THE UNIVERSITY OF CALGARY

Enhanced anti-inflammatory effects of NCX-1015 in the rat

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

NOVEMBER, 2002

UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Enhanced anti-inflammatory effects of NCX-1015 in the rat" submitted by Fusun Turesin in partial fulfilment of the requirements of the degree of Master of Science in Mucosal Inflammation.

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Abstract

I tested the hypothesis that a novel nitric oxide (NO)-releasing derivative of prednisolone (NCX-1015) has enhanced anti-inflammatory effects. This hypothesis was tested in the carrageenan-airpouch model in rats. Injection of carrageenan into the rat airpouch resulted in infiltration of neutrophils and increases in nitrite/nitrate, prostaglandin E_2 and leukotriene B_4 in the airpouch exudate. Prednisolone or NCX-1015 was injected into the airpouch 1 h before the carrageenan injection. 6 h after carrageenan administration exudate samples were collected. NCX-1015 reduced neutrophil infiltration and inflammatory mediator release more potently than prednisolone. Co-administration of prednisolone with the NO donor diethylenetriamine NONOate further inhibited leukocyte infiltration compared to prednisolone alone. Moreover, the negligible induction of apoptosis in exudate cells suggests that the anti-inflammatory effects of NCX-1015 do not involve induction of apoptosis.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr. John L. Wallace whose support and guidance were continuous throughout each stage of this thesis. I also would like to acknowledge the financial support I received from him over the last few years.

I'd like to thank Webb McKnight and Mike Dicay for their continuous help, encouragement and providing a perfect atmosphere in the lab.

I appreciate the support and guidance of my committee, Dr. Andre Buret, Dr. Kamala Patel and Pierre-Yves von der Weid.

Li Ma and Shari Laliberte thank you for all your help, but most of all for your friendship.

Finally, I'd like to thank my family Aziz, Seyhan and Zeynep Turesin for their love and continuous support.

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List of Symbols, Abbreviations and Nomenclature

AA	Arachidonic acid
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
AP-1	Activator protein-1
ATP	Adenosine tri-phosphate
cGMP	Cyclic guanosine monophosphate
CREB	cAMP response binding element
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
DETA-NONOate	Diethylenetriamine NONOate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
eNOS	Endothelial nitric oxide synthase
EDRF	Endothelium-derived relaxing factor
FLAP	5-lipoxygenase-activating protein
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GI	Gastrointestinal
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanylate cyclase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
h	Hour
HPA	Hypothalamic-pituitary-adrenal axis
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin

iNOS	Inducible nitric oxide synthase
L-NAME	L^G-nitro-L-arginine methyl ester
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
mRNA	Messenger RNA
min	Minute
NADPH	Nicotinamide-adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drug
PBS	Phosphate-buffered saline
PG	Prostaglandin
RNA	Ribose nucleic acid
SNAP	S-nitroso-N-acetylpenicillamine
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TBST	Tris-buffered saline with tween
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumour necrosis factor-α
TX	Thromboxane
VCAM	Vascular cell adhesion molecule

CHAPTER 1: INTRODUCTION

1.1. INFLAMMATION: THE ROLE OF NITRIC OXIDE, PROSTAGLANDINS AND LEUKOTRIENES

'It sounds like the irony of fate that I should be ordered by my doctor to take nitroglycerin internally.'

Alfred Bernhard Nobel (Lincoln et al., 1997)

The history of nitric oxide (NO) in vascular biology goes beyond its discovery as endothelium-derived relaxing factor (EDRF). It started with the use of amyl nitrite and nitroglycerine for the treatment of angina. In the 1860s, the hypotension induced by inhaled amyl nitrite was explained by the effect of amyl nitrite on the heart. However, its use was not recommended in medicine because of the intensity of its action. In 1871, Brunton was the first to propose that the hypotension induced by amyl nitrite is not due to its effect on heart, but due to its dilatation of the vessels (9, 90, 170).

A century later, Diamond & Blissard (63), and later Murad et al (193) published that NO activates guanylate cyclase. Moreover, Furchgott and Zawadski reported that an intact endothelium is required for relaxation of vascular smooth muscle by acetylcholine. Stimulation of the endothelium was proposed to cause the release of an endogenous substance, named as EDRF (89). Meanwhile, Ignarro and his colleagues had reported that NO causes relaxation of vascular smooth muscle and the production of cGMP by the smooth muscle (129).

Previously, the endogenous synthesis of nitrate and nitrite was shown in human intestine, but it was thought to be due to intestinal microflora (262). In

1981, the same laboratory showed that both germ-free and conventional rats could produce nitrate (105). In 1985, it was reported that lipopolysaccharide (LPS)-stimulated macrophages synthesize nitrate and nitrite (258). These studies made it obvious that mammalian cells can synthesize nitrogen oxides and made NO a candidate for the regulation of vascular tone.

In 1987, EDRF was identified as NO (130, 204). The Nobel Prize in Medicine was awarded to Furchgott, Murad and Ignarro for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system.

Prostaglandins (PGs) and leukotrienes (LTs) are potent eicosanoid mediators that are involved in numerous homeostatic functions and inflammation. Ulf von Euler discovered a substance in seminal fluid with smooth-muscle-stimulating activity. This substance was given the name 'prostaglandin', because its source of activity was thought to be the prostate gland (75). Another breakthrough in prostaglandin research came in the late 1950s. Sune Bergstrom and Bengt Samuelsson isolated PGs, determined their structure and demonstrated that they were derived from an essential fatty acid, arachidonic acid (25). Although initially discovered in the 1940s and described as the slow-reacting substance of anaphylaxis, LTs were chemically defined by Samuelsson (237).

Bergstrom and Samuelsson were awarded the Nobel Prize in 1982 for their discoveries concerning prostaglandins and related biologically active substances.

1.1.1. BIOLOGY OF NO

NO, a soluble gas with a half-life of ~ 6-30 s, is enzymatically formed from the terminal guanidine-nitrogen of L-arginine by a group of enzymes called nitric oxide synthases (NOS). The amino acid L-citrulline is formed as a by-product of the reaction (123,205). Nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide, flavin adenine dinucleotide and tetrahydrobiopterin are the

co-factors required for NO biosynthesis. Several isoforms of NOS have been identified, the activity of the constitutive isoforms being calcium/calmodulin-dependent (37), whereas the activity of the inducible forms is calcium-independent (290). The enzymes are classified into three isoforms: eNOS, nNOS and iNOS (reviewed in 147, 249).

eNOS is a constitutive enzyme present primarily in endothelium. nNOS is a neurally associated constitutive NOS. These NOSs rapidly generate picomolar amounts of NO that is involved in a variety of physiological functions via the activation of the soluble guanylate cyclase (GC). GC forms guanosine cyclic 3', 5'-monophosphate (cGMP) which in turn regulates protein phosphorylation, ion channel conductivity and phosophodiesterase activity (reviewed in 241). This way, NO produces fast and transient responses in target cells such as smooth muscle cells and platelets.

iNOS is an inducible enzyme the expression of which in macrophages, neutrophils, endothelium and epithelium is induced by LPS and cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (27, 82, 109, 290). iNOS can produce relatively large concentrations of NO (nanomolar) for a long time and therefore, has been associated with pathophysiological events.

1.1.2. PHYSIOLOGICAL ROLES OF NO RELEVANT TO INFLAMMATION

Among the physiological roles of NO is the maintenance of microvascular homeostasis. This protective role is mainly achieved by the capacity of NO to inhibit leukocyte (150) and platelet adhesion (218) to endothelium as well as to inhibit leukocyte superoxide anion production (49). Endothelial dysfunction characterized by decreases in NO production has been known to cause a variety of circulatory disorders, including myocardial ischemia/reperfusion (174), circulatory shock and trauma (164), hypercholesterolemia and atherosclerosis

(87, 202). Therefore, the use of NO donors was found to be an important therapeutic strategy for the treatment of these diseases (269).

1.1.2.1. NO-MEDIATED MODULATION OF MICROVASCULAR PERMEABILITY

One of the hallmarks of an inflammatory response is an increase in vascular fluid and protein leakage. There are conflicting data available with regard to the role played by NO in the regulation of microvascular permeability. Inhibition of NO synthesis with N^G-nitro-L-arginine methyl ester (L-NAME) resulted in an increase in microvascular fluid and protein fluxes in cat intestine (151) and rat mesentery (157, 158, 160). Moreover, in these studies, it was noted that NO donors, antioxidants and CD11/CD18, intercellular adhesion molecule-1 (ICAM-1) and P-selectin monoclonal antibodies were able to inhibit this process, suggesting a leukocyte adhesion and oxidant-dependent microvascular dysfunction that could be reversed by NO. Furthermore, it has also been demonstrated that NO administration via spermine-NO (134) or sodium nitroprusside (SNP) (4) was able to block histamine-induced vascular protein leakage in rat mesentery. However, in some other studies of microvasculature such as hamster cheek pouch, NO donors were found to increase permeability, and NOS inhibitors prevented this increase in permeability induced by histamine (289).

1.1.2.2. NO-MEDIATED MODULATION OF LEUKOCYTE ADHESION

The recruitment of leukocytes to a site of inflammation is largely determined by events that take place in the post capillary venules. When leukocytes move out of the capillaries, the hemodynamic forces in the venules favour the outward movement of these cells to the vessel wall and factors

produced either by endothelial cells or leukocytes promote the adhesive interactions among these cells (206).

For a leukocyte to adhere, it must initially establish a low-affinity binding state where the flow of blood acts on these cells to induce a rotational motion. A family of adhesion molecules called "selectins" mediates this rolling. The firm adhesion of leukocytes, however, is mainly due to the interaction between leukocyte integrins and immunoglobulin superfamily of adhesion molecules (206). As leukocyte-endothelial cell adhesion is considered to be a rate-controlling step in leukocyte recruitment, interference with this interaction has been considered as a potential therapeutic strategy with clinical applications to a number of acute and chronic inflammatory diseases.

Inhibition of NO synthesis promotes P-selectin-dependent leukocyte rolling, suggesting that NO is a homeostatic regulator of leukocyte rolling (60). Moreover, in several studies the delivery of exogenous NO was found to decrease leukocyte rolling in acute inflammatory conditions induced by oxidants and mast cell degranulation (152), ischemia/reperfusion (94), and TNF-α (154). Recently, these results were confirmed with eNOS-deficient mice. It was found that leukocyte rolling induced by thrombin was significantly increased in eNOS-deficient mice largely due to an up-regulation of P-selectin in the intestinal venules. (166).

NO also inhibits the firm adherence of leukocytes to the endothelium. Kubes et al. have shown that inhibition of NOS with L-NAME resulted in a rapid increase in leukocyte adhesion in feline mesenteric vasculature, and an antibody against β_2 integrin prevented this adhesive interaction (150). It was shown that basal NO release is decreased in ischemia/reperfusion; subsequently resulting in increased neutrophil adherence to endothelial cells (174). Therefore, the effect of NO donors on leukocyte influx elicited by ischemia/reperfusion was determined in a number of studies. It was found that NO donors attenuated the leukocyte adherence and emigration in post-capillary venules and coronary endothelium elicited by ischemia/reperfusion and prevent myocardial necrosis

(153, 157, 165). The role of NO in modulating leukocyte adhesion was also assessed in eNOS-deficient mice following superfusion of the mesentery with thrombin. It was found that leukocyte adhesion was significantly higher than the wild type mice suggesting that eNOS-derived NO plays an important role for modulating leukocyte adhesion induced by thrombin (166).

This anti-adhesive effect of NO has several components. One potential mechanism to explain the enhanced neutrophil influx with L-NAME could be related to the reduced blood flow and associated hemodynamic forces that normally push leukocytes through blood vessels (139). Secondly, NO downregulates expression of cell adhesion molecules such as P-selectin, ICAM-1, and vascular cell adhesion molecule-1 (VCAM-1) (60, 61) via inhibition of the transcription factor nuclear factor-κB (NF-κB) (61) and inhibition of protein kinase C activation (194). Thirdly, NO inhibits leukocyte actions by inhibiting the cytoassembly of NADPH oxidase, thereby attenuating the release of superoxide radicals by activated leukocytes (49). Finally, NO also inhibits mast cell activation (233) and administration of NOS inhibitors to rats was found to enhance mast cell degranulation with subsequent increase in leukocyte adherence to the vascular endothelium (145).

1.1.2.3. NO AS AN INHIBITOR OF PLATELET ADHESION AND AGGREGATION

The endothelium not only regulates the vascular tone but also plays an important role in preventing thrombus formation by inhibiting platelet aggregation and adhesion. Platelet aggregation occurs following activation by thrombin, collagen and ADP (229). NO released from endothelial cells increase cGMP levels in platelets and prevent platelet aggregation both *in vitro* and *in vivo* (220). NO also inhibits platelet adhesion to endothelial cells (218). These studies suggest that NO released from the endothelium plays an important role by maintaining the endothelium thromboresistant.

1.1.2.4. NO AS AN ANTIOXIDANT

NO has an important role in maintaining the normal barrier function of the microvasculature and it is an important modulator of the adhesive interactions between leukocytes and endothelium. One of the mechanisms underlying this response is the fact that suppression of NO synthesis enhances oxygen-derived free radical-mediated functional changes in the microvascular endothelium, such as alterations in microvascular permeability (284) and leukocyte adhesion (259).

Several published reports have indicated that an important function of NO is to act as a scavenger of cytotoxic superoxide anions (91, 160). The prevailing view suggests that under normal conditions, the flux of NO greatly exceeds the rate of superoxide production. When the balance between NO and superoxide is tipped in favor of superoxide (e.g., increased production of superoxide by leukocytes and endothelial cells and a corresponding decline in the synthesis of NO from eNOS), superoxide accumulates and allows for an enhanced generation of hydrogen peroxide (Figure 1). Then, these two reactive oxygen metabolites (O_2^- and H_2O_2) can rapidly initiate an inflammatory state in venules (40).

Another antioxidative effect of NO can be produced via the induction of extracellular superoxide dismutase, which is located at the outer cell membranes of vascular smooth muscle cells. The up-regulation of superoxide dismutase represents a mechanism through which the formation of peroxynitrite will be reduced, as superoxide will be dismutated to hydrogen peroxide (88).

1. $NADPH + O_2 \longrightarrow NADPH^+ + O_2^-$
2. $O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$
3. $O_2^- + NO^\cdot \longrightarrow ONOO^-$

Figure 1.1. The generation of reactive oxygen metabolites that react with NO, NADPH, Nicotinamide adenine dinucleotide phosphate; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; $ONOO^-$, peroxynitrite. (Adapted from reference 74)

1.1.2.5. iNOS AND INFLAMMATION

Relatively high concentrations of NO produced by iNOS have been reported have been implicated as part of the pathogenesis of many diseases, such as sepsis (117). NO-derived from iNOS appears to be important for the killing of bacteria by inflammatory cells; however it also contributes to sepsis-induced hypotension. Several studies have shown protective effect of iNOS inhibitors in rodent models of sepsis (261,285) whereas others have reported deleterious effects of iNOS inhibition (115). During sepsis, elevated levels of leukocyte-endothelial adhesion was reported in iNOS-deficient mice suggesting that iNOS-derived NO may be a potential regulator of leukocyte recruitment in this model (124).

Chronic colitis is also associated with the up-regulation of iNOS. Using the trinitrobenzene sulfonic acid (TNBS)-induced model of colitis in rats, Zingarelli et al (291) have shown that iNOS inhibitor, mercaptoethylguanidine administration attenuated clinical, macroscopic and histological signs of colitis. The same group also reported that TNBS induced 90% mortality in wild-type mice and 38% mortality in iNOS-deficient mice and iNOS-deficient mice had a 100% resolution of colitis 7 days after the induction of colitis (292) whereas McCafferty et al (182) reported that inflammation in iNOS-deficient mice were identical to wild-type mice in response to TNBS.

It has been proposed that NO produced by iNOS causes tissue injury through generation of peroxynitrite (155). Under normal conditions NO is oxidized rapidly by oxygenated haemoglobin to produce nitrite and nitrate (9). When the concentration of superoxide increases in the cell, NO reacts with superoxide to form peroxynitrite. The oxidizing potential of peroxynitrite facilitates lipid peroxidation and induces protein damage by tyrosine nitration (21).

At high concentrations, NO also has a potential to interact with any enzyme that contains iron-sulfur moiety. Examples of such enzymes that are

inactivated by NO are ribonucleotide reductase of DNA synthesis (167) and cytochrome c oxidase (50). NO also cause nitrosylation inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (35). The interaction of NO with these enzymes result in energy depletion, DNA damage and ultimately cell death, thus may contribute to cytotoxic effects of NO during inflammation.

1.1.3. BIOLOGY OF PROSTAGLANDINS

Cyclooxygenases (COX, also referred to as prostaglandin G/H synthases) are the key enzymes required for the synthesis of PGs from AA. AA is released from the membrane phospholipids pool by the activation of phospholipase A_2. COX enzyme catalyzes the oxygenation of AA to form PGG_2, which is subsequently reduced to form PGH_2. PGH_2 is converted to other PGs and thromboxane A_2 (237). The PG produced varies depending on the particular downstream enzyme present in a particular cell type. Prostacyclin (PGI_2), the main cyclooxygenase product of endothelial cells, is formed by prostacyclin synthetase. Thromboxane (TXA_2), the main product of platelets, is formed by thromboxane synthetase. PGD_2 and PGF_2 are formed from PGH_2 by their respective synthase enzymes in a variety of cell types. PGE_2, on the other hand, is formed both by PGE_2 isomerase and non-enzymatic pathways (186).

COX is expressed in cells in two distinct forms: COX-1 and COX-2. In 1972, Flower and Vane showed that COX preparations from the brain were more sensitive to paracetamol than those from the spleen and they proposed that there might be different isoforms of COX (83). It is now known that paracetamol is a weak inhibitor of both COX-1 and COX-2 *in vitro*; however, it has potent anti-pyretic and analgesic actions with very weak anti-inflammatory activity. Recently, Chandrasekharan et al. demonstrated the presence of a variant of COX-1, which they have named COX-3, which is sensitive to acetaminophen. Thus, inhibition

of COX-3 could represent a primary mechanism by which these drugs decrease pain and possibly fever (42).

Both COX-1 and COX-2 essentially carry out the same enzymatic reaction, however, many of the 'housekeeping' effects of COX seem to be carried out by the COX-1 enzyme. The functional role of COX-1 is consistent with its tissue expression. COX-1 is expressed in most tissues under basal conditions (246). However endothelial cells, platelets and kidney tubule cells are notable in that they express particularly large amounts of COX-1. It is the only isoform expressed in platelets and responsible for the maintenance of essential physiological functions, such as integrity of gastrointestinal mucosa, platelet activity and vascular homeostasis (reviewed in 71).

COX metabolites are released in high amounts locally at the site of inflammation or systemically after infection. Although initially it was believed to be due to an increase in supply of AA, it was later demonstrated that the increase in PG formation following exposure to inflammatory stimuli was due to an increase in COX-2 expression (178). COX-2 expression could be induced in neutrophils, monocytes and endothelial cells by many cytokines including IL-1 and TNF-α (54). When COX-2 expression is induced in cells, a proportionately larger amount of PGE_2 is produced by non-enzymatic conversion (71, 186). Although it has restricted expression under basal conditions, the highest levels of COX-2 were found in the brain, vas deferens and renal cortex (186).

