ATP) to

cyclic adenosine monophosphate (cAMP). The activation of adenylyl cyclase via β-AR involves a third protein, which serves to couple the β-AR-subtypes to the catalytic enzyme. These coupling proteins are called guanine nucleotide regulatory proteins (G-proteins; G_s for stimulation, G_i for inhibition), they consist of three subunits (α-, β-, and γ-) and are essential for receptor-mediated activation of adenylyl cyclase (147,148). The sequence of events is generally believed to be as follows:

-1: β-AR agonists bind to either β_1- , β_2- or β_3-AR;
-2: The resulting receptor-agonist complex will have high affinity for, and bind to G_s protein;
-3: Formation of the receptor-agonist-G_s protein complex facilitates the exchange of GDP for GTP on the G_s protein;
-4: The complex between G_s and GTP dissociates from the receptor-agonist complex and interacts with the catalytic subunit of adenylyl cyclase, thereby promoting the conversion of ATP to cAMP;
-5: cAMP causes the activation of an intracellular protein called cAMP-dependent protein kinase, which phosphorylates a variety of intracellular proteins, or cAMP binds to cAMP-responsive element binding protein (CREB), and subsequently to cAMP responsive elements (CRE) present in the DNA, regulating gene-expression, ultimately leading to a pharmacological response (135,149).

The activation of the β-AR and subsequent signal transduction is depicted schematically in figure 3. In the sequence of events via which the β-AR modulates the immune response, the elevation of the (intracellular) second messenger cAMP is thought to play a pivotal role (121,122,150,151). Although the phenomenon of β-AR agonist-mediated immunoregulation has been described before, there are still many questions unanswered about the exact mechanisms by which the β-AR agonists modulate an immune reaction. Moreover, most studies on this subject have been performed *in vitro* and limited data is available *in vivo*. In addition, therapeutical implications for the use of β-AR agonists as anti-inflammatory agents in treatment of acute- and systemic inflammatory disorders are still obscure.

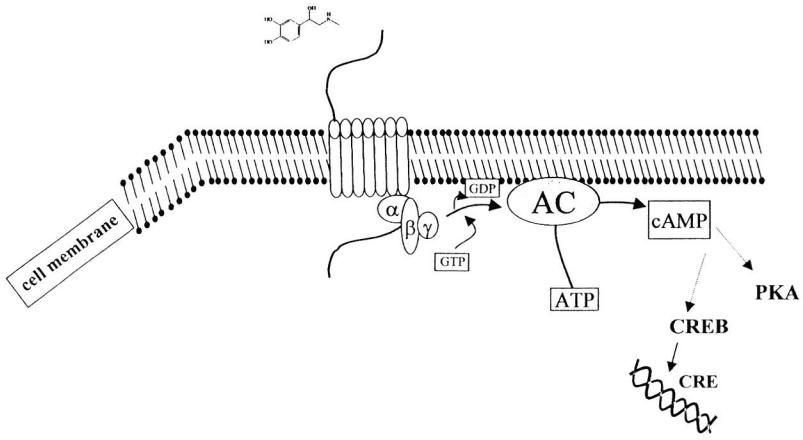

Figure 3. *Activation of the β-AR by the endogenous ligand epinephrine (adrenaline), and subsequent intracellular signalling. See text for explanation.*

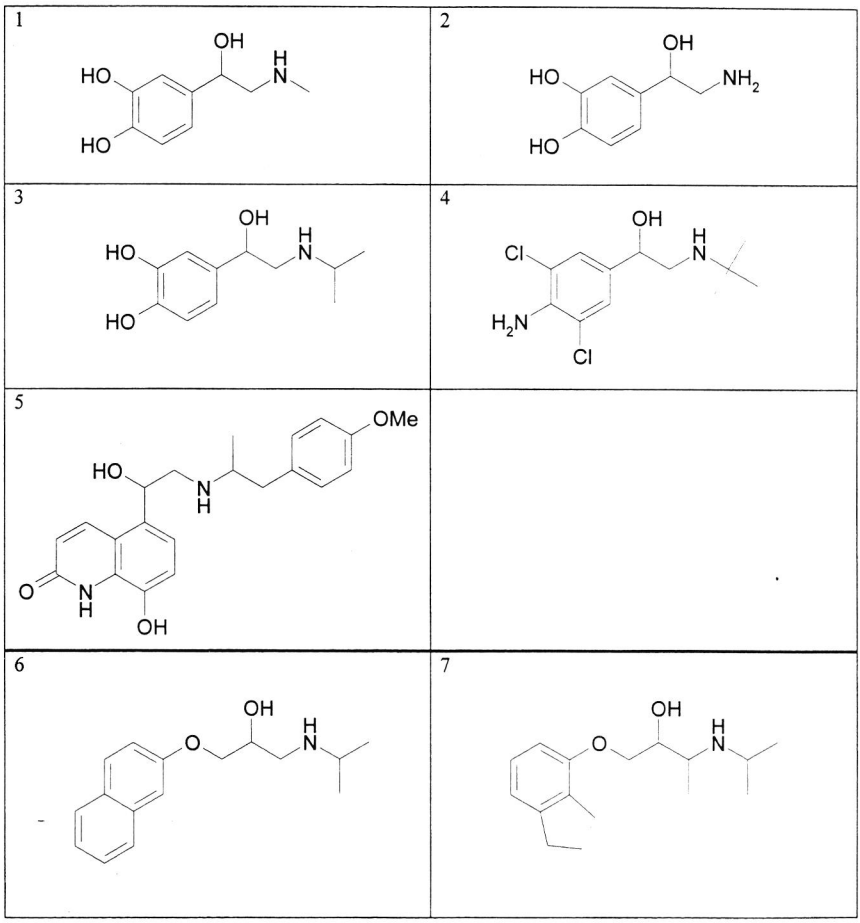

Figure 4. *Some important ligands (agonists and antagonists) for the β-AR. The endogenous agonists epinephrine (adrenaline,1) and norepinephrine (noradrenaline,2), the non-selective agonist isoprenaline (isoproterenol,3), the selective β_2-AR agonists clenbuterol and TA2005 (4,5), the non-selective β-AR antagonist propranolol(6), and the selective β_2-AR antagonist ICI-118551 (7).*

SCOPE AND OUTLINE OF THE THESIS

Although some aspects of the regulation of the immune response by adrenergic agents have been described, a number of issues remained to be elucidated. Therefore, the general objective of the research described in this thesis was to study in more detail the immunomodulatory actions of β-AR agonists during an acute inflammatory response.
The important issues which were addressed are:

- The expression of β-adrenoceptors on different macrophages, and the suppression of macrophage activation by β-agonists.
- The subtype of the β-AR on macrophages, which is responsible for the observed anti-inflammatory effects
- The differential regulation of various cytokines by β-agonists during an inflammatory response.
- The reproducibility *in vivo* of the observed *in vitro*-effects.
- The beneficial effect of suppressed macrophage activation by β-agonists on liver-functioning.
- The importance of timing- and way- of administration of β-agonists when applied during an acute systemic inflammatory response.

An acute inflammatory response was initiated either *in vitro* or *in vivo*, using LPS, and used as a model to study the immunomodulatory properties of particularly β-AR agonists. *In vitro* the U937 monocytic cell-line, differentiated into macrophage- like cells, and primary isolated porcine macrophages and liver cells were used to study the effects of several (potential) anti-inflammatory cAMP-elevating agents. In addition, rats were used for *in vivo* studies for therapeutic potential of β-adrenoceptor agonists during an acute inflammatory response.
In chapter 2 and 3 the expression of β-adrenoceptors was confirmed and quantified on U937 cells and on primary isolated porcine macrophages, respectively. Additionally, the suppression of LPS-induced release of some important pro-inflammatory cytokines and the enhanced release of an anti-inflammatory cytokine, after incubation with β-AR agonists or other anti-inflammatory agents was demonstrated. In chapter 4, the mechanism by which the β-AR agonists modulate the release of pro- and anti-inflammatory cytokines in U937-cells was studied in more detail. The anti-inflammatory effect of β-AR agonists appeared to be signalled solely via the β_2-AR-subtype. The importance of stereoselective ligand recognition by the β_2-AR was emphasised and shown to be detectable at receptor-binding, efficacy- (cAMP), and response- (cytokines) level. The effects of β_2-AR agonists on cytokine mRNA expression during LPS-induced activation of macrophages was object of study in chapter 5. The differential effects of cAMP-elevating agents in general, and β_2-AR agonists in particular, on liver cell functioning during an inflammatory response have been studied using isolated porcine liver cells, as described in chapter 6. In chapter 7, the gap between *in vitro* cell studies and *in vivo* animal experiments was bridged, using the β_2-AR agonist clenbuterol. Clenbuterol appeared to be the most potent inhibitor of cytokine release amongst some other β-AR agonists

and anti-inflammatory agents. Moreover, the modulation of cytokine release *in vitro* was reproducible in rats *in vivo.* In addition, the timing of clenbuterol-administration and therapeutical consequences for the anti-inflammatory potency was investigated. The β_2-AR agonist was found to suppress systemic inflammation, and associated liver-failure *in vivo* in endotoxemic rats is described in chapter 8.

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CHAPTER 2

SUPPRESSION OF THE ACUTE INFLAMMATORY RESPONSE OF PORCINE ALVEOLAR- AND LIVER MACROPHAGES

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ABSTRACT

During infection and inflammation drug disposition and hepatic metabolism are markedly affected in mammals. Pro-inflammatory mediators play an important role in the suppression of (cytochrome-P450-mediated) drug metabolism. Inflammatory mediators like cytokines, nitric oxide (NO), reactive oxygen species (ROS) and eicosanoids are released by activated macrophages from various sources, including liver and lung.

It was the aim of this study to investigate ways to suppress the activation of macrophages during the onset of the inflammatory cascade. Therefore porcine lung and liver macrophages were isolated, and incubated with lipopolysaccharide (LPS) to initiate an acute inflammatory response, represented by the release of high amounts of tumour necrosis factor-α (TNF-α) into the culture medium. Additionally the primary macrophages were coincubated with phosphodiesterase-IV- (PDE-IV)-inhibitors or β-adrenoceptor agonists that in previous studies demonstrated strong suppressive effects on TNF-α release.

Especially the β-adrenoceptor agonists showed to be very potent TNF-α suppressants, which indicates that the β-adrenoceptor might be an interesting target for suppression of activation of macrophages. This was strengthened by the observation that the β-adrenoceptor expression was not altered during the onset of the inflammatory cascade.

INTRODUCTION

Inflammatory mediators, like cytokines, nitric oxide (NO), reactive oxygen species (ROS) and eicosanoids are released during infection and inflammation by activated macrophages from various sources, including liver and lung (8,9,15). The release of these mediators is intended as defence against invading pathogens, however excessive release can be detrimental to the host and aggravate disease leading to e.g. shock and multiple organ failure (15,27). Therefore it can be of therapeutical interest to suppress early macrophage activation during an inflammatory response.
Among (pro-)inflammatory mediators TNF-α is released as one of the first and most potent initiators of the inflammatory cascade (2,36,38). Its detrimental effect on the functioning of various cell types and organs has been shown extensively (20,36,42). For this reason the suppression of the release of this cytokine was chosen as potential beneficial therapeutic target.
During an inflammatory reaction and during infections drug disposition and hepatic metabolism are markedly affected in mammals (24,25,34). Changes in drug disposition may have great clinical consequences such as potential toxicity or diminished therapeutical effects (40,41). The crucial role of pro-inflammatory mediators, especially TNF-α, in the suppression of (cytochrome-P450-mediated-) drug metabolism has been demonstrated before (4,6,26). It is assumed that the activation of liver macrophages (Kupffer cells) is an important factor in the dysfunctioning of the liver during inflammation (8,20,21).
Substantial research on the effect on drug dispostion and metabolism has been investigated *in vitro* and *in vivo* using porcine models of infection and inflammation (23-25,28). Therefore primary macrophages were isolated from pigs (lung and liver) to test the *in vitro* anti-inflammatory effects of phosphodiesterase-IV-(PDE-IV)- inhibitors (pentoxyfylline and rolipram) and β-adrenoceptor agonists (clenbuterol and salbutamol). Lipopolysaccharide (LPS) was used as a model compound that induces a general inflammatory response both *in vitro* and *in vivo* (15,30). In addition, we studied the properties of the β-adrenoceptor present on macrophages, and their maintenance during an LPS-induced inflammatory response.

MATERIAL AND METHODS

Chemicals

β-adrenoceptor agonists: clenbuterol and salbutamol; phosphodiesterase-IV-inhibitors: pentoxifylline and rolipram, lipopolysaccharide (*Escherichia coli* O111:B4), and 3-(4,5-dimethyl-thiazol-2-y)2,5-diphenyltetrazolium bromid (MTT) were obtained from Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands. ^{125}I-iodocyanopindolol (^{125}I-ICYP) a radioligand for the β-adrenoceptor was purchased from Amersham Life Science (Buckinghamshire, UK).

Isolation of liver cells

Liver cells were isolated from pigs, based on the method of Seglen (31), and modificated as described by Monshouwer (23). In short, castrated male pigs (Great Yorkshire x Dutch Landrace) were used, aged approximately 12 weeks (30-40 kg) obtained from the University's breeding farm. The animals were killed by an i.v. injection with pentobarbital-sodium (150 mg/kg bw). After isolation the livers were immediately exsanguinated using icecold saline, followed by collagenase perfusion. The resulting liver cell suspension, a mixture of hepatocytes (parenchymal cells) and non-parenchymal cells, was subsequently centrifuged for 5 minutes at 200g at 4°C. The supernatant was removed and the pellet was resuspended in PBS (Life Technologies, Breda, The Netherlands). This cell suspension was centrifuged for 2 minutes at 50 g, 4°C. The resulting pellet contained hepatocytes and the supernatant was used for the further isolation of Kupffer cells.

Isolation of liver macrophages (Kupffer cells)

The procedure for Kupffer cell isolation was based on the method as described by Smedsrød and Pertoft (35) with some modifications. Supernatants (see isolation of liver cells) containing non-parenchymal cells were transferred to four 50-ml tubes (Micronic, Lelystad, the Netherlands) followed by centrifugation at 50*g* for 2 minutes at 4°C. This procedure was repeated twice to discard remaining hepatocytes. After the final step, supernatant was centrifuged at 200*g* in a swing out rotor for 10 minutes at 4°C. Supernatant was discarded and resulting pellets were resuspended in PBS to a final volume of 40 ml. The non-parenchymal cell suspension was carefully placed on top of a two layer (60% and 25% v/v) percoll-gradient, and centrifuged for 15 minutes at 400*g* (4°C). After centrifugation the cell suspension in between the two layers of percoll was collected and diluted with PBS. This cell suspension, containing both Kupffer cells and endothelial cells, was centrifugated at 200*g* for 10 minutes and the resulting pellet was diluted with Williams medium E (without serum) (Sigma). After a final wash step, cells were diluted in Williams medium E (without serum) to a concentration of $2x10^6$ cells/ml. To remove endothelial cells, cells were plated on tissue culture plates at 37°C and 5%CO_2 for 30 minutes followed by a single wash step discarding the non-adherent endothelial cells. Viability of Kupffer cells was $\geq$95% as determined by trypan blue exclusion assay. Subsequently cells were cultured in William's E, supplemented with 10% (v/v) fetal calf serum (Life Technologies), glutamine (1.67 mM), and gentamicin (50 µg/ml).

Isolation of alveolar macrophages

Alveolar macrophages were isolated based on a method as described by Cruijsen (7), with some modifications. In short, lungs were isolated from the same pigs as described in the section 'isolation of liver cells'. After isolation the lungs were placed on ice. Lung lavage was performed by adding icecold sterile PBS (50 ml) to each lobe. The lobes were gently massaged for 3 minutes, and subsequently the PBS was aspired and transferred to sterile centrifugation tubes. This procedure was repeated once. The tubes were centrifuged for 10 minutes at 100g (4°C). The

resulting pellets were resuspended in PBS and again centrifuged for 10 minutes at 100g (4^0C). This washing procedure was repeated once. The resulting pellet, containing mainly alveolar macrophages, was then resuspended in RPMI-1640 (Sigma) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 2 mM L-glutamine (all Life Technologies), but no serum. The cell suspension was adjusted to a concentration of $1x10^6$ cells/ml. Cells were plated on tissue culture plates and incubated at 37^0C and 5%CO_2 for 90 minutes. This procedure resulted in a further purification of the alveolar macrophage culture. Subsequently the culture medium was replaced by RPMI-1640, supplemented as described above, with additional 10% (v/v) fetal calf serum (Life Technologies). Viability of alveolar macrophages was $\geq$95% as determined by trypan blue exclusion assay.

Cell cultures and incubations

Macrophages (liver- or alveolar, $1x10^6$ cells per well) isolated on separate occasions from three pigs were used for experiments the day after the isolation. Cells were incubated with 1 or 10 µg/ml LPS alone, or in combination with the following test substances: the β-agonists clenbuterol or salbutamol (10^{-6}M, 10^{-8}M), or the PDE-IV inhibitors: pentoxifylline and rolipram (10^{-3}-10^{-8}M). Incubations were performed in triplicate. All stock solutions were prepared on the day of the experiment in phosphate buffered saline (PBS, Life Technologies). Controls were treated similarly and incubated with either the test substance alone or vehicle (PBS). Culture medium was collected at various time intervals during incubation. After removal of culture medium the cells were lysed in 1 ml NaOH (0.1 M) and used for protein determination by the modified method of Bradford (Bio-Rad, München, Germany). The amount of cytokines in the culture medium was expressed per total amount of cell protein.
For TNF-α determinations in the culture medium of the macrophages a pig TNF-α ELISA has been used, according to protocol as provided by the manufacturer (Endogen, Woburn, MA).

Radioligand binding studies

Liver macrophages (Kupffer cells) isolated on separte occasions from three pigs were cultured in 24-wells plastic culture plates (Corning Costar, Badhoevedorp, The Netherlands) at a density of $1x10^6$ cells/well. Cells were cultured at 37^0C in a humidified atmosphere of 95% air 5% CO_2. The day after isolation the culture medium was changed, and additionally either LPS or vehicle (PBS) was added to the wells. After 24 hrs the culture medium was replaced and saturation binding assays were performed (in PBS) in triplicate to determine the total β-adrenoceptor expression. The radioligand binding studies (in PBS) were performed according to standard procedures. For saturation increasing concentrations of the specific β-adrenoceptor ligand ^{125}I-ICYP (1.95- 125 pM) were added. Reactions were performed at 4^0C for 3 hrs. Then the incubation buffer was aspirated and cells were washed several times with PBS in order to separate bound from unbound radioligand. Subsequently cells were lysed using 1ml of 0.1 M NaOH per well, and radioactivity was counted using the Wallac LKB clinigamma counter. Non-specific binding was defined as the amount of ^{125}I-ICYP binding, measured in the presence of 500 µM isoproterenol. Fitting of ligand-binding and -displacement data, to obtain

Bmax-, and Kd-values, was performed using Graphpad Prism software (San Diego, USA).

Statistical analysis
Values presented are means ± standard deviation. For statistical analysis Student's *t* test was performed. The mean values of two groups (LPS-treated vs. LPS/drug treated) were considered significantly different if $p < 0.05$.

RESULTS

Primary macrophage isolation
Isolations of both alveolar and liver macrophages from healthy pigs resulted in large quantities of viable cells as determined by trypan blue exclusion tests (viability >95%). Purity of the culture was determined by visual inspection of morphology, and typically >90%.

Modulation of LPS-induced TNF-α release by alveolar macrophages
Alveolar macrophages were cultured at densities of $1x10^6$ cells/well. The day after the isolation the cells were incubated with LPS (1.0 µg/ml) together with either clenbuterol, salbutamol or pentoxyfylline ($1x10^{-6}$, and $1x10^{-8}$M respectively) for 24 hrs. These cells were found to respond to the addition of LPS by the release of high concentrations of TNF-α into the culture medium, compared to control incubations (figure 1). When the cells were simultanously incubated with two differnt concentrations of either clenbuterol, salbutamol or pentoxyfylline a concentration-dependent suppression of the LPS induced TNF-α release was observed (figure 1). The suppression of TNF-α release was most pronounced in the presence of the ß-agonist clenbuterol ($1x10^{-6}$M), resulting in a decrease of more than 50%. Neither clenbuterol, salbutamol nor pentoxyfylline were found to have any effect on TNF-α release in control incubations without LPS (data not shown).

Modulation of LPS-induced TNF-α release by liver macrophages (Kupffer cells)
Kupffer cells were cultured at densities of $1x10^6$ cells/well. The day after the isolation the cells were incubated with LPS (10 µg/ml) together with either clenbuterol ($1x10^{-6}$M), rolipram $1x10^{-4}$M) or pentoxyfylline ($1x10^{-3}$M) during 24 hrs. Time concentration profile of TNF-α release into the culture medium in reaction to the incubation with LPS is presented in figure 2. TNF-α levels reached a rather sharp maximum at approximately 4 hours after adding LPS, returning to a plateau level after 24 hrs of incubation. When the cells were simultaneously incubated with LPS and clenbuterol, rolipram or pentoxyfylline respectively, a significant suppression of the release of TNF-α was found for all three compounds. The suppression of TNF-α release lasted for the entire incubation period of 24 hrs. The degree of suppression of the TNF-α release was comparable for these compounds, however the phosphodiesterase-IV inhibitors pentoxyfylline and

rolipram were used in much higher concentrations (1000- and 100-fold respectively) than the ß-agonist clenbuterol.
In control incubations using only clenbuterol, rolipram or pentoxyfylline (without LPS) no effect on the TNF-α release by Kupffer cells could be observed (data not shown).

ß-adrenoceptor expression and receptor affinities on liver macrophages.
Kupffer cells were used in receptor-binding studies with a specific ligand for the ß-adrenoceptor ^{125}I-iodocyanopindolol. Saturation studies were performed to study specific, non-specific and total binding. Receptor affinity (Kd) and receptor expression (Bmax) were determined using a one-site receptor binding model. In table 1 the obtained values for these parameters are summarized. It was found that primary porcine Kupffer cells express a substantial number of ß-adrenoceptors, as represented by the amount of ligand capable of binding to the receptor on the cells, and the number of sites per cell (receptor binding places, assuming 1:1 binding of receptor and ligand). ^{125}I-ICYP binds with high affinity (28±13 pM) to the receptors at these liver macrophages.
In order to investigate the effect of incubation with LPS on the expression of ß-adrenoceptors on porcine liver macrophages the cells were incubated for 24 hrs with LPS before the receptor-binding studies were performed. It was found that both Kd and Bmax were increased after the 24 hr LPS incubations, but this effect was statistically not significant. The affinity of binding as well as the amount of ligand bound, and the binding sites per cell were in the same order of magnitude as obtained on untreated Kupffer cells (table 1).

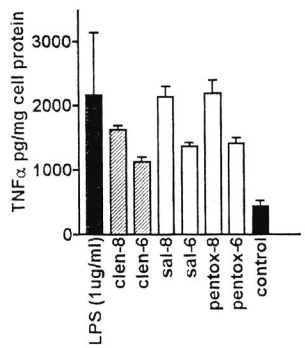

Figure 1.
Effect of different concentrations of β-agonists and PDE-IV inhibitors on LPS induced TNF-α release by porcine alveolar macrophages. TNF-release wasmeasured from 1x10^6 cells/well at 24 hrs. Bars indicate: LPS only (1 μg/ml); LPS in combination with either (β-agonists clenbuterol (clen, striped bars, 10^{-6} and 10^{-8}M) or salbutamol (sal, 10^{-6} and 10^{-8}M), or the PDE-IV inhibitor pentoxyfylline (pentox, 10^{-6} and 10^{-8}M). Data are means ± SD. for triplicate determinations from one of a series of two experiments.