1.1.4. PROSTAGLANDINS AND INFLAMMATION

COX products, mainly PGE_2 are well-known mediators of the cardinal features of inflammation-pain, edema, erythema and warmth. Therefore, pharmacological inhibition of COX by nonsteroidal anti-inflammatory drugs (NSAIDs) attenuates acute inflammation (29,265). One of the best studied inflammatory roles of PGs is edema. PGs are thought to cause edema by acting

synergistically with other inflammatory mediators. The mechanism by which vasodilating PGs enhance edema is related to their capacity to increase blood flow in inflamed tissue. Increased blood flow increases intravenular hydrostatic pressure that aids the outward flow of plasma through the venules (287).

One of the most important inflammatory diseases associated with COX-2 induction is arthritis. Aspirin and other NSAIDs have been used clinically to reduce the swelling of joints affected by rheumatoid arthritis suggesting that prostaglandins contribute to the swelling in this disease (138). In animal models of arthritis, COX-2-associated increase in PGE_2 production was detected (7). In collagen-induced arthritis model, COX-2-deficient mice had significant reductions in synovial inflammation and joint destruction, whereas arthritis in COX-1-deficient mice was indistinguishable from controls suggesting a role for COX-2 in this model (185).

In a carrageenan-induced pleurisy model of rat, an increase in COX-2 expression in the cellular infiltrate and an increase in PGE_2 in the pleural exudate was observed (141). Similarly, an increase in COX-2 expression was also noted in carrageenan-induced paw edema in rats (178). Treatment with celecoxib as well as other NSAIDs or PGE_2 monoclonal antibody administration reduced the exudate formation in these models suggesting a role for COX-2-mediated PGE_2 in carrageenan-induced inflammation (58, 98, 141, 215).

1.1.5. BIOLOGY OF LEUKOTRIENES

LTs are 5-lipoxygenase (5-LO) products of AA metabolism. Upon stimulation of the cell, 5-LO translocates to the cell membrane, where 5-lipoxygenase-activating protein (FLAP) presents AA to 5-LO. 5-LO exhibits both a dioxygenase activity that converts AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and a dehydrase activity that transforms 5-HPETE to the unstable

intermediate LTA_4 (99). The formation of LTA_4 is the last common step in the synthesis of LTB_4 and of the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4). In contrast to prostaglandins, leukotrienes are produced predominantly by inflammatory cells like neutrophils, eosinophils, macrophages and mast cells. In neutrophils and macrophages, hydrolysis of LTA_4 by LTA_4 hydrolase yields LTB_4. In eosinophils and mast cells, the conjugation of LTA_4 with glutathione yields LTC_4, which subsequently forms LTD_4 and LTE_4 through extracellular metabolism (232).

1.1.6. LEUKOTRIENES AND INFLAMMATION

LTs play an important role in the allergic and inflammatory response in both animals and humans (237). Originally described as slow-reacting substance of anaphylaxis, the cysteinyl leukotrienes are produced by a variety of cells in the airways including eosinophils and mast cells. These mediators exert many of the clinical features of the asthma such as bronchoconstriction, increased mucus secretion and increased permeability of postcapillary venules and airways. Therefore, LT receptor antagonists and inhibition of LT formation have been shown to be effective for the treatment of asthma (59, 207, 236).

LTB_4 is one of the most potent chemotactic agents for neutrophils. LTB_4 causes the release of lysosomal enzymes from neutrophils and it also mediates neutrophil-endothelial cell adhesion (reviewed in 239). The major function of the release of LTB_4 by neutrophils is to further promote neutrophil-induced inflammation. Synovial fluids of patients with rheumatoid arthritis contain high levels of LTB_4 produced mainly by infiltrating neutrophils (5). LTB_4 was shown to be required for the development of collagen-induced arthritis in the mouse by utilizing a potent and selective receptor antagonist (106). LTB_4 receptor, FLAP or 5-LO-deficient mice have reduced inflammatory responses compared to wild-

type mice in different inflammation models emphasizing the role played by LTB_4 (43,102, 116).

1.2. NO-NSAIDS: A NEW LOOK FOR AN OLD CLASS OF DRUGS

'In research there is always a "climate" of experience, which acts as a background to important discoveries.'

John R. Vane, Nobel Lecture, 1982

The story of aspirin is a remarkable one. More than 2000 years ago, Hippocrates prescribed willow bark to relieve fever and pain. In 1763, Edward Stone reported to the Royal Society of London of his successful experiments with willow bark to reduce fever in fifty patients. In 1828, Johann Buchner isolated salicin (the basis of a class of drugs called salicylates), the active compound in willow bark. In the late 1800s, salicylates had become a standard drug for the treatment of arthritis. Finally in 1897 German chemist Felix Hoffmann developed aspirin by acetylating the salicylic acid and forming a better tasting alternative. In 1915 aspirin became available without a prescription (270).

Over the next hundred years, numerous research articles have been published about aspirin. One of the most important pieces of research about it came in 1971 when John R. Vane, a pharmacologist at the Royal College of Surgeons in London, discovered that aspirin works mainly by inhibiting the synthesis of prostaglandins (270). In 1982 Vane won the Nobel Prize in Physiology and Medicine.

Each year about 80 billion aspirin tablets are consumed. Being the most popular analgesic, anti-inflammatory and anti-pyretic drug and being considered as a lifesaver in certain cardiovascular and cerebrovascular diseases, aspirin is rightly named as a 'wonder drug' (67).

1.2.1. NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Nonsteroidal anti-inflammatory drugs (NSAIDs) provide effective control of pain and inflammation, especially in arthritis. The therapeutic effects of NSAIDs are achieved through the inhibition of the cyclooxygenase enzyme (COX), which catalyzes the conversion of arachidonic acid to various eicosanoids, including PGs and thromboxanes (270).

Despite their efficacy as analgesic and anti-inflammatory drugs, the toxicity of NSAIDs in the gastrointestinal tract has been a major clinical problem. The major side effects associated with repeated NSAID use are gastrointestinal perforations, ulcers, and hemorrhages. Such adverse effects account for 16,500 deaths in the United States per year among rheumatoid arthritis patients alone (252). This is greater than the number of deaths attributed to asthma, cervical cancer and Hodgkin disease combined and comparable to the annual death toll from AIDS in the USA (252).

In the first half of the 20^{th} century, the mechanisms leading to the gastrointestinal toxicity of NSAIDs was a mystery. Vane first proposed in 1971 that inhibition of prostaglandin synthesis is the reason for the ulcerogenic properties of NSAIDs. Later, it was noted that NSAIDs cause a reduction in gastric mucosal blood flow followed by hemorrhagic lesions (273). This reduction of blood flow was thought to contribute to the ulceration process by interfering with the ability of mucosa to neutralize back-diffusing acid and dilute toxic substances that had entered the mucosa.

Wallace et al. showed that neutrophil adhesion to the gastric endothelium is an important part of the pathogenesis of NSAID-induced gastric damage, as adherent neutrophils not only cause capillary obstruction and limit the blood flow to the gastric mucosa but they may also cause endothelial and epithelial injury via the release of reactive oxygen metabolites and proteases (272).

Meanwhile NO was identified as EDRF (130). It was thought that NO with its vasodilatory and anti-aggregatory actions might be an important factor regulating gastric mucosal integrity. NO, in fact, plays an important role in protecting in the maintenance of gastric mucosal integrity (reviewed in 74). A paper published in 1989 provided one more evidence to the qualities that NO has and demonstrated that topical application of NO donors can protect against indomethacin- and ethanol-induced gastric damage in an *ex vivo* chamber preparation of the rat stomach (175). While the importance of this finding was yet to be seen, the forthcoming studies mainly concentrated on understanding the underlying mechanisms of NSAID-induced gastric damage.

Interestingly, NO was also shown to be an anti-adhesive molecule. Inhibitors of nitric oxide synthesis cause leukocyte adhesion to the endothelium (150), emphasizing the similarity between prostacyclin and nitric oxide not only as vasodilatory and anti-aggregatory but also as anti-adhesive substances.

1.2.2. STRATEGIES TO REDUCE GASTRIC TOXICITY OF NSAIDs

Over the years, a number of strategies have been taken to reduce the incidence of NSAID gastropathy. Anti-ulcer therapies such as histamine-2-receptor antagonists and enteric coating of the NSAIDs to reduce their topical irritant effects in the stomach and formulation of prodrugs that require hepatic metabolism do not significantly reduce the incidence of perforation and hemorrhages (103, 283). Another strategy is co-administration of a PG analogue, misoprostol. Misoprostol reduces the incidence of NSAID-induced stomach and duodenal ulcers; however, a high incidence of adverse effects, such as diarrhea, and its high cost limit its clinical use (93, 183).

Selective COX-2 inhibitors were introduced as anti-inflammatory drugs with the potential for less gastrointestinal toxicity (178). Previously, it was believed that NSAIDs inhibit a single COX enzyme. A second isoenzyme, COX-

2, was discovered in 1991 (286). COX-1 is constitutively expressed in the gastrointestinal tract and is considered to be responsible for maintaining mucosal integrity. COX-2, on the other hand, is induced by pro-inflammatory mediators and involved in the amplification of inflammation and pain (71). Therefore, it was hypothesized that a selective inhibitor of COX-2 would provide significant anti-inflammatory and analgesic function but without gastrointestinal side effects (178).

Selective COX-2 inhibitors are well tolerated by animals and humans after acute or chronic administration (178,276). However, recent studies show that COX-2 has physiological roles other than mediating inflammation and pain. COX-2 is strongly induced at sites of gastric ulceration and selective COX-2 inhibitors exacerbate ulceration and delay the healing of gastric ulcers (187). Moreover, NSAID-induced gastric damage requires the inhibition of both COX-1 and COX-2 and administration of a selective COX-2 inhibitor resulted in an increased leukocyte adhesion to the vascular endothelium of rats (277). The chronic administration of selective COX-2 inhibitors also exacerbates colitis and causes colonic perforations (184, 224). Therefore, it is questionable at the moment whether COX-2 inhibitors are safe to use when there is a pre-existing inflammation in the gastrointestinal tract.

Another concern with respect to the use of selective COX-2 inhibitors is the fact that COX-2 is constitutively expressed in kidney and its suppression can impair kidney function and contribute to hypertension (148). In fact, hypertensive rats treated with a COX-2 inhibitor (celecoxib) exhibited significantly increased plasma arginine-vasopressin and urea levels and elevated blood pressure (196). Moreover, COX-2 inhibitors cannot replace aspirin as a cardioprotective drug as platelets produce thromboxane A_2 via COX-1 (276). More recently, it was also reported that cyclooxygenase-2 plays a protective role in ischemia-reperfusion of heart most probably via the restoration of blood flow through the production of prostaglandins in response to hypoxia and its inhibition by selective COX-2 inhibitors exacerbates myocardial injury in the rabbit (227).

1.2.2.1. NO-NSAIDs

The observation that NO donor (sodium nitroprusside) significantly reduced the severity of gastric damage induced by an NSAID (175) strengthened the notion of NO being a gastroprotective agent. Therefore, another approach was taken with hopes to reduce the gastrointestinal toxicity of NSAIDs: a NO-releasing moiety was linked to these compounds. The rationale behind this approach was to take advantage of the similar gastroprotective properties that prostaglandins and NO share and to compensate for the loss of prostaglandins by adding NO. In this way, NO was thought to counteract the reduced gastric blood flow and increased leukocyte adherence in the gastric microcirculation that NSAIDs cause. Being a scavenger of superoxide anion (229) and a regulator of the secretion of mucus by gastric epithelial cells (34), NO also has other properties that may improve the tolerability of NO-NSAIDs in the gastrointestinal tract. In fact, it was later shown the oral administration of these drugs to rats was shown to be GI-safe without altering systemic blood pressure (273). Moreover, one such drug, NO-aspirin, was also shown to reduce increase in leukocyte adherence in response to N-formyl-Met-Leu-Phe in rat mesenteric venules (278).

1.2.2.2. COX-INDEPENDENT ACTIONS OF NO-NSAIDs

NO-NSAIDs also exert COX-independent actions. Fiorucci et al. first demonstrated that, similar to NO (68), NO-aspirin also caused S-nitrosylation/inhibition of interleukin 1β-converting enzyme (ICE)-like proteases, resulting in protection against apoptosis induced by TNF-α (78, 79). Fiorucci et al. later extended these observations in an *in vivo* model of hepatitis. Mice were injected with concanavalin A, which results in a T_H1-like response and Fas/Fas ligand –mediated liver cell death. NO-aspirin (100 mg/kg), but not aspirin, reduced plasma levels of IL-18, IFN-γ and IL-1β via S-nitrosylation/inhibition of

caspases. NO-aspirin also prevented concanavalin A-induced up-regulation of Fas, Fas ligand and IL-2R up-regulation in lymphocytes. Treatment of mice with NO-aspirin completely abolished the apoptotic response and protected against acute liver failure.

Another interesting feature of NO-NSAIDs is that they inhibit iNOS expression. Flurbiprofen nitroxybutylester reduced LPS-induced nitrite production and iNOS expression in a macrophage cell line without directly affecting the enzyme activity (48). This result is in agreement with NO being an inhibitor of NF-κB activation (211). This finding further explains the enhanced anti-inflammatory properties of NO-NSAIDs.

1.3. GLUCOCORTICOIDS AS ANTI-INFLAMMATORY DRUGS

'Perhaps chance will at some time give us what all our efforts have not been able to achieve.'

The remarks of the prize-awarding judge, the philosopher Montesquieu, on the results of a scientific competition by Bordeaux Academy of Science entitled 'What is the importance of adrenals?' in 1716. (From The Nobel Prize in Physiology or Medicine 1950 presentation speech by G. Liljestrand)

The Italian anatomist Eustachi first described the adrenal glands in 1563 (77). Despite the continued scientific interest, the function of adrenal glands remained a mystery until 1855 when Thomas Addison described the symptoms of adrenocortical deficiency (46). In the early 1930s, the syndrome of excess glucocorticoids secreted by adrenal tumors was defined and published by Cushing (57).

In 1935, the first adrenal gland hormone cortisone was isolated independently by Edward C. Kendall and Tadeus Reichstein. At this time, Philip.S. Hench was trying to find a treatment for rheumatoid arthritis. He had noticed that in the presence of jaundice or during pregnancy, the severe pain of arthritis decreased and even disappeared. He suspected that an adrenal hormone might be the common substance causing this improvement. In 1948, Hench tried cortisone on 15 arthritic patients. Cortisone ameliorated the symptoms of rheumatoid arthritis and became a key drug in the treatment of rheumatoid arthritis (172). Kendall, Hench and Reichstein received the Nobel Prize in Physiology or Medicine in 1950 for discoveries concerning hormones of the adrenal cortex, their structure and biological effects.

1.3.1. GLUCOCORTICOIDS

Since their introduction for the treatment of rheumatoid arthritis, glucocorticoids revolutionized the treatment of inflammatory diseases. Glucocorticoids inhibit many aspects of an inflammatory response including cytokine production, adhesion molecule expression, and leukocyte migration, and they continue to be the most effective anti-inflammatory therapy available for the treatment of rheumatoid arthritis and asthma. The major drawback associated with their use is their systemic adverse effects. In this section, the molecular mechanisms that account for the anti-inflammatory effects of glucocorticoids, their side effects and novel glucocorticoids with special emphasis on NO-releasing glucocorticoid (NCX-1015) are discussed.

1.3.1.1. MECHANISM OF ACTION OF GLUCOCORTICOIDS

Because of their lipophilic nature, glucocorticoids passively diffuse through the cell membrane where they bind to the glucocorticoid receptor (GR) located in the cytoplasm of cells (288). The inactive GR is associated with a protein complex consisting of two molecules of 90 kDa heat shock protein (hsp90), a 59 kDa immunophilin protein and various other inhibitory proteins. hsp90 molecules act as a molecular chaperone and they prevent the nuclear localization of GR (20, 216).

When glucocorticoid binds to GR, GR undergoes a conformational change and hsp90 dissociates from the complex. The activated glucocorticoid-GR complex subsequently translocates into the nucleus and it binds to glucocorticoid response elements (GREs) located in the promoter regions of glucocorticoid-responsive genes. The binding of glucocorticoid-GR complex to DNA either increases (transactivates) or decreases (transrepresses) target gene expression (16). Glucocorticoids also are able to decrease the transcription of pro-

inflammatory genes, which have no identifiable GREs in their promoter regions, indicating that there are other ways to account for the glucocorticoid anti-inflammatory activity (140).

The major anti-inflammatory effect of glucocorticoids is the transrepression of pro-inflammatory genes. This inhibitory effect appears to be largely due to the inhibition of transcription factors such as activator protein-1 (AP-1) (135) and nuclear factor-κB (NF-κB) (13). NF-κB plays a critical role in regulating the expression of many pro-inflammatory cytokines (TNF-α, IL-1, IL-6, GM-CSF), adhesion molecules (ICAM-1, V-CAM-1, E-selectin), and enzymes (iNOS, COX-2, 5-LOX, PLA_2) involved in the inflammatory procesess, therefore inhibition of NF-κB activity is instrumental for the glucocorticoid activity (reviewed in 17). AP-1, on the other hand, regulates the expression of IL-2, IL-2 receptor, IL-4, IL-8, IFN-γ and metalloproteinases (reviewed in 84, 231).

In 1990s, it was recognized that the GR could regulate gene expression by directly interacting with nuclear factors without binding DNA. Direct protein-protein interactions between GR and AP-1 and between the p65 subunit of NF-κB have been demonstrated to prevent their binding to DNA (135,221). Glucocorticoids can also inhibit NF-κB via the induction of IκB, the cytoplasmic inhibitor of NF-κB (13). More recently, GR has been shown to prevent c-Jun phosphorylation, which subsequently inhibits the signaling cascade that leads to the activation of AP-1 (39). Therefore, glucocorticoids may affect either the activation or binding of these nuclear factors. AP-1 and NF-κB are involved in the regulation of many pro-inflammatory genes; therefore by inhibiting their binding to DNA glucocorticoids prevent the augmentation of inflammation.

Another mechanism through which glucocorticoids affect gene expression is by enhancing transcription of ribonucleases, thus resulting in reduced stability of mRNA. Inhibition of IL-1, IL-2, IL-6, GM-CSF, and inducible COX-2 by glucocorticoids was suggested to occur via this mechanism (6, 26, 32, 225).

Transcription of genes is secondary to the uncoiling of DNA after acetylation of its histone residues. The acetylation of histone is mediated by the enzymatic action of molecules like cyclic AMP response element binding protein (CBP). CBP is activated by the binding of transcription factors such as AP-1 and NF-κB (136, 201). As activated GR also interacts and activates CBP as well, competition between the transcription factors and the GR for the limited binding sites may be another mechanism for the anti-inflammatory effects of glucocorticoids (2, 137).