DISCUSSION

In this study we have shown that two different classes of therapeutics, phosphodiesterease-IV (PDE-IV)- inhibitors and ß-adrenoceptor agonists, are capable of suppressing the endotoxin induced TNF-α release by isolated porcine macrophages.
Although the effects of PDE-IV inhibitors on TNF-α have been demonstrated before on various cells of the immune system, like (human) monocytes (10,33), there are hardly any data available on primary macrophages, especially not from larger mammals. Both pentoxyfylline and rolipram are capable of suppressing the LPS-induced TNF-α release by primary macrophages. This effect seemed to be concentration dependent. These data are in agreement with previous studies by other authors using different models (5,10,33), and underline the capability of PDE-IV inhibitors to act as anti-inflammatory agents.

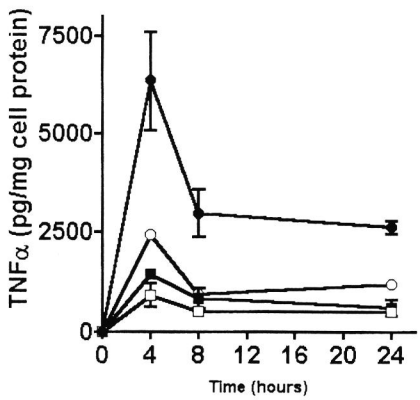

Figure 2.
Time-concentration profiles of LPS-induced TNF-α release by porcine liver macrophages (Kupffer cells) and the effect of different concentrations of (β-agonists and PDE-IV inhibitors.
TNF release was measured after exposure of cells to LPS only (10 μg/ml, ●), or LPS in combination with either the β-adrenoceptor agonist clenbuterol (10^{-6} M, ❍), or the phoshodiesterase inhibitors rolipram (10^{-4} M, ■) or pentoxyfylline (10^{-3} M,). Data are means ± SD for triplicate determinations from one of a series of three experiments.

The other class of therapeutics used in this study, the ß-adrenoceptor agonists clenbuterol and salbutamol, are better known for their use as bronchodilators in (human) therapy. However, evidence is accumulating that β-agonists possess anti-inflammatory properties as well (3,12,19,32). ß-adrenoceptor agonists proved to be also potent modifyers of cytokine release by activated macrophages (14,18). In figure 1 and 2 it is shown that the ß-agonists salbutamol and clenbuterol were able to suppress TNF-α release (concentration-dependent) both from alveolar and liver macrophages. These data are in accordance with findings by other researchers using different cell systems (16,19,37). Present data offer additional proof of the potent anti-inflammatory effects of this type of drugs. This appoints the ß-adrenoceptor as an interesting target for anti-inflammatory therapy.
As shedding of receptors during (the onset) of an inflammatory reaction is a common phenomenon (39), we investigated the expression of ß-adrenoceptors after incubation with the endotoxin LPS. Shedding would obviously diminish the potential of these receptors as targets for anti-inflammatory therapy.
However, no significant effect was observed on the expression of ß-adrenoceptors on liver macrophages after 24 hrs exposure to LPS, compared to untreated Kupffer cells (table 1). Although in this study the upregulation of the β-adrenoceptors was found to be not significant, other researchers reported recently a significant rise in the amount of β-adrenoceptors on Kupffer cells *in vivo* during the late stage of sepsis (13). The expression of β-adrenoceptors on Kupffer cells is in the same order of magnitude as was found on other macrophages or macrophage-like cells (13,18,29). The considerable standard deviation of these data are common normal for primary isolated macrophages (especially when isolated from several animals) and also found in other studies (13).

Table 1. β-adrenoceptor binding study data with porcine liver macrophages.
Incubations were performed in triplicate using ^{125}I-ICYP as specific ligand for the β-adrenoceptor and $1x10^6$ Kupffer cells per incubation. Binding affinities and receptor expression were determined at untreated Kupffer cells and after 24hr LPS incubation (10μg/ml). Data are means ± s.d. of three different pigs.

Treatment	Kd($x10^{-12}$M)	Bmax($x10^{-15}$mol ^{125}I-ICYP/$1x10^6$ cells)	β-receptors(sites/cell)
control	28±13	3.0±0.75	1782±443
+LPS	50±25	4.0±1.8	2402±1089

Both classes of drugs used in this study affect different pathways leading to suppressed TNF-α release. Pentoxyfylline, rolipram, clenbuterol and salbutamol are all capable of raising the intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP). PDE-IV inhibitors evoke a rise in cAMP by preventing the enzymatic breakdown of cAMP. The ß-agonists initiate a rise in cAMP by activating the production of cAMP by adenylate cyclase via their G_s-protein coupled receptor. The elevated levels of intracellular cAMP have shown to be involved in the supression of TNF-α release (5,10). It is interesting to note that there is a considerable difference in the amount of active compound necessary to achieve significant suppression of TNF-α release. In this study it was found that the ß-agonists could be used in much lower concentrations (up to 100 times less) than the PDE-IV inhibitors. The clinical implications of the two different *in vitro* pharmacological approaches described in this paper are not fully clear yet. However, it is evident that the majority of endotoxin that enters the circulation is cleared by the liver and mainly associated with the Kupffer cells (11). Moreover the majority of TNF-α that is released after LPS injection appears to be released from within the liver (1). Other researchers also already pointed out that the liver is an important pharmacological target for the suppression of cytokine release to prevent liver failure during inflammatory reactions (20,21). In addition, this modulation of pro-inflammatory cytokine release may protect against reperfusion injury, e.g. taking place after colic in horses.

On the contrary, complete suppression of TNF-α release could also alter the immunological defense of the organism or remove the beneficial physiological functions of this cytokine. However, the detrimental effects of excessive TNF-α release are more dominant and far more often reported than the benificial effects. Additionally, the compounds tested in this study are capable of concentration-dependent suppression of TNF-α release, herewith offering a way to control (excessive) TNF-α release.

Whether the suppression of TNF-α release by Kupffer cells has the intended preventive effect against altered drug metabolism and drug disposition is currently under investigation *in vitro* using primary isolated liver cell cultures as developed by Hoebe *et al* (17). Ultimately, the protective effect against organ dysfunctioning of this cytokine suppression has to be tested *in vivo*, using animal models for endotoxemia and infection as described before (19,22,25).

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CHAPTER 3

PARTICIPATION OF ß-ADRENERGIC RECEPTORS ON MACROPHAGES IN MODULATION OF LPS-INDUCED CYTOKINE RELEASE

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ABSTRACT

For several years it is known that ß-adrenergic receptor agonists have anti-inflammatory effects. However, little is known about the role of β-adrenergic receptors on macrophages in the modulation of cytokine production by β-agonists during inflammation. In this study, the presence of β-receptors on PMA-differentiated U937 human macrophages, and the participation of these receptors in the modulation of LPS-mediated cytokine production by β-agonists was investigated. Total β-receptor expression on undifferentiated (monocyte) and PMA-differentiated U937 cells was established using receptor binding studies on membrane fractions with a radio ligand. The expression of β-receptors proved to be significantly lower on monocytes than on macrophages, additionally a predominant expression of β2-receptors was found. Production of the cytokines TNF-α, IL-6, and IL-10 by LPS-stimulated differentiated U937 cells was measured in time. Peak concentrations for TNF-α, IL-6 and IL-10 occurred at 3, 12 and 9 hrs, respectively. When differentiated U937 cells were incubated with both LPS and the β-agonist clenbuterol the production of TNF-α and IL-6 was significantly reduced. However the production of IL-10 was increased. To study the mechanism of modulation of cytokine production in more detail, U937 macrophages were incubated with LPS/clenbuterol in combination with selective β1- and β2- antagonists. These results indicated that the β2- and not the β1-receptor is involved in the anti-inflammatory activity of clenbuterol.

INTRODUCTION

Macrophages are cells of the innate immune system and are a major source of inflammatory cytokines (1,2). These cells are present with different morphology in all parts of the body, for example in liver, brain and lungs. During the onset of acute inflammation and in the progress of chronic inflammation, cytokines play a pivotal role (3). Pro-inflammatory cytokines such as tumour Necrosis factor-α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6) aggravate the state of disease (e.g. induce tissue injury) (3,4). Anti-inflammatory cytokines such as interleukin-10 (IL-10) attenuate the inflammatory response by inhibiting release of pro-inflammatory cytokines (3).
Many studies have indicated that cAMP-elevating agents are able to modify the inflammatory cascade in a beneficial way. This anti-inflammatory effect was reflected in a reduced cellular secretion of inflammatory mediators like histamine, prostaglandins and cytokines (e.g.5,6,7). Levels of intracellular cAMP can be elevated by phoshodiesterase-4 (PDE4) inhibitors (6,8), or by inducers of the enzyme adenylate cyclase (8) . Also β-adrenoreceptor agonists (β-agonists) are able to elevate cAMP levels and have shown anti-inflammatory effects (5,7,9,10,11). Several studies have been performed in order to reveal nature and mechanism of anti-inflammatory effects of β-agonists. These studies were focussed on different β-agonists (5,10), and used different cell types (9,10,11). However, little has been published about the role of β-adrenoreceptors (β-receptors) on macrophages especially in the modulation of cytokine production by β-agonists during inflammation. Aim of this study was to investigate the presence of β-receptors on PMA-differentiated U937 human macrophage cells. Special interest was paid to participation of these receptors in modulation of lipopolysaccharide (LPS)-mediated cytokine production by β-agonists. Cytokine production was induced by incubating differentiated U937 cells with LPS, being a part of the outer membrane of Gram-negative bacteria that induces a general inflammatory response both *in vitro* and *in vivo* (3,12). Production of TNF-α, IL-6, and IL-10, was measured in time after addition of LPS to culture medium. To study in more detail the mechanism of modulation of cytokine production, U937 macrophages were incubated with LPS/clenbuterol in combination with selective antagonists for the β-receptors. Atenolol was used as a selective β1- antagonist and ICI-118,551 as a selective β2-antagonist.

MATERIALS AND METHODS

Chemicals

^{125}I-Iodocyanopindolol, (^{125}I-ICYP, 78 Gbq/mmol): Amersham (Buckinghamshire, UK), ICI-118.551: Research Biochemicals Int. (RBI, Natick, MA, USA), isoproterenol, atenolol, clenbuterol and all other chemicals and solutions: Sigma Chemical Co. (St. Louis, MO, USA).

Materials
Cell harvester (MPR-24): Brandel (Gaithersburg, USA). Equipment for radio ligand binding studies, Wallac 1450 microbeta liquid scintillation counter, and Victor 1420 multilabel counter: Wallac Oy (Turku, Finland).

Culturing U937 cells
U937 cells (human monocyte like, histocytic lymphoma) from 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 ,5% CO_2. U937 cells were either used as monocytes or differentiated into macrophages using phorbol myristate acetate (PMA, 10 ng/ml) according to standard procedures (13,14). The PMA-differentiated macrophages were allowed to recover for 48 hours, during which culture medium was replaced every day.

Isolation of Membrane Fractions and Receptor Binding Studies
Membrane fractions were isolated according to standard procedures (15) from either U937 monocytes or macrophages. In short, cells were washed with ice-cold phosphate buffered saline (PBS, pH=7.4). Subsequently homogenized in ice-cold buffer: 150 mM NaCl, 20 mM Tris-HCl and 1 mM EDTA, pH 7.5, containing PMSF and aprotinin as protease inhibitors . Homogenates were centrifuged at 100,000 x g, 30 min at 4°C. Membrane pellet was suspended in a reaction mixture (50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM EDTA: pH 7.5). Saturation binding assays and studies of competition between β-antagonists and radioligand were performed according to standard procedures. Membrane fractions were used at a concentration of 100 µg membrane protein per incubation. For saturation increasing concentrations of ^{125}I-ICYP (1.95- 125 pM) were added. Reactions were performed at 37°C for 60 min. The reaction was terminated by rapid vacuum filtration, radioactivity retained on glass fibre filters was counted. Non specific binding was defined as the amount of ^{125}I-ICYP binding measured in the presence of 100 µM isoproterenol. Competition studies between a β1-antagonist or β2-antagonist, and ^{125}I-ICYP for binding to membrane fractions (displacement studies) were performed with constant radioligand concentrations (62.5 pM) and varying concentrations of competitors (0.0 - 30 µM for atenolol and 0.0 - 20 µM for ICI-118,551). Reaction conditions were the same as for saturation studies. Fitting of ligand-binding and -displacement data, to obtain Bmax-, Kd- and logEC50- values, was performed using Graphpad Prism software (San Diego, USA).

Macrophage Activation and Cytokine Assays
U937 macrophages were cultured at a concentration of 1×10^6 cells/well. Cells were incubated respectively with LPS (*E.Coli* 0111:B4, 1µg/ml), LPS and β-agonist clenbuterol (10^{-6}M), and LPS/clenbuterol together with either atenolol (10^{-6}M) or ICI-118,551 (10^{-6}M). At regular time intervals, until 24 hours after incubation with LPS, culture medium was collected for determination of TNFα, IL-6 and IL-10 concentrations. After removal of culture medium the cells were lysed in 1.0 ml NaOH (0.1 M) and used for total cell protein determination by

method of Bio-Rad (modified Bradford). Cytokine levels in culture medium were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits for determination of these cytokines were purchased from CLB (Amsterdam, The Netherlands). Kits were used according to manufacturers protocol.

Statistical Evaluation
All values presented are means ± S.D. For statistical analysis the two-tailed Student's t-test was performed. The mean values of two groups were considered significantly different if $P < 0.05$.

RESULTS

Expression Levels of ß-Receptors
A saturable binding of the non-specific ß-agonist ^{125}I-ICYP was found on both cells. Non-specific binding was linear with increasing concentrations of radioligand and represented <12% of total binding around the Kd value (fig. 1A). A significant higher expression of ß-receptors was found on differentiated cells. Bmax values on monocytes and macrophages were 17.8±1 and 35.4±1 fmol/mg cell protein respectively, corresponding with approximately 2700 and 5000 sites/cell. Kd values for ^{125}I-ICYP on monocytes and macrophages were 10±4 pM and 24±2 pM, respectively. Displacement studies showed that antagonists inhibited the binding of the radioligand in a concentration dependent way (fig. 1B). LogEC50 values for atenolol of -7.4±0.05 and -7.7±0.1, and for ICI-118,551 of -8.5±0.1 and -8.4±0.1, on monocytes and macrophages respectively, were significantly different. The logEC50 values between undifferentiated and differentiated cells were similar.

Effect of ß-Agonist and -Antagonists on LPS-Induced Cytokine Release
Addition of LPS to differentiated U937 cells resulted in rapid release of TNFα in culture medium, reaching 2360±525 pg/mg cell protein, at 3 hrs and returning to control levels at 24 hrs after start of the incubation (fig 2A). A dramatic inhibition of TNFα release was observed when cells were incubated with LPS in combination with clenbuterol. At 3 hours the concentration was 468±117 pg/mg cell protein. Atenolol did not affect the inhibition of TNFα release. However, clenbuterol induced inhibition was less dramatic in the presence of ICI-118,551 (peak 1551±153 pg/mg cell protein). Clenbuterol, atenolol or ICI-118,551 in the absence of LPS had no effect on the release of TNFα and the other cytokines.
A steady increase of IL-6 was detected until 12 hours after addition of LPS (fig. 2B). Highest concentration was 1940±192 pg/mg cell protein. Only a slight decrease of IL-6 concentration was seen between 12 and 24 hours. When LPS was added together with clenbuterol, a steady increase of IL-6 was observed only until 6 hours. Between 6 and 24 hours the concentration of IL-6 remained at a steady level. Antagonists did not significantly alter the effect of clenbuterol.
Incubation of the U937 macrophages with LPS resulted in a peak concentration of IL-10: 2666±834 pg/mg cell protein, at 9 hours after addition of LPS (fig. 2C). Combination of LPS and clenbuterol initiated IL-10 release to a higher level as

when incubated with LPS alone. Highest concentration : 3615 ± 994 pg/mg cell protein. Additional atenolol initiated a substantial rise of IL-10, that exceeded levels of prevous incubations: peak concentration of 4716±429 pg/mg cell protein. Additional ICI-118,551 resulted in the same concentration curve as the curve for LPS alone.

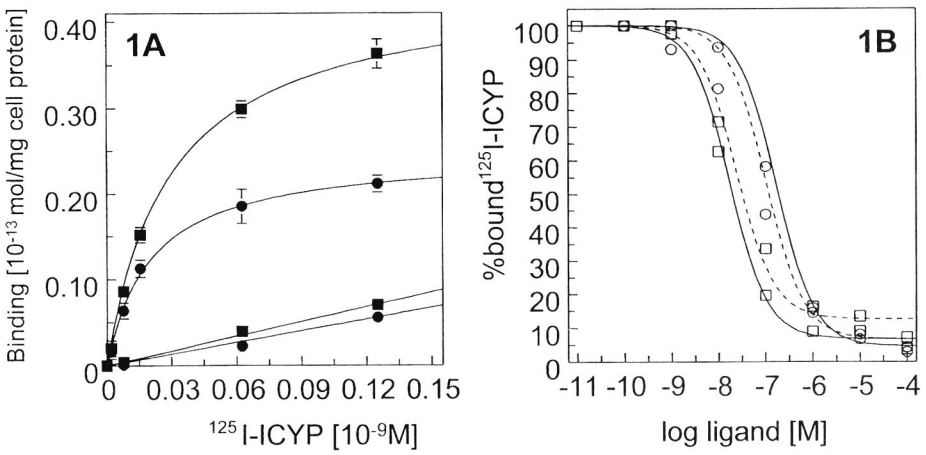

Figure 1.
Total and aspecific binding (A) to U937 monocytes (●) and macrophages (■), Bmax is 17.8±0.1 and 35.4±0.1 fmol/mg, respectively. Displacement (B) of ^{125}I-ICYP by atenolol (β1-antagonist,○) and ICI-118,551(β2-antagonist,□) from U937 monocytes ( ) and macrophages (- -) .

DISCUSSION

A majority of cytokines that are secreted after activation of macrophages can be detrimental to the host and aggravate the state of disease (3). This can lead to a more complicated (chronic) inflammation or even to shock, as is seen in sepsis. Therefore, pharmacological intervention in order to control the inflammatory cascade in a beneficial way is subject of many studies. For example, drugs that are able to elevate intracellular cAMP levels were proved to be potent suppressors of pro-inflammatory cytokine release. In this study β-agonists were chosen. Partly because they are able to elevate cAMP levels (via β-receptors), but also because they are already therapeutically used for treatment of diseases like asthma. However, untill now not all of their working mechanisms are fully understood. Main interest of this study was the mechanism how β-agonists are able to achieve their anti-inflammatory effect, in particular the secretion of which cytokines they are able to modulate. Three macrophage derived cytokines were studied: TNFα, IL-6, and IL-10. Because PMA-differentiated U937 cells are accepted as an *in vitro* model for human macrophages, and have been used by others to analyze the process of infection (16), these cells were used in this study.

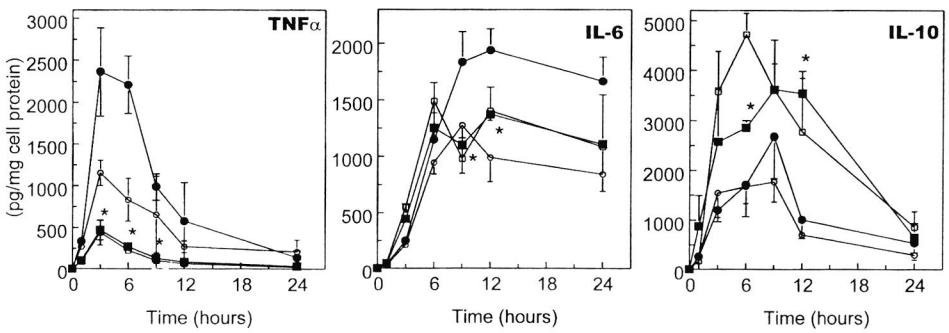

Figure 2.
Release of pro-inflammatory cytokines TNFα, and IL-6 and anti-inflammatory cytokine IL-10 by U937 macrophages. Concentrations in culture medium during incubation with LPS (1 μg/ml, ●), LPS and clenbuterol (10^{-6}M,■), LPS/clenbuterol and atenolol (10^{-6}M, □), and LPS/clenbuterol and ICI-118,551(10^{-6}M, ○). Data from one out of three representative experiments are shown (data points are means ±S.D.). * significant difference of LPS/clenbuterol vs. LPS ($P<0.05$).

To investigate the target for β-agonists, first the expression of β-receptors was determined. Although U937 monocytes were not further used in the cytokine assays, we were interested in the effect of PMA-mediated differentiation on the expression of β-receptors. A significant (two fold) increase was found on differentiated cells. This is in contrast to the results of Radojcic et al. (14). Although receptor numbers and affinities were in the same order of magnitude, Radojcic et al. found a decrease in β-receptor number after PMA treatment. Apart from small differences in experimental setup, major difference between the studies is that Radojcic et al, treated U937 cells for 24 hours with PMA. In this study cells were treated with PMA overnight and recovered from treatment for 48 hours. It is assumed that cells were in different condition at the moment of β-receptor determination. Competition binding assays indicated no differences in logEC50 on monocytes and macrophages for atenolol and ICI-118,551. However, logEC50 values between the two antagonists were significantly different. Aproximately 10 times more atenolol was needed to displace 50% of the radioligand than ICI-118,551. The affinity for their receptor subtypes is in the same order of magnitude based on (17): low affinity but high specificity. From these results it is not possible to give an exact ratio of the β1:β2-receptors on these cells, however the data indicate a predominant expression of β2-receptors.
Then attention was paid to the effect that is achieved via these receptors. Incubation of PMA-differentiated U937 cells with LPS resulted in release of all three cytokines measured. The LPS induced release of these cytokines was affected by β-agonist clenbuterol. In particular the effect on TNFα was rather dramatic as clenbuterol almost completely prevented TNFα release. These observations are in accordance with previous studies on effect of β-agonists on TNFα with different cell types (9,10,11). Use of antagonists revealed that the effect of clenbuterol on TNFα release is probably signalled via the β2-receptor, as ICI-118,551 was able to abolish partly the effect, whereas atenolol had no effect.
The effect of clenbuterol on anti-inflammatory cytokine IL-10 was completely different. LPS induced a release of IL-10 which was higher in the presence of clenbuterol. This result is in accordance with Suberville et al (18) using the β-agonist isoproterenol. However these data are in contrast to Haskó et al (19) who observed an inhibition by isoproterenol on IL-10 induction, but this could be due to the fact that IL-10 concentrations were measured only at 24 hours after LPS incubation. In our study it is observed that the effect of clenbuterol on IL-10 was even more pronounced in the presence of a β1-antagonist. An explanation might be that clenbuterol is not highly specific for the β2-receptor and occupation of the β1-receptor by atenolol results in more binding of clenbuterol at the β2-receptor. The effect of clenbuterol on IL-10 release also seemed to be signalled via the β2-receptor, as a β2-antagonist abolished the effect. Results on both TNFα and IL-10 supply additional prove to studies that claim that release of these cytokines can be regulated via cAMP dependent pathways (6).
Compared to the curves for TNFα and IL-10, the time-concentration curves for IL-6 were rather different. Maximum IL-6 concentrations were reached later and remained high for at least 24 hours after LPS incubation. Clenbuterol modified

the IL-6 response in two ways, firstly maximum IL-6 concentrations were reached earlier, secondly maximum concentrations were much lower. Both antagonists were not able to abolish the effect of clenbuterol. Studying IL-6 using LPS as an inducer is rather complicated. IL-6 release is not only induced by LPS, but also by TNFα or IL-1β (20), and regulated by even more mediators, like PGE_2 (21). So, in this study, it is strongly suggested that the onset in release of IL-6 is induced directly (by LPS) and additional release is mediated indirectly by other mediators like TNFα, as it was found that U937 cells release high levels of TNFα after LPS stimulation. The observed effect of clenbuterol on IL-6 release might be due to the inhibitory effect of clenbuterol on TNFα release. This idea is supported by the fact that clenbuterol only in a later phase inhibited LPS induced IL-6 release. An interesting difference between IL-6 and the two other cytokines, was that IL-6 remained high until at least 24 hours after incubation, whereas TNFα and IL-10 rapidly disappeared from the culture medium. Further research on the regulation of cytokine expression is necessary to explain this difference. In conclusion the observed effect of the β-agonist clenbuterol on the LPS induced cytokine release is signalled probably via the β2-receptors on these macrophages. The effects on TNFα and IL-10 appeared to be directly regulated via the β-receptors, but the effect on IL-6 seemed to be an indirect effect that was provoked by earlier changes in mediator release initiated by the β-agonist. Important observation was that although release of not all cytokines was directly regulated by β-agonists, the release can still be modulated by these agents. This branched effect of β-agonists appears to lead to a controlled suppression of an inflammatory response. Knowing more about the β-receptors on macrophages could be helpful in effective treatment of inflammation, with applications in not only asthma, but also for example, in rheumatoid arthritis and infections.

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CHAPTER 4

STEREOSELECTIVITY AT THE β_2-ADRENOCEPTOR ON MACROPHAGES IS A MAJOR DETERMINANT OF THE ANTI-INFLAMMATORY EFFECTS OF β_2-AGONISTS

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ABSTRACT

Previous research has shown that β-adrenoceptor (β-AR) agonists have potent anti-inflammatory capabilities, e.g. represented by suppression of release of the proinflammatory cytokines.

Aim of this research was to determine whether the effects of β-agonists on LPS-induced TNFα and IL-10 release are influenced by their different stereochemistry. In addition the role of the β-AR subtypes was studied. The effect of two stereoisomers of the selective β_2-AR agonist TA2005 ((R,R)- and (S,S)-) on the LPS-induced TNFα and IL-10 release by U937 macrophages was compared. The (R,R)-stereoisomer was 277 times more potent in inhibiting the TNFα release than the (S,S)-form. The (R,R)-stereoisomer also appeared to be more potent in increasing the IL-10 release. In radioligand binding studies the affinity of (R,R)-TA2005 for the β-adrenoceptor was 755 times higher than the (S,S)-TA2005 stereoisomer. In addition, the elevation of intracellular cAMP in U937 cells appeared to be stereoselective: (R,R)-TA2005 was more potent in elevating intracellular cAMP. The effect of both stereoisomers on the LPS-induced TNFα release could almost completely be antagonized by preincubation with the selective β_2-AR-antagonist ICI-118551. Further evidence that the effect of the β-agonists is mediated via the β_2-adrenoceptor subtype exclusively was acquired by incubation of U937 cells with selective β_1- and β_3-agonists. None of these receptor subtype agonists showed significant

suppressive effect on TNFα release. This study provides additional proof that the anti-inflammatory effects of β_2-agonists are mediated via the β_2-adrenoceptor and indicates that these effects are highly dependent on the stereoselectivity of the ligand.

INTRODUCTION

The β_2-adrenoceptor (β_2-AR) agonists are well known for their application as bronchodilators in asthma. The anti-inflammatory effects of β_2-AR agonists are an additional and therapeutically interesting property of these compounds. The main anti-inflammatory capabilities that have been studied are suppression of histamine release by mast cells, modulation of inflammatory cytokine release by several cells of the immune system, and modulation of release of prostaglandines or nitric oxide (NO) (1-4). Especially in acute inflammatory disorders, due to infection, endotoxemia, or during sepsis, a vast amount of mediators (prostaglandines, cytokines) are released during the onset of the inflammatory reaction. The release of these mediators is intended to defend the organism against the infectious agents. However, in excessive amounts they induce several detrimental side effects like organ failure, shock or eventually even death (5-7). The capability of β_2-AR agonists to modulate release of inflammatory mediators both *in vitro* and *in vivo* (8,9) may offer a potential therapeutical instrument for the treatment of (acute) inflammatory disorders to suppress the inflammatory reaction and eventually to prevent the detrimental side effects mentioned above. Although various information has become available on the anti-inflammatory effects of the β_2-AR agonists, not much data is available on the cellular mode of action. Bisonette *et al.* indicated that the anti-inflammatory effects are probably selective β_2-AR mediated (10). Others have pointed out that the ability to raise intracellular cAMP via the β_2-AR might be involved in the anti-inflammatory effects, since a similar mechanism has been reported for other drugs like phosphodiesterase-IV- (PDE-IV) inhibitors that are able to increase intracellular cAMP and hence inhibit release of proinflammatory cytokines (11-14).
One aspect that has not been studied in this respect is the concept of stereoselectivity. Since it is known that the binding of ligands and subsequent effects mediated via the β_2-AR can be influenced by stereoselectivity (15,16), it was supposed that this could also be an important aspect in the anti-inflammatory effects of β_2-AR agonists.
The primary objective of the present research was to study stereoselectivity in the anti-inflammatory activities of β_2-AR agonists at the levels of drug-receptor interaction, efficacy (second messenger cAMP) and final response (modulation of endotoxin-induced cytokine release). Secondary objective was to verify that the effect is mediated via the β_2-AR subtype only.
Macrophages are an important source of inflammatory mediators (7,17). In the present study the U937-derived macrophage cell line was used as test system. This U937 (human) cell line has been used previously to study the effects of β_2-AR agonists in a lipopolysaccharide (LPS, endotoxin) induced model of acute inflammation (18-20). Two stereoisomers, the (R,R) and (S,S)-stereoisomer, of the

selective β_2-AR agonist TA2005 (developed as a long acting β_2-AR stimulant, structure see Fig. 1) were evaluated by comparing their effects at the various levels (receptor, efficacy, response). The suppressive effect on the LPS-induced release of the pro-inflammatory cytokine TNFα and the enhancing effect on the release of the anti-inflammatory cytokine IL-10 were studied. These cytokines are both recognized for their crucial role during inflammation (21-24).
The results presented in this report show that stereoselectivity can substantially influence the anti-inflammatory mechanism and effects of β_2-AR agonists.

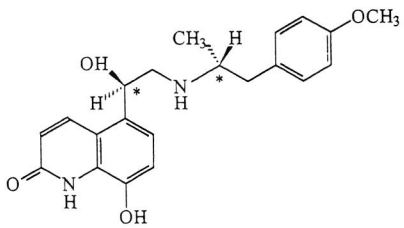

Fig. 1 The structure of the (*R,R*)-stereoisomer of TA2005; *asterisks* mark the two chiral carbon atoms

METHODS

Chemicals

^{125}I-Iodocyanopindolol, (^{125}I-ICYP, (78 GBq/mmol)) was purchased from Amersham Buckinghamshire, UK. ICI-118.551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropyl-aminobutane-2-ol) was obtained from Research Biochemicals Int. (RBI, Natick, MA, USA). (R,R)-TA2005 (8-hydroxy-5-[(1R)-1-hydroxy-2-[N-[(1R)-2-(p-methoxy-phenyl)-1-methylethyl] amino] ethyl] carbostyril, hydrochloride), and its (S,S)-stereoisomer (Tanabe, Seiyaku Co., Ltd, Osaka, Japan) were a kind gift of Dr. David Donnell, 3M Health Care Limited (Loughborough, UK). CGP12177A ((-)-4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one), PMSF (phenylmethylsulfonylfluoride), and aprotinin were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands), and xamoterol was acquired from Tocris/Biotrend (Cologne, Germany).

Culturing and differentiation of U937 cells.

U937 cells (human monocyte like, histocytic lymphoma) from 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, 5% CO_2. U937 cells were differentiated into a macrophage-like cell type using phorbol myristate acetate (PMA, 10 ng/ml) according to standard procedures (19,25). The PMA-differentiated macrophages recovered for 48 hours, during which culture medium was replaced daily.

Isolation of membrane fractions and β-AR binding studies

Membrane fractions were isolated from U937 macrophages as described previously (19,26). In short, cells were washed with ice-cold phosphate buffered saline (PBS, pH=7.4), and subsequently homogenized in ice-cold buffer (150 mM NaCl, 20 mM Tris-HCl and 1 mM EDTA, pH 7.5, containing the protease inhibitors PMSF and aprotinin (300 µM, and 200 U/l respectively, both obtained from Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Homogenates were centrifuged at 100,000 x g, 30 min at 4°C. Membrane pellet was suspended in a reaction mixture (50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM EDTA: pH 7.5). Membrane fractions were stored at –80 °C until further use. Competition assays (displacement studies) between β-AR agonists (10^{-13}M - 10^{-4}M) and radioligand (^{125}I-ICYP, 10 pM) were performed according to standard procedures. Membrane fractions were used at fixed amounts of 100 µg membrane protein per incubation. Assays were performed for 2 hours at room temperature. The reaction was terminated by rapid vacuum filtration using a cell harvester (MPR-24, Brandel, Gaithersburg, USA), radioactivity retained on glass fibre filters (Wallac Oy, Turku, Finland) was counted. Fitting of ligand binding data, to obtain K_i-values, was performed using Graph Pad Prism (Graph Pad Prism San Diego, CA,USA).

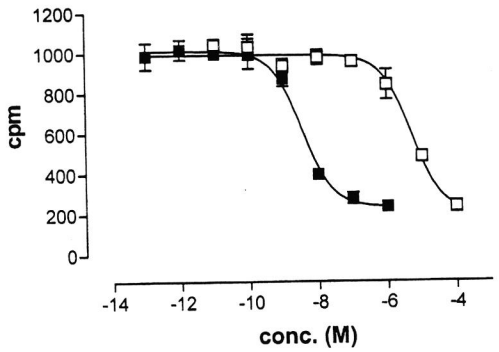

Fig. 2 Radioligand displacement studies using fixed amounts of U937-macrophage membrane fractions and 10 pM [^{125}I]iodo-cyanopindolol. The radioligand was displaced according to a one-site competition model using concentration ranges of the (*R,R*)- and (*S,S*)-stereoisomers of TA2005 (β_2-AR agonists). K_i-values of (*R,R*)- (*black squares*) and (*S,S*)-TA2005 (*white squares*) were 2.53±1.1 nM, and 1.91±0.9 µM, respectively (ratio between these values is 755). Data are means ± SD of three independent experiments with duplicate samples

Measurement of intracellular cAMP formation

U937 macrophages were cultured in 12-wells cell culture plates (Corning, Schiphol, The Netherlands) at a concentration of 1×10^6 cells/well. Cells were incubated with LPS (*E.coli* 0111:B4, 1 µg/ml), and LPS together with either (R,R)-TA2005 or (S,S)-TA2005 (10^{-10}M - 10^{-5}M) respectively. In addition, cells that were used for antagonism studies were preincubated for 1 hour with the β2-AR antagonist ICI-118,551 (10^{-7}M) before LPS and TA2005 were added. After 30 minutes incubation at 37°C, the culture medium was aspirated and cells were immediately placed on ice. Icecold 70% (v/v) ethanol was added (1 ml/well) to extract cAMP from the cells. Subsequently, the ethanol solutions were transferred to tubes, and ethanol was evaporated at 45°C, under a constant stream of N_2. The residues in the tubes were resuspended in PBS and cAMP levels were determined by ELISA using a cAMP assay kit (Cayman Chemical, Ann Arbor, USA). Data were expressed as pmol cAMP per one million cells.

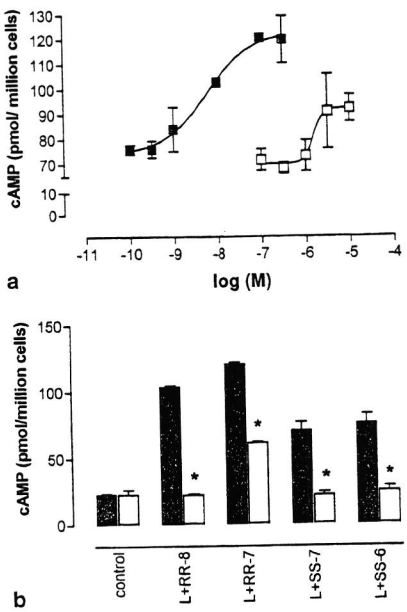

Fig. 3 **a** Stimulation of intracellular cAMP production after incubation of U937 macrophages with fixed amounts of LPS (1 µg/ml) and concentration ranges of (*R,R*)- and (*S,S*)-TA2005 stereoisomers [*black squares:* (*R,R*), *white squares:* (*S,S*)]. Intracellular cAMP levels were determined after 30-min incubation at 37°C with the β_2-agonists. Curves were fitted according to a sigmoidal dose-response model (variable slope). Respective EC_{50}-values for (*R,R*)- and (*S,S*)-TA2005 were 6.22 nM and 1.54 µM (ratio between these values is 248). Data are means ± SD (n=4). **b** Intracellular cAMP production in U937 macrophages initiated by stereoisomers of TA2005 is blocked (and returned to control levels) by preincubation with the selective β_2-AR antagonist ICI-118551. Cells were incubated with LPS (1 µg/ml) and TA2005 only (*black bars; RR-8* (*R,R*)-TA2005 [1×10^{-8} M], *SS-7* (*S,S*)-TA2005 [1×10^{-7} M]), or preincubated for 1 h with the antagonist ICI-118551 and subsequently incubated with LPS and TA2005 (*white bars*). Data are means ± SD (n=3). *Significant inhibition of TA2005-induced cAMP production after preincubation with ICI-118551

Macrophage activation and cytokine assays

U937 macrophages were cultured at a concentration of 1×10^6 cells/well (12-wells plate cell culture plates). Cells were incubated with the endotoxin LPS (*E.coli* 0111:B4, 1 µg/ml), or LPS together with concentration ranges of the β-AR agonists xamoterol, TA2005 (R,R and S,S), and CGP12177A ($1x10^{-11}$-$1x10^{-5}$M) . In addition, the cells were preincubated for 1 hour with ICI-118,551 (10^{-7}M) before LPS and the β-agonists were added. Culture medium was collected after 3 hrs of incubation for determination of TNFα concentrations, and after 6 hours for IL-10 determination. TNFα and IL-10 levels in culture medium were determined by ELISA, using TNFα and IL-10 ELISA kits (CLB, Amsterdam, The Netherlands).

Statistical evaluation

The values presented are means ± S.D. or means ± SEM. For statistical analysis the two-tailed Student's t-test was performed. The mean values of two groups were considered significantly different if $P < 0.05$.

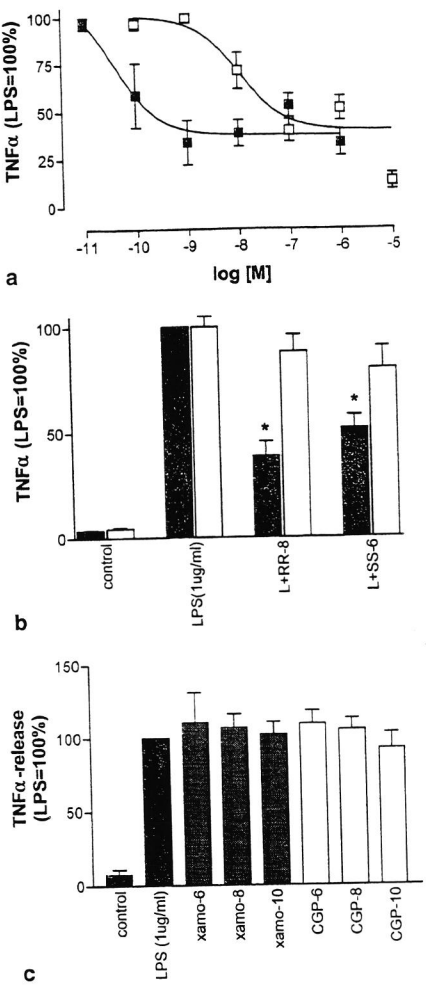

Fig. 4 **a** Inhibition of TNFα release from U937 macrophages with LPS (1 µg/ml) and concentration ranges of *(R,R)*- (*black squares*) and *(S,S)*-TA2005 (*white squares*). Concentrations of TNFα were determined in the culture medium after 3-h incubation at 37°C with LPS and the β_2-AR agonists. Curves were fitted according to sigmoidal dose-response curve (Hill slope =–1). Respective IC_{50}-values for *(R,R)*- and *(S,S)*-TA2005 were 38.7 pM and 10.8 nM (ratio between these values is 277). Data are means ± SEM (n=6). **b** Inhibition of LPS-induced TNFα release from U937 macrophages by stereoisomers of TA2005 [*(R,R)*- at 1×10^{-8} M, *(S,S)*- at 1×10^{-6} M; *black bars*] is blocked by preincubation with the selective β_2-AR antagonist ICI-118551 (10^{-7} M). Cells were preincubated for 1 h with the antagonist and subsequently incubated with LPS and TA2005 (*white bars*). Data are means ± SEM (n=6). *Significant reversal of TA2005-induced inhibition of TNFα release after preincubation with ICI-118551. **c** TNFα release from U937 macrophages with LPS (1 µg/ml; *black bar*), or LPS in combination with several concentrations of the selective β_1- and β_3-AR agonists xamoterol (*grey bars*) and CGP12177A (*white bars*). TNFα release was not significantly inhibited by the selective β_1- or β_3-agonist. Concentrations of TNFα were determined in the culture medium after 3-h incubation at 37°C with LPS and the β-AR agonists. Data are means ± SEM (n=6)

RESULTS

A receptor binding study was performed to determine the affinity for the β-AR of the (R,R)- and (S,S)-stereoisomers of TA2005. In a competition assay using a radiolabelled ligand for the β-AR (^{125}I-ICYP) both stereoisomers were able to displace the radioligand according to a one-site competition model (Fig. 2). The (R,R)-stereoisomer had a much higher affinity for the β-AR than the (S,S)-form. Respective K_i values were 2.53 ± 1.1 nM for (R,R)-TA2005 and 1.91 ± 0.9 μM for (S,S)-TA2005, yielding a stereoselectivity index of 755.

Next, the effects at signal transduction level were studied. Therefore the intracellular cAMP levels were determined after incubation of the cells with concentration ranges of (R,R)-TA2005 and (S,S)-TA2005, with or without LPS. Both stereoisomers were able to increase the basal intracellular cAMP-levels in a dose-dependent manner, as well in the experiments with additional LPS, as in the experiments without LPS. Addition of only LPS (1 μg/ml) to the cell cultures did not alter the intracellular cAMP-levels significantly. Also at this level stereoselectivity was found in the ability to increase intracellular cAMP. The (R,R)-stereoisomer was much more potent than the (S,S)-form, represented by the lower concentrations needed for (R,R)-TA2005 to accomplish its effect, and the higher levels of cAMP achieved (Fig. 3A). EC_{50} values of (R,R)- and (S,S)-TA2005 were 6.22 ± 3.38 nM and 1.54 ± 0.44 μM (means ± SEM), respectively (ratio of stereoselectivity based on these values is 248). Preincubation of the cells with the selective β2-AR antagonist ICI-118551 prevented the increase of cAMP followed by incubation with the stereoisomers (Fig. 3B).

When U937 macrophages were incubated with LPS the cells responded by release of TNFα into the culture medium. The peak concentration of TNFα was detected at three hours of incubation with LPS. When the U937 cells were incubated with LPS together with concentration ranges of (R,R)- and (S,S)-TA2005 a concentration-dependent inhibition of LPS-induced TNFα release was found at 3 hours after incubation (Fig. 4A). The effect was found to be stereoselective because the (R,R)-stereoisomer inhibited TNFα release at much lower concentrations than the (S,S)-stereoisomer, as evident from the IC_{50}'s of 38.7 pM and 10.8 nM, respectively (stereoselectivity index 277). When the cells were preincubated with the β_2-AR antagonist ICI-118551 the inhibitory effects of (R,R)- and (S,S)-TA2005 were blocked (Fig. 4B). Figure 4C shows that agonists selective for the β1- or the β3-AR did not suppress the LPS-induced TNFα-release significantly. When cells were incubated with only agonists or antagonist (without LPS) no effect on the TNFα-release was observed (data not shown).

Upon incubation with LPS U937 cells released IL-10 into the culture medium, reaching peak concentrations at 6 hours after LPS incubation. When the U937 macrophages were incubated with concentration ranges of the two stereosiomers of TA2005 a concentration-dependent increase in the release of the anti-inflammatory cytokine IL-10 was found (Fig. 5A). A considerable difference in the effect of (R,R) and (S,S)-TA2005 was found, represented by concentrations needed and the extent of the increase. For (S,S)-TA2005 only at concentrations >1μM a significant additional increase of IL-10 could be found, in contrast to

incubations with (R,R)-TA2005 where at 0.1 nM a significant additional release of IL-10 was found. After preincubation of the cells with the β2-AR antagonist ICI-118551 part of the increased release by the stereosiomers was partially blocked (Fig. 5B). No effect on IL-10 release was observed when cells were incubated with only agonists or antagonist, in the absence of LPS (data not shown).

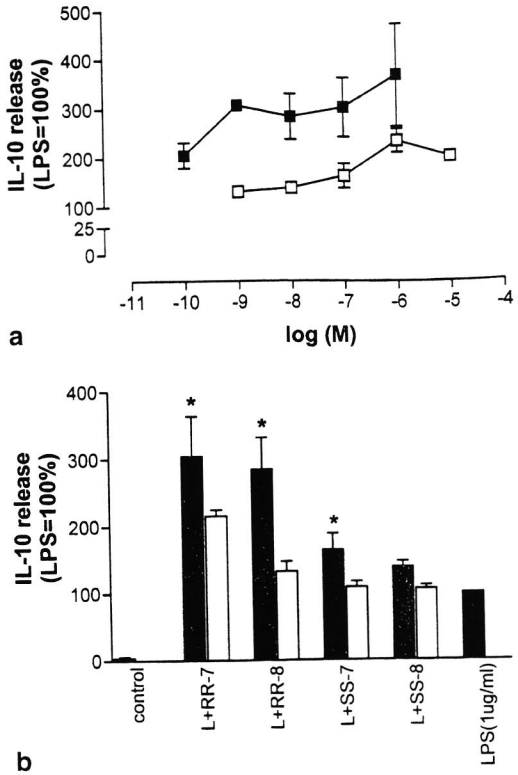

Fig. 5 **a** Increase of LPS-induced (1 μg/ml) IL-10 release from U937 macrophages incubated with concentration ranges of (*R,R*)-(*black squares*) and (*S,S*)-TA2005 (*white squares*). Concentrations of IL-10 were determined in the culture medium after 6-h incubation at 37°C with LPS and the β_2-AR agonists. Data are means ± SEM (n=4). **b** Reversal of TA2005-induced increase of IL-10 release from U937 macrophages. The increased release of LPS-induced (1 μg/ml) IL-10 release by TA2005 (*black bars*) is partially inhibited after preincubation with the selective β_2-AR antagonist ICI-118551 (10^{-7} M; *white bars*). Cells were preincubated for 1 h with the antagonist and subsequently incubated with LPS and TA2005 (*RR-8* (*R,R*)-TA2005 [1×10^{-8} M], *SS-7* (*S,S*)-TA2005 [1×10^{-7} M]). Data are means ± SEM (n=4). *Significant increase compared to incubations with only LPS

DISCUSSION

In this study it was shown that stereoselectivity at the β-AR substantially influences the mechanism and extent of the modulation of LPS-induced cytokine release, initiated by β_2-AR agonists. The concept of stereoselectivity in ligand binding and effects mediated via the β_2-AR is well known (15,16,27). However, this phenomenon had not been investigated yet for the anti-inflammatory properties of β-AR agonists. Moreover, the mechanisms by which the anti-inflammatory effects of β-AR agonists are achieved at cellular level are still poorly characterized. The aim of this research was to study stereoselectivity in the effects of a selective β_2-AR agonist (TA2005) at three separate levels: drug-receptor interaction, effectuation (effect at intracellular signaltransduction level) and final response. As an end-point for the anti-inflammatory effects of the β_2-AR agonists the modulation of the pro-inflammatory cytokine TNFα and the anti-inflammatory cytokine IL-10 were chosen. In addition, the receptor subtype selectivity (β_1-, β_2-, or β_3-AR) of the effects was investigated.