1.3.1.2. ANTI-INFLAMMATORY EFFECTS OF GLUCOCORTICOIDS

The inhibition of pro-inflammatory cytokine production by glucocorticoids is one of the major mechanisms of glucocorticoids anti-inflammatory action. Among those cytokines are IL-1 (6), IL-2 (203), IL-3 (56), IL-4 (44), IL-5 (190), IL-6 (3, 6), IL-8 (266), IL-12 (30), TNF-α, IFN-γ (3), granulocyte colony-stimulating factors (G-CSF) and GM-CSF (171). Glucocorticoids do not only block the cytokines themselves, they may also inhibit cytokine receptors as in the case of IL-2 receptor (Grabstein et al., 1986).

Glucocorticoids inhibit the synthesis of several inflammatory mediators by an inhibitory effect on enzyme induction. For example, they inhibit prostaglandin, leukotriene, and platelet activating factor synthesis by inhibiting the arachidonic acid formation. This inhibition appears to involve a distinct mechanism, the induction of lipocortin-1, a protein that inhibits phospholipase A_2 (210, 212). Glucocorticoids have also been shown to have direct inhibitory effects on the cytokine–induced expression of COX-2 and phospholipase A_2 in monocytes and other inflammatory cells (100). Metalloproteinases are also among the enzymes inhibited by glucocorticoids (65).

INOS is another important enzyme involved in the inflammatory process. Glucocorticoids are potent inhibitors of iNOS enzyme (66). There is evidence that glucocorticoids not only prevent NF-κB and AP-1 binding to iNOS promoter

region but they also enhance the degradation of iNOS protein and destabilize iNOS mRNA (146, 149, 156). Intriguingly, there is also evidence that NO itself can inhibit iNOS expression and activity (250).

Glucocorticoids inhibit exudation of plasma and migration of leukocytes into the site of inflammation. Glucocorticoids inhibit exudation of plasma in more than one way. Firstly, they decrease the blood flow to the inflamed area mainly via vasoconstriction and inhibition of vasodilatory substances and they also inhibit endothelial cell permeability by interfering with histamine and bradykinin production (213). The up-regulation of adhesion molecules plays an important role in the migration of leukocytes to sites of inflammation. The expression of many adhesion molecules on endothelial cells and on leukocytes is inhibited by glucocorticoids either by reducing their expression or by inhibiting pro-inflammatory cytokines. Among these adhesion molecules are ICAM-1, E-selectin (55) CD18 (36), and L-selectin (198). Moreover, chemotactic factors such as IL-8 (161) and eotaxin (169) are also inhibited by glucocorticoids.

1.3.1.3. ADVERSE EFFECTS OF GLUCOCORTICOIDS

It was estimated that 1% of the human genome can be affected by glucocorticoids (213). Considering the diversity of their actions, it is not surprising that glucocorticoids have a wide array of side effects. Harvey Cushing was first to describe many of the symptoms that result from excessive tissue exposure to glucocorticoids (57). Prolonged exposure to high doses of glucocorticoids result in osteoporosis, hypertension, diabetes, suppression of HPA axis, weight gain, hyperlipidemia, myopathy, cataracts and glaucoma, growth retardation and opportunistic infections (46).

1.3.1.4. GLUCOCORTICOIDS AND OSTEOPOROSIS

Osteoporosis is one of the major limitations of chronic glucocorticoid therapy. In 1950s, clinical trials with cortisone resulted in a large number of osteoporotic fractures (31). In a more recent study, about two thirds of patients receiving chronic glucocorticoid therapy were diagnosed with osteoporosis and many of these patients were also reported to develop fractures (223). It was also documented that treatment with low-dose prednisone for 20 weeks decreases the trabecular bone of the lumbar spine by 8% (162).

The pathogenesis of glucocorticoid-induced osteoporosis involves several different mechanisms. Glucocorticoids are known to suppress bone formation by inhibiting osteoblast replication and they also inhibit bone matrix synthesis by decreasing the expression of type 1 collagen synthesis, osteopontin, and fibronectin (279). An increase in bone resorption was also related to glucocorticoid therapy (64). Together, these changes lead to osteoporosis via reduced bone formation. Finally, glucocorticoids decrease the renal and intestinal absorption of calcium and this way indirectly contribute to the bone loss (52, 95). The inhibitory effect of glucocorticoids on the synthesis and secretion of sex hormones also contributes to the pathogenesis of osteoporosis as these hormones inhibit osteoclast formation (133).

1.3.1.5. GLUCOCORTICOIDS AND HYPERTENSION

Glucocorticoids also play an important role in the regulation of blood pressure by modulating the vascular smooth muscle tone. Soon after the introduction of cortisone, hypertension was noted as a complication of the glucocorticoid therapy (255).

Glucocorticoids either potentiate the effects of vasoconstrictor substances such as angiotensin II and catecholamines (214, 268) or they may augment the reabsorption of sodium by proximal tubule of kidney via increasing the expression of Na^{+}, K^{+} adenosine triphosphatase and Na^{+}-H^{+} exchangers (112, 163). Moreover, glucocorticoids also suppress the production of vasodilator substances as in the case of prostaglandins and NO (76, 280). More recently, it was shown that glucocorticoids suppress eNOS expression in kidney and this decrease in expression of eNOS contributes to the hypertension as basal release of NO maintains Na^{+} and water homeostasis in kidney (173).

1.3.1.6. GLUCOCORTICOIDS AND HYPERGLYCEMIA

Glucocorticoid use is associated with hyperglycemia in patients without known diabetes mellitus and in diabetic patients. Clinical studies indicate that 10-20% of previously undiagnosed diabetic patients develop diabetes over the first few years of glucocorticoid use (126).

As their name suggests, glucocorticoids have glucose-regulating properties and they play a protective role against glucose deprivation. Glucocorticoids in excess have long been known to increase blood glucose and liver glycogen levels by stimulating gluconeogenesis. Supraphysiological concentrations of glucocorticoids induce the enzymes that regulate glucose metabolism such as glucose-6-phosphatase resulting in a hyperglycemic state (53). In addition, glucocorticoids also result in the inhibition of insulin release from pancreatic β cell, which also account for the diabetogenic action of glucocorticoids (62).

1.3.1.7. GLUCOCORTICOIDS AND HPA AXIS

Secretion of glucocorticoids by adrenal glands is under control of the hypothalamic-pituitary-adrenal (HPA) axis. Bodily insults, such as inflammation,

pain or mental stress causes the activation of HPA axis. These stimuli act on hypothalamus, which then releases corticotropin-releasing hormone (CRH). CRH causes pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH stimulates adrenal cortex to release glucocorticoids such as cortisol. Once in the blood cortisol acts on its target organs and elicits numerous metabolic effects such as increased blood glucose levels, mobilization of amino acids and fatty acids (199). The HPA axis, like many other endocrine systems, is regulated by a negative feedback mechanism. Administration of exogenous glucocorticoids inhibits the release of ACTH and CRH and causes decreased stimulation of the adrenal glands by ACTH, which results in adrenal atrophy (86).

1.3.1.8. GLUCOCORTICOIDS AND THYMUS GLAND

Research concerning the effect of adrenal gland on the immune system goes back to 1936 when Selye showed that stress-induced involution of lymphoid tissue was mediated by adrenal glands (247). Similarly, administration of ACTH to mice caused a reduction in the thymus and lymph node weight (70). An example of this dramatic response was demonstrated by a single intraperitoneal injection of dexamethasone (5 mg/kg) to adrenalectomized rats. It was shown that 48 h after the injection there was 50% reduction in thymus gland weight and 80% reduction in the thymocyte population (51). Glucocortioid-induced atrophy of the thymus gland occurs by apoptosis of immature thymocytes (reviewed in 12).

1.3.1.9. NOVEL GLUCOCORTICOIDS

In 1970s, topical steroids with local anti-inflammatory effects were developed to prevent the systemic side effects of glucocorticoids. Nasal and inhaled glucocorticoids such as budesonide, fluticasone propionate and

mometasone furoate improved the treatment of asthma and rhinitis (18, 19). Although the swallowed fraction of these drugs is eliminated by hepatic metabolism, there still is a risk for systemic side effects at high doses (18). Therefore a search for locally metabolized glucocorticoids with marked anti-inflammatory effects has started. These so-called soft steroids were shown to have very poor efficacy as they were being metabolized before they can exert any anti-inflammatory action (19).

It was proposed that many of the anti-inflammatory effects of glucocorticoids result from the binding of a GR monomer to transcription factors causing gene repression whereas the endocrine and metabolic effects are likely to be due to the binding of GR dimers to DNA resulting in the transactivation of genes (2, 118). Therefore it was proposed that if a glucocorticoid selectively transrepresses, systemic side effects could be avoided. Heck et al. (118) has demonstrated that transactivation and transrepression can be dissociated through mutations in the DNA binding domain of the GR. These mutations prevented the GR to dimerize and bind to DNA and resulted in the inhibition of transactivation of GRE-dependent promoters. Transrepression of an AP-1-dependent promoter, however, functioned as effectively as the wild type GR.

Recently, a novel class of glucocorticoids called dissociated steroids has been described. These drugs exhibit higher transrepression and weaker activation of GRE-dependent genes. *In vitro* studies revealed that one such glucocorticoid, RU 24858, did not affect GRE-dependent transactivation in murine fibroblast cells (24). However, it efficiently repressed TNF-α-induced IL-6 secretion in murine fibroblasts and LPS-induced IL-1β secretion from monocytes (24, 271). Moreover, this compound has shown more enhanced anti-inflammatory activity than prednisolone in a granuloma model and an ear edema model in rats (271). A recent study, however, reported that although RU 24858 has comparable anti-inflammatory activity to the budesonide, systemic side effects still do occur after 7-day oral treatment of rats (22).

Another class of novel glucocorticoids was obtained by the addition of a NO-releasing moiety via nitrooxymethylbenzoate side chain to the glucocorticoid prednisolone (NCX-1015) (Figure 4.1). NCX-1015 has been shown to be more potent than prednisolone in reducing zymosan-induced neutrophil extravasation into the peritoneal cavity of mice. Furthermore, NCX-1015 was more effective in decreasing zymosan-induced iNOS expression in infiltrated cells and KC production in the peritoneal cavity (208). In addition, NCX-1015 suppressed the clinical signs of collagen-induced arthritis in rats to a greater degree than prednisolone, with milder side effects on bone (209). NCX-1015 not only has enhanced anti-inflammatory properties, it also exerts bronchodilatory effects. It was reported that NCX-1015 relaxes pre-contracted guinea pig trachea more effectively than the parent drug in a cGMP-dependent way (263).

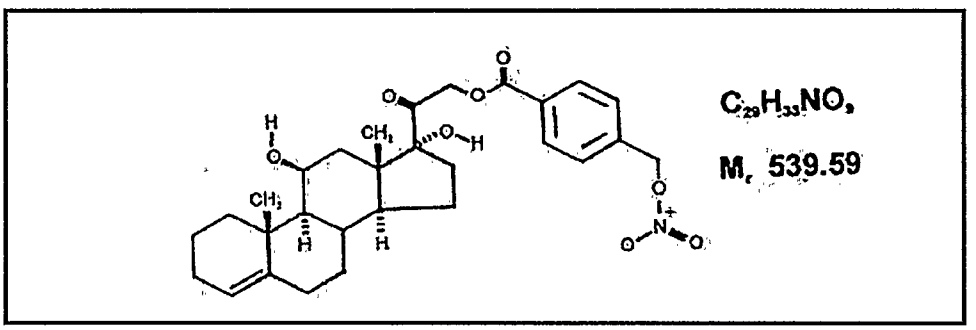

Figure 1.2. The chemical structure of NCX-1015 (208)

1.4. OBJECTIVES

Three studies have been compiled to form the body of thesis. The major objectives of the work contained herein are:

1. To characterize carrageenan-induced airpouch inflammation (i.e., leukocyte infiltration and inflammatory mediator release).

2. To determine the anti-inflammatory effects of NCX-1015 in the carrageenan-airpouch model and to understand the mechanisms that account for the enhanced anti-inflammatory effects of NCX-1015 compared to prednisolone.

3. To examine some of the systemic effects on NCX-1015 (i.e., blood pressure and plasma glucose levels).

CHAPTER 2: MATERIALS AND METHODS

2.1. ANIMALS

Male, Wistar rats (175-200 g) were obtained from Charles River Breeding Farms (Montreal, Canada). The rats were housed in the Animal Care Service of the University of Calgary and were fed standard laboratory chow and water ad libitum. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. CARRAGEENAN-AIRPOUCH MODEL

Edwards et al. first characterized the rat airpouch model in 1981 (72). They demonstrated that when subcutaneous connective tissue was disrupted with repeated injections of air, the cavities develop a lining histologically similar to the synovial membrane. Rat airpouch offers several other advantages including the ability to evaluate both acute and chronic phases of an inflammatory reaction. It also allows easy sampling of the exudate and reproducible measurements of cellular and humoral components of local inflammation. Carrageenan is a sulphated polysaccharide extracted from the algae *Chondrus crispus* (66). Injection of carrageenan into the airpouch results in a local inflammatory reaction characterized by exudate formation and increased leukocyte infiltration (243). Therefore, this model has been extensively used to test the anti-inflammatory effects of many experimental drugs (23, 97, 275)

In this study, an airpouch was produced by the subcutaneous injection of 20 ml of air on the back of the rats on the first day. On the third day, an

additional 10 ml of air was injected into the airpouch. Five days after the first injection another 10 ml of air was injected at the same site. On the sixth day, 2 ml of either sterile saline (0.9% w/v) or 1% w/v solution of type IV lambda carrageenan in sterile saline was injected into the pouch (275). Before obtaining the samples, rats were killed with an overdose of 2% w/v sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON, Canada) and 1 ml of sterile saline containing 10 units of heparin (Leo Pharma Inc., Ajax, Ontario, Canada) was injected into the pouch. All injections were performed under 5% (v/v) halothane (Halocarbon Laboratories, River Edge, NJ, USA) anesthesia. 1, 2, 4, 6 and 24 h after the carrageenan injection, a small incision in the airpouch was made and the exudate was collected into sterile tubes.

The volume of the exudate was measured gravimetrically. Next, an aliquot of the exudate was used to quantify the number of leukocytes using a Coulter Particle Counter (Coulter Electronics, Beds. U.K.). Data are expressed as total number of cells in the exudates. Differential cell counts were also performed using Wright's stain for characterization of the leukocytes. In addition, the percentage of viable cells and the rate of apoptosis were determined by trypan blue staining and a cell death-detection ELISA kit, respectively. These assays were done as soon as the exudate samples were recovered. Finally, the rest of the exudate was centrifuged at 400g for 10 minutes (Beckman Centrifuge Model J2-MI, Palo Alto, CA, USA). Cell pellets were frozen on dry ice and kept at –70 °C for western blot experiments. The supernatant was also collected and kept frozen at –70 °C until the nitrite (NO_2^-), prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) concentrations were measured. PGE_2 and LTB_4 concentrations were determined using specific ELISAs and NO_2^- concentrations were determined using the Griess reaction. The results are expressed as total NO_2^-, PGE_2 and LTB_4 in the exudates.

2.2.1. GRIESS REACTION

In this study, the concentration of nitric oxide in the airpouch exudate was quantified indirectly as nitrite (NO_2^-) using the Griess reaction. This procedure involves nitrosation of sulfanilamide that results in a diazonium salt under acidic conditions. This product with an aromatic amine, N-1-naphtylethylenediamine, produces an azo dye complex that can be detected colourimetrically at 540 nm (200).

Briefly, NO_3^- in the supernatant was reduced to NO_2^- by incubation with 50 µl of 1 U/ml nitrate reductase and 35 µl of 110 µM β-NADPH at room temperature for 30 min. 100 µl of both 1% sulfanilamide in 5 % H_3PO_4 and 0.1 % N- (1-naphtyl)-ethylenediamine dihydrochloride were incubated with 100 µl of the reduced sample for 10 min at room temperature and the absorbance was measured at 540 nm with a microplate reader (Molecular Devices Co., Sunnyvale, CA, U.S.A.). Nitrite concentrations were calculated by comparing to a standard curve of known concentrations of sodium nitrate (1-100 µM) prepared in phosphate buffered saline (PBS) (0.01M, pH: 7.4) (96, 108).

2.2.2. PGE_2 DETERMINATION

PGE_2 concentrations in the cell-free airpouch exudates were determined using an ELISA kit (Medicorp, Montreal, Quebec, Canada). This kit operates on the basis of competition between PGE_2-horseradish peroxidase conjugate and PGE_2 in the sample for a limited number of binding sites on the antibody-coated plate. Briefly, the sample and the enzyme conjugate were added to the microplate and incubated for 1 h. After washing the plate to remove any unbound reagents, the bound enzyme conjugate was detected by the addition of substrate, 3,3', 5,5' tetramethylbenzidine and hydrogen peroxide. The plate was read spectrophotometrically at 450 nm with a microplate reader (Spectra Max

Plus, Molecular Devices Corporation, Sunnyvale, CA, USA) and PGE_2 concentrations of the samples were calculated by using a standard curve constructed by measuring the absorbance of known concentrations of PGE_2.

2.2.3. LTB_4 DETERMINATION

LTB_4 levels in the cell-free airpouch exudates were determined using an ELISA kit obtained from Cayman Chemical Company (Ann Arbor, MI, USA). This assay is based on the competition between LTB_4 and LTB_4-acetylcholinesterase conjugate for a limited amount of LTB_4 antibody. The antibody-LTB_4 complex, then binds to a rabbit IgG mouse monoclonal antibody that has been previously attached to the well. After washing the plate to remove any unbound reagents, the substrate for the enzyme acetylcholineesterase, acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid), was added. The product, 5-thio-2-nitrobenzoic acid, gives strong absorbance at 412 nm. The intensity of the colour formation was, and then measured spectrophotometrically with a microplate reader (Spectra Max Plus, Molecular Devices Corporation, Sunnyvale, CA, USA) and LTB_4 concentrations of the samples were determined by using a standard curve constructed by measuring the absorbance of known concentrations of LTB_4.

2.2.4. WESTERN BLOT ANALYSIS

Cell pellets were incubated for 30 minutes in 0.5 ml of lysis buffer containing a protease inhibitor cocktail, 1% (v/v) Triton X-100, 10 mM Tris, 1 mM EDTA and 1 mM phenylmethyl sulfonyl floride and centrifuged at 10,000 g (Beckman Centrifuge Model J2-MI, Palo Alto, CA, USA) for 10 min at 4 °C. Protein concentrations of the supernatants were determined with a colourimetric assay based on the Bradford dye-binding procedure (Bradford, 1976). The assay was carried out according to the manufacturer's instructions. Briefly, 200

μl of the dye reagent supplied by the manufacturer was added to 10 μl of sample and the absorbance was read at 595 nm with a microplate reader (Spectra Max Plus, Molecular Devices Corporation, Sunnyvale, CA, USA). The protein concentration of the samples was calculated by comparing to a standard curve of bovine serum albumin (0.1-0.5 mg/ml).

The supernatant of the lysate was mixed with sample buffer (4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris-HCl, pH: 6.8) and boiled for 5 minutes. Samples (50 μg protein/lane) and pre-stained SDS-PAGE molecular weight control markers were resolved by gel electrophoresis on 10% SDS-polyacrylamide gel for 1 h at 100 V at room temperature in running buffer (25 mM Tris, 192 mM glycine and 0.1 % (w/v) SDS, pH: 8.3) and transferred electrophoretically to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, U.S.A.) in transfer buffer (25 mM Tris and 192 mM glycine, pH: 8.3). After the transfer, the membrane was blocked for an hour in 5% (w/v) blotting grade blocker non-fat dry milk in Tris-buffered saline (20 mM Tris, 100 mM NaCl, pH: 7.2) with 0.05% Tween 20 (TBST) and then incubated overnight with a primary antibody in 5% (w/v) blotting grade dry milk in TBST at 4°C. The blot was washed for 1 h with TBST. Then, it was incubated for 1 h with a horseradish peroxidase-conjugated IgG in 5% (w/v) dry milk in TBST. After washing the blot for 1 h, the proteins were detected by enhanced chemiluminescence using a Hyperfilm and chemiluminescence reagent (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, U.K.). The X-ray film was analyzed with a calibrated imaging densitometer (Model GS 710) and Quantity One software (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

2.2.4.1. ANTIBODIES

5-LO antibody (1:2,000; rabbit-anti-human polyclonal) was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). COX-1 antibody (1:200; rabbit-anti-human polyclonal), COX-2 antibody (1:200; rabbit-anti-human polyclonal)

and eNOS antibody (1:200; rabbit-anti-human polyclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). iNOS antibody (1:10,000; rabbit-anti-mouse polyclonal) was obtained from BD Pharmingen (Mississauga, ON, Canada). The secondary antibody (1:10,000; donkey-anti-rabbit IgG, horseradish peroxidase-conjugated) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, U.S.A.).

2.2.5. CELL VIABILITY

The trypan blue dye exclusion method was used to determine the percentage of viable cells in the airpouch exudate. Briefly, cells from the exudate were re-suspended in PBS (0.01M, pH:7.4). A fraction of this cell suspension was then diluted with 0.4% (w/v) trypan blue solution. The numbers of viable and non-viable cells were determined by counting with a hemocytometer 5-15 minutes after the dilution. The cells that did not take up trypan blue within this period were considered viable, whereas the cells that were stained with trypan blue were considered non-viable.

2.2.6. APOPTOSIS

The rate of apoptosis of the exudate cells was determined using a cell death detection ELISA (Roche Molecular Biochemicals, Manheim, Germany). This is a photometric enzyme-immunoassay that determines histone-associated-DNA-fragments (mono- and oligonucleosomes) that form after the induction of apoptosis. The assay was carried out according to the instructions of the supplier. Briefly, the exudate cells were diluted to a concentration of 1×10^5 cells/ml. Then, they were centrifuged for 10 min at 200g (Eppendorf Centrifuge Model 5415C, Rexdale, Ontario, Canada) re-suspended in a lysis buffer and incubated for 30 min at room temperature. The lysate was again centrifuged for 10 min at 200g and the supernatant was incubated at room temperature with

biotinylated anti-histone and peroxidase-conjugated anti-DNA antibodies. After the addition of substrate, the colour development was measured at 405 nm with a microplate reader (Spectra Max Plus, Molecular Devices Corporation, Sunnyvale, CA, USA).

2.3. EFFECTS OF PREDNISOLONE AND NCX-1015

The airpouch was produced in male Wistar rats (175-200 g) as described in the previous section. The vehicle dimethylsulfoxide (DMSO) or one of the drugs, prednisolone or NCX-1015 was injected subcutaneously into the airpouch at doses ranging from 0.8-28 μmol/kg one hour before the carrageenan injection. Then, all rats were injected with 2 ml of 1% w/v solution of carrageenan in sterile saline subcutaneously. The rats were euthanized 6 h after carrageenan administration and samples were collected as described above.

2.3.1. RELEASE OF NO_2^- FROM NCX-1015

This experiment was designed to determine the amount of release of NO_2^- from NCX-1015 into the airpouch in the absence of carrageenan-induced inflammation. The airpouch was produced in male Wistar rats (175-200 g) as described above. These rats were administered the vehicle (DMSO) or NCX-1015 at doses 0.8 or 28 μmol/kg directly into the airpouch. An additional group of rats received 1.4 μmol/kg diethylenetriamine NONOate (DETA-NONOate) in the same way. DETA-NONOate is a NO-nucleophile complex that releases NO spontaneously in physiological solutions without metabolic activation and has been shown to cause vasorelaxation via the release of NO (191). 1 h after the injection of test drugs, all rats were injected with 2 ml of sterile saline and the exudates were collected 6 hours after the saline injection. The exudates were centrifuged at 400 g for 10 minutes and the supernatant was frozen immediately

on dry ice and later kept at -70 °C until the NO_2^- concentrations were determined via the Griess reaction.

2.3.2. EFFECTS OF COMBINING PREDNISOLONE WITH A NO DONOR

Rats received either DETA-NONOate or prednisolone at doses of 1.4 μmol/kg and 2.8 μmol/kg, respectively or a combination of both into the airpouch. 1 h later, all rats received 2 ml of 1% w/v solution of carrageenan in sterile saline into the airpouch. The dose of DETA-NONOate was selected so that it would be equimolar number to NCX-1015 in terms of the NO-releasing moieties. 6 h after the carrageenan injection, exudates were collected and leukocyte counts, NO_2^-, PGE_2 and LTB_4 determinations were performed as described above.

2.3.3. EFFECTS OF DMSO ON CARRAGEENAN-INDUCED INFLAMMATION

Rats were administered with either DMSO or sterile saline 1 hour before the carrageenan injection. Then, all rats received 2 ml of 1% w/v solution of carrageenan in sterile saline into the airpouch. 6 h later, exudates were collected and leukocyte number, NO_2^-, PGE_2 and LTB_4 determinations were performed as described above.

2.4. EFFECTS OF PREDNISOLONE AND NCX-1015 ON SYSTEMIC BLOOD PRESSURE AND PLASMA GLUCOSE

Male Wistar rats (175-200 g) were anesthetized with 5% (v/v) halothane. Their abdomen were shaved and scrubbed with a disinfectant. The midline abdomen was opened (4-6 cm incision) and the contents were exposed by using a retractor. The intestines were held back using saline-moistened gauze

sponges to allow good visualization of the descending aorta. A 2-0 silk suture was placed beneath the aorta and traction applied to restrict the blood flow. The aorta was then carefully punctured by using a 21-gauge needle. The tip of the catheter of a radiotelemetry probe (Data Sciences International, St. Paul, Minnesota, USA) was slid under the tip of the needle until the end of the catheter is inside the vessel. The puncture site was dried and sealed by applying tissue adhesive and a cellulose patch over the puncture site. The area was observed for the leakage and the catheter function was verified by checking its tone with an AM radio tuned to 550 kHz. The abdominal cavity was flushed with sterile saline and the device was carefully placed on top of the intestines, parallel to the long axis of the body. Finally the radiotelemetry probe was secured by closing the abdominal incision and incorporating the tabs on the implant into the closure using 3-0 sutures in a simple uninterrupted pattern. After the surgery, rats were put in a warm environment and they were monitored in animal facility for 1 week.

After 1 week, blood pressure was recorded for 1 h both on the first day without any drug treatment and on the second day before the drug injections started. Then, rats were injected intraperitoneally with vehicle (DMSO), prednisolone (28 μmol/kg) or NCX-1015 (2.8 or 28 μmol/kg) twice-a-day at 12 hour intervals. Everyday for 6 days, 2 h after the morning administration of test drugs, blood pressure was monitored for 1 h.

One week after the beginning of the experiment, rats were injected with 2% w/v sodium pentobarbital (0.1 ml/100 g) and 5 ml of blood was obtained via cardiac puncture for the determination of plasma glucose levels. The blood was centrifuged at 400g and the cell precipitate and the plasma were quickly separated to prevent consumption of glucose by blood cells. The supernatant was frozen on dry ice and then kept at -70 °C until the glucose determination was done. Finally, the adrenal glands and thymus gland were removed from these rats and both wet and dry weights were recorded.

Glucose concentrations were determined using a glucose reagent kit (Sigma Diagnostics, Inc., St. Louis, MO, USA). The assay is based on the

hexokinase method (Carroll et al., 1970). As part of the assay, glucose is first phosphorylated by adenosine triphosphate in the reaction catalyzed by hexokinase. The glucose-6-phosphate formed is then oxidized to 6-phosphogluconate, a reaction catalyzed by glucose-6-phosphate dehydrogenase. The NADPH formed reduces phenazine methosulfate, which subsequently reduces iodonitrotetrazolium chloride. Reduced iodonitrotetrazolium forms a colourful product that could be detected at 520 nm with a microplate reader. A glucose standard of known concentration was used for the calculation of glucose concentration in the samples.

2.5. MATERIALS

DMSO was obtained from EM Science (Darmstadt, Germany). NCX-1015 (prednisolone 21-[(4′nitrooxymethyl) benzoate] was obtained from NicOx (Sophia Antipolis, France). DETA NONOate was obtained from Cayman Chemical (Ann Arbor, MI, USA). Trypan blue was purchased from Mallinckrodt Organic Reagents (Pointe-Claire, Quebec, Canada). Sodium nitrate was purchased from (ICN Biomedicals Inc. Aurora, Ohio, USA). All reagents and pre-stained SDS-PAGE molecular weight control markers used in western blot studies were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Prednisolone, type IV lambda carrageenan, nitrate reductase and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.6. STATISTICAL ANALYSIS

Data are expressed as the mean ± SEM. Comparisons between two groups of data were performed using a Student's unpaired t-test. Comparisons among three or more groups were made by using one-way ANOVA followed by

Dunnett's multiple-comparison test or Student-Newman-Keuls test using Instat version 3.00 (GraphPad Software, San Diego, CA, U.S.A.). A P value of <0.05 was considered to as significant.

CHAPTER 3: RESULTS

3.1. CARRAGEENAN-INDUCED INFLAMMATION

Injection of carrageenan into the airpouch resulted in a time-dependent accumulation of leukocytes (Figure 3.1). The leukocyte infiltration into the airpouch was evident at 4 h after the carrageenan injection and the numbers of leukocytes were significantly higher compared to saline controls. The number of leukocytes in the airpouch exudates continued to increase at 24 h (results not shown) and differential staining indicated that the majority of the cells were neutrophils (>90 %) at all time points examined.

The increase in leukocyte infiltration into the airpouch was paralleled by an increase in nitrite/nitrate, PGE_2 and LTB_4 levels. The concentration of nitrite/nitrate in the airpouch exudates were almost undetectable in saline-injected rats, however, carrageenan administration resulted in a progressive increase in nitrate/nitrite levels in the airpouch exudates. The increase in nitrate/nitrite is at least partly due to increased production of iNOS in the leukocyte infiltrate, as the protein expression of this enzyme is increased at 4 h after carrageenan administration and increases further at 6 h coinciding with the increases in nitrite/nitrate concentration in the airpouch exudate (Figure 3.2A,B).

A marked increase in the exudate levels of PGE_2 was observed at 6 h after carrageenan administration compared with the vehicle-treated group (Figure 3.3A). COX-1 protein expression in the cellular infiltrate progressively increased over time (Figure 3.3B). However, the expression of COX-2 protein was first detected at 4 h and markedly increased 6 h after the carrageenan injection (Figure 3.3C). The majority of the PGE_2 produced in the airpouch in response to carrageenan administration is likely to be derived from COX-2, as has been demonstrated previously in studies utilizing selective COX-2 inhibitors (275).

In contrast to the changes in nitrate/nitrite and PGE_2, there was not a progressive increase in LTB_4 in the airpouch with time after administration of

carrageenan (Figure 3.4A). Rather, LTB_4 concentrations in the airpouch were maximal 2h after the carrageenan injection. At 4 and 6h after carrageenan administration, although still significantly greater than in the control group, there was a gradual decrease in LTB_4 levels. However, 5-LO expression in the infiltrating leukocytes was first detectable at 4 h and its levels did not change significantly at 6 h after the carrageenan injection (Figure 3.4B).

We have also determined the rate of necrosis in the exudate cells. The majority of cells (> 90%) were found to be viable, with no sign of necrosis (Figure 3.5). Moreover, unlike vehicle-treated group, carrageenan administration did not result in any significant apoptosis in cells recovered from the exudates (Figure 3.6).

3.2. EFFECTS OF PREDNISOLONE AND NCX-1015

Injection of prednisolone into the airpouch at doses of 8.3 or 28 μmol/kg significantly inhibited leukocyte infiltration (Figure 3.7). Although prednisolone and NCX-1015 were equally effective in reducing leukocyte infiltration into the airpouch at the highest dose tested, NCX-1015 was more effective than prednisolone at doses of 2.8 and 8.3 μmol/kg (Figure 3.7).

The potent anti-inflammatory action of NCX-1015 was also observed in terms of its inhibitory effects on nitrate/nitrite, PGE_2 and LTB_4 accumulation in the airpouch exudate. The increase in nitrate/nitrite levels by carrageenan was significantly reduced by prednisolone at doses $\geq$ 8.3 μmol/kg, whereas NCX-1015 was effective at a dose as low as 2.8 μmol/kg (Figure 3.8).

To determine the amount of nitrate/nitrite that was contributed by NCX-1015, we measured the nitrite/nitrate concentration in the airpouch exudates of rats treated with NCX-1015 at the lowest and the highest doses tested (0.8 and 28 μmol/kg) in the absence of inflammation. Negligible levels of nitrite/nitrate

suggest that NCX-1015 did not contribute to the levels of NO species observed in carrageenan-induced inflammation (Figure 3.9).

Similar to its effect on leukocyte infiltration, NCX-1015 significantly inhibited PGE_2 and LTB_4 production in the airpouch at a much lower dose than prednisolone whereas the same degree of inhibition was achieved with the highest dose of prednisolone (28 μmol/kg) (Figure 3.10 and 3.11).

The effect of DMSO on inflammatory mediator content of the exudate was also determined. Rats received either saline or DMSO 1 h before carrageenan injection. Our results suggest that DMSO may partly account for the decreases observed in nitrate/nitrate levels as it caused significant reduction of nitrate/nitrite compared to saline-treated group. However, DMSO did not have any significant effect on PGE_2 or LTB_4 levels (results not shown).

In order to understand the underlying mechanism for the observed anti-inflammatory effects of NCX-1015, we have determined the rate of necrosis and apoptosis in the cells of exudates recovered from rats treated with prednisolone or NCX-1015 (8.3 μmol/kg). More than 90% of the cells were viable and none of the drugs induced apoptosis at the doses tested in this study (Figure 3.12 and 3.13).

3.2.1. THE EFFECT OF COMBINING PREDNISOLONE WITH AN NO DONOR

Prednisolone attenuated carrageenan-induced leukocyte infiltration into the airpouch by approximately 40%. DETA-NONOate reduced leukocyte infiltration by approximately 20%. The combination of both drugs, on the other hand, elicited more than a 50% reduction in leukocyte infiltration (Figure 3.14).

3.3. THE EFFECTS OF PREDNISOLONE AND NCX-1015 ON SYSTEMIC BLOOD PRESSURE AND PLASMA GLUCOSE LEVELS

As shown in Figure 3.15, intraperitoneal injection of vehicle, prednisolone or NCX-1015 at a dose of 28 μmol/kg did not induce any significant change in the systemic blood pressure values over the course of 1 week of daily treatment. Daily administration of prednisolone (28 μmol/kg) did not affect adrenal gland dry weight. However, it significantly reduced thymus gland dry weight by approximately 50%. NCX-1015, at the highest dose tested (28 μmol/kg), reduced adrenal gland dry weight by approximately 40% and thymus gland dry weight by 87%. A lower dose of NCX-1015 (2.8 μmol/kg) was also used in this experiment as this dose has been shown to have potent anti-inflammatory effects in the carrageenan-induced inflammation. NCX-1015 at 2.8 μmol/kg dose did not significantly affect adrenal gland dry weight and it reduced the thymus gland dry weight only by approximately 20% (Figure 3.16 and 3.17). As for the plasma glucose levels, prednisolone (28 μmol/kg) did not have any significant effect whereas NCX-1015 at both doses tested (2.8 and 28 μmol/kg) increased plasma glucose levels significantly compared to vehicle-treated groups (Figure 3.18).

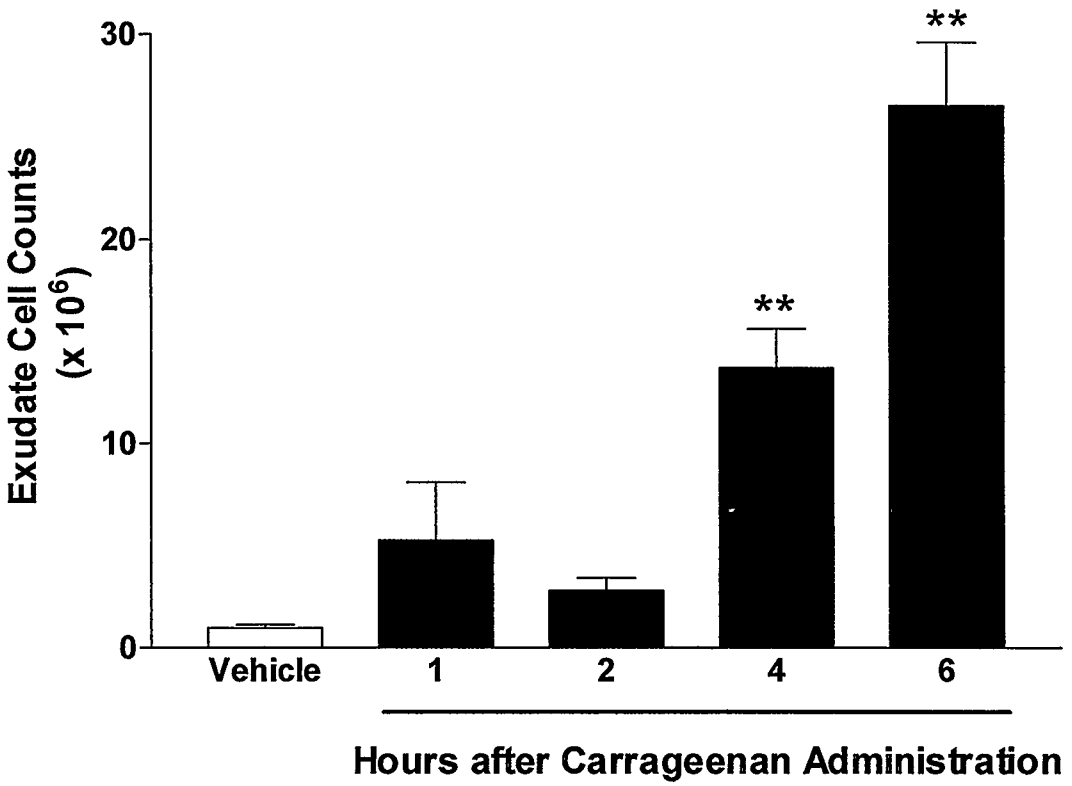

Figure 3.1. Time-course of leukocyte infiltration after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. Each bar represents the mean ± SEM of 5 rats per group. **P<0.01 compared to the vehicle-treated group.