When the affinities of two stereoisomers of the selective β_2-AR agonist TA2005 for the β-AR were compared in a receptor binding study it was found that the (R,R)-isomer had a 755-times higher affinity than the (S,S)-isomer. The higher affinity of the (R,R)-TA2005 for the β-AR is in line with the stereochemistry of the endogenous ligand adrenaline (epinephrine) which has R-configuration at the benzylic position. An important intracellular secondary messenger that has been reported to be involved in the pathway leading to anti-inflammatory effects of several drugs is cAMP (11,12,28). For example, phosphodiesterase-IV (PDE-IV) inhibitors inhibit the enzymatic breakdown of cAMP, and it has been shown that elevation of intracellular levels of cAMP had a suppressive effect on TNFα release and augmented IL-10 release (14,28,29). In this study the cAMP levels were determined in U937 macrophages upon incubation with the two stereoisomers. Both stereoisomers were able to increase cAMP in a concentration-dependent manner. However, the (R,R)-isomer of TA2005 increased intracellular cAMP at much lower concentrations. Furthermore the (R,R)-isomer elevated cAMP to much higher intracellular levels than the (S,S)-isomer, which behaved as a partial agonist in this assay. This effect on cAMP of the (R,R)- and (S,S)-stereoisomers of TA2005 could be antagonized by preincubation with the selective β_2 -AR antagonist ICI-118551.

Subsequently, it was shown that the stereoselectivity detected at receptor- and effectuation-level can also be found at the response-level. The two stereoisomers of TA2005 differed in their ability to suppress the release of TNFα, or to increase the release of IL-10. The ratios for stereoselective inhibition of TNFα release and induction of IL-10 release are similar to the order of magnitude of the ratio of affinities for the receptor. These results clearly show that the stereoselectivity in binding at the receptor is reflected by the modulation of LPS-induced cytokine release. The effects of these β_2-AR agonists on TNFα and IL-10 release are in accordance with previously reported results on the effects of epinephrine, the endogenous ligand for the β-AR, on plasma concentrations of TNFα and IL-10 during human endotoxemia (30).

In addition, when the receptor subtype selectivity was investigated, it was shown that β-agonists selective for the β_1- or β_3 -AR were not able to supress the TNFα release. The effects on TNFα release of both TA2005 stereoisomers could be antagonized after preincubation with ICI-118551, a selective β_2-AR antagonist. Together with the results on the β_1- and β_3-agonists these data indicate that the anti-inflammatory effect is solely mediated via the β_2-AR on this cell type. The effect of (R,R)- and (S,S)-TA2005 on IL-10 was only partially antagonized by the selective β_2-antagonist ICI-118551. This observation might be explained by kinetics of mRNA- and protein-synthesis after LPS incubation, since IL-10 is synthesized and released later than TNFα when monocytes or macrophages are incubated with LPS (13,31). Moreover, the release of IL-10 can also be influenced by TNFα and other inflammatory mediators via feedback mechanisms (7), therefore it is assumed that the effect of β_2-AR agonists on IL-10 is partly indirect mediated.
In conclusion, besides the clear stereoselectivity in the effects of β_2-AR agonists, the fact that these compounds are able to suppress the LPS-induced release of a pro-inflammatory cytokine and to increase the release of an anti-inflammatory cytokine, offers interesting perspective for anti-inflammatory therapy.

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CHAPTER 5

MODULATION OF LPS-INDUCED TNFα -AND IL-10 mRNA EXPRESSION BY A ß-ADRENOCEPTOR AGONIST IN U937-MACROPHAGES

Manuscript in preparation

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ABSTRACT

TNFα and IL-10 exert key roles in several acute and chronic inflammatory disorders. The therapeutic modulation of production and release of these cytokines continues to be subject of intensive research. The β_2-adrenoceptor agonists have been shown to be potent modulators of inflammatory cytokine release. However, the exact intracellular mechanisms leading to the altered releases of the inflammatory peptides have not yet been fully elucidated. In the present study, the expression levels of TNFα and IL-10 in resting-, and lipopolysaccharide (LPS)-activated U937 macrophages have been studied after incubation with the β_2-adrenoceptor β_2-AR agonist clenbuterol and dibutyryl-cAMP (db-cAMP).

The β_2-AR agonist was demonstrated to evoke a decrease in LPS-induced TNFα mRNA expression, which is comparable with previously published results on protein expression. In addition, clenbuterol increased the LPS-induced IL-10 mRNA expression, and also appeared to alter the kinetic profile of IL-10 mRNA expression. These data indicate that the modulation of LPS-induced TNFα and IL-10 production by β_2-AR agonists appears to be regulated at the transcription level.

INTRODUCTION

The pivotal role of the inflammatory mediators TNFα and IL-10 during the onset and development of an (acute) inflammatory reaction is generally acknowledged (1,2). Both TNFα and IL-10 are released relatively early (within several hours) during the onset of an inflammatory response. The pro inflammatory cytokine TNFα is generally assumed to play an initiating role in the development of the inflammatory cascade. Due to this, this cytokine is often referred to as major trigger of detrimental side-effects of an inflammatory reaction. In contrast, the cytokine IL-10 possesses anti-inflammatory characteristics. Therapeutic modulation of the production and release of these and other cytokines might be important in the control of (excessive) inflammatory reactions.
Previously, we have shown that β_2-adrenoceptor (β_2-AR) agonists are able to modulate the LPS-induced cytokine release at protein level both *in vitro* in culture medium of macrophage cell lines, or primary isolated porcine macrophages, and *in vivo* in the blood of endotoxemic rats (3-5). The intracellular mechanisms behind the observed modulation by β_2-AR agonists of LPS-induced cytokine release have not been yet been fully elucidated.
In the present study, the expression of TNFα and IL-10 mRNA will be studied in U937 cells, a human monocyte-derived macrophage cell line. The effects of the selective β_2-AR agonist clenbuterol on the expression of TNFα and IL-10 mRNA in both resting-, and LPS-activated U937 macrophages will be investigated.

MATERIAL AND METHODS

Chemicals

Clenbuterol, phorbol myristate acetate (PMA), dibutyryl-cAMP (db-cAMP), and LPS (*E.Coli* 0111:B4) were all obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands).

Culturing and differentiation of U937 cells

U937 cells (human monocyte like, histocytic lymphoma) from 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, 5% CO_2. U937 cells were differentiated into a macrophage-like cell type using PMA (10 ng/ml) according to standard procedures (3,6). Until use of the PMA-differentiated macrophages in experiments (at least 48 hours after PMA-challenge) the culture medium was replaced daily.

Macrophage activation and RNA isolation

U937 macrophages were cultured at a concentration of 1×10^6 cells/well in 12-wells cell culture plates. Cells were incubated with the endotoxin LPS (1 µg/ml), or simultaneously with LPS and db-cAMP (10µM) or the β-AR agonist clenbuterol (1 µM). Control incubations consisted of either vehicle (PBS), clenbuterol, or db-cAMP only.

At several time intervals after the start of the incubations (0.5, 1, and 2 hours), cells were put on ice, the culture medium was aspirated, and the cells were washed once with icecold PBS (Life Technologies). Subsequently, cells were lysed using Trizol® reagent (Life Technologies). Samples were stored at -20°C until further analysis. The RNA was extracted from the Trizol®-samples according to the protocol as provided by the manufacturer.

cDNA synthesis and oligonucleotides

Aliquots of each total RNA extraction were reversely transcribed into cDNA, with the use of AMV reverse-transcriptase according to the manufacturers protocol (Promega Benelux B.V., Leiden, The Netherlands). From the resulting cDNA mixture 2.5 µl was used for amplification by PCR (see below).
The following oligonucletides specific for human GAPDH, TNFα, and IL-10 were synthesized (Eurogentec, Luik, Belgium):
Primer-sequences:
GAPDH-upper: 5'-AGA-TCA-TCA-GCA-ATG-CCT-CC-3'
GAPDH-lower: 5'-TAC-ATG-ACA-AGG-TGC-GGC-TC-3'
IL-10-upper: 5'-CAC-GCT-TTC-TAG-CTG-TTG-AGC-3'
IL-10-lower: 5'-ACC-CAG-GCT-GGA-GTA-CAG-G-3'
TNFα-upper: 5'-CCT-CAG-CCT-CTT-CTC-CTT-CC-3'
TNFα-lower: 5'-AGG-AGG-TTG-ACC-TTG-GTC-TG-3'

PCR

Polymerase chain reaction (PCR) was performed on the Geneamp® PCR system 9700 (Perkin Ellmer, Applied biosystems, USA), all reagents necessary were obtained from Promega Benelux B.V. The primers used (as described above) resulted in fragments of 747 bp (GAPDH), 413 bp (TNFα) and 496 bp (IL-10), respectively.
The resulting PCR mixtures were analysed on 1.5 % agarose gels. Quantification of mRNA was performed using the Fluor-S™ Multi-imager, in combination with Multianalyst-software (version1.1) for image analysis systems (Bio-Rad laboratories, Hercules, CA, USA).

Statistical evaluation

The values presented are means ± S.D. For statistical analysis the two-tailed Student's t-test was performed. The mean values of two groups were considered significantly different if $P < 0.05$.

RESULTS

The incubation of U937 macrophages with LPS (1µg/ml) evoked a strong increase of both TNFα and IL-10 intracellular mRNA expression, compared to control incubations (PBS- treated macrophages) that showed no significant expression of respective mRNA.
The increase in LPS-induced mRNA expression appeared to be time-dependent. TNFα-mRNA was found to reach its maximum between 30 to 60

minutes after LPS incubation (data not shown). The LPS-evoked increase in IL-10-mRNA appeared to reach its maximum one hour later than TNFα, at 2 hours after start of the incubations.
Clenbuterol completely inhibited the LPS-induced increase in TNFα-mRNA in U937. In addition, in U937-cells incubated with LPS together with db-cAMP, the expression levels of TNFα-mRNA were much lower compared to cells that had been treated with only LPS (Fig. 1). In control experiments of U937-cells incubated with either clenbuterol or db-cAMP only, no expression of TNFα-mRNA was detected.

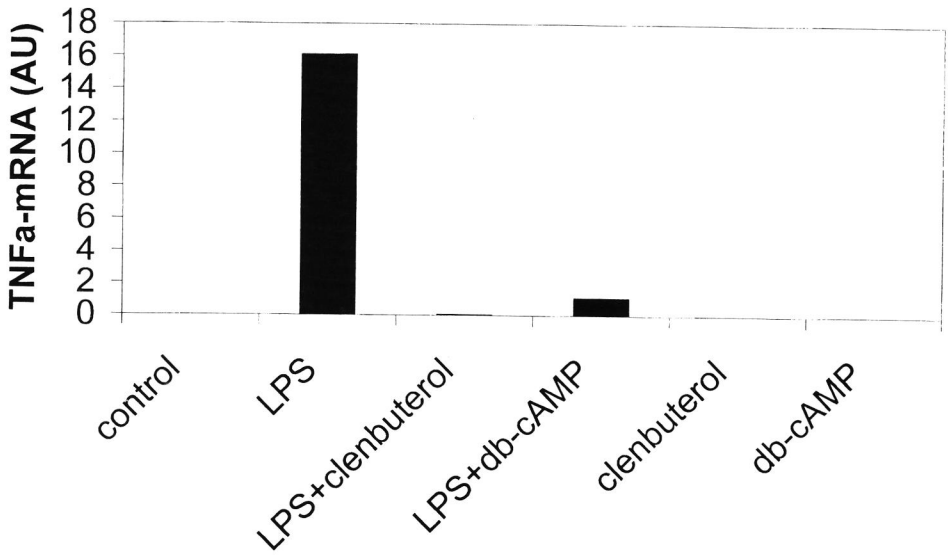

Figure 1
Increase of TNFα mRNA in U937 cells. LPS (1 μg/ml) induced increase in TNFα-mRNA expression is inhibited by the β-AR agonist clenbuterol (1 μM), and by db-cAMP (10 μM). Data on TNFα-mRNA expression are adjusted for GAPDH expression.

The experiments with U937 cells in which the intracellular LPS-induced mRNA levels of IL-10 were investigated, showed that co incubation with clenbuterol further increased the LPS-induced IL-10-mRNA expression. Moreover, clenbuterol appeared to alter the timing of mRNA-expression in these cells, since a significant increase of IL-10 mRNA was already detected after 30 minutes of co incubation with clenbuterol and LPS, whereas the expression of IL-10-mRNA at that timepoint was not yet detectable in incubations with only LPS (Fig 2). In control experiments, it was shown that cells incubated with clenbuterol only, no modulation of intracellular IL-10 mRNA expression was found.

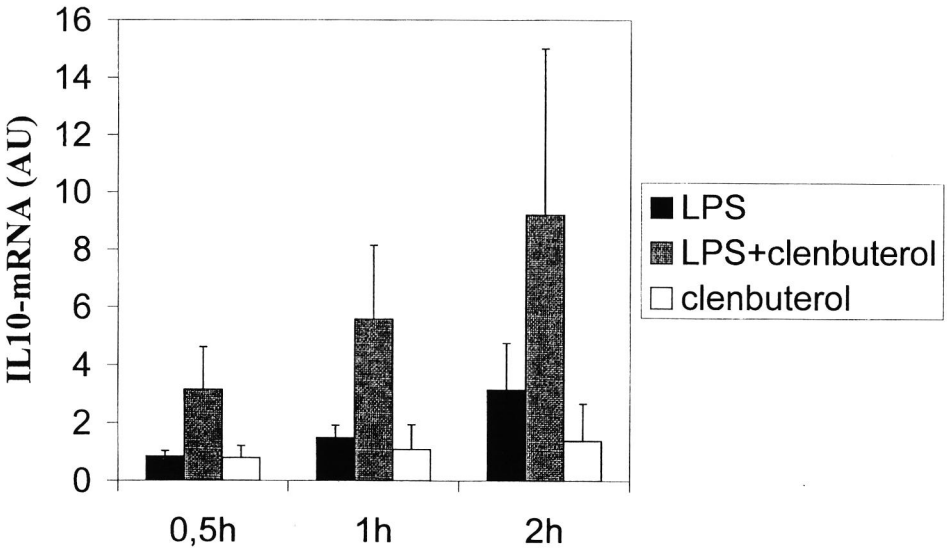

Figure 2
Increase of IL-10 mRNA in U937 cells. LPS (1 µg/ml) induced increase in IL-10-mRNA expression is further increased by the β-AR agonist clenbuterol (1 µM), and also the rate of IL-10mRNA synthesis appears to be affected by clenbuterol. Data on IL-10mRNA expression are adjusted for GAPDH expression, and standardized relative to control incubations (PBS=1).

DISCUSSION

It has been shown previously, that β_2-AR agonists are able to modulate LPS-induced cytokine release at protein level. However, the intracellular mechanisms leading to the anti-inflammatory effects of β_2-AR agonists have not yet been fully elucidated. It has been suggested that stimulation of cAMP-dependent signal transduction pathways in macrophages are important for the regulation of cytokine-release by various other anti-inflammatory agents (7-9). Furthermore, the β_2-AR agonists are capable to potently increase intracellular cAMP, as we have demonstrated before (10).
In the present study, the expression of TNFα-mRNA in U937 macrophages was found to be up regulated by LPS. When these cells were co incubated with LPS and the β_2-AR agonist clenbuterol, it was found that the LPS-induced induction of TNFα-mRNA was completely blocked. A similar result was found when U937 macrophages were co incubated with LPS and the synthetic cAMP derivative db-cAMP. These results show that two different stimuli that both induce intracellular cAMP-levels had the same inhibiting effect on LPS-induced

cytokine-mRNA. These results are in accordance with Hetier et al. (11), who demonstrated a decrease in LPS-induced TNFα-mRNA in microglial cells when these cells were coincubated with the non-selective β-AR agonist isoproterenol. In addition, Eigler et al (7) obtained comparable results, using phosphodiesterase-inhibitors as inucers of cAMP dependent pathways, in primary human mononuclear cells. Therefore, it can be concluded that inhibition of intracellular TNFα-mRNA levels contributes to the previously reported inhibition of LPS-induced TNFα production at protein level (3,10) via a cAMP-dependent mechanism. Thus β_2-AR agonists modulate LPS-induced TNFα production at the transcription level in U937-cells.
Regarding the modulation of LPS-induced IL-10 production by β_2-AR agonists, a similar mechanism is proposed. The β_2-AR agonist clenbuterol was found to increase the LPS-induced IL-10 mRNA expression in U937 cells. This regulation at the transcriptional level most likely explains the increase in IL-10 release by β_2-AR agonists observed previously (3,10). These results on IL-10 are in accordance with Suberville et al (12), who demonstrated that LPS induced IL-10 from mouse peritoneal macrophages is inhibited by the non-selective β-AR agonist isoproterenol at both protein and mRNA level, although in that case a two hour preincubation with isoproterenol was needed in order to achieve maximal IL-10 induction. Eigler et al (7) demonstrated that other cAMP-elevating agents regulate LPS-induced IL-10 release at the transcriptional level.
In the present study, we found a change in the production rate of IL-10 mRNA caused by clenbuterol. Clenbuterol caused a shift in the production of this cytokine, IL-10 mRNA becoming already detectable at 30 minutes after the start of the co incubations. This finding is in accordance with the previously observed change in IL-10 (protein) time-concentration profile in the culture medium of macrophages (3) and in blood of endotoxemic rats (chapter 8). In the *in vivo* study clenbuterol was found to accelerate the systemic release of IL-10 in rats.
In conclusion, modulation by β_2-AR agonists of the cytokine mRNA-expression levels most likely contributes to the previously observed modulation of inflammatory cytokine release from U937 macophages and other immune cells at the protein level.

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CHAPTER 6

DIFFERENTIAL EFFECTS OF CAMP ELEVATING AGENTS ON ISOLATED PORCINE KUPFFER CELLS AND HEPATOCYTES DURING AN INFLAMMATORY RESPONSE

Submitted

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ABSTRACT

The pro-inflammatory agents TNFα and nitric oxide (NO) are generally recognized as important mediators of liver-failure during a systemic inflammatory response. Suppression of the release of these compounds might prevent this inflammation-associated liver-damage. In this study primary isolated porcine liver macrophages (Kupffer cells) and hepatocytes were used as model system to test compounds that act via different mechanisms to elevate intracellular cAMP, since stimulation of cAMP-dependent signal transduction pathways have been associated with anti-inflammatory effects. Selective agonists for the adenosine-2A receptor (CGS21680), the β_2-adrenoceptor (clenbuterol) , and a selective phosphodiesterase-IV inhibitor (rolipram) elevated intracellular cAMP levels in Kupffer cells and were found to inhibit LPS-induced TNFα release. LPS-induced NO-release was not affected in monocultures of hepatocytes, but could partially be decreased in cocultures of hepatocytes and Kupffer cells, indicating a Kupffer cell associated mechanism of inhibition. Since the liver is an important source of inflammatory mediators, the balance between cAMP-elevation and inhibition of inflammatory mediators is proposed to be very important in this organ.

INTRODUCTION

Multiple organ failure (MOF) is often observed during (acute) systemic inflammatory diseases, like severe infections, sepsis and SIRS (1,2). MOF in general, and the inflammation-associated damage of the liver in particular, are important determinants of the outcome of a systemic inflammatory response and the eventual survival of patients, and are therefore of prime clinical importance (3). Pro-inflammatory mediators which are released in excessive amounts during systemic inflammation, e.g. cytokines (4,5), reactive oxygen- and nitrogen-intermediates (6-8), play a role as key mediators involved in organ failure (9). The resident macrophages of the liver, Kupffer cells, are generally recognized for their important role during the onset of a systemic inflammatory response, partly due to their excessive release of pro-inflammatory mediators (10,11). For example, the release of TNFα by Kupffer cells is regarded as crucial in the impairment of hepatocyte activity (5,12). Nitric oxide (NO), which is also produced in vast amounts in the liver during an inflammatory response, is (at high concentrations) held responsible for cytoxicity and the decreased biotransformation capacity (8,13).
TNFα is released during the initial phase of an inflammatory response, and inhibition of release of this pleiotropic cytokine is assumed to prevent the development of the inflammatory cascade (4,14,15). The potential beneficial effects of TNFα inhibition might therefore be due to the prevention of additional release of inflammatory mediators, like other cytokines and NO from liver cells (14), and the prevention of impaired liver function.
Several strategies have been designed to suppress the release of inflammatory mediators in order to prevent or reduce associated organ failure. One approach to modulate inflammatory mediator release is the elevation of intracellular cAMP, for example by stimulation of G-protein coupled receptors or inhibition of phosphodiestereases in cytokine producing cells, which has been shown to be succesful in different experimental setups (16-19).
Although Kupffer cells represent an important population of resident macrophages in the body, and are an important source of inflammatory mediators (20,21), relatively little attention has been paid to this cell-type with respect to the role of cAMP in the control of cytokine release. In the present study, several compounds were compaired for their effectiveness to elevate cAMP and subsequently inhibit LPS-induced TNFα release from isolated Kupffer cells. In addition, the effect of the cAMP-elevating agents on the release of NO from primary liver cell cultures was determined. The compounds used have been selected for their different mechanisms to elevate intracellular cAMP. Increased synthesis of intracellular cAMP was achieved by activation of adenylyl cyclase via activation of the adenosine-2A receptor or the β_2-adrenoceptor. Another approach that was investigated to accumulate intracellular cAMP is initiated via inhibition of enzymatic breakdown of cAMP by inhibiting phosphodiesterase-IV (PDE-IV) activity, or addition of exogenous cAMP.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS, *E.coli* 0111:B4), clenbuterol, rolipram, dibutyryl-cAMP, 3,3-diaminobenzidine, EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), were obtained from Sigma (St. Louis, MO, USA). CGS21680 was purchased from RBI (Natick, MA, USA). Myoclone super plus fetal calf serum (FCS, endotoxin less than 10EU/ml) and glutamine were obtained from Life Technologies (Breda, The Netherlands). Percoll was purchased from Pharmacia (Upsala, Sweden).

Isolation of liver cells

Porcine liver cells were isolated using a method adapted from Seglen (22), as described by Izeboud (23). In short, livers were isolated from castrated male pigs (Great Yorkshire x Dutch Landrace), aged approximately 12 weeks (30-40 kg) obtained from the University's breeding farm. After isolation, livers were immediately exsanguinated using icecold saline, followed by collagenase perfusion. The resulting liver cell suspension, a mixture of hepatocytes (parenchymal cells) and non-parenchymal cells, was subsequently centrifuged for 5 minutes at 200g at 4°C. The supernatant was removed and the pellet was resuspended in PBS (Life Technologies, Breda, The Netherlands). This cell suspension was centrifuged for 2 minutes at 50 g at 4°C. The resulting pellet contained hepatocytes and the supernatant was used for the preparation of Kupffer cells.