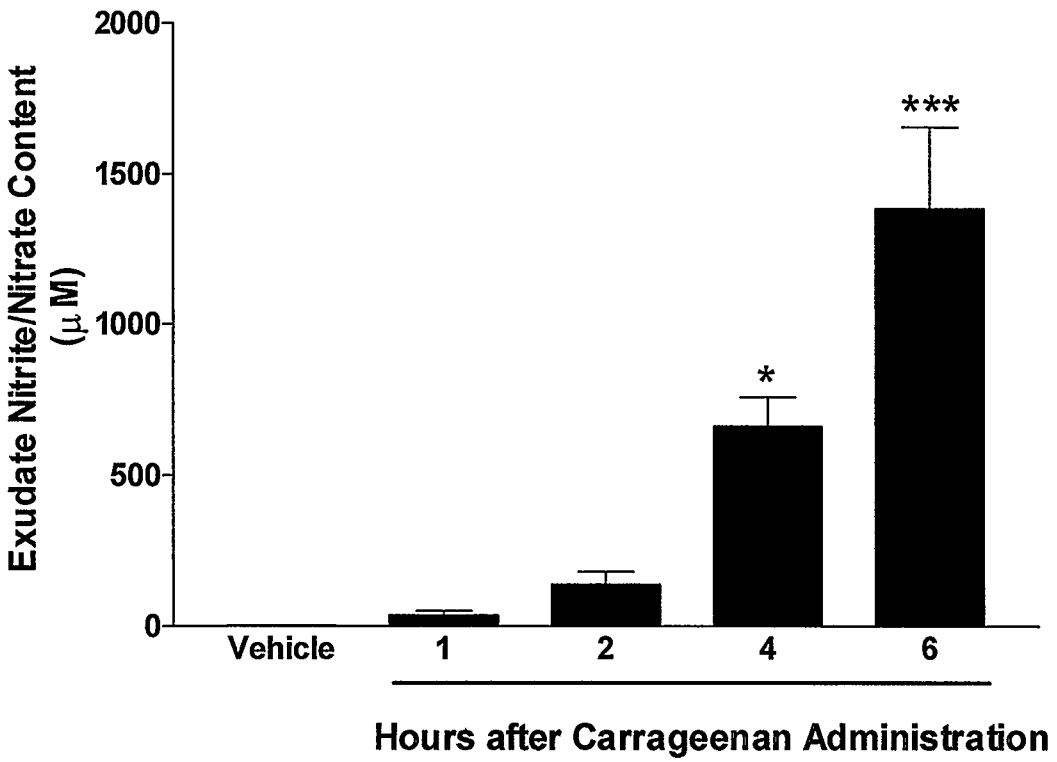

Figure 3.2A. Time-course of nitrite/nitrate accumulation in the exudate after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. Each bar represents the mean ± SEM of 5 rats per group. *P<0.05 and ***P<0.001 compared to the vehicle-treated group.

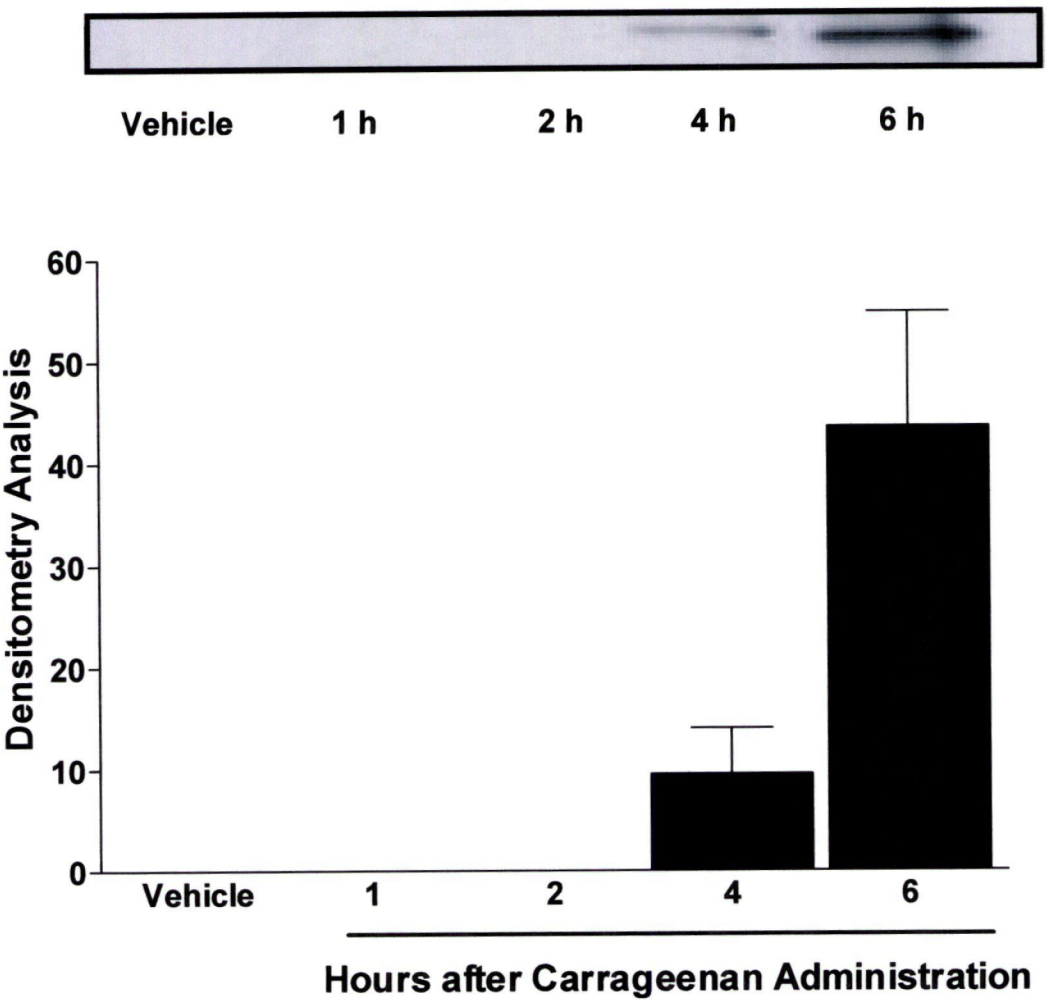

Figure 3.2B. Western blot analysis of iNOS protein (130 kDa) expression in the cellular infiltrate at different time points after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. A representative blot is shown on top and the results of densitometric analysis (expressed in arbitrary units) are shown at the bottom. Each bar represents the mean ± SEM of 4 rats per group.

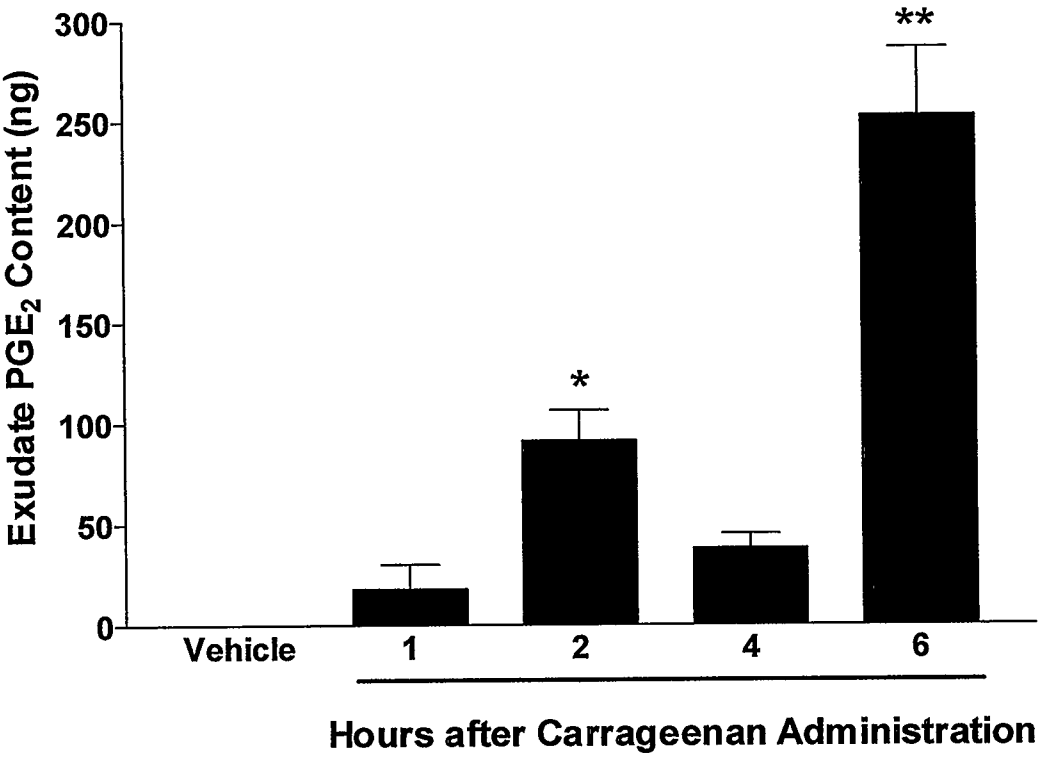

Figure 3.3A. Time-course of PGE_2 accumulation in the exudate after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. Each bar represents the mean ± SEM of 5 rats per group. *P<0.05 and **P<0.01 compared to the vehicle-treated group.

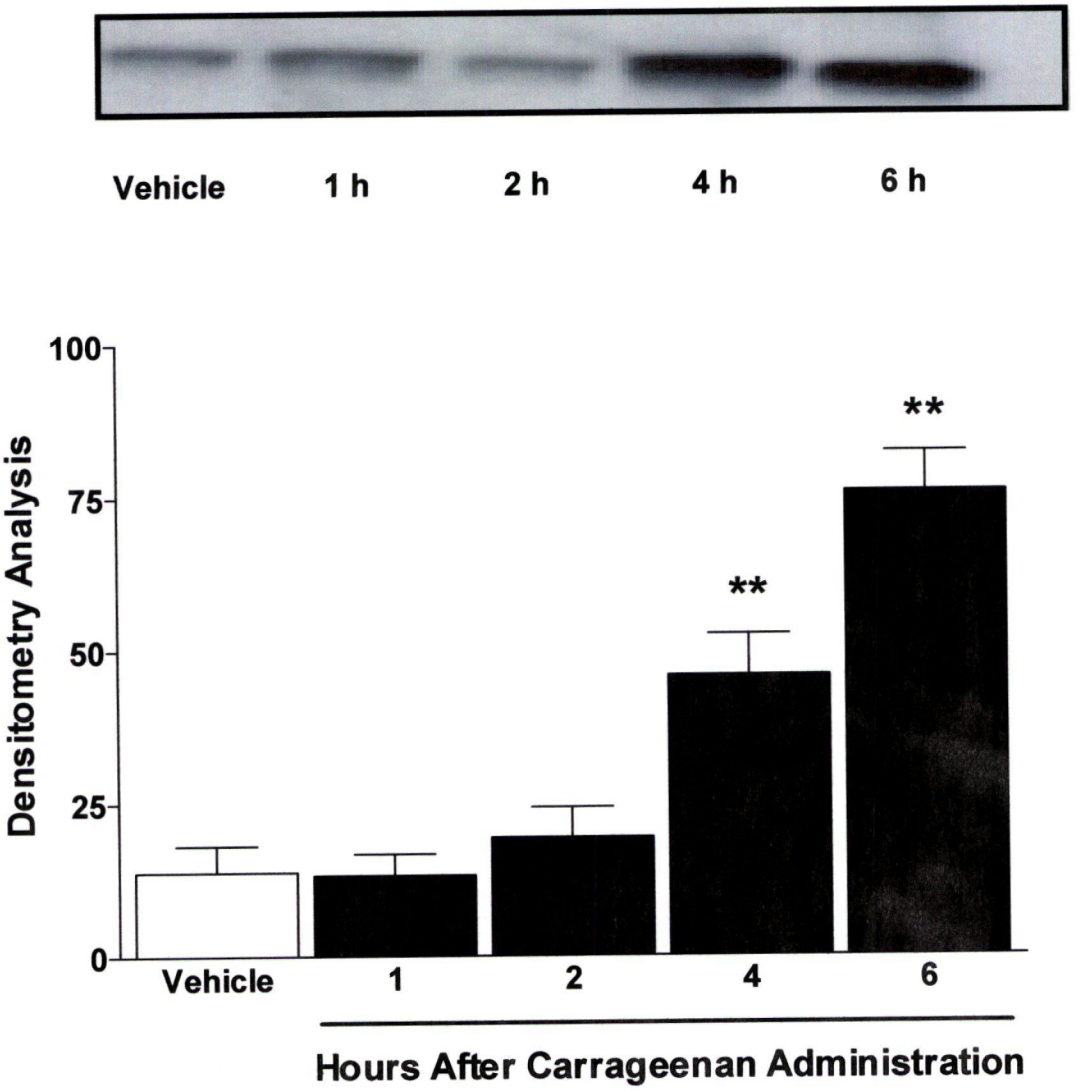

Figure 3.3B. Western blot analysis of COX-1 (70 kDa) expression in the cellular infiltrate at different time points after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. A representative blot is shown on top and the results of densitometric analysis (expressed in arbitrary units) are shown at the bottom. Each bar represents the mean ± SEM of 4 rats per group. **P<0.01 compared to the vehicle-treated group.

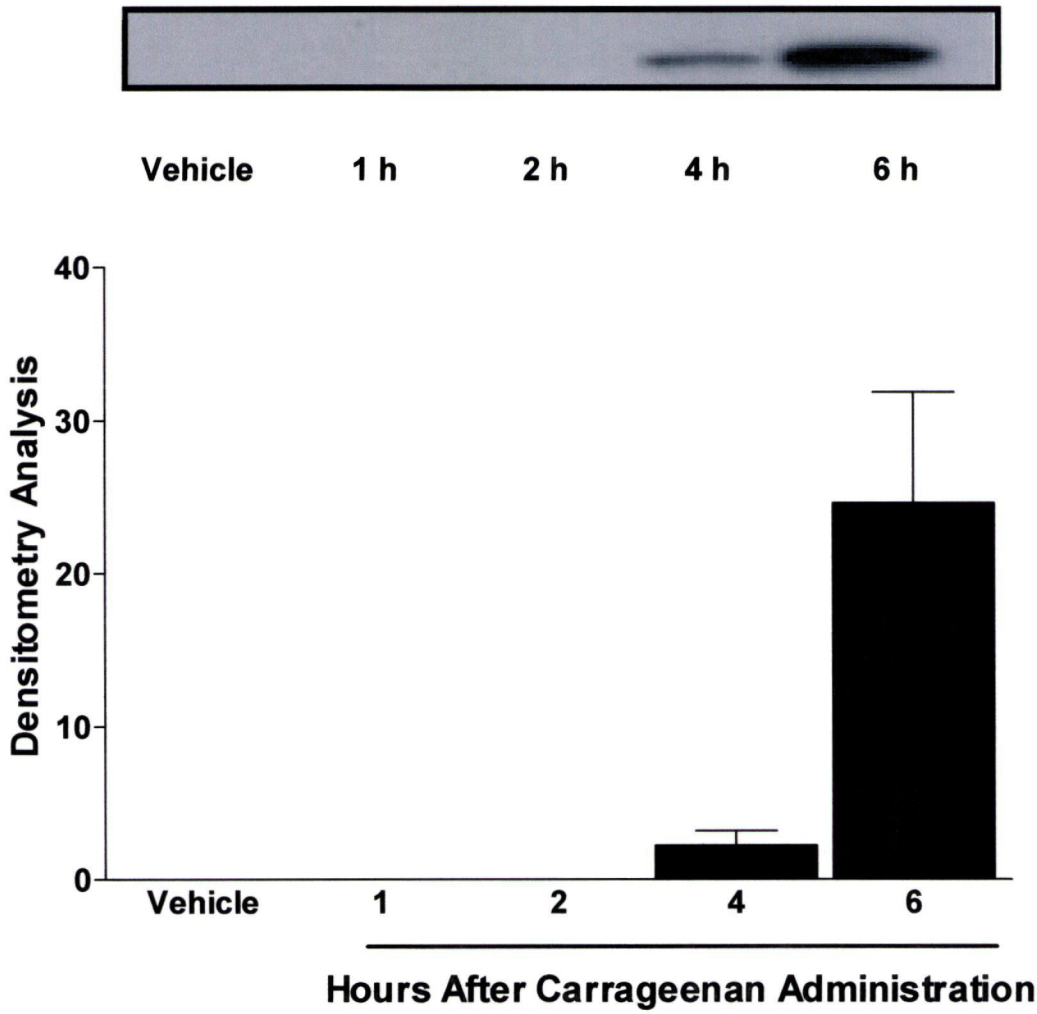

Figure 3.3C. Western blot analysis of COX-2 (80 kDa) expression in the cellular infiltrate at different time points after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. A representative blot is shown on top and the results of densitometric analysis (expressed in arbitrary units) are shown at the bottom. Each bar represents the mean ± SEM of 4 rats per group.

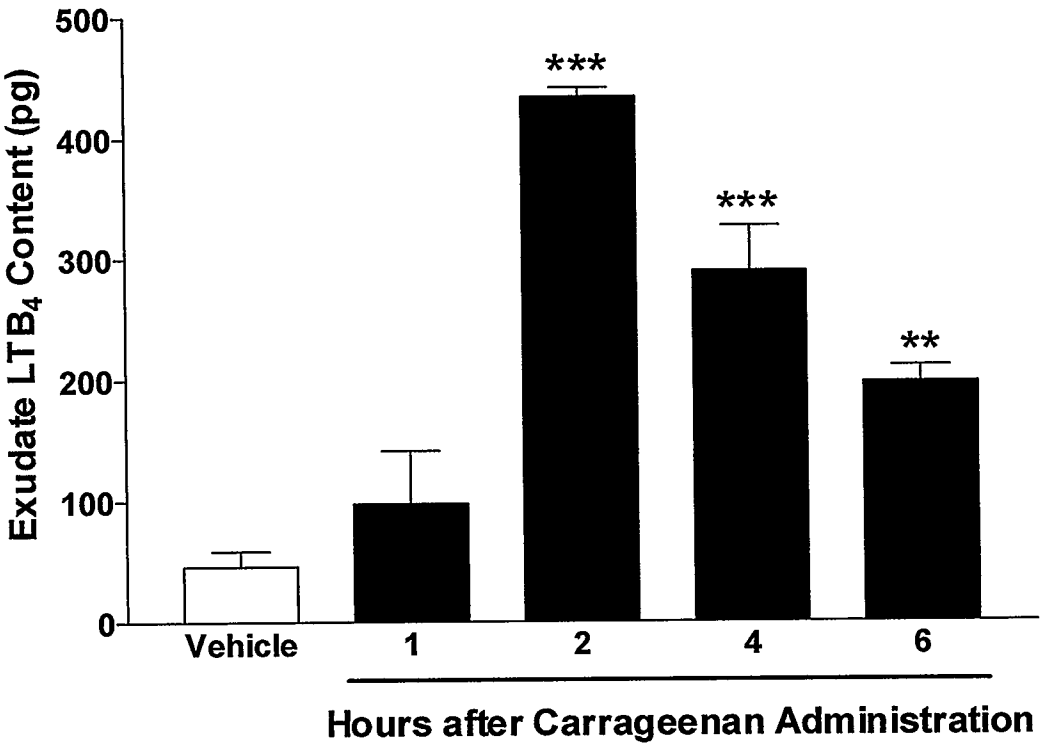

Figure 3.4A. Time-course of LTB_4 accumulation in the exudate after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. Each bar represents the mean ± SEM of 5 rats per group. ** $P<0.01$ and ***$P<0.001$ compared to the vehicle-treated group.

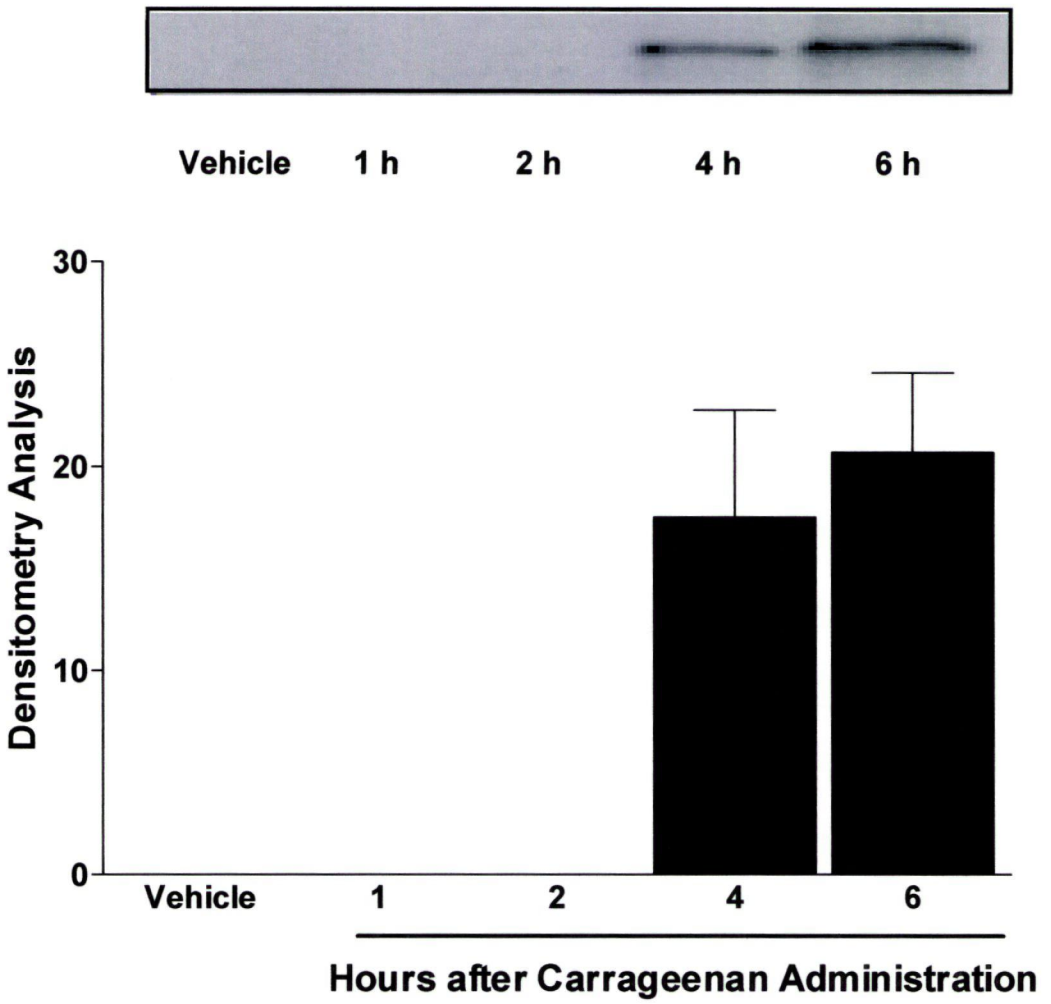

Figure 3.4B. Western blot analysis of 5-LO (78 kDa) expression in the cellular infiltrate at different time points after carrageenan administration into the airpouch. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. A representative blot is shown on top and the results of densitometric analysis (expressed in arbitrary units) are shown at the bottom. Each bar represents the mean ± SEM of 4 rats per group.

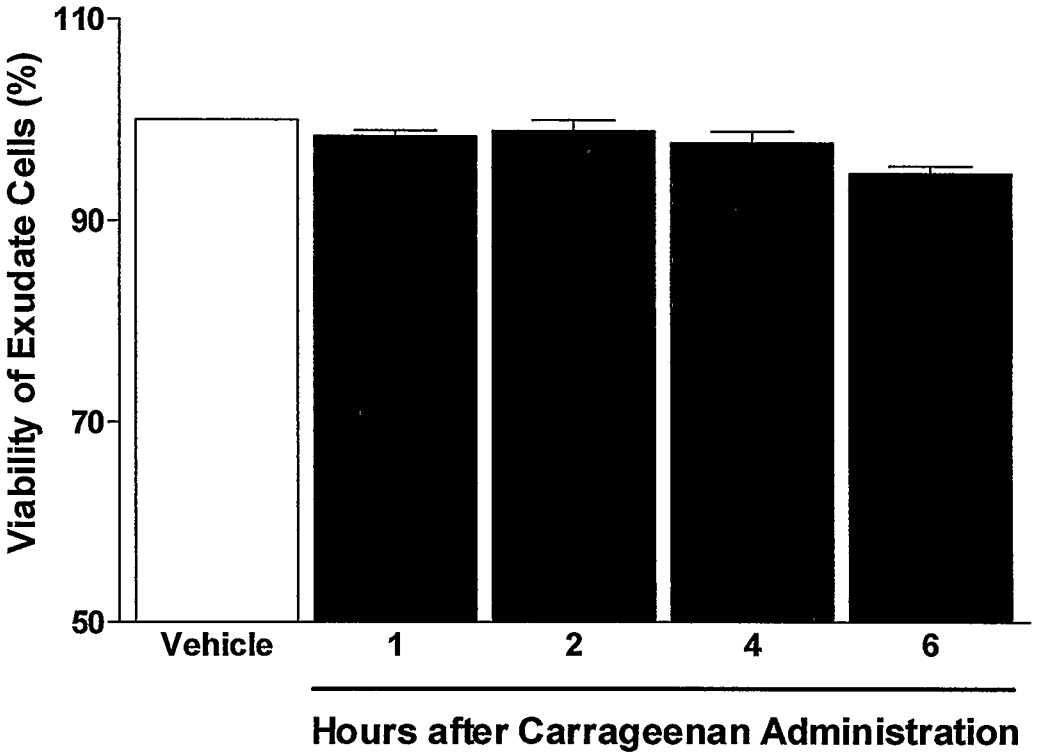

Figure 3.5. The viability of exudate cells after carrageenan administration into the airpouch was determined via trypan blue staining. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. Each bar represents the mean ± SEM of 5 rats per group.

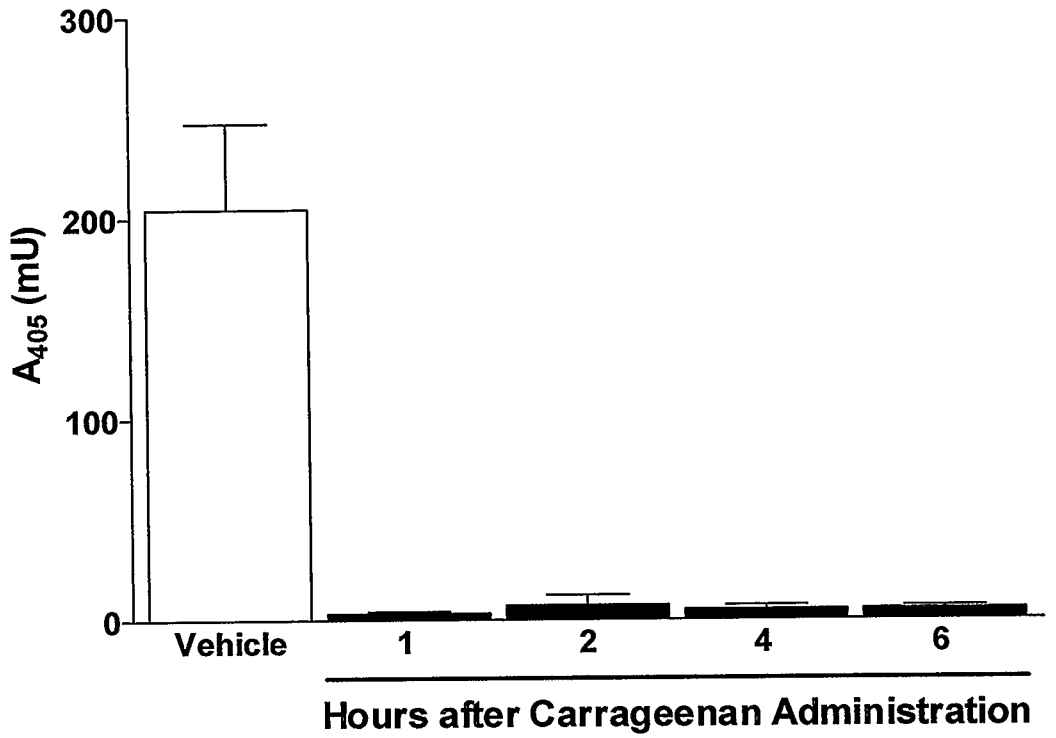

Figure 3.6. The apoptosis of exudate cells after carrageenan administration into the airpouch were determined via an ELISA that detects apoptotic nucleosomes. Exudates from the vehicle-treated rats were collected 6 h after carrageenan injection. Each bar represents the mean ± SEM of 5 rats per group.

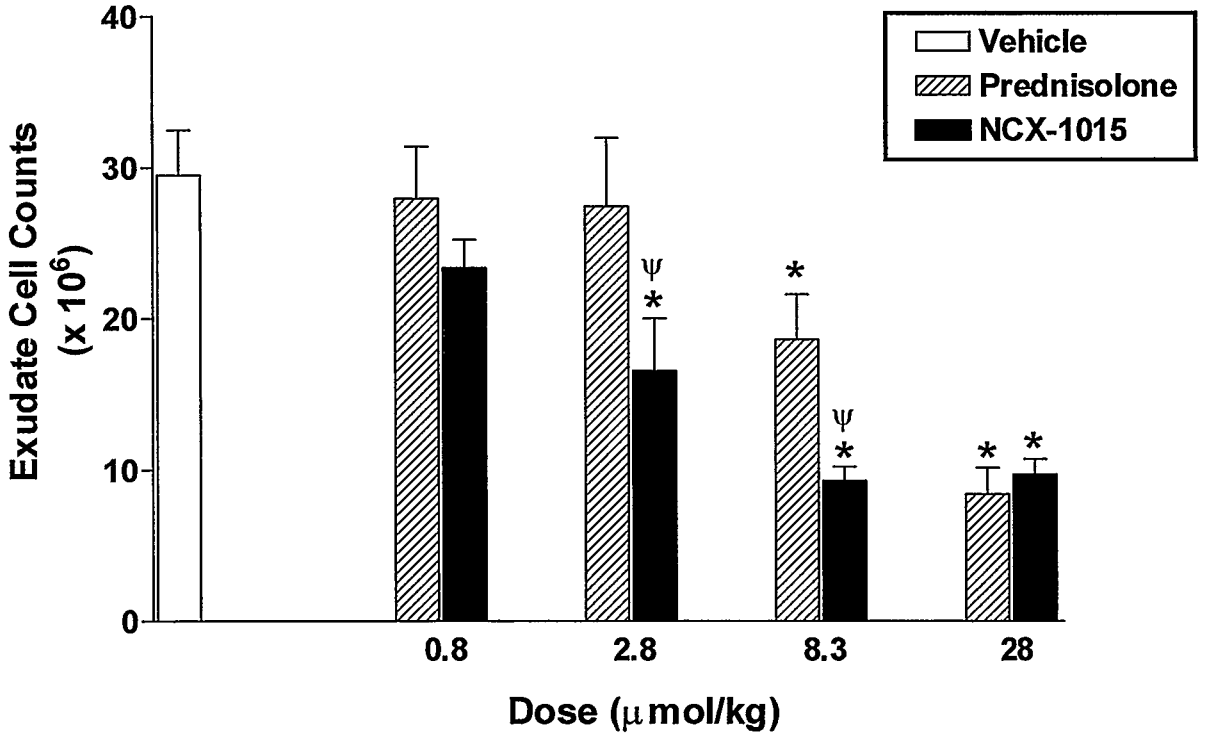

Figure 3.7. The effect of prednisolone and NCX-1015 on leukocyte counts in the airpouch exudate. The vehicle (DMSO), prednisolone or NCX-1015 was injected into the airpouch 1 h before carrageenan administration. The exudate fluid was collected 6 h after carrageenan administration. Each bar represents the mean ± SEM of 4-8 rats per group. *$P<0.05$ compared to the vehicle-treated group and $\psi P<0.05$ compared to the corresponding prednisolone group.

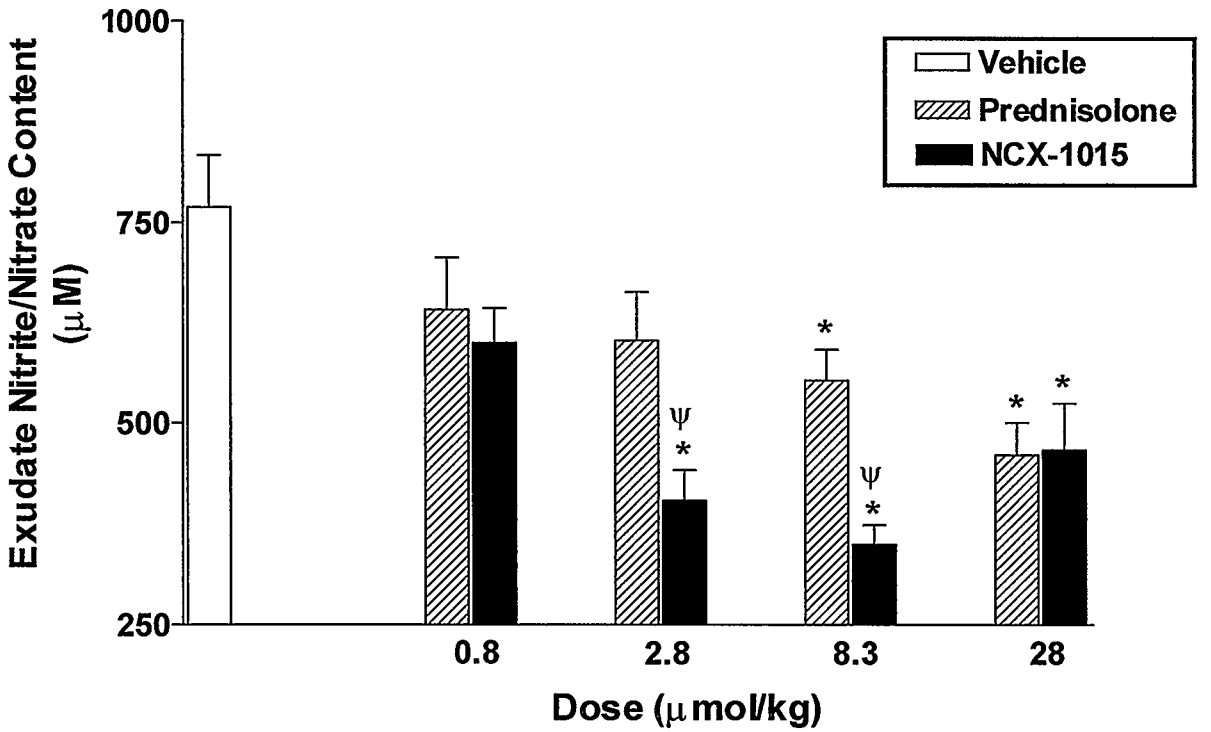

Figure 3.8. The effect of prednisolone and NCX-1015 on nitrite/nitrate concentration in the airpouch exudate. The vehicle (DMSO), prednisolone or NCX-1015 was injected into the airpouch 1 h before carrageenan administration. The exudate fluid was collected at 6 h after carrageenan administration. Each bar represents the mean ± SEM of 4-7 rats per group. *P<0.05 compared to the vehicle-treated group and ψP<0.05 compared to the corresponding prednisolone group.

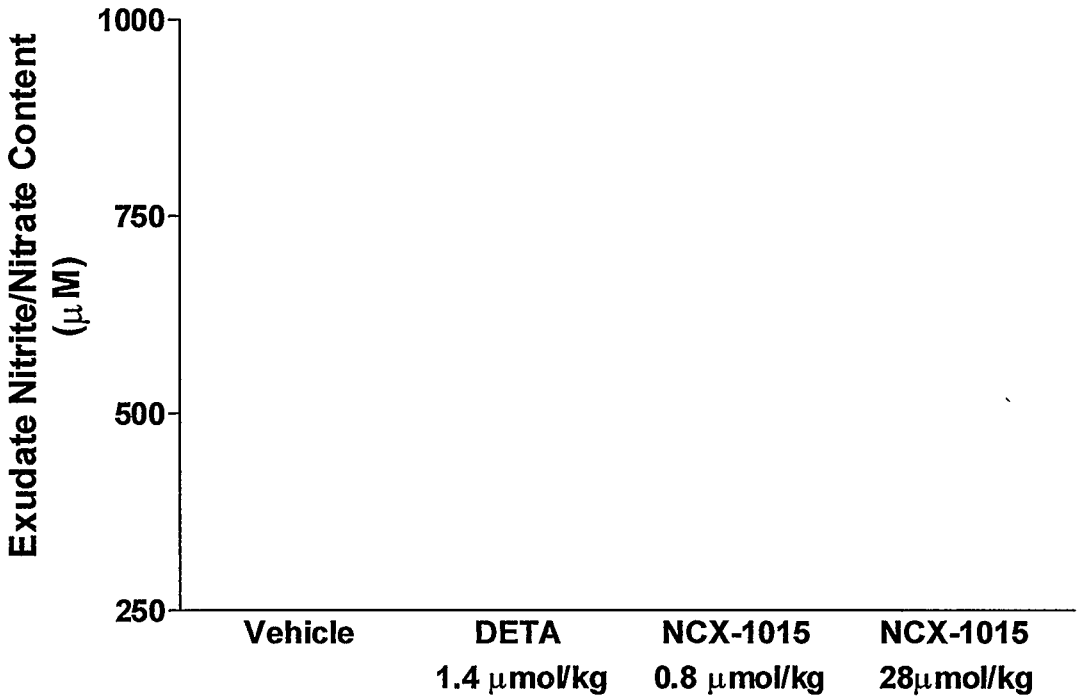

Figure 3.9. The effect of DETA-NO (DETA) and NCX-1015 on nitrite/nitrate concentration in the airpouch exudate in the absence of carrageenan-induced inflammation. The vehicle (DMSO), DETA-NO or NCX-1015 was injected into the airpouch 1 h before the saline administration. The exudate fluid was collected at 6 h after saline administration.