The procedure for Kupffer cell isolation was performed as described before (23). In short, supernatants (resulting from the liver cell isolation step) containing non-parenchymal cells were transferred to sterile centrifugation tubes (Micronic, Lelystad, the Netherlands) and several density-gradient centrifugation steps, using percoll, were applied to purify the cells. After a final wash step, the resulting cells (Kupffer cells and endothelial cells) were diluted in William's E (without serum) to a final concentration of $2x10^6$ cells/ml. To remove endothelial cells, cells were plated on tissue culture plates at 37°C and 5%CO_2 for 30 minutes followed by a single wash step discarding the non-adherent endothelial cells. Subsequently, cells were cultured in William's E, supplemented with 10% (v/v) fetal calf serum (Life Technologies), glutamine (1.67 mM), and gentamicin (50 µg/ml).

Cell cultures and incubations

Cells were isolated on different occasions from separate pigs, and used for experiments on the day after the isolation. Monocultures of hepatocytes were incubated at a density of 2 million cells per well in 6-wells cell-culture plates (Corning, Schiphol, The Netherlands) in William's E containing 5% v/v FCS. Co-incubations of hepatocytes and Kupffer cells (referred to as cocultures) were cultured in 6-wells cell culture plates at a density of 2 million cells each (1:1 ratio) in William's E containing 5% v/v FCS. Monocultures of Kupffer cells were cultured at a density of 1 million cells per well in 24-well cell culture plates in William's E containing 10% FCS (v/v). Intracellular cAMP levels were

determined after 30 and 90 minutes of incubation. TNFα concentrations in the culture medium were measured at 4 hours after LPS challenge. NO concentrations in culture supernatants and cell culure viability were determined after 24 hours incubation.

Measurement of intracellular cAMP formation
Cells were incubated with LPS (10 µg/ml), or LPS together with various cAMP-elevating agents (10^{-4}-10^{-5}M). After 30 or 90 minutes incubation at 37°C, the culture medium was aspirated and cells were immediately placed on ice. Icecold 70% (v/v) ethanol was added (1 ml/well) to extract cAMP from the cells. Subsequently, the samples were transferred to tubes, ethanol was evaporated (45°C, under a constant stream of N_2). The residues in the tubes were dissolved in PBS and cyclic AMP was determined by ELISA using a commercially available cyclic AMP assay kit (Cayman Chemical, Ann Arbor, USA). Data are expressed as fmol cyclic AMP per one million cells.

Determination of TNFα
TNFα was measured using the PK(15) cell line according to the method as described by Bertoni *et al.* (24). PK(15) cells were purchased from the American Type Culture Collection (CCL-33). Samples were titrated in 3-fold dilutions and for each plate a positive control (3 fold dilutions of recombinant pig TNFα (Endogen, Woburn, MA)) and negative control (incubations with medium deprived of TNFα) were measured. Cytotoxicity / inhibited proliferation was measured indirectly using the MTT-assay (see cell-viability assays) based on Denizot and Lang (25).

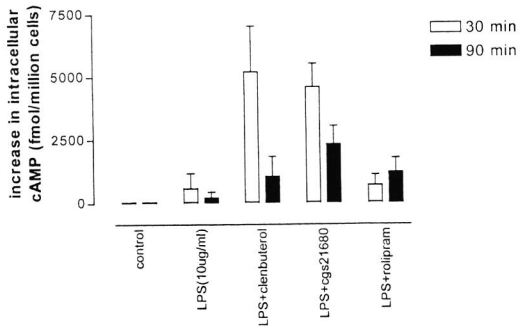

Figure 1
Increase of intracellular cAMP-concentrations in porcine Kupffer cells (compared to control). Kupffer cells were incubated for 30 minutes (white bars) or 90 minutes (black bars) with LPS only (10µg/ml) or LPS simultaneously with either the β_2-adrenoceptor agonist clenbuterol (1µM), the adenosine-2A receptor agonist CGS21680 (1µM), or the phosphodiesterase inhibitor rolipram (10µM), n=4 _ ± S.E.

Cell Viability assays
Cell viability was measured indirectly using the MTT-assay (25). At the end of the incubation, 50 µl of a 3 mg/ml MTT-solution (in PBS) was added to each well. The test was terminated after 3 hrs by the aspiration of culture medium and addition of 100 µl of isopropanol (Mallinckrodt Baker, Deventer, The Netherlands), containing 0.5% (w/v) SDS (Sigma), and 36 mM HCl (Merck, Darmstadt, Germany). Plates were shaken thoroughly, and optical densities were determined spectrophotometrically at 590 nm using a spectrophotometer (Victor^2-multilabel counter, Wallac Oy, Turku, Finland).

No determinations
After 24 hours of incubation with or without LPS, samples were collected and stored at -70°C until further analysis. The NO-production was determined by measuring the amount of NO_2^- in samples, according to the Griess reaction (26).

Statistical analysis
Values presented are means ± standard error or standard deviation. For statistical analysis Student's *t* test was performed. The mean values of two groups were considered significantly different if $P < 0.05$.

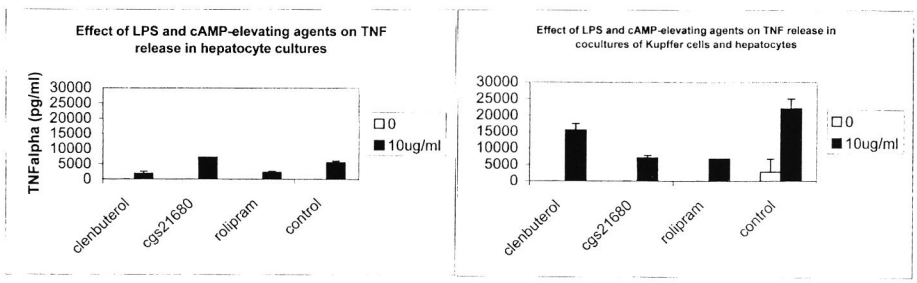

Figure 2A and 2B
Effect of LPS (10µg/ml) and cAMP-elevating agents on TNFα release in monocultures of hepatocytes (2A) and cocultures of hepatocytes and Kupffer cells (2B). Isolated cells were incubated for 4 hours with compounds only (white bars) or in combination with LPS (10µg/ml, black bars). Clenbuterol (1µM), CGS21680 (1µM), and rolipram (10µM) inhibited TNFα release, n=4 _ ± S.D.

Table I. Inhibition of LPS induced TNFα-release by porcine Kupffer cells

Compound	*Mechanism of cAMP elevation*	IC_{50} *(M)*
db-cAMP	passive transport into cell (diffusion)	$2.3 \pm 0.3 \times 10^{-6}$
clenbuterol	β_2-adrenoceptor activation	$3.3 \pm 1.2 \times 10^{-8}$
cgs21680	adenosine-2A receptor activation	$1.4 \pm 1.1 \times 10^{-8}$
rolipram	phosphodiesterase-IV inhibition	$9.8 \pm 0.7 \times 10^{-8}$

TNFα concentrations were determined in culture supernatants at 4 hr after LPS challenge when this cytokine reaches peak concentrations in the culture medium (n=6, $\bar{x} \pm$ s.d.).

RESULTS

Incubation of isolated porcine liver cells with two different receptor agonists, the selective adenosine-2A-receptor agonist CGS21680, and the selective β_2-adrenoceptor agonist clenbuterol, initiated a time- and concentration-dependent rise in intracellular cAMP. In addition, the selective PDE-IV-inhibitor rolipram initiated a time- and concentration-dependent rise of cAMP in both Kupffer cells and hepatocytes. Incubation of primary liver cells with LPS did not significantly alter intracellular cAMP. In Figure 1, the increase of intracellular cAMP (compared to control) caused by these agents in Kupffer cells is represented. The receptor agonists were found to increase cAMP at a faster rate and to higher levels than the PDE-IV inhibitor. High concentrations of the pro-inflammatory cytokine TNFα were measured in culture media of primary liver cells after incubation with LPS, and TNFα levels were found to be highest after 4 hours of LPS challenge. TNFα appeared to be released predominantly by Kupffer cells, since the levels in monocultures of Kupffer cells or cocultures of Kupffer cells and hepatocytes were much higher than in monocultures of hepatocytes. The release of TNFα from Kupffer cells was inhibited by the three compounds that elevated intracellular cAMP, clenbuterol, CGS21690, and rolipram. The potency of the respective compounds to inhibit LPS-induced TNFα-release is represented as IC_{50}-values in table I, and shown in figures 2A and 2B.

Primary hepatocytes release vast amounts of NO when incubated with LPS, as determined by NO_2^- concentrations in the culture medium at 24 hours after LPS challenge. The release of NO was increased when hepatocytes were cocultured with Kupffer cells. By contrast, no NO-production was detected in monocultures of Kupffer cells. The cAMP-elevating agents were found to suppress NO-release in cocultures of hepatocytes and Kupffer cells. No significant effect of cAMP-elevating agents on NO-release was found in monocultures of hepatocytes, as represented in figure 3A and 3B. To test whether the different agents were cytotoxic at the concentrations used, general cell-culture viabilty was assessed. Only LPS appeared to impair cell-viability after 24hr incubation.

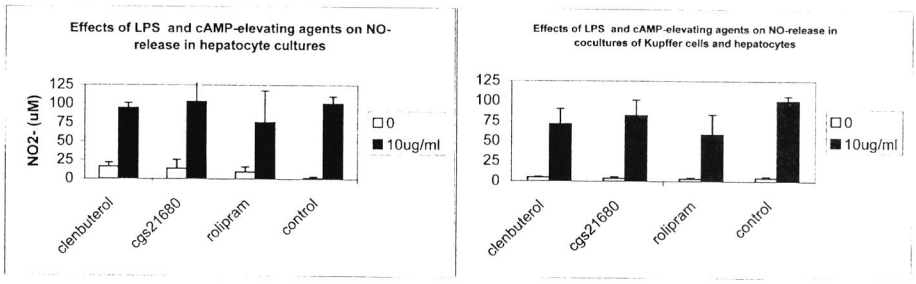

Figure 3A and 3B
Effect of LPS (10µg/ml) and cAMP-elevating agents on NO-release (as determined by NO_2^--concentrations in culture medium) in monocultures of hepatocytes (3A) and cocultures of hepatocytes and Kupffer cells (3B). Isolated cells were incubated for 24 hours with compounds only (white bars) or in combination with LPS (10µg/ml, black bars). NO-release is represented as percentage of maximum response (10µg/ml LPS=100%, and equals 17.8 µM NO_2^- in hepatocyte cultures or 50.4 µM NO_2^- in cocultures). Clenbuterol (1µM), CGS21680 (1µM), and rolipram (10µM) inhibited NO-release only in cocultures of hepatocytes and Kupffer cells, not in monocultures of hepatocytes, n=6 _ ± S.D.

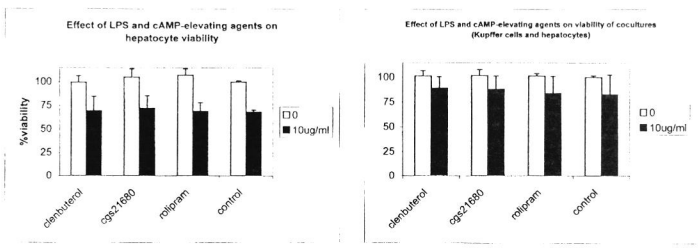

Figure 4A and 4B
Effect of LPS (10µg/ml) and cAMP-elevating agents on cell-culture viability in monocultures of hepatocytes (4A) and cocultures of hepatocytes and Kupffer cells (4B). Isolated cells were incubated for 24 hours with compounds only (white bars) or in combination with LPS (10µg/ml, black bars), and after 24 hours cell-viability was determined. Viability is represented as percentage of maximum viability (control=100%). LPS was found to decrease cell-culure viabilty after 24 hours, no additional cytotoxicity of Clenbuterol (1µM), CGS21680 (1µM), and rolipram (10µM) was observed, n=4 _ ± S.D.

DISCUSSION

Impaired liver function is often observed during (acute) systemic inflammatory diseases, like generalized infections, sepsis or SIRS (1,3,27). TNFα and NO are produced and released systemically in excessive amounts by the liver (12,28), and both agents are important mediators involved in organ failure (4-8). Several strategies have been designed to suppress the release of inflammatory mediators, or inhibit their effects. One way to modulate inflammatory mediator release is by induction of cAMP dependent signal transduction pathways, which has been shown to be a succesful approach in different experimental setups (16-19).

Based on our earlier observation that administration of the β_2-adrenoceptor agonist clenbuterol was able to inhibit LPS-stimulated TNFα release in vivo in the rat (29) and the importance of the liver from an immunological point of view, we hypothesized that this effect was largely due to a non-specific action in the Kupffer cells. Using pigs, apart from being an attractive model in general, offered the additional advantage that kupffer cells can be isolated in relatively large quantities. In the present study, several cAMP-elevating compounds were demonstrated to suppress LPS-induced TNFα release from porcine liver macrophages, as well in monocultures of Kupffer cells as in cocultures of Kupffer cells and hepatocytes. Intracellular cAMP was increased faster via receptor agonists (CGS21680 for the adenosine 2A receptor, and clenbuterol for the β_2-adrenoceptor) than via diffusion of the exogenous derivative cAMP (db-cAMP) or PDE-IV-inhibition (rolipram). The cAMP-inducing compounds varied in their potency to inhibit TNFα, as was demonstrated by the differences in IC_{50}-values of the compounds. Present finding might be explained by the rate and magnitude of cAMP-elevation, which is determined by the respective receptor-affinities of clenbuterol and CGS21680, as well as by the rate-limiting step of diffusion into the cell for rolipram and db-cAMP.

In addition to the effect on TNFα, the effect of cAMP-induction on NO-production was studied. Remarkably, the LPS-induced NO-release was found to be only partially inhibited by these cAMP-elevating agents in cocultures and no effect was found in monocultures of hepatocytes. Recently Hoebe *et al.* (accepted for publication) in our laboratory demonstrated that the parenchymal cells and not the Kupffer cells are the main source of NO in pig liver. This is in contrast to the previous idea that the macrophages in the liver are the major source of NO, but in accordance with findings in other species, including human (30,31). However, these results indicate that cAMP-elevating agents exert their effect via inhibition of a Kupffer cell derived initiator of hepatic NO-release in cocultures of hepatocytes and Kupffer cells. TNFα is often regarded as one of the main mediators that induces iNOS-expression and NO- release. The observation that only partial inhibition of NO-release was found might be explained by the fact that at the concentrations of the compounds used no complete inhibtion of TNFα release was obtained. In addition, NO-release by hepatocytes can be affected by more than just TNFα or TNFα-initiated factors. Interestingly, Mustafa et al (32) demonstrated a direct inhibitory effect of cAMP on nitric-oxide synthase expression and subsequent NO-release, and recently

Samardzic et al. demonstrated that cAMP differentially regulates NO-production (33). Another factor that initiates or aggravates NO-release, demonstrated by Hoebe *et al*, is the coculturing of hepatocytes and Kupffer cells, which was also observed in the present study.
Current results emphasize the complex interactions between intracellular cAMP (e.g. elevated by β_2-AR agonists, adenosine2A-receptor agonists, or PDE-IV inhibitors) and the production of TNFα and NO. In addition, during an inflammatory response changes in intracellular cAMP-levels have been observed in liver cells (34,35). The liver is not only an important source of inflammatory mediators but also a major target for their actions. These effects may have severe consequences such as generalized organ failure, and are important for the prognosis of the patient. Our results show that these effects may be inhibited at the level of the Kupffer cells by mechanisms that appear to be rather non-specific.
Presently we are concentrating on the possible clinical applications of low dosis of β_2-AR agonists in patients suffering from a systemic inflammatory response.

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CHAPTER 7

THE ß-ADRENOCEPTOR AGONIST CLENBUTEROL IS A POTENT INHIBITOR OF THE LPS-INDUCED PRODUCTION OF TNF AND IL-6 *IN VITRO AND IN VIVO*

Inflamm. Res. 1999;48(9):497-502

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ABSTRACT

Objective and Design: To investigate the suppressive effects of the β-agonist clenbuterol on the release of TNF-α and IL-6 in a lipopolysaccharide (LPS)-model of inflammation, both *in vitro* and *in vivo*.
Material and Subjects: human U-937 cell line (monocyte derived macrophages), and male Wistar rats (200-250 g).
Treatment: U-937 macrophages were incubated with LPS at 1 µg/ml, with or without 1.0 mM-0.1 nM test drugs (clenbuterol and other cAMP elevating agents) for 1-24 hrs. Rats were administered either 1 or 10 µg/kg clenbuterol (or saline) orally, one hour before intraperitoneal administration of 2mg/kg LPS.
Methods and results: TNF-α and IL-6 time-concentration profiles were determined both in culture media and plasma, using ELISA's and bioassays.LPS-mediated release of both cytokines was significantly suppressed by clenbuterol.
Conclusions: The β-agonist clenbuterol very potently suppresses the LPS-induced release of the pro-inflammatory cytokines TNF-α and IL-6 both *in vitro* and *in vivo*.

INTRODUCTION

The important regulatory role of the cytokines tumor necrosis factor-" (TNF-") and interleukin-6 (IL-6) during various acute and chronic inflammatory processes is widely recognized [1-4].

Several potential strategies are currently under investigation aiming to modulate the synthesis and release of these cytokines or to block (part of-) their effects before or after receptor interaction. Regulatory mechanisms leading to diminished pro-inflammatory cytokine production are of immediate clinical interest in the treatment of many chronic and acute inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, and sepsis.
Results from different studies, mostly *in vitro*, indicate that the adrenergic system is also involved in the regulation of an inflammatory response [5-8]. It has been shown that elevated intracellular levels of the second messenger cAMP are associated with a reduced release of pro-inflammatory mediators, or an induction of anti-inflammatory mediators [9,10]. One way to induce elevated intracellular cAMP levels is by inhibiting the phosphodiesterase (PDE) IV enzyme which breaks down cAMP [11,12]. Some of the PDE-inhibitors, including xanthine derivatives such as pentoxifylline, have already found their way into the clinic.
The induction of adenylate cyclase, directly or via stimulation of G-protein coupled receptors (e.g. β-adrenoceptors), is an alternative way to increase cAMP levels and hence inhibit cytokine production. Several agents known for their cAMP elevating capability via the adenylate cyclase pathway have been tested for their anti-inflammatory activity. Among these are forskolin [11], $-agonists [6,13], adenosine agonists [14], and catecholamines [5,7,15].
Although β-agonists are better known for their use as bronchodilators in asthma, they also show some anti-inflammatory characteristics. These anti-inflammatory effects have been studied mainly *in vitro* [8,11,16,17]. In these studies different cell lines have been used, producing sometimes contrasting results [13,17-20]. Data about the effect of $-agonists on the inflammatory response *in vivo* is very scarce [13,21].
Macrophages are a major source of (pro-) inflammatory cytokines [19,22,23]. We were interested in therapeutic suppression of pro-inflammatory cytokines during inflammation. First goal was to compare the effect of some cAMP-elevating agents on the LPS stimulated release of the cytokines TNF-" and IL-6. Therefore several $-agonists and other cAMP-elevating agents were evaluated *in vitro*, using the human monocyte derived macrophage U-937 cell line. The second goal of this study was to correlate the *in vitro* findings with *in vivo* data obtained from an animal model of acute inflammation: i.e. LPS treated (conscious) rats, monitoring TNF-" and IL-6 in plasma.

MATERIALS AND METHODS

Chemicals

Adenosine, clenbuterol, terbutaline, (+/-)-isoproterenol, pentoxifylline, rolipram, phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (*Escherichia coli* O111:B4), 3-(4,5-dimethyl-thiazol-2-y)2,5-diphenyltetrazolium bromid (MTT) were obtained from Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands.

Culturing U-937 cells

U-937 cells (a human monocyte like, histocytic lymphoma cell line, CRL- 1593.2) were purchased from the American Type Culture Collection (Rockville, Maryland,

USA). The cells were grown in plastic flasks (75cm^2, Corning Costar, Badhoevedorp, The Netherlands) in RPMI-1640 (Sigma Chemical Co., St. Louis, MO), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS, Life Technologies, Breda, The Netherlands), and 2 mM of L-glutamine (Life Technologies) at 37EC in a humidified atmosphere of 95% air-5% CO_2. The cells were seeded at densities of 0.1H10^6 cells/ml and subcultured when the cell concentration reached 1.0H10^6 cells/ml.

Differentiation of U-937 cells and incubations

U-937 cells are monocytes which can be easily differentiated into macrophages, and are very often used as model for human macrophages. Differentiating the U-937 monocytes into macrophages was based on the method of Sajjadi *et al.* [14]. When the cells were prepared for experiments they were seeded at a concentration of 1H10^6 cells/well in 12-wells cell culture plates and incubated overnight with 10 ng/ml PMA, added to the culture medium. The U-937 cells were allowed to recover from PMA-treatment for 48 hrs, during which the culture medium was changed every day. After this recovery period the U-937 macrophages were incubated with 1 µg/ml LPS alone, or in combination with the following test substances: adenosine; β-agonists: clenbuterol, terbutaline, (+/-)-isoproterenol; PDE-IV inhibitors: pentoxifylline and rolipram.

All stock solutions were prepared on the day of the experiment in phosphate buffered saline (PBS, Life Technologies). Controls were treated similarly and incubated with either the test substance alone or vehicle (PBS). In the comparative study of the several cAMP elevating agents culture medium was collected at 3 hrs after incubation with LPS. In the experiments with only LPS and clenbuterol (4-amino-α-[t-butylaminomethyl]-3,5-dichlorobenzyl-alcohol hydrochloride, at 1 µM, 10 nM, or 0.1 nM) culture medium was collected at regular time intervals, for 24 hrs, and tested in an ELISA for TNF-", and IL-6 concentrations. ELISA kits were purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Kits were used according to protocol provided by the manufacturer. After removal of culture medium the cells were lysed in 1 ml NaOH (0.1 M) and used for protein determination by the modified method of Bradford (Bio-Rad, München, Germany). The amount of cytokines in the culture medium was expressed per total amount of cell protein.

Cell viability determination/ MTT-assay

In order to test for possible cytotoxic effects of clenbuterol, alone or in combination with LPS, the cell viability was determined by MTT-assay based on Denizat and Lang [24]. In short, the differentiated U-937 cells were incubated for 3, 6, and 24 hrs with 10, and 1 µM clenbuterol, and with the combination of 1 µM clenbuterol and 1 µg/ml LPS. At the end of the incubation, 250 :l of a 3 mg/ml MTT-solution (in PBS) was added to each well. The test was terminated after 3 hrs by the aspiration of culture medium and addition of 500 :l of isopropanol (Mallinckrodt Baker, Deventer, The Netherlands), containing 0.5% (w/v) SDS (Sigma), and 36 mM HCl (Merck, Darmstadt, Germany). Plates were shaken thoroughly, and

optical densities were determined spectrophotometrically at 590 nm using a spectrophotometer (Victor^2-multilabelcounter, Wallac Oy, Turku, Finland).

Animals
Male Wistar rats (200-250 g U:WU (CPB)) were purchased from the Utrecht University central animal facilities (GDL, Utrecht, The Netherlands). The animals were housed in macrolon cages, and received food and water *ad libitum*. Room temperature was kept constant at 22 EC, and light was maintained at a 12-hr cycle. The animal experimentation guidelines of our institute were followed.