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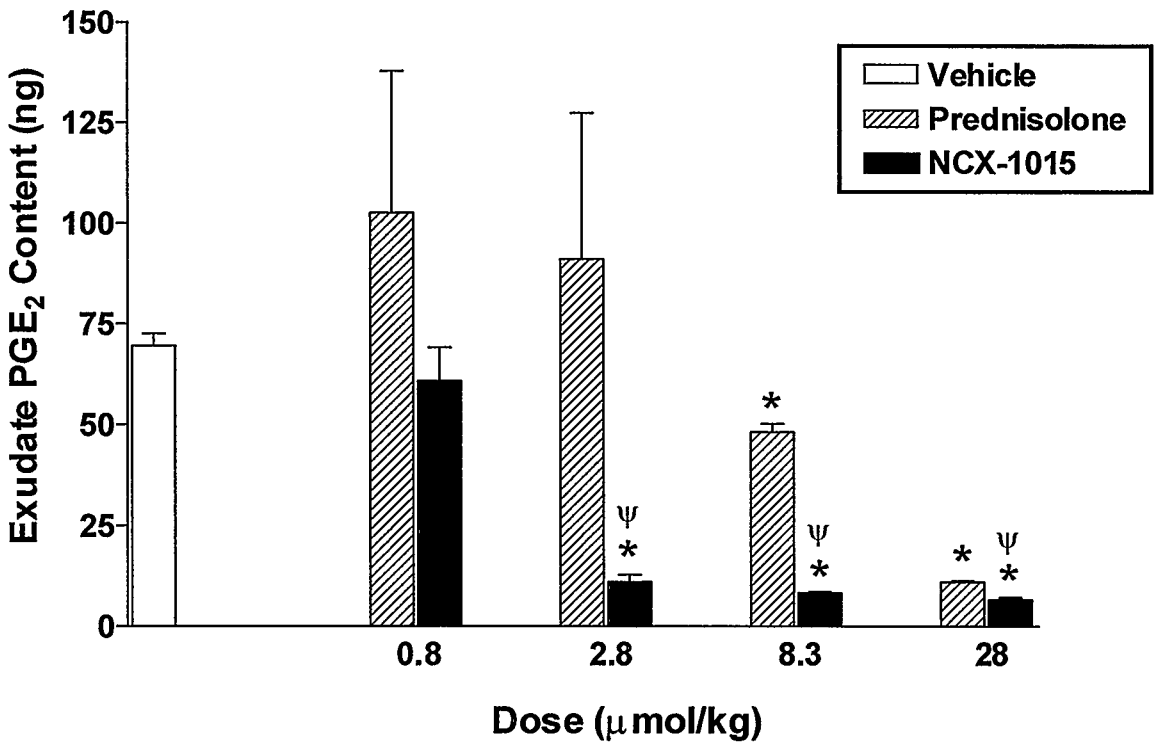

Figure 3.10. The effect of prednisolone and NCX-1015 on PGE_2 levels in the airpouch exudate. The vehicle (DMSO), prednisolone or NCX-1015 was injected into the airpouch 1 h before carrageenan administration. The exudate fluid was collected 6 h after carrageenan administration. Each bar represents the mean ± SEM of 4 rats per group. *$P<0.05$ compared to the vehicle-treated group and ψ$P<0.05$ compared to the corresponding prednisolone group.

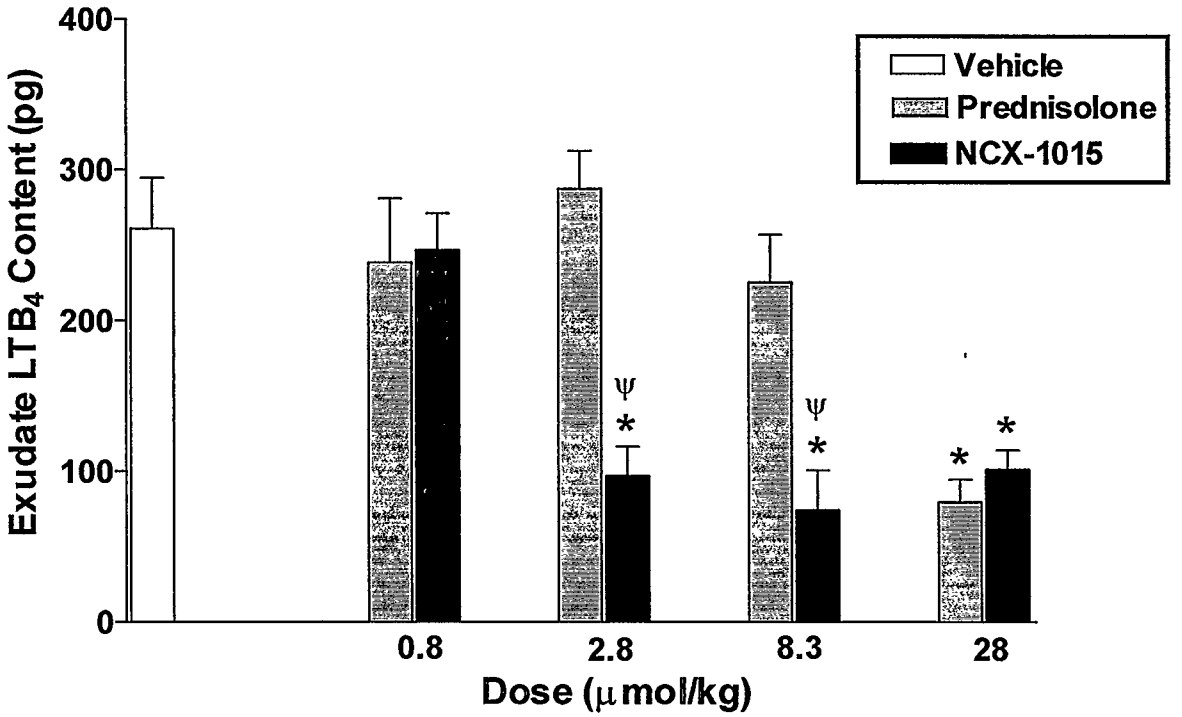

Figure 3.11. The effect of prednisolone and NCX-1015 on LTB_4 levels in the airpouch exudate. The vehicle (DMSO), prednisolone or NCX-1015 was injected into the airpouch 1 h before carrageenan administration. The exudate fluid was collected 6 h after carrageenan administration. Each bar represents the mean ± SEM of 4 rats per group. *$P<0.05$ compared to the vehicle-treated group and ψ$P<0.05$ compared to the corresponding prednisolone group.

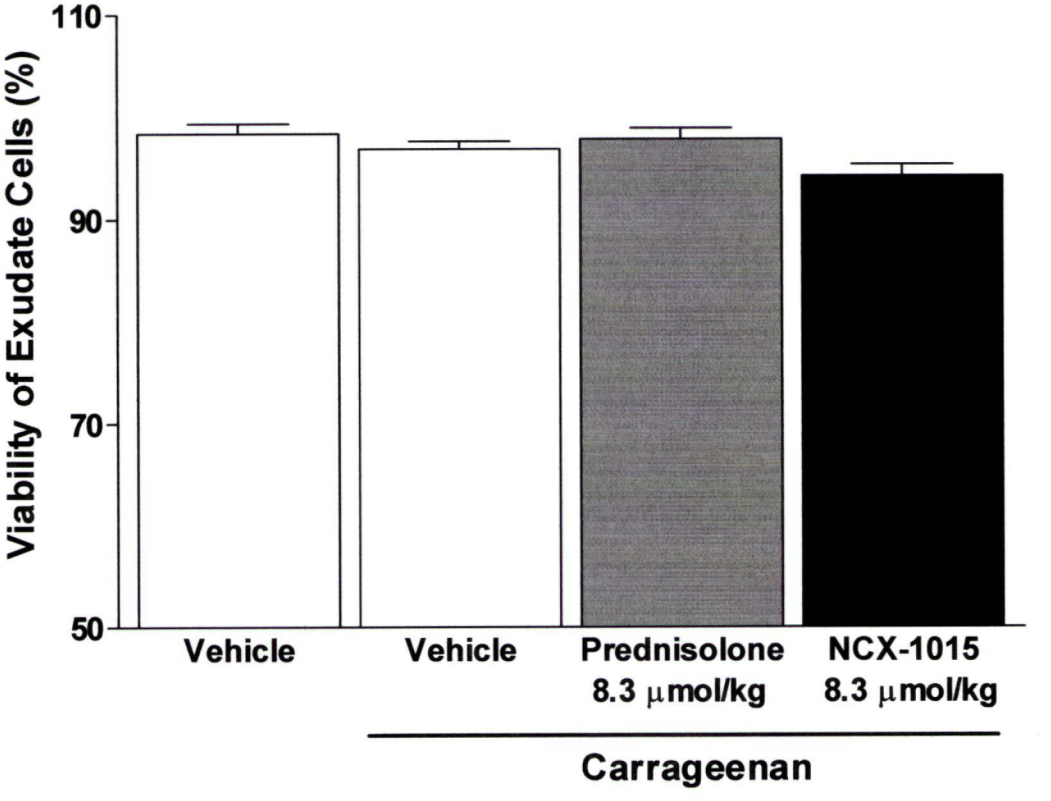

Figure 3.12. The effect of prednisolone or NCX-1015 on the viability of exudate cells was determined via trypan blue staining. The vehicle (DMSO), prednisolone or NCX-1015 was injected into the airpouch 1 h before saline or carrageenan administration. The exudate fluid was collected 6 h after carrageenan administration. Each bar represents the mean ± SEM of 4 rats per group.

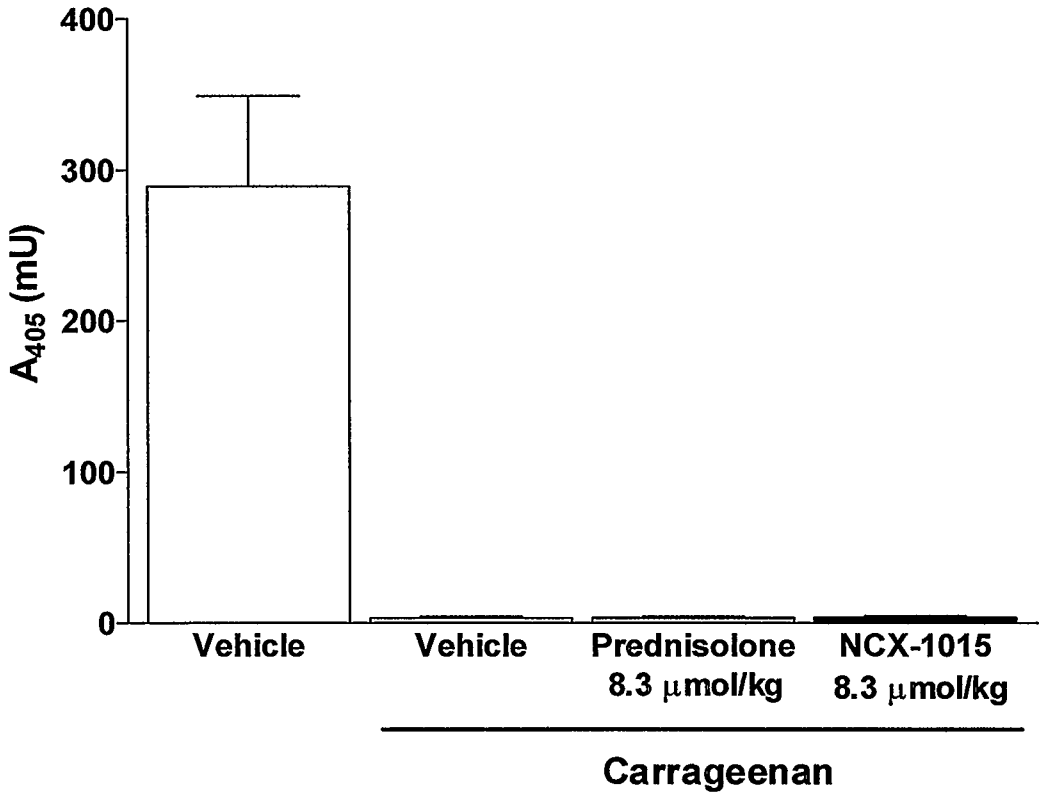

Figure 3.13. The effect of prednisolone or NCX-1015 on the apoptosis of exudate cells was determined via an ELISA that detects apoptotic nucleosomes. The vehicle (DMSO), prednisolone or NCX-1015 was injected into the airpouch 1 h before saline or carrageenan administration. The exudate fluid was collected 6 h after carrageenan administration. Each bar represents the mean ± SEM of 4 rats per group.

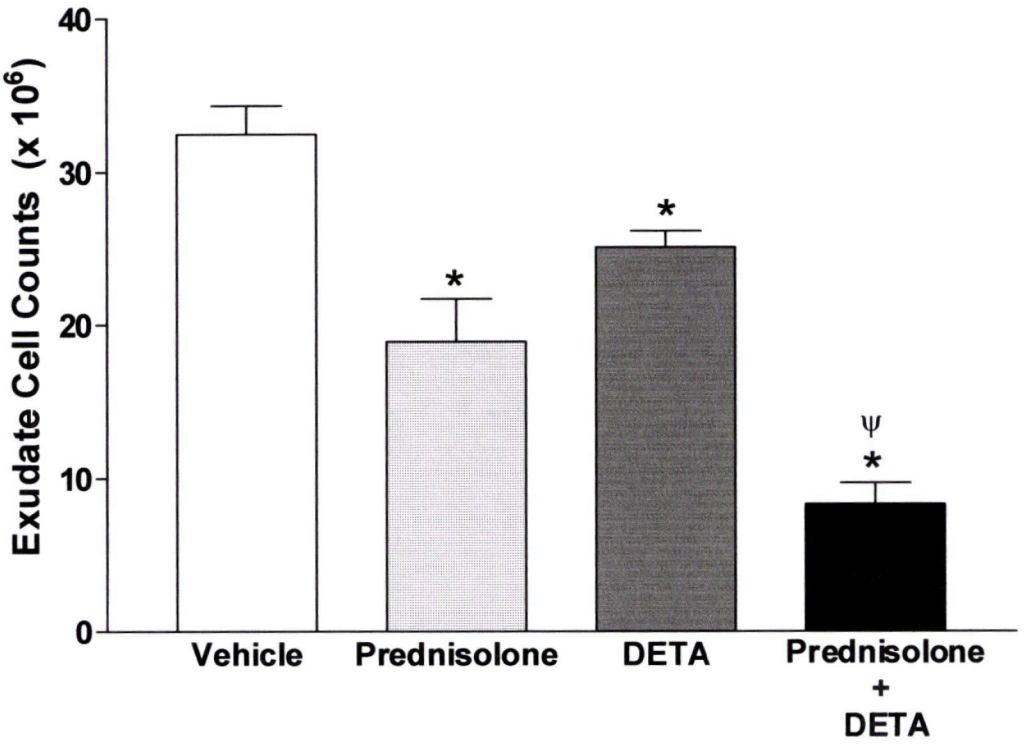

Figure 3.14. The effect of prednisolone and an NO donor, DETA-NONOate (DETA), alone or in combination, on leukocyte infiltration in response to carrageenan. The vehicle (DMSO), prednisolone (2.8 μmol/kg) or DETA-NONOate (1.4 μmol/kg) was injected into the airpouch 1 h before the carrageenan injection. The exudate fluid was collected 6 h after carrageenan administration. Each bar represents the mean ± SEM of 6 rats per group. *$P<0.05$ compared to the vehicle-treated group and ψ$P<0.05$ compared to prednisolone group.

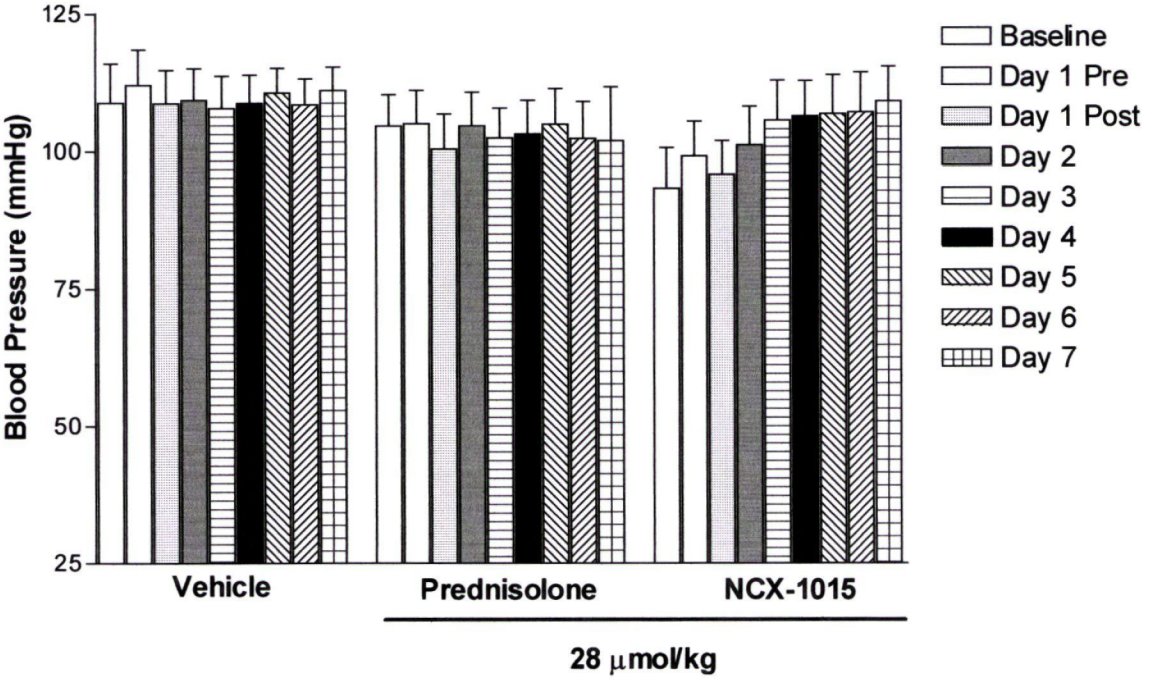

Figure 3.15. The effect of chronic administration of prednisolone and NCX-1015 on systemic blood pressure. Blood pressure was recorded for 1 hour on the first day without any drug treatment (baseline) and on the second day before (day 1 pre) and after (day 1 post) the drug injections started. Rats were injected intraperitoneally with vehicle (DMSO), prednisolone (28 μmol/kg) or NCX-1015 (28 μmol/kg) twice-a-day. Everyday for 6 days, 2 hours after the morning administration of test drugs, blood pressure was monitored for 1 hour. Each bar represents the mean ± SEM of 5 rats per group.