Experimental setup for plasma TNF-α and IL-6 measurements in rats
Twenty rats, divided in five groups of four each, were fasted the night before the experiment (only water available). The first group received only LPS, the second and third group received LPS and different doses of clenbuterol. The fourth and fifth group were controls to study the effect of saline or clenbuterol only. Just before the experiment a control blood sample was taken (t=0). At the start of the experiment the rats received orally either drug vehicle (saline) or clenbuterol (1 or 10 :g/kg bodyweight), by gavage. One hour later they were injected intraperitoneally with either vehicle or LPS (2 mg/kg bodyweight). Blood samples were withdrawn from the tail at 1,2,3, and 4 hrs after LPS challenge. Blood was collected in heparinised microvials (Sarstedt, Nhmbrecht, Germany), and centrifuged for 10 min at 4EC. The plasma was stored at -80EC until assayed.

Determination of rat plasma TNF-α concentration by a PK(15)-cells based bioassay
TNF-" was measured using the PK(15) cell line according to the method as described by Bertoni *et al.* [25]. The assay is based on the concentration dependent cytotoxic effect of TNF-" on PK(15) cells. PK(15) cells were purchased from the American Type Culture Collection (CCL-33). Cytotoxicity / inhibited proliferation was measured indirectly using the MTT-assay as described above. Samples were titrated in 3-fold dilutions and for each plate a positive control (3 fold dilutions of recombinant rat TNF-α (Sanvertech, Heerhugowaard, The Netherlands)) and negative control (incubations with medium deprived of TNF-α) were measured.

Determination of rat plasma IL-6 concentration by a B9-cells based bioassay
IL-6 was measured with a murine hybridoma B9 cell line according to the method as described by Helle *et al.* [26]. B9 cells were a kind gift from the CLB (Amsterdam, The Netherlands). The assay is based on the IL-6 dependent proliferation of B9 cells. Proliferation was measured indirectly using the MTT-assay. Samples were titrated in 3-fold dilutions and for each plate a positive control (3 fold dilutions of recombinant rat IL-6 (Sanvertech, Heerhugowaard, The Netherlands)) and a negative control (wells incubated with medium deprived of IL-6) were measured.

Statistical analysis

Values presented are means " standard error or standard deviation. For statistical analysis Student's *t* test was performed. The mean values of two groups (LPS-treated vs. LPS/clenbuterol treated) were considered significantly different if $P < 0.05$.

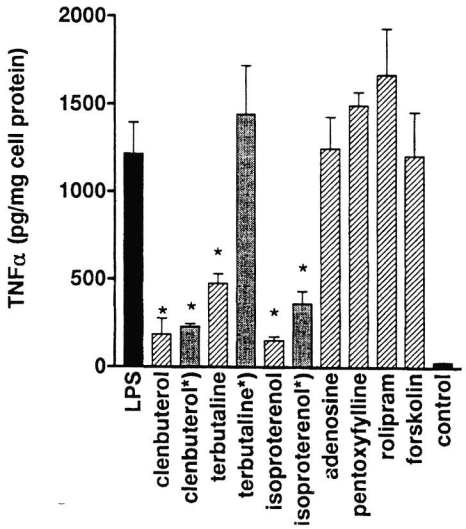

Fig. 1. Inhibitory effect of different compounds on TNF-α release by differentiated U-937 cells at 3 h after LPS incubation. U-937 cells were incubated with only LPS (1 μg/ml), or LPS simultaneously with different concentrations (1 μM or * 10 nM) of the inhibitors. Controls received only vehicle (PBS). Data are means ± SEM for triplicate determinations from the second of a series of three experiments.

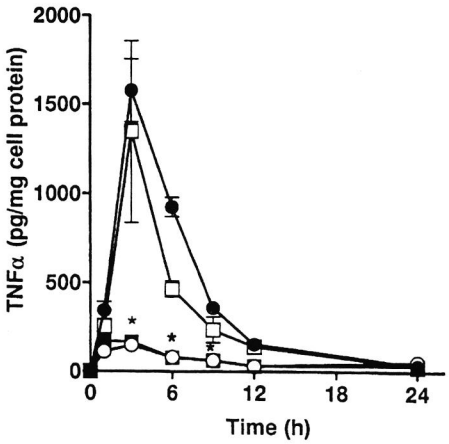

Fig. 2. Time-course of LPS-induced TNF-α release by differentiated U-937 cells. Effect of simultaneous incubation with different concentrations of clenbuterol. U-937 cells were incubated with only LPS (1 μg/ml ●), or LPS together with clenbuterol (1 μM ○; 10 nM ■; 0.1 nM □). Data are means ± SEM for triplicate determinations from the third of a series of three experiments.

RESULTS

Modulation of the LPS induced cytokine release in U-937 cells

Undifferentiated U-937 cells, growing in suspension, were succesfully stimulated to differentiate to a macrophage-like cell type by overnight incubation with PMA. After a recovery period of 48 hrs, these cells were found to respond strongly to the addition of LPS (1 μg/ml) by releasing TNF-α and IL-6 into the medium.

When U-937 macrophages were incubated with LPS together with several cAMP-elevating agents a strong suppression of the LPS-induced TNF-α release by β-agonists (clenbuterol, terbutaline, and isoproterenol, at 1 μM) was found at 3 hrs after incubation (figure 1). The other compounds (adenosine, pentoxifylline, and rolipram) were not able to suppress the TNF-α release at this concentration. These

compounds needed to be added at much higher concentrations (1 mM, 0.1 mM) to achieve the same inhibition of TNF-α-release (data not shown). The β-agonists were also tested at 10 nM concentration, at this concentration clenbuterol was shown to be the most potent suppressor of LPS-induced TNF-α release.
As a result of these findings the effect of clenbuterol on the LPS-induced TNF-α and IL-6 release was studied in more detail by measuring concentration-time profiles of these cytokines. The concentration-time profiles for TNF-α and IL-6 were found to be different (figures 2 and 3). TNF-α levels reached a rather sharp maximum at approximately 3 hrs after adding LPS and had returned to basal levels within 24 hrs of exposure to LPS (figure 2). The increase in IL-6 concentration started later than that of TNF-α and showed a biphasic response (figure 3). A first plateau was seen at approximately 6 hrs after adding LPS, and a second, higher, plateau at 12 hrs after adding LPS. Clenbuterol was found to reduce the release of both TNF-α and IL-6 in a concentration-dependent way. This effect lasted for at least 24 hrs. Neither clenbuterol itself nor saline were found to have any effects on cytokine release.

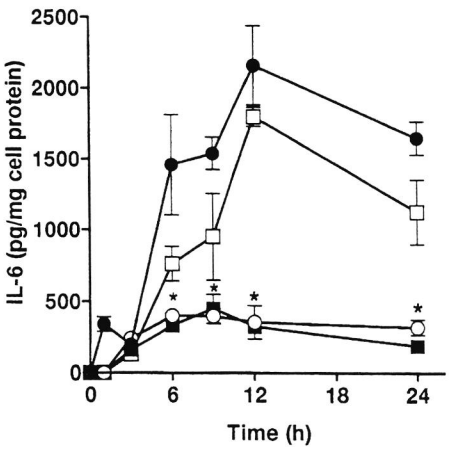

Fig. 3. Time-course of LPS-induced IL-6 release by differentiated U-937 cells. Effect of simultaneous incubation with different concentrations of clenbuterol. U-937 cells were incubated with only LPS (1 μg/ml ●), or LPS together with clenbuterol (1 μM ○; 10 nM ■; 0.1 nM □). Data are means ± SEM for triplicate determinations from the third of a series of three experiments.

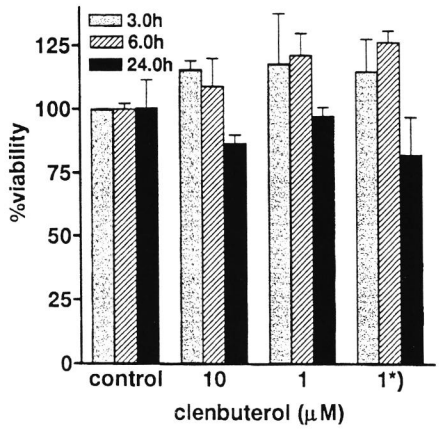

Fig. 4. Effect of different concentrations of clenbuterol and LPS on the viability of differentiated U937-cells in time. U937-cells were incubated with different concentrations of clenbuterol (10, and 1 μM, and * 1 μM + 1 μg/ml LPS). At 3, 6 and 24 h after start of the incubations the cell viability was determined using the MTT-assay. Viability was expressed as percentage of control. Data are means ± SD for triplicate determinations from a representative experiment.

Cytotoxicity studies

In order to exclude possible non-specific cytotoxic effects as explanation for the effect of clenbuterol on the LPS stimulated cytokine release, cell viability was tested at 3, 6, and 24 hrs. Clenbuterol (1 and 10 μM) and a combination of clenbuterol and LPS were incubated with differentiated U-937 cells. Using the MTT cytotoxicity assay, it was found that none of the concentrations tested was significantly cytotoxic (figure 4).

In vivo effects of clenbuterol on the LPS induced cytokine release

Following LPS (2 mg/kg i.p.) administration to rats, high plasma levels of TNF-α and IL-6 were detected. Plasma profiles of both cytokines were slightly different. For TNF-α, peak plasma concentrations were found between 1 and 2 hrs after LPS injection (figure 5). TNF-α had been cleared from the plasma at 3 hrs after LPS administration. Compared to TNF-α the IL-6 concentration in plasma reached its peak somewhat later (between 2 and 3 hrs). IL-6 levels were back to control values at 4 hrs after LPS injection (figure 6).

The administration of clenbuterol one hour before injecting LPS resulted in an almost complete block of the LPS-induced release of TNF-α and IL-6. Neither saline nor clenbuterol itself were found to have any effect on cytokine release *in vivo*.

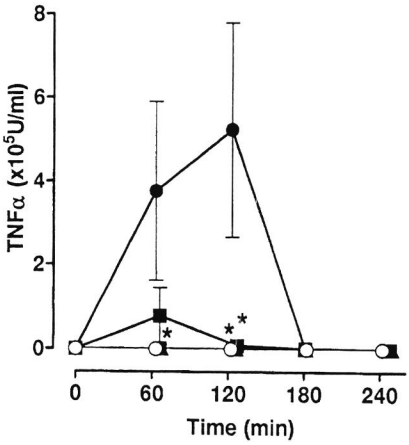

Fig. 5. Effect of clenbuterol on the LPS induced plasma levels of TNF-α. Rats were administered orally either saline or clenbuterol 1 h before intra-peritoneal injection of LPS or saline. Control (saline orally and intraperitoneally; ○), LPS (saline orally and LPS intraperitoneally; ●), LPS and clenbuterol (clenbuterol orally 1 μg/kg □, or 10 μg/kg ■, and LPS intraperitoneally), clenbuterol (orally 10 μg/kg , saline intraperitoneally; ▲). Data are means of 4 animals ± SEM.

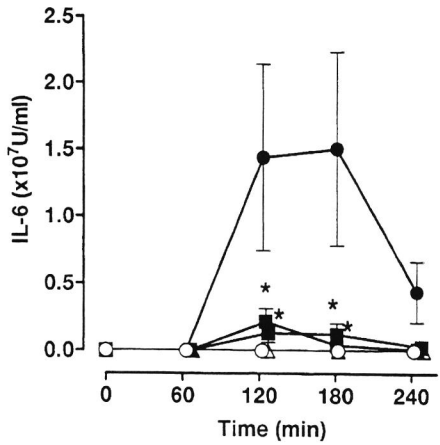

Fig. 6. Effect of clenbuterol on the LPS induced plasma levels of IL-6. Rats were administered orally either saline or clenbuterol 1 h before intra-peritoneal injection of LPS or saline. Control (saline orally and intraperitoneally; ○), LPS (saline orally and LPS intraperioneally; ●), LPS and clenbuterol (clenbuterol orally 1 μg/kg □, or 10 μg/kg ■, and LPS intraperitoneally), clenbuterol (oral 10 μg/kg, saline intraperitoneally; ▲). Data are means of 4 animals ± SEM.

DISCUSSION

The results of the present study demonstrate that clenbuterol is a very potent inhibitor of the LPS-induced release of TNF-α and IL-6 both *in vivo* (rats) and *in vitro* (U-937 cells). When the suppressive effect of some eminent cAMP-elevating agents on the LPS-induced pro-inflammatory cytokine release was compared, it was found that the β-adrenoceptor agonists were more potent than adenosine, and phosphodiesterase- inhibitors (pentoxifylline, rolipram). The concentration of clenbuterol, necessary to cause this effect, was remarkably low compared to other β-agonists and other compounds of the classes tested.

Our *in vivo* results show that clenbuterol, when given one hour before LPS, is able to block the release of the pro-inflammatory cytokines TNF-α and IL-6 almost completely. The clenbuterol dose needed to produce such an effect (1 μg/kg) is in the same order of magnitude as that has been used previously in human therapeutics (asthma), this even without taken into account a correction for species-extrapolation from man to rat.

Clenbuterol is a β-adrenoceptor agonist with relatively high affinity for β2-adrenoceptors, developed as a long acting, orally active compound. Like most other β2-agonists the main indication for use is in asthma therapy where the compound produces relaxation of bronchial smooth muscle. In addition, it has been shown that some β2-agonists posses anti-inflammatory properties [6,8,27]

There have been some other studies suggesting an effect of β-adrenoceptor agonists on the release of cytokines. Most papers describe *in vitro* effects using different cell types such as RAW 264.7 (murine macrophage cell line) [17], HL-60 (human leukemic cell line) [16], or PBMCs (freshly isolated human monocytes) [8]. In these studies, β-agonists such as isoproterenol and salbutamol were found to inhibit the release of TNF-α. In addition, the stimulation of IL-10 and inhibition of IL-1β by β-agonists were reported by Haskó *et al.* [17], and Yoshimura *et al.* [8], respectively. However in contrast to our study most authors use a much higher β-agonist concentration.

As far as we know there are only two papers describing an effect of β-agonists on LPS-induced IL-6 release [13,20]. Remarkably, von Patay *et al.* [20] report a synergistic effect of isoproterenol on the LPS induced IL-6 release by rat thymic epithelial cells *in vitro*. Yoshimura *et al.*[8] studied the effect of four different β-agonists, including clenbuterol, on LPS stimulated human peripheral blood mononuclear cells (PBMCs). They also found an inhibition of the release of TNF-α by LPS treated PBMCs, at concentrations comparable to these used in the present study. As far as we know there are no such data in this respect on the human U-937 cell line.

By contrast, *in vivo* data seem to be very scarce in the literature and apparently only studies in mice are described. Szabó *et al.* [13] studied the effect of isoproterenol on inflammatory mediator production after LPS stimulation. Using this β-agonist, at a 1000-fold higher dose (10 mg/kg) compared to our study, they found that LPS induced TNF-α release was inhibited. However, in contrast to our findings, the authors describe an increase in LPS induced IL-6 production after isoproterenol pretreatment. Sekut *et al.* [21] also used mice, and studied the effect of orally administered salmeterol (β-agonist) on TNF-α release (not IL-6) after

intraperitoneal injection of LPS. They also showed an inhibitory effect of this β-agonist on TNF-α-release, but a 100-fold higher dose of salmeterol (0.1 mg/kg) was used to achieve this effect. They determined that the best effect (highest inhibition of TNF-α-release) was reached when salmeterol was given at 1 hour before LPS injection.
A possible explanation for the difference in magnitude of effect of different β-agonists on cytokine release and a different effect on separate cytokines was already suggested by Sekut *et al.* [21], who argued that more specific β2-adrenoceptor agonists had different effects on cytokine release than non-selective β-agonists. Furthermore, the difference in response of different cell types on β-agonist stimulation might also be explained by the varying β-adrenoceptor distribution on different cells of the immune system [28,29].
Additionally, it is likely that pharmacokinetic properties of the compounds tested *in vivo* are of importance. Altogether this may explain why clenbuterol (high affinity, and more specific for the β2-adrenoceptor) is having such a potent *in vivo* inhibitory effect on cytokine release.
Our *in vitro* data are well in accordance with the *in vivo* findings, although the kinetics of cytokine release appeared to be slower *in vitro* than *in vivo*. Macrophage cells are a major source of LPS-induced TNF-α and IL-6 release. Studies that inactivated macrophages *in vivo* [19,22] observed a dramatic decrease in plasma levels of LPS induced cytokine release, compared to animals with active macrophages.The results of these studies and the present study suggest that the potent systemic effects of clenbuterol are for a large part achieved by an action on macrophages.
In conclusion, we demonstrated that the β-agonist clenbuterol is a very potent inhibitor *in vitro* and *in vivo* of the LPS induced release of the pro-inflammatory cytokines TNF-α and IL-6. Although it seems logical to conclude that the compound exerts its effect via cAMP, additional mechanisms may also play a role. In the present study clenbuterol was given one hour before LPS challenge. Whether our findings may be of therapeutic value needs to be further investigated. A negative aspect might be that low doses of β-agonists could suppress systemic immune reactions (possibly even when administered locally). This could result in a higher susceptibility of patients for infections.

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CHAPTER 8

SUPPRESSION OF THE INFLAMMATORY RESPONSE AND PREVENTION OF LIVER–FAILURE BY THE ß$_2$-ADRENOCEPTOR AGONIST CLENBUTEROL IN ENDOTOXEMIC RATS

Submitted

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ABSTRACT

The effects of administration of the β-adrenoceptor (β-AR) agonist clenbuterol to endotoxemic rats were investigated. For this, a model of lipolysaccharide (LPS)-induced acute systemic inflammation was used. The β-AR agonist was administered before-, during, and after LPS-challenge in order to investigate suppression of an acute inflammatory response, and eventually to reduce or prevent associated liver-failure. Oral administration of clenbuterol one hour before-, or intravenously at the same time as LPS-challenge resulted in a marked reduction of plasma levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6. Additionally, a change both in plasma-level and in plasma time-concentration profile of the anti-inflammatory cytokine IL-10 was found. Moreover, clenbuterol-administration prevented LPS-induced liver damage, as represented by lowered concentrations of several parameters for liver-failure (AST,ALT, Bilirubine) at 24 hours after LPS-challenge, and improved hepatic tissue morphology. When clenbuterol was administered one hour after LPS-challenge, TNFα-release was no longer inhibited. However, liver-failure could still be prevented, which puts the general idea of the key role of this cytokine in organ-failure in another perspective. Remarkably, pretreatment of rats with the β_2 -AR antagonist propranolol augmented LPS-induced liver failure, suggesting

a role of endogenous adrenoceptor-agonists in prevention of organ-failure during systemic inflammation.
The results indicate that β_2 -AR agonists might offer an additional tool in the treatment of (acute) systemic inflammatory disorders and prevention of subsequent organ failure.

INTRODUCTION

Systemic inflammatory reactions, e.g. sepsis, septic shock, and systemic inflammatory response syndrome (SIRS), are characterised by high concentrations of inflammatory mediators in the blood of patients. High concentrations of these mediators are indicative for a poor prognosis and affect the recovery of patients (1-3). In addition, pro-inflammatory cytokines are thought to be involved in (multiple-) organ failure as has been shown in many studies involving the liver, kidneys, and other organs (4-6).
During the past few years different therapeutic approaches have been developed to modulate the inflammatory response in order to prevent or reduce associated organ-failure (7). Suppression of the activation of monocytes and macrophages, a major source of inflammatory mediators, seems to be a succesfull therapeutic approach (8-10). Unfortunately, to date many of the anti-inflammatory therapies that were effective in animal studies did not prove to be succesful in clinical trials (as reviewed e.g. in (9,11)). For example, phosphodiesterase-IV inhibitors were thought to be promising compounds for anti-inflammatory therapy. However, recently Quezado *et al.* (12) showed that administration of pentoxifylline as continuous infusion during Gram-negative shock could be harmful and caused increased mortality in dogs. Other anti-cytokine approaches like anti-TNFα antibodies, or administration of cytokine receptor antagonists did not result in the desired suppression of the inflammatory response or the faster recovery of patients or test-animals (13,14).
During the last decade, more attention was paid to the anti-inflammatory properties of β_2-adrenoceptor (β_2-AR) agonists, compounds that are therapeutically well known for their use as bronchodilators in asthma and COPD. The results of these, mostly *in vitro,* studies showed that β_2-AR agonists are potent suppressors of the release of pro-inflammatory cytokines and on the other hand increase the release of the anti-inflammatory cytokine IL-10 (15-19).
As several other anti-inflammatory approaches did not prove to be succesful in clinical trials, there are several reasons to continue the research on the anti-inflammatory capabilities of β_2-AR agonists. For example, β_2-AR agonists can be used at much lower doses, thus decreasing the risk of side-effects. However, their *in vivo* effects during sytemic inflammation have not been evaluated thoroughly. In a previous study (18) we demonstrated that the β_2-AR agonist clenbuterol was effective *in vivo* in lowering TNFα and IL-6 levels in a lipopolysaccharide-(LPS) model in rats.
The present study was designed to test the hypothesis that major actors in the inflammatory cascade, like the cytokines TNFα, IL-1β, IL-6 and IL-10, are modulated by β_2-AR agonists *in vivo* in the same way as has been

demonstrated *in vitro*. In addition, the effect of timing of administration of the drug during the onset of the systemic inflammatory response was investigated. Furthermore, the effect of the β_2-AR antagonist propranolol on the modulation of the inflammatory reaction by clenbuterol was studied in order to see whether the effects of the agonist are mediated via the β-AR *in vivo*. Additionally, parameters for liver-failure were studied, since organ failure in general and liver-failure in particular are often reported and associated with systemic inflammation. Therefore, the transaminases asparagine-amino-transferase (AST) and alanine-amino-transferase (ALT), and total bilirubine (Tbil) were measured and hepatic tissue morphology was examined.

MATERIAL AND METHODS

Chemicals

Clenbuterol, propranolol, and lipopolysaccharide (LPS, *E. coli*, serotype 0111:B4) were obtained from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands).

Animals

Male Wistar rats (200-250g) were purchased from Charles River Inc. (Sulzfeld, Germany). Animals were housed in macrolon cages in groups of four, and received food and water *ad libitum*. Room temperature was kept constant and light was maintained at a 12-h cycle. The study protocol was approved by the Ethical Committee for Experiments on Animals of our institute.

Experimental design and treatment of groups

Rats were randomly divided into eight groups of four animals. The rats were fasted the night before the experiment (only water available, *ad libitum*). The experimental design is visualised in figure 1 (outline of treatments and laboratory evaluations).

Saline was used for administrations ('saline for infusion', Braun AG, Melsungen, Germany) either as control, or as vehicle for the following doses: LPS-solution (2 mg/kg b.w., intraperitonally), clenbuterol-solution (20 µg/kg b.w., orally or intravenously), and propranolol (25 µg/kg b.w., orally).

Blood samples from all rats were drawn from the tail at 1, 2, 3, 4, and 6 hours after LPS challenge. Blood was collected in heparinised microvials (Sarstedt, Nümbrecht, Germany), and centrifuged for 10 minutes. Plasma was stored at -20°C until analysis.

On the second day a 24 hour bloodsample was collected and rats were sedated using O_2/CO_2 (1:2), subsequently exsanguinated, and immediately the livers were isolated and frozen in liquid nitrogen.

Determination of different parameters in plasma

Plasma concentrations of rat TNFα, IL-1β, IL-6, and IL-10 were determined by ELISA (samples obtained at 1 until 6 hours after LPS challenge). ELISAs were used according to the protocol as provided by the manufacturer (Biosource

International Inc., Camarillo, CA, USA). The 24 hour plasma samples were analysed for AST, ALT, and Tbil using a Hitachi 911 autoanalyser and Boehringer Mannheim reagents .

Fig. 1. Outline of treatments and laboratory evaluations

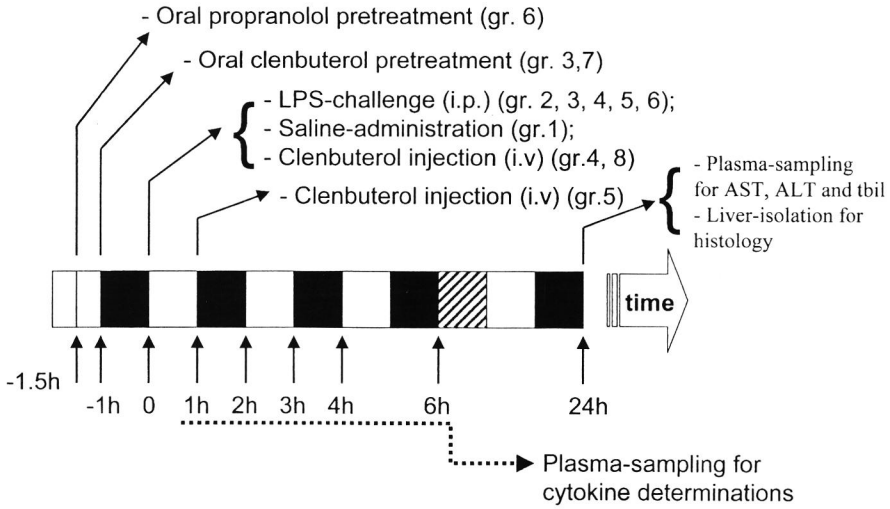

Figure 1
Outline of treatments and laboratory evaluations.
The first (control) group was orally administered saline and one hour later injected intra-peritonally with saline. The second group was injected intraperitonally with LPS-solution (2 mg/kg b.w., in saline). Group three received a clenbuterol-solution orally (20 µg/kg b.w., in saline) one hour before the intraperitonal injection of LPS. The fourth group was administered LPS intraperitonally and clenbuterol intravenously (20 µg/kg b.w., tail-vein) at the same time. Group five was injected with LPS intraperitonally and one hour later with clenbuterol intravenously. The sixth group was administered propranolol orally (25 µg/kg b.w., in saline); 30 minutes later these rats were administered clenbuterol orally, one hour later LPS was injected intraperitonally. Group seven received clenbuterol only, and group eight was administered intravenously only clenbuterol.

Histology
The frozen livers were used to make hepatic tissue specimens (0.5x0.5 cm). The pieces were embedded in Tissue-Tek® (Miles Inc., Elkhart, IN, USA). Subsequently slices were cut (7 µm) using a freeze-microtome at -24 ° C (Leica, Jung CM 3000 , Rijswijk, The Netherlans).
The slices were fixated for 15 minutes in aceton (Merck, Darmstadt, Germany) and stored at -20 ° C until further anaysis. Slices were used for Haemotoxiline/Eosine colouring (HE, Merck) and embedded on glass slides in mounting medium (Dako, Carpinteria, CA, USA). The hepatic tissue specimens were analyzed histomorphologically using a light microscope (Axiolab, Zeiss, Jena, Germany), equipped with a camera (3CCD, DXC-930P, Sony Electronics Inc., Japan), camera adaptor (CMA-D2, Sony), and digital still recorder (DKK-700P, Sony). Pictures of hepatic tissue were made at 900x magnification.

Statistical analysis.
All data on cytokine-levels reported are means ± S.D. Student's *t*-test was performed for comparison of means. Differences were considered statistically significant for $P < 0.05$. The plasma levels of AST, ALT, and Tbil respectively, from clenbuterol-treated groups (3, 4 and 5) were compared to the LPS-only challenged group (2) using the Mann-Whitney U test (unpaired, nonparametric, one-tailed P-value). Differences were considered significant if $P < 0.05$. Statistics were performed using the software package Graphpad Instat (Graphpad software Inc., San Diego, CA, USA).

RESULTS

Modulation of plasma cytokine concentrations
TNFα
Clenbuterol administration to rats clearly decreased the LPS-induced systemic TNFα release. However, the timing of administration of clenbuterol appeared to be of great influence on the inhibition of TNFα release by this β-AR agonist. In plasma of rats that were pretreated with clenbuterol orally one hour before administration of LPS (gr.3) or treated intravenously at the same time as LPS (gr.4), plasma TNFα levels were very low, comparable to the saline-group (gr.1). In contrast, rats that were treated with clenbuterol after LPS administration (one hour later, group 5) the same high plasma TNFα concentrations were found as in the LPS-only group (gr.2) (fig. 2A). To test whether the impact of clenbuterol on TNFα release in endotoxemic rats is a receptor-mediated process the β-AR antagonist propranolol was administered before clenbuterol administration. Propranolol pretreatment was shown to block the clenbuterol-initiated inhibition of TNFα-release completely.
IL-1β
Clenbuterol administration to LPS-challenged rats was also found to decrease the systemic release of the proinflammatory cytokine IL-1β. Although the way of administration did not appear to alter the inhibition of IL-1β, timing of administration was shown to be more crucial for the magnitude of inhibition. The

plasma time-concentration of LPS-induced IL-1β differed from the other pro-inflammatory cytokine TNFα with respect to the cumulation of IL-1β in the system. The main characteristic of clenbuterol administration to rats during the onset of the inflammatory response was the prevention of cumulation of IL-1β in the blood compared to rats that had received LPS only (fig. 2B). When clenbuterol was administered orally one hour before LPS challenge or simultaneously with LPS administration (gr. 3 and 4) the plasma-concentrations of IL-1β remained at control level. Administration of clenbuterol one hour after injection of LPS (gr.5) resulted in lower plasma-concentrations, i.e. less cumulation of IL-1β, compared to the rats that received solely LPS (gr.2). The β-AR antagonist propranolol was able to block the inhibitory effect of clenbuterol on the release of IL-1β completely.

IL-6

The effect of the β-AR agonist clenbuterol on systemic levels of IL-6 during the onset of an inflammatory response in rats appeared to be sensitive to both time- and way- of administration. Remarkably the strongest inhibitory effect on LPS-induced IL-6 release was found when clenbuterol was injected intravenously at the same time when LPS was administered (fig. 2C). In this group (gr.4) the IL-6 levels remained close to control concentrations (as observed in the saline-group 1) and no systemic cumulation of IL-6 was found as was seen in group 2 that only received LPS.

When clenbuterol was administered before or after LPS-administration (gr. 3 and 5) IL-6 was still detectable in plasma, however the systemic cumulation of IL-6 was lower compared to the LPS-group that had not received clenbuterol (gr.2).

Pretreatment with the receptor-antagonist propranolol before clenbuterol administration blocked the inhibitory effect of clenbuterol on IL-6 release.

IL-10

The effect of clenbuterol on the systemic release of the anti-inflammatory cytokine IL-10 was antipodal to the inhibitory effects that were observed on the release of the pro-inflammatory cytokines. Interestingly, the application of clenbuterol before or simultaneously with LPS-injection evoked both an increase of the plasma IL-10 concentrations and altered the time-concentration profile of IL-10 release (groups 3 and 4) compared to the rats that had received solely LPS (-2) (fig. 2D).

When clenbuterol was administered after LPS-challenge no significant effect on the systemic IL-10 levels was found compared to the LPS-rats (gr.2). When rats were pretreated with the receptor-antagonist propranolol before clenbuterol and LPS administration (gr.6) no increase in plasma-IL10 levels was found, as was observed in other rats (gr. 3 and 4). Instead, systemic IL-10 concentrations appeared to be even lower compared to IL-10 concentrations that were measured in rats that had only received LPS (gr.2).

Clenbuterol administration, either intravenously or orally, in control rats (group 7 and 8) did not alter basal TNFα, IL-1β, IL-6 or IL-10 levels (data not shown).

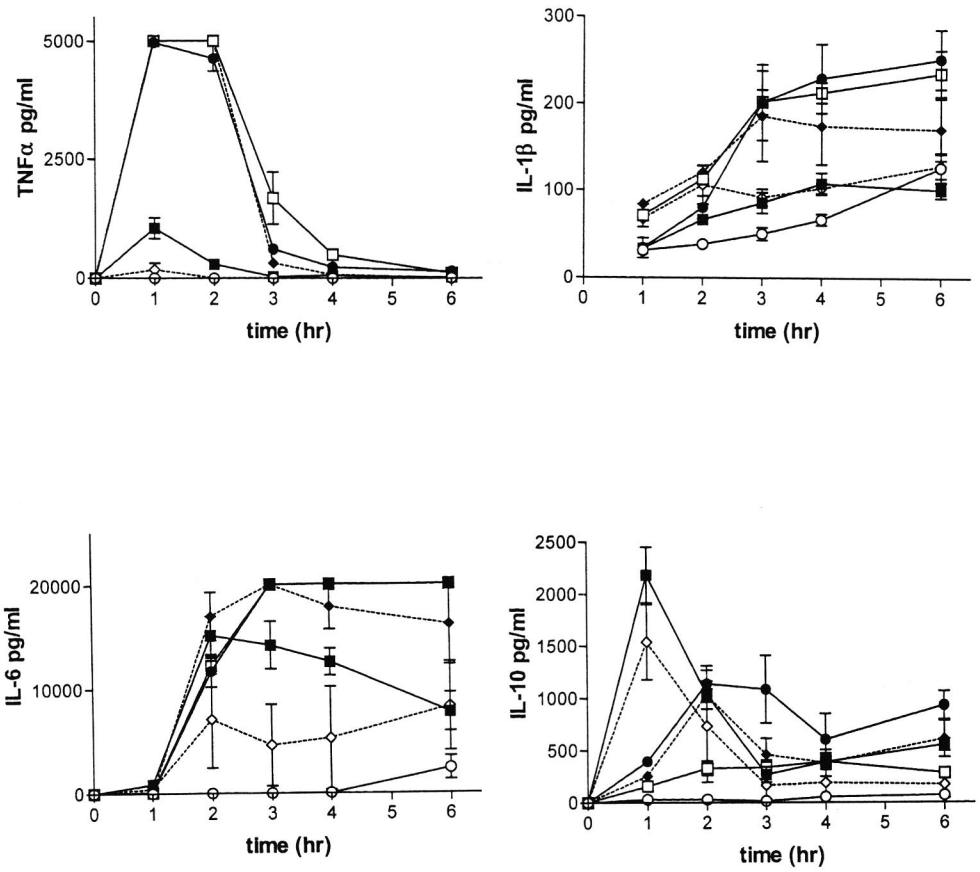

Figure 2 A,B,C,D
Time concentration profiles of the pro-inflammatory cytokines TNFα (2A), IL-1β (2B), IL-6 (2C), and the anti-inflammatory cytokine IL-10 (2D) in rat plasma. ❍=control group (n=4, saline orally), •=LPS (2 mg/kg b.w. intraperitonally), ■= clenbuterol administered orally (20 µg/kg b.w.) one hour before LPS-challenge, ◊ ◊, dotted line = clenbuterol administered intravenously (20 µg/kg b.w.) simultaneously with LPS challenge, ◆, dotted line = clenbuterol administered intravenously (20 µg/kg b.w.) one hour after LPS challenge. Additonally, the effect of the β-AR antagonist propranolol (□ = propranolol orally, 25 µg/kg b.w.) on the modulation of cytokine concentrations by the β-AR agonist clenbuterol is shown.

Plasma parameters for liver function:
In order to evaluate the effect of clenbuterol on endotoxemia-induced organ failure three parameters for liver-functioning were determined in plasma (fig. 3). At 24 hours after LPS challenge the plasma enzyme activities of AST and ALT were significantly higher in LPS treated rats compared to control (saline, group 1) or LPS/clenbuterol treated groups (gr. 3, 4 and 5). The treatment of rats with propanolol prior to clenbuterol/LPS treatment not only abrogated the hepato-protective effect of clenbuterol but appeared to increase the LPS-induced elevated levels of AST and ALT (fig. 3A,B). Tbil concentrations in plasma at 24 hours after LPS administration were higher in group 2 that was treated with LPS compared to the saline- or LPS/clenbuterol treated rats (gr. 1, 3, and 4). Pretreatment of rats with propranolol prior to clenbuterol/LPS administration (gr. 6) evoked higher Tbil concentrations in plasma compared to clenbuterol/LPS treated rats (fig.3C).

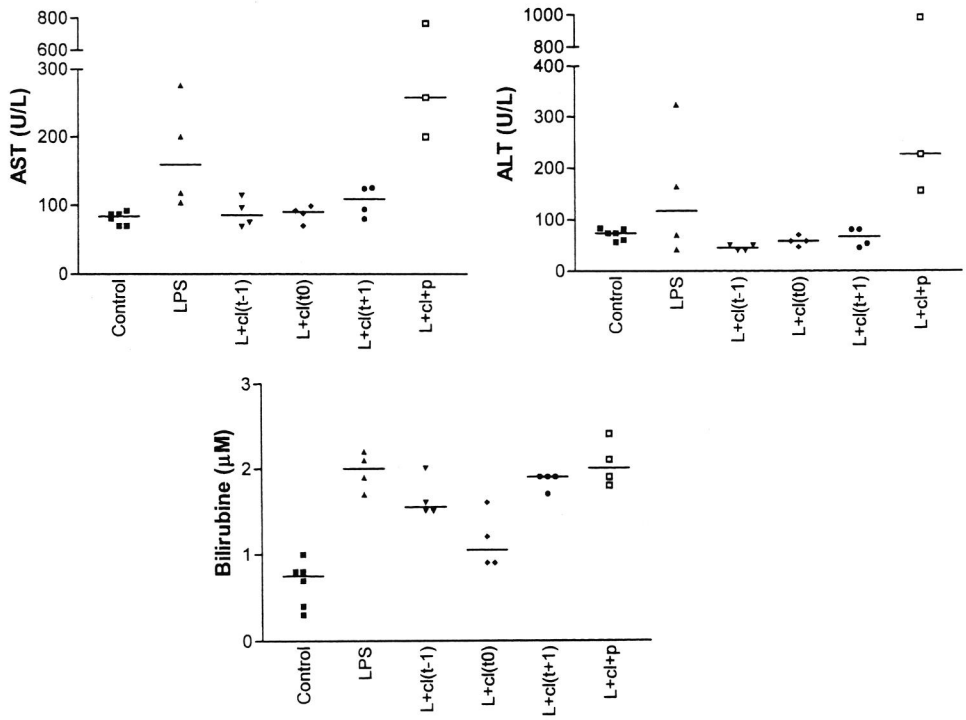

Figure 3 A,B,C
AST (U/ml), ALT (U/ml), and total bilirubine (Tbil, µM) -levels in rat plasma at 24 hours after LPS-inducd endotoxemia (with or without administration clenbuterol and propranolol). Values of each animal per group and group-medians are shown. Bar labels: L=LPS (2mg/kg bodyweight); cl=clenbuterol (20 µg/kg b.w.); t-1, t0 and t+1 indicate the times of clenbuterol administration: one before, simultaneously, or one hour after LPS-challenge; p=propranolol (25 µg/kg b.w.).

Liver histology

General hepatic tissue morphology was determined by light-microscopic analysis, using the liver-slices that had been prepared from all rats used in the experiments.

Liver-tissue of animals that were injected with only LPS was severely damaged. Hepatic tissue damage was represented by clearly visible inflammatory foci, infiltration of neutrophils, and necrotic tissue, especially in the periportal region (fig. 4). Inflammatory foci and tissue necrosis was absent in saline treated animals and in livers of animals that had been injected with LPS in combination with clenbuterol. Clenbuterol prevented liver-damage in all endotoxemic rats when administered before-, as well when administered simultaneously- , or after LPS-challenge.

The liver-protective effect that was obtained when the β-adrenoceptor agonist clenbuterol was administered during an acute inflammatory response could be neutralized by pretreatment with the antagonist propranolol. Remarkably, propranolol appeared to augment the LPS-induced liver-damage, represented by the abundant inflammatory foci observed in hepatic tissue of rats that were pretreated with propranolol, as shown in fig. 4D.

DISCUSSION

In the present study the potent anti-inflammatory action of clenbuterol was demonstrated *in vivo* when administered to endotoxemic rats. This β-AR agonist has been demonstrated before to modulate *in vivo* the LPS-induced TNFα and IL-6 release when rats were pretreated with clenbuterol before LPS-challenge (17). The present research showed that clenbuterol was not only able to suppress the release of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) but also increased the release of the anti-inflammatory cytokine IL-10. Additionally, it was shown that timing of administration is more important than way of administration to achieve modulation of systemic cytokine-levels. The effects of clenbuterol on LPS-induced cytokine release were less profound when clenbuterol was administered one hour after LPS- challenge. The effects of this synthetic β-AR agonist on cytokine release are in agreement with the modulation of the immunesystem by the endogenous ligand for the adrenoceptor epinephrine (adrenaline) (20,21).

Pretreatment with the β-AR antagonist propranolol blocked the modulation by clenbuterol of LPS-induced cytokine levels. This observation clearly indicated that the effects of clenbuterol on systemic cytokine concentrations are mediated via the β-AR, which is in accordance with previous *in vitro* findings (15,17,19).

Studies using β-AR agonists *in vivo* as anti-inflammatory agents are scarce. Although the suppressive effects of the β-AR agonist clenbuterol on TNFα and IL-1β are in correspondence with similar studies (18,21,22), the results on IL-6 are contradictory : increase (16) as well as suppression was found (18). The additional increase of endotoxin induced IL-10 release is in line with other *in vivo* findings using epinephrine (21,22).

With respect to timing- and way of administration, in studies on *in vivo* anti-inflammatory effects of β-AR agonists the animals were pretreated with these compounds (16,18) and anaesthesized (16) before LPS-challenge. Such treatment obviously limits the applicability of these drugs under clinical conditions. In the present study however, strong effects on cytokine levels were obtained when clenbuterol was administered simulatenously with LPS, and without the animals being sedated or pretreated.
More interesting even, are present results of clenbuterol administration on several parameters for liver-failure during endotoxemia. The administration of clenbuterol during the LPS-induced systemic inflammatory response prevented high levels of AST, ALT, and Tbil that are normally associated with an endotoxin injection and are characteristic parameters for organ-failure. Remarkable are the observations that although the effect of clenbuterol on cytokine-levels was less profound when administered later during the process of the inflammatory response, prevention of liver damage could still be obtained. Further proof of the prevention of LPS-induced liver-damage by clenbuterol was obtained after examination of hepatic tissue. Rats that had been injected with LPS were found to have severly damaged hepatic tissue at 24 hours after LPS-injection. In contrast, necrotic tissue and inflammatory foci were absent in rats that had been treated with the β-AR agonist during the acute inflammatory response. Remarkably, the liver-damage appeared to be augmented in propranolol-pretreated endotoxemic rats regarding the high AST and ALT levels and severely impaired hepatic tissue compared to the rats of other groups. It has been demonstrated before that catecholamine-concentrations in blood are elevated during acute inflammatory disorders and infections (23-25). Therefore, it is tempting to speculate that the more pronounced organ-damage in propranolol-pretreated rats during endotoxemia not only antagonized the protective effect of the agonist clenbuterol, but might additionally have blocked the effects of endogenous ligands (like epinephrine) for the β-AR.
Present findings are of interest with respect to the relation between high concentrations of pro-inflammatory cytokines and organ failure. For example, administration of clenbuterol one hour after LPS-challenge did not prevent the high plasma-concentrations of TNFα, but AST and ALT-levels and hepatic tissue morphology were comparable to healthy controls. The general assumption that organ-failure during a systemic inflammatory response is due to high systemic concentrations of pro-inflammatory cytokines (especially TNFα) (6,26,27), seems therefore less certain.
In conclusion, this study demonstrated that the β-AR agonist clenbuterol is a potent modulator of *in vivo* cytokine release, both when administered before or during an acute inflammatory response. Additionally, clenbuterol has been shown to prevent or reduce organ-failure in endotoxemic rats, and the blocking of this protective effect by the β-AR antagonist propranolol underlined the essential role of the β-AR in this respect. These results offer ideas for therapeutical applications of β-AR agonists in areas where they have not been used so far, such as prevention of organ-failure in SIRS- or sepsis patients, or as profylaxis during surgery where there is a huge risk of initiating SIRS, sepsis

or septic shock. A β-AR agonist like clenbuterol which targets and modulates the release of several inflammatory mediators and prevents organ-failure, might have a better chance of succes in (clinical) trials than previously applied anti-inflammatory agents.

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Figure 4 A,B,C,D
Light microscopic images of hepatic tissue (900x-magnification). In panel A hepatic tissue from a control (saline-treated) rat is shown. In panel B the liver of a LPS-challenged endotoxemic rat is presented, clearly visible are the inflammatory foci (necrotic tissue). When rats were administered clenbuterol during the inflammatory response the liver damage could be prevented as can be seen in panel 4C, the hepatic tissue of a rat that was challenged with LPS and treated with clenbuterol (i.v.) one hour after LPS-injection, no inflammatory foci or necrotic tissue was observed in these livers. The liver-protective effect that was obtained after the administration of the β-adrenoceptor agonist clenbuterol during an acute inflammatory response, was neutralized by pretreatment with the antagonist propranolol. Propranolol appears to augment the LPS-induced liver-damage, regarding the abundant inflammatory foci observed in hepatic tissue of rats that were pretreated with propranolol, as shown in panel 4D.

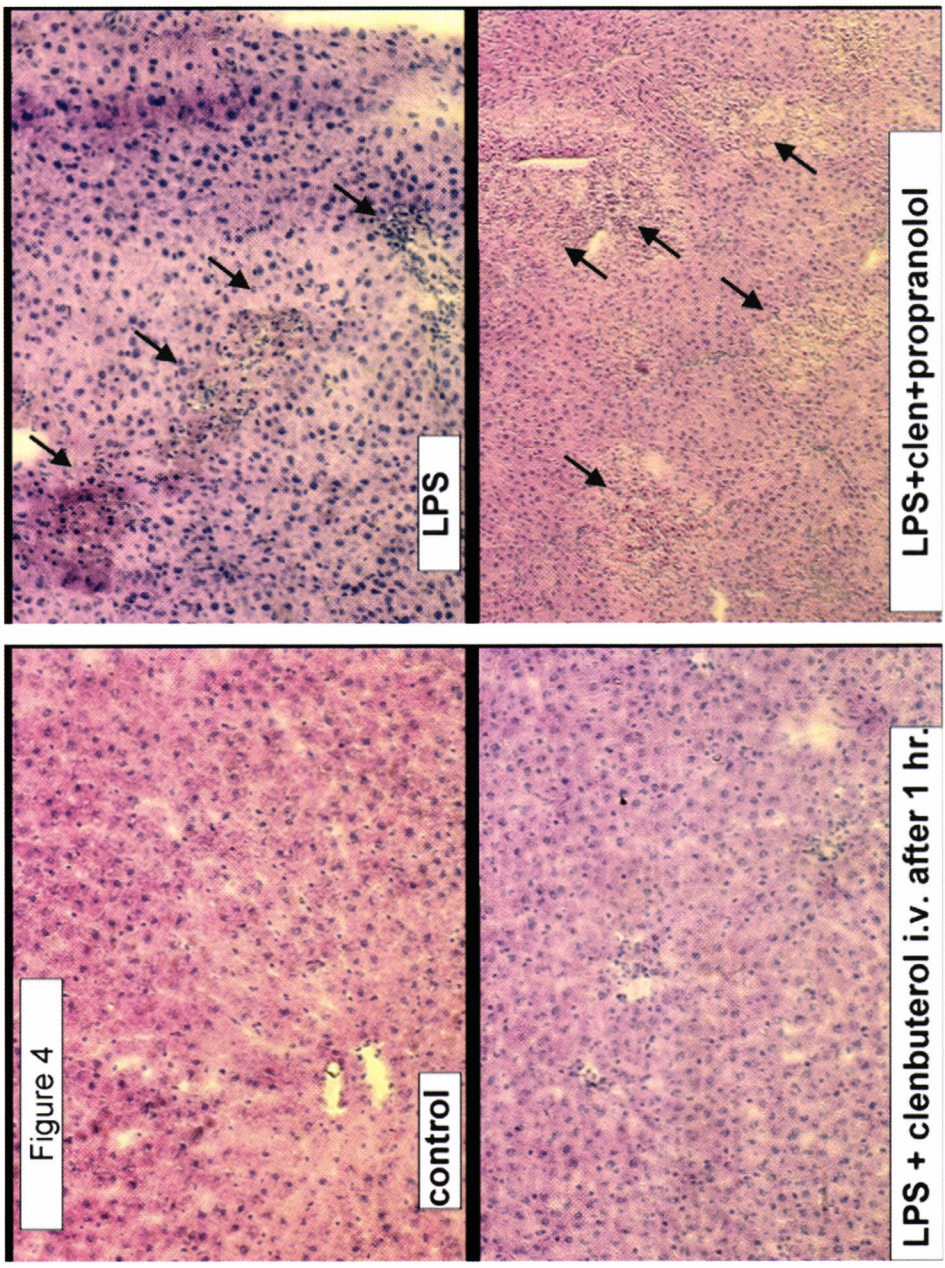
Figure 4
control
LPS
LPS + clenbuterol i.v. after 1 hr.
LPS+clen+propranolol

CHAPTER 9

SUMMARIZING DISCUSSION

Acute generalised inflammatory disorders are characterised by high (systemic) concentrations of inflammatory mediators and may progress into (multiple-) organ failure. Inflammatory mediators, including cytokines, lipid mediators, and reactive oxygen- and nitrogen-intermediates, determine for a major part the progress of inflammation and are thought to be key mediators in the initiation of organ damage during systemic inflammation.
During the past decades, extensive research has been performed to develop anti-inflammatory strategies in order to modulate a (systemic) inflammatory response and hence reduce the risk of shock, organ failure and eventually death. Although quite a number of compounds were found to possess promising anti-inflammatory capabilities in *in vitro* models, only few of these compounds appeared to have this strong potential *in vivo* in laboratory animals or in clinical trials (1-3).
Several studies have provided evidence for the modulation of immune function by catecholamines via adrenoceptor-mediated mechanisms (reviewed by Madden *et al*, and Rosman and Brooks (4,5)). This is consitent with the observation that the concentrations of catecholamines in blood appear to rise during the course of an inflammatory response (6,7), which might implicate an active role for these agents during inflammation.
The research described in this thesis focusses on the immuno-modulatory properties of β-adrenoceptor agonists during an LPS-induced inflammatory response. The effects of β-AR agonists on inflammatory cytokine release and the effects on liver-functioning during inflammation have been studied in detail. The results of these investigations are summarized in this chapter.

Modulation of inflammatory mediator release by β-AR agonists
In chapters 2-5 it has been shown that β_2-AR agonists are potent inhibitors of the production and subsequent release of pro-inflammatory cytokines in isolated macrophages and U937 cells. For the β_2-AR agonist clenbuterol it was demonstrated in chapters 7 and 8 that this compound also exerts this effect *in vivo* in rats.
The release and the time-concentration-profile of the anti-inflammatory cytokine IL-10 was modulated by β_2-AR agonists both *in vitro* and *in vivo* (chapters 2, 4 and 8), thus supporting the hypothesis that these compounds have potent and rather selective anti-inflammatory properties. *In vivo* studies in which β-AR agonists are used as anti-inflammatory agents are scarce. Although the demonstrated effects on TNFα, IL-1β, and IL-10 have been reported before (8,9), the results on IL-6 are contradictory. Some studies demonstrated an increased production (10) which is in contrast to the results described in this

thesis. Such differences might be explained by the different cell systems and experimental setups that have been used.
In order to study the mechanisms by which β-agonists initiate their immunomodulation, selective agonists and antagonists for the β-adrenoceptor were tested. In chapters 2, 4 and 8 it is described how β-AR agonists act specifically via the β-AR to modulate an inflammatory response, since pretreatment with antagonists for the β-AR (e.g. propranolol) could reverse the anti-inflammatory actions. Using the selective antagonist for the β_2-AR (ICI-118551), it was demonstrated that β-agonists act solely via the β_2-AR subtype to achieve immunomodulation. This β_2-AR-subtype selectivity is elucidated in more detail in chapter 4 in which is described that selective β_1 - or β_3 -agonists have no effect on LPS-induced cytokine release. Furthermore, the anti-inflammatory action of β-agonists was found to be highly dependent on the stereoselectivity of the ligand, a characteristic feature for the β_2-AR (11,12), as demonstrated using (R,R-)TA2005 a pure stereoisomer and selective β_2-AR agonist.
Our studies provide further evidence for the idea that induction of cAMP levels in cytokine releasing cells are an important and common mechanism by which β_2-AR agonists and phosphodiesterase type IV inhibitors exert their anti-inflammatory action, as demonstrated in chapter 4 and 6. Substantially increased cAMP levels in the LPS-activated macrophage cell line (U937) or isolated porcine liver macrophages were found to be directly associated with modulated cytokine release by β_2-AR agonists. The effects of β_2-AR-agonists on LPS-induced TNFα and IL-10 production in U937 cells was shown in chapter 5 to be regulated at the mRNA level.
The delicate balance between concentrations of different inflammatory mediators (like TNFα and NO) and the critical action of cAMP-elevating agents (β_2-AR agonists, phosphodiesterase inhibitors) acting via (liver-) macrophages on this equilibrium, is described in chapter 2 and 6.

Clinical relevance
Recently, doubt has risen about the concept and usefulness of the cytokine-suppressive approach in the treatment of (acute) systemic inflammatory disorders (1,13). The reasons for failure of a number of anti-inflammatory drugs during clinical trials, as argued by the authors, is based on recent findings that the initial hyper-inflammatory phase during SIRS and sepsis is followed by a hypo-inflammatory phase. Usually, when SIRS and sepsis patients are submitted at intensive care, the hyper-inflammatory phase is already past and what is left is a hypo-inflammatory stage where it is of no use to interfere with anti-inflammatory drugs and thereby suppressing the reactivity of the immune system.
Based on the results described in chapters 7 and 8, it is hypothesized that the need for critical monitoring of (systemic) cytokine levels during inflammatory diseases, and the importance of the timing of application of (potential) anti-inflammatory agents, are crucial to obtain succesful therapy.
Another point of doubt is the choice of models to test anti-inflammatory agents. According to van der Poll and van Deventer (13), a sytemic inflammatory

response usually does not start systemically but instead is initiated locally (organ or body cavity) and subsequently spreads throughout the body leading eventually to a systemic inflammatory response. Therefore, a therapeutic approach directed against pro-inflammatory cytokines present in the system (blood compartment) might not be the best strategy and should be replaced by an approach that is directed against the original source of inflammation. These arguments address the classical problem that every therapist rather cures the causes than the symptoms of a disease. Potent anti-inflammatory agents, like the β_2-AR agonists described in this thesis, are important and needed to treat inflammatory disorders. However, the search for therapies to cure a disease rather than its symptoms obviously has to continue.
An additional argument for the failure of anti-inflammatory drugs in clinical trials was proposed by Cirino (14), who commented on the current trends in inflammation research. He argued that due to the great complexity of the biochemical, pharmacological, immunological, and pathological processes that are involved in inflammation, there is a tendency to investigate in depth the pathogenetic role played by one single agonist at a time rather than to analyze the effects of multiple factors in the initiation of tissue damage. This approach is justified, since such complex models may be fraught with many technical and interpretational difficulties. However, there is a dilemma that many of the mediators produced operate in a network, and most of the time in an experimental system more than one enzyme or protein is induced. This might be one of the reasons why current anti-inflammatory therapies for the treatment of sepsis and SIRS have failed in clinical trials.
The approach, as is shown in this thesis using the β_2-AR agonist clenbuterol, where the plasma levels of several cytokines are modulated simultaneously, might have more beneficial effects. Moreover, in chapter 8 is shown that application of β_2-agonists during an ongoing inflammatory response had substantially less effect on plasma cytokine levels in rats, whereas inflammation-associated liver-failure was still prevented. Since the inflammatory cascade is complex, compounds (like β_2-AR agonists) that target and modulate the release of several inflammatory mediators might have a better chance of success in (clinical) trials than previously applied anti-inflammatory agents.

Endogenous ligands for the β-AR as hepatoprotective agents during inflammation ?

In the *in vivo* study described in chapter 8 using clenbuterol, liver damage was augmented in LPS-challenged rats that had been pre treated with propranolol. Propranolol is an antagonist for the β-AR, and its effects in combination with clenbuterol in the LPS treated rats show that the effect of clenbuterol was both specific and receptor dependent. LPS-challenged rats developed severe liver failure during the evolution of the systemic inflammatory response. The fact that propranolol augmented this liver failure (represented by increased AST- and ALT-levels and damaged hepatic tissue) could be explained by the fact that blocking of the β-AR probably resulted in an suppression of the protective effects of endogenous ligands during an inflammatory response. The previously reported observation that catecholamine concentrations increase during an

inflammatory response (6,7,15), could perhaps be explained as a defense mechanism of the organism to prevent systemic inflammation associated organ-failure.

CONCLUSIONS

- Catecholamines play an active role during an inflammatory response and must therefore be regarded as an important group of mediators which take part in maintaining the balance between mediators of inflammation during an inflammatory response.
- Synthetic catecholamines like β_2-adrenoceptor agonists ought to be regarded as important immunomodulatory drugs.
- The β-AR agonists appear to be most effective in modulating an acute inflammatory response when they are applied as selective β_2-AR subtype agonists. Moreover, efficacy is also improved by racemic purity of the drug (i.e. R-configuration as shown for TA2005, which is in accordance with the endogenous ligand adrenaline (epinephrine), having R-configuration at the benzylic position).
- The (synthetic, pure) β_2-AR agonists might be a better choice to treat acute inflammatory disorders than the endogenous ligand for the β-AR epinephrine (adrenaline), because they will evoke maximal effect and presumably less side-effects because of selectivity for the receptor-subtype and lower doses needed.

FUTURE PERSPECTIVES

- Present results offer ideas for therapeutical applications of β_2 -AR agonists in treatment of acute inflammatory disorders. Already mentioned is the potential application in diseases like sepsis sand SIRS. A request for a pilot clinical trial in a small group of human sepsis patients is submitted to the medical ethical committee (Dr. A.F. Grootendorst, Medical Centre Rijnmond Zuid). In this pilot trial, the effects of a 4-hour infusion of a β-agonist on systemic concentrations of inflammatory mediators are planned to be studied.
- To obtain effective therapy, the time- and dose- dependency for the applications of β_2-AR agonists in acute inflammatory disorders has to be studied thoroughly. Effective therapy depends on many intra- and inter-individual variabilities, including the type- and progress- of the systemic inflammatory response.
- To date, the β_2-AR agonists are best known for their application as bronchodilators in asthma and COPD (in particular when patients are short of breath, tightness of chest). Possibly, the immunomodulatory action of these compounds are in part responsible for the acutely improved condition when asthma- and COPD-patients use β_2-agonists. The anti-inflammatory properties of β_2-AR agonists contributes to the treatment of asthma, COPD, and chronic inflammatory diseases in general.

- Present results also offer ideas for therapeutical applications of β_2-AR agonists in areas where they have not been used so far, such as:
  * Prevention of organ-failure in SIRS or sepsis patients
  * As profylaxis during surgical operations, where there is a huge risk of initiating SIRS, sepsis or septic shock.
  * In other areas of human and veterinary medicine where a great risk of encountering acute systemic inflammatory disorders exists.
- Potential beneficial therapy with β_2-agonists in other chronic immune diseases which are characterised by high (local) levels of inflammatory cytokines (e.g. rheumatoid arthritis) remains to be investigated.
- To improve the understanding of the way how β_2 -agonists modulate an inflammatory response, and to obtain effective therapy, the fundamental mechanisms of inflammatory diseases as well as β_2-agonist action still needs to be studied in more detail. For example, the intracellular modulation of signal transduction pathways during an inflammatory response needs further investigation.

The β_2-AR agonists have potent immunomodulatory properties, represented by the effects on pro- and anti-inflammatory cytokine release, and the protection of liver-failure during acute systemic inflammatory disorders. The results described in this thesis emphasize the importance of the adrenoceptor-system in modulating immune-activity, and implicate a potential role for β_2-AR agonists in the treatments of various (acute, systemic) inflammatory disorders.

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SAMENVATTING

Het onderzoek dat in dit proefschrift beschreven wordt, richt zich op het bedwingen van acute ontstekingsreacties en in het bijzonder op het onderdrukken van immuuncel-activatie. Om dit doel te bereiken zijn met name β_2-adrenoceptor agonisten gebruikt. Deze groep van medicijnen zijn afgeleid van de moleculaire structuur van adrenaline.

Ontstekingsreacties zijn een natuurlijke afweer van het lichaam tegen lichaamsvreemde indringers (zoals bacteriën en andere micro-organismen). Echter, op het moment dat een ontstekingsreactie te heftig is, kunnen er allerlei ongewenste bijwerkingen als gevolg van deze immuunreactie optreden. Voorbeelden hiervan zijn lokale ontstekingsreacties die zich via het bloed uitbreiden over het hele lichaam tot een 'systemische' ontsteking (zoals sepsis ('bloedvergiftiging') en systemisch inflammatoir respons syndroom, SIRS). Tijdens een zo uit de hand gelopen heftige afweerreactie, waar het hele lichaam bij betrokken raakt, worden allerlei weefsels en organen beschadigd. Bovendien bestaat het gevaar van shock en zelfs overlijden als gevolg van de hoge concentraties van allerlei ontstekings-mediatoren die afgegeven worden door immuuncellen zoals macrofagen.
Zowel sepsis als SIRS zijn in toenenemende mate verantwoordelijk voor het overlijden van patiënten op Intensive Care Units. Momenteel bestaat er nog geen bevredigende (medicinale) therapie voor de behandeling van deze ziektes.

In de onderzoeken die beschreven worden in dit proefschrift is gebruik gemaakt van verschillende modelsystemen waarmee ontstekingsreacties nagebootst worden. De "*in vitro*" modellen bestaan uit kunstmatig gekweekte cellen (macrofaag-achtige cellijn U937, of macrofagen geïsoleerd uit de lever en longen van biggen: Kupffercellen en alveolair macrofagen). De "*in vivo*"-experimenten zijn uitgevoerd met proefdieren (ratten). Door de modelsystemen bloot te stellen aan lipopolysaccharide (LPS, een molecuul dat aan de buitenkant van bacteriën zit) kan kunstmatig een ontstekingsreactie uitgelokt worden. Deze (acute) ontstekingsreacties worden met name gekenmerkt door de uitstoot van allerlei ontstekingsmediatoren (o.a. cytokines) die vervolgens gemeten kunnen worden in het kweekmedium van de cellen of in het bloed van de dieren.
In deze modellen zijn vervolgens verschillende klassen van verbindingen (kleine moleculen) getest op hun vermogen om de ontstekingsreactie te kunnen onderdrukken. De klassen van verbindingen waren allen geselecteerd op hun vermogen om de concentraties van het intracellulaire signaal-molecuul cAMP te kunnen verhogen. Alle geselecteerde moleculen bleken in staat te zijn om in de

immuuncellen de concentratie van cAMP te verhogen en daarmee een ontstekingsreactie te beteugelen.
In de hoofdstukken 2 tot en met 6 is beschreven hoe de verschillende stoffen de acute ontstekingsreactie beïnvloeden in de celkweeksystemen. Met name de β_2-adrenoceptor agonisten bleken zeer sterk de acute onstekingsreactie te moduleren en te bedwingen. Het werkingsmechanisme van deze verbindingen in de onderdrukking van een onstekingsreactie is daarom in meer detail onderzocht. De belangrijkste resultaten van deze studies met cellen waren dat de β_2-adrenoceptor agonisten de uitstoot van pro-inflammatoire mediatoren (interleukines -1,-6 en TNF-alpha) remmen in zowel de immuun cellijn U937 als in macrofagen geïsoleerd uit biggen (long- en levermacrofagen). De afgifte van een ontstekingsremmend cytokine (interleukine-10) bleek gestimuleerd te worden door de β_2-agonisten. Om deze effecten te bereiken activeren deze agonisten selectief bepaalde β-adrenoceptoren, namelijk de β_2-adrenoceptoren. Daarnaast speelt de ruimtelijke (moleculaire) bouw van de β_2-agonisten (chiraliteit), en de zuiverheid (zuivere stereo-isomeer) bij de ontstekings-remmende werking van deze moleculen een belangrijke rol. De expressie van β-adrenoceptoren is op de verschillende celtypen gekwantificeerd.
Door de afgifte van onstekingsmediatoren te remmen en de concentraties in het bloed te moduleren zouden de schadelijke gevolgen van de heftige afweerreactie, waar het hele lichaam bij betrokken is, beperkt kunnen worden. Vervolgonderzoek in proefdieren (ratten) wees uit dat de remming van ontsteking door β_2-agonisten niet alleen op celniveau mogelijk was, maar dat een systemische ontsteking ook *in vivo* geremd kon worden (hoofstukken 7 en 8). Dit is een interessante bevinding, want een diermodel staat uiteraard dichter bij de werkelijkheid dan celkweeksytemen. Uit een studie, waarbij parameters voor orgaanfalen tijdens een systemische onsteking in ratten werden bekeken, bleek tevens dat de β_2-agonist clenbuterol orgaanfalen als gevolg van de ontsteking kon beperken. Deze bevinding toonde dus nog duidelijker het gunstige effect van de behandeling met deze verbindingen tijdens een acute ontstekingsreactie (sepsis) aan.

Opmerkelijk genoeg zijn β_2-agonisten in de medische en farmaceutische praktijk tot nu toe vrijwel alleen bekend en toegepast voor de lokale behandeling van astma (als broncho-dilatoren). Bij deze aandoeningen zorgen de β_2-adrenoceptor agonisten voor verwijding van de luchtwegen om de benauwdheid te verminderen, hetgeen een heel ander effect en toepassing is. Uit de medisch-wetenschappelijke literatuur zijn studies bekend waaruit blijkt dat (chemische) signalen vanuit het sympatisch zenuwstelsel (met name adrenaline en noradrenaline) in staat zijn om het immuunsysteem te beïnvloeden via adrenerge receptoren (adrenoceptoren). De studies in dit proefschrift beschrijven voor een deel de achterliggende mechanismen waarmee de β_2-adrenoceptor agonisten (chemisch gezien van adrenaline afgeleid) hun effect veroorzaken in modellen voor acute (systemische) ontstekingsreacties. Daarnaast is het nut van deze β_2-agonisten bij de behandeling van systemische ontstekingsreacties in proefdieren aangetoond

door het bedwingen van de heftigheid van de reactie en het beperken van orgaanfalen (m.n leverfalen). Deze resultaten openen perspectieven voor nieuwe toepassingen van deze medicijnen, bijvoorbeeld in de behandeling van acute systemische ontstekingsreacties zoals sepsis en SIRS.

NAWOORD

Tijdens de bijna vier jaar die mijn promotie-onderzoek in beslag hebben genomen ben ik met veel mensen in aanraking gekomen die allen in meer of mindere mate betrokken zijn geweest bij het totstandkomen van dit proefschrift. Uit deze grote groep mensen wil ik een aantal personen met name noemen die wetenschappelijk, praktisch of persoonlijk zeer betrokken zijn geweest.
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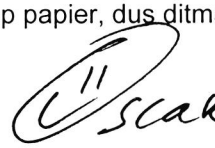
Tot slot...Marijke, ik weet dat ik te veel klets, ook op papier, dus ditmaal zonder omhaal van woorden: jij bent voor mij 'Magic'.



CURRICULUM VITAE

Cornelis Alexander (Oscar) Izeboud is geboren op 26 Augustus 1970 te Zeist. Na de middelbare school doorlopen te hebben (C.S.W. in Middelburg, Willem de Zwijger College in Bussum) startte hij in 1990 met de studie Scheikundige Technologie aan de Technische Universiteit in Delft. In 1992 stapte hij over naar de studie Scheikunde aan de Universiteit van Leiden, alwaar hij na het behalen van de propaedeuse in 1993 vervolgde met de bovenbouwstudie Bio-Farmaceutische Wetenschappen. Zijn hoofdvakstage werd uitgevoerd bij de afdeling Biofarmacie (Prof. Dr. Th.J.C. van Berkel) van het Leiden-Amsterdam Center for Drug Research. Onder begeleiding van Dr. M. van Oosten en Dr. J.Kuiper werd onderzoek verricht naar de expressie van TNFalpha-receptoren door levercellen in een proefdiermodel voor sepsis. In augustus 1996 studeerde hij af aan de faculteit voor Wiskunde en Natuurwetenschappen. In september van hetzelfde jaar trad hij in dienst als AIO (promovendus) bij TNO-Pharma in Zeist. Het in dit proefschrift beschreven onderzoek werd uitgevoerd op de afdeling Farmacologie (AAMF, Dr. R.F. Witkamp). Een belangrijk deel van het promotie-onderzoek werd tevens uitgevoerd bij de afdeling Veterinaire Farmacologie, Farmacie en Toxicologie (Prof. Dr. A.S.J.P.A.M. van Miert) van de Universiteit Utrecht. In 1998 werd zijn aanstelling als AIO uitgebreid met de functie 'project-management trainee'. Deze aanstelling mondde in 1999 uit in een authorisatie als 'studydirector' op het gebied van farmacologie en analyse. In de hoedanigheid van trainee en later als projectleider was hij betrokken bij studies voor externe opdrachtgevers ('contract research' onder GLP-voorwaarden, met name voor farmaceutische industrie).
Sinds juli 2000 is hij werkzaam als account manager op de afdeling Sales bij SPECS and BioSPECS te Rijswijk. In deze hoedanigheid is hij verantwoordelijk voor contacten van SPECS met farmaceutische- en biotech-bedrijven in Noord-Amerika.

Curriculum Vitae in ENGLISH

Cornelis Alexander (Oscar) Izeboud is born on August 26th in Zeist, The Netherlands. In 1990 he started to study Chemical Engineering at the Technical University in Delft. In 1992 he switched to Chemistry at Leiden University. In 1993, he continued with the specialization Bio-Pharmaceutical Sciences. His major trainee-ship was performed at the Biopharmaceutics division of the Leiden-Amsterdam Center for Drug Research, during which the expression of TNFalpha-receptors on liver cells was investigated in an animal-model for sepsis. After graduation (M.Sc.) in August 1996 he started as a Ph.D.-student at TNO-Pharma in Zeist (C.R.O.). The research described in this thesis was performed at the department of Pharmacology of TNO-Pharma and at the department of Veterinary Pharmacology, Pharmacy and Toxicology of the University of Utrecht. In 1998 his position as Ph.D.-student was expanded to project-management trainee. In 1999 he was authorized to operate as study-director in the field of pharmacology and analysis in contract research studies (GLP-conditions) mainly for external customers from the pharmaceutical industry. Since July 2000 he is working as account manager at the sales department of SPECS and BioSPECS in Rijswijk. In this job he is involved in the contacts with pharma- and biotech industry in North America.

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