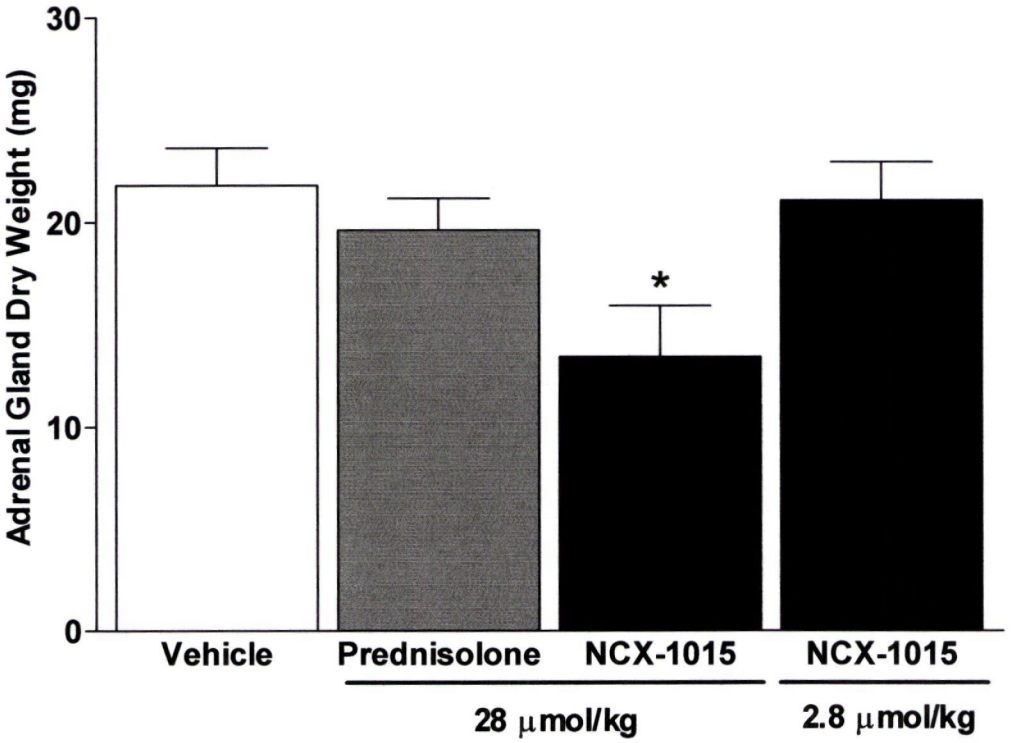

Figure 3.16. The effect of chronic administration of prednisolone and NCX-1015 on adrenal gland dry weight. Rats were injected intraperitoneally with vehicle (DMSO), prednisolone (28 μmol/kg) or NCX-1015 (2.8 or 28 μmol/kg) twice-a-day for a week. Each bar represents the mean ± SEM of 5 rats per group. *P<0.05 compared to the vehicle-treated group.

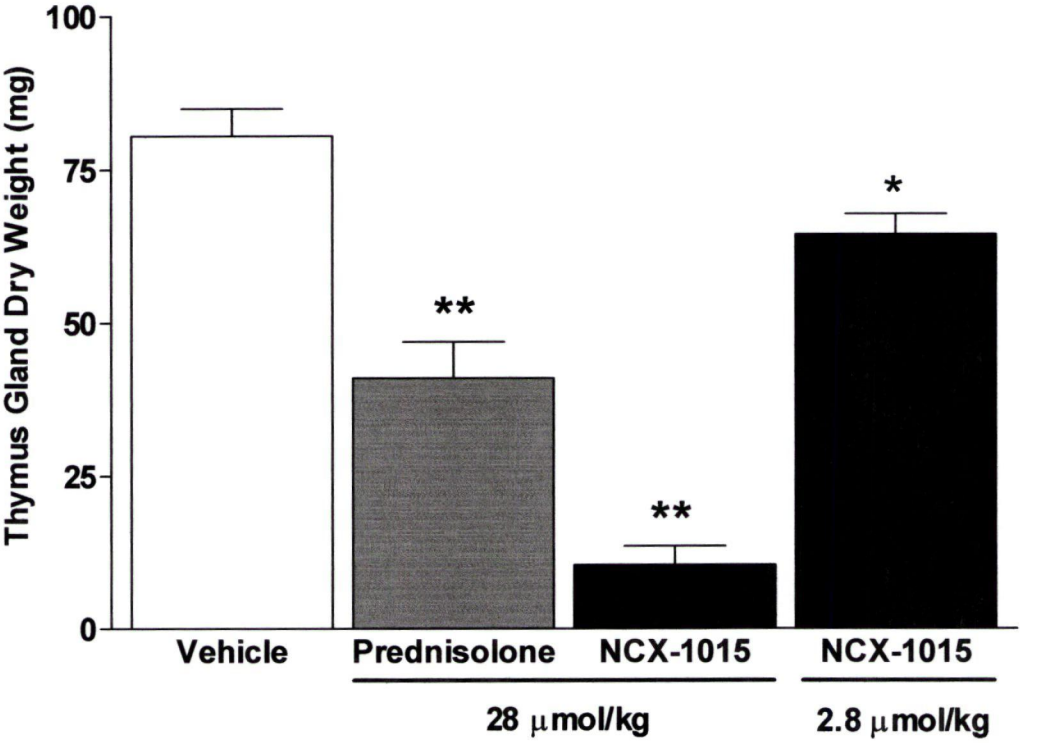

Figure 3.17. The effect of chronic administration of prednisolone and NCX-1015 on thymus gland dry weight. Rats were injected intraperitoneally with vehicle (DMSO), prednisolone (28 μmol/kg) or NCX-1015 (2.8 or 28 μmol/kg) twice-a-day for a week. Each bar represents the mean ± SEM of 5 rats per group. **P<0.01 and *P<0.05 compared to the vehicle-treated group.

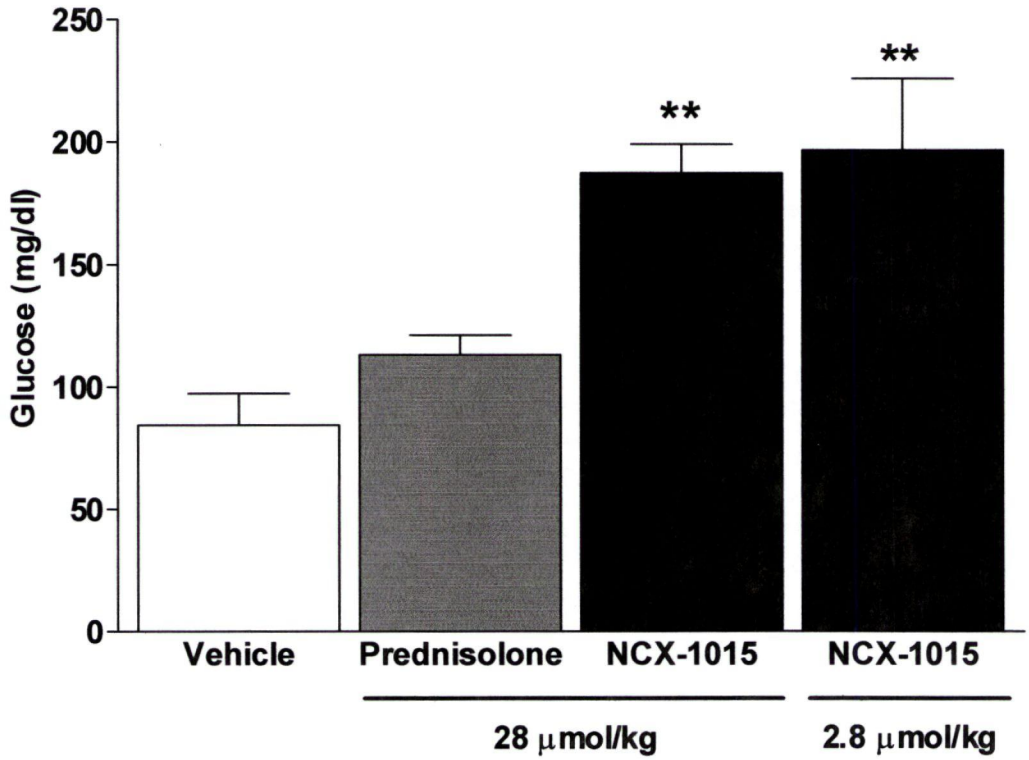

Figure 3.18. The effect of chronic administration of prednisolone and NCX-1015 on plasma glucose levels. Rats were injected intraperitoneally with vehicle (DMSO), prednisolone (28 μmol/kg) or NCX-1015 (2.8 or 28 μmol/kg) twice-a-day for a week. Each bar represents the mean ± SEM of 5 rats per group. **P<0.01 compared to the vehicle-treated group.

CHAPTER 4: DISCUSSION OF THE RESULTS

An airpouch is formed by the disruption of the subcutaneous connective tissue in rats by repeated injections of air (72). The resulting cavity, the airpouch, has a lining composed of fibroblasts and macrophages between the cavity and the underlying vasculature of the subcutaneous tissues. Mast cells are also found deep to the lining of the airpouch. Carrageenan injection into the airpouch induces inflammation by activating the alternative pathway of the complement system and causing subsequent mast cell degranulation (242, 251). It also results in exudate formation and leukocyte infiltration. There is a consensus in the literature that the majority of infiltrating cells are neutrophils during the first 24 h after carrageenan injection into the airpouch (15, 243).

We found that after carrageenan injection, nitrate/nitrite and PGE_2 content of the exudate increased in parallel with the increase in neutrophil extravasation. Carrageenan stimulation also results in the induction of iNOS and COX-2 protein expression both in our study and in different models of carrageenan-induced inflammation (128, 235). In this study we also observed an increase in the expression of COX-1. Although COX-1 contributes to the carrageenan-induced inflammation in the rat paw, the increase in PGE_2 observed in this model was shown to occur via COX-2, as a selective inhibitor of COX-2 completely reduced PGE_2 in the exudate (275). 5-LO induction and subsequent increases in LTB_4 concentrations were also observed in other carrageenan-induced inflammation models (222). In the present study, we found that LTB_4 concentration peaked at 2 h after the carrageenan injection and gradually decreased thereafter suggesting that LTB_4 may be degraded. Overall, our data indicate that iNOS, COX-2 and 5-LO enzymes expressed in migrating leukocytes contribute to the observed increases in nitrate/nitrite, PGE_2, and LTB_4 although we can not exclude the possibility that cells of the airpouch lining can also express these enzymes (235).

NO-NSAIDs are a novel group of drugs synthesized by the ester linkage of a NO-releasing moiety to conventional NSAIDs. NO released from these drugs have decreased gastric toxicity and improved anti-inflammatory efficacy. Moreover, NO-NSAIDs also gained new activities independent of COX inhibition. A similar approach was followed to create a novel glucocorticoid. A nitrooxy-methylbenzoate derivative of prednisolone was synthesized (NCX-1015) (208). NCX-1015 released NO dose-dependently both *in vitro* in human platelet rich plasma and *in vivo* when it was injected into the mouse peritoneal cavity (208). We proposed that NCX-1015 has enhanced anti-inflammatory effects in carrageenan-induced inflammation in the rat.

Previous studies demonstrated that NCX-1015 inhibited neutrophil extravasation and the release of the chemokine, KC, more potently than prednisolone in zymosan-induced peritonitis in mice (208). Similarly, in the present study, we have shown that administration of NCX-1015 resulted in potent inhibition of leukocyte infiltration, and inflammatory mediator release (i.e., nitrate/nitrite, PGE_2, LTB_4) in the carrageenan-airpouch model.

A number of factors may contribute to the enhanced anti-inflammatory effects of NCX-1015. Our experiments have shown that co-administration of prednisolone with a NO donor, DETA-NONOate, reduced carrageenan-induced leukocyte infiltration by approximately 50% compared to prednisolone alone, suggesting that NO donor has an additive effect.

NO acts as a physiological regulator of inflammation, therefore there are some possible mechanisms through which NO may act synergistically with prednisolone to bring the potent anti-inflammatory effects. Firstly, NO is considered as an anti-adhesive molecule and inhibition of NO production results in increased leukocyte rolling and adhesion in different experimental models (reviewed in 139). Carrageenan-induced inflammation was characterized by increased production of superoxide anions via NADPH oxidase and mast cell degranulation (235, 251). NO inhibits neutrophil superoxide anion production via a direct effect on NADPH oxidase (49) and exogenous NO can prevent leukocyte

adhesion induced by increased levels of superoxide (229). Furthermore, NO also inhibits leukocyte recruitment and microvascular permeability elicited by mast cell degranulation (92). In the present study, emigrating neutrophils contributed to the increases in inflammatory mediator content of the exudate via the increased expression of iNOS, COX-2 and 5-LO. Therefore, suppression of neutrophil extravasation by NCX-1015 may explain its enhanced anti-inflammatory effects.

The inhibitory effect of NO on NF-κB may also contribute to the enhanced anti-inflammatory effects of NCX-1015 as the activation of NF-κB is crucial for the neutrophil extravasation and also for inflammatory mediator formation (54, 120). NO has been shown to activate protein phosphatases in peripheral blood mononuclear cells suggesting the possibility that NO may inhibit NF-κB via dephosphorylation of IκBα. In fact, the NO donor sodium nitroprusside was shown to inhibit tumor necrosis factor-α (TNF-α)-induced NF-κB activation in human vascular endothelial cells (254). NO also prevented the degradation of IκBα and increased its mRNA expression (211). Oxidants such as hydrogen peroxide have been shown to activate NF-κB (reviewed in 248). As NO scavenges superoxide anion, it can also prevent superoxide to forming the dismutation product hydrogen peroxide (127). Finally, NO donors were shown to inhibit DNA binding activity of the p50 subunit of NF-κB, suggesting another mechanism for NO to modulate the expression of NF-κB-responsive genes (180).

The suppression of nitrite/nitrite, PGE_2 and LTB_4 production may also be due to enhanced inhibition of the enzymes. Although the present study does not address that, there is some evidence suggesting that NO may have an inhibitory effect on the iNOS enzyme. NO donors and NO-NSAIDs inhibited iNOS expression in vascular smooth muscle cells and in macrophages induced by cytokines or LPS without affecting enzyme activity (48, 142). NO also inhibits iNOS via direct inhibition of the iNOS enzyme (107). Paul Clark et al. reported that NCX-1015 inhibited iNOS protein expression more potently than

prednisolone in emigrated cells in zymosan-induced peritonitis model in mice, suggesting that the inhibitory action of NCX-1015 on nitrate/nitrite may be due to a stronger suppression of iNOS expression compared to prednisolone (208).

NO was also suggested to interact directly with the COX-2 enzyme (potentially by S-nitrosation of cysteine residues in the catalytic domain of the enzyme) and inhibition of NO production resulted in inhibition of PG generation in a macrophage cell line and in the carrageenan airpouch in rats (234, 235). However, this is a matter of debate since the results obtained vary according to the cell type considered and experimental conditions (28, 111, 114).

CD163 is expressed by monocytes and macrophages under the exquisite regulation of glucocorticoids and its expression correlates with glucocorticoid receptor activation. Therefore it was suggested that glucocorticoid-induced expression of CD163 could be used as an indication of glucocorticoid potency (125). NCX-1015 more potently induced CD163 expression on peripheral blood mononuclear cells suggesting that NCX-1015 may have higher glucocorticoid activity than prednisolone (208).

When neutrophils die via necrosis, for example during the abscess formation, cells burst and release their pro-inflammatory mediators into their microenvironment. Therefore, necrosis of neutrophils not only augments the existing inflammation, it also leads to tissue injury. During apoptosis cells shrink, develop blebs on their surface and break into membrane-wrapped fragments. The also have the chromatin in their nucleus cleaved into mono- and oligo-nucleosomes. Apoptosis of neutrophils is associated with a decrease in cellular functions of neutrophils. For example, they can no longer phagocytose or degranulate. Moreover, as membrane integrity is preserved during apoptosis, tissues are protected from the noxious contents of neutrophils (reviewed in 256). Induction of apoptosis in bronchoalveolar neutrophils reduces the synthesis of LTB_4 in the lung, and therefore exerts anti-inflammatory effects (45). In order to understand whether apoptosis was involved as a mechanism for the observed reduction in exudate cells and inflammatory mediator release, we determined the

rate of necrosis and apoptosis in the infiltrated cells. Our results suggest that the majority of infiltrated cells in the exudates were viable and there was no induction of apoptosis in response to carrageenan or to the test drugs. Therefore, the observed anti-inflammatory effects of NCX-1015 involve mechanisms other than induction of apoptosis.

Other than having enhanced anti-inflammatory effects, a NO-releasing glucocorticoid may have other possible advantages. First among these is NO has a rapid onset of action. Although glucocorticoids are known to have potent effects via direct protein-protein interactions, most of their anti-inflammatory effects involve genomic actions and therefore have a long latency (reviewed in 38). In the ovalbumin-induced anaphylaxis model in Hooded-Lister rats, intraperitoneal administration of equimolar doses of NCX-1015 and prednisolone were equally effective in decreasing circulating leukocyte numbers within 3 h of ovalbumin challenge. However, NCX-1015 was also able to reduce leukocyte numbers within an hour of ovalbumin challenge, whereas prednisolone had no effect (unpublished results).

NCX-1015 induced significant relaxation of guinea pig airway smooth muscles in a concentration dependent way (263). This result suggests that NO-glucocorticoids may combine an effective anti-inflammatory therapy with the bronchodilatory effects of NO for the treatment of asthma and chronic obstructive pulmonary disease.

Long-term glucocorticoid treatment is associated with increased blood pressure (255). A NO-releasing glucocorticoid may have vasodilatory actions, therefore may counteract the hypertensive effects of the glucocorticoids. Previous studies have shown that unlike a standard NO donor (SNP), equimolar dose of NO-NSAIDs do not elicit a change in the blood pressure of animals; however, NO released by NO-aspirin reduced adrenergic vasoconstriction in perfused rat tail artery preparation (227). Therefore, we determined the effect of prednisolone and NCX-1015 on the blood pressure of rats. Systemic

administration of either prednisolone or NCX-1015 at a dose of 28 μmol/kg did not significantly alter the blood pressure of rats in our study.

Another potential advantage of a NO-glucocorticoid may be the decreased adverse effects. For example, a NO donor could counteract prednisolone-induced bone loss (282). Oral administration of NCX-1015 versus prednisolone resulted in less bone and cartilage erosion (209).

Therefore, we determined the effect of NCX-1015 on adrenal and thymus gland weights and also on plasma glucose levels. Unlike prednisolone, the highest dose of NCX-1015 (28 μmol/kg) significantly reduced adrenal gland weight. With a lower dose of NCX-1015 to 2.8 μmol/kg this effect was not observed. Therefore, it is possible that using a more potent glucocorticoid like NCX-1015, an overall dosage could be decreased and therefore some of the adverse effects could be avoided. As for the thymus gland weight, NCX-1015 reduced thymus gland weight dose-dependently and plasma glucose levels significantly increased in response to both doses of NCX-1015. Similar to glucocorticoids, the NO donor S-nitroso-N-acetylpenicillamine has been shown to induce DNA degradation in thymocytes *in vitro* (101). Therefore, the NO moiety may contribute to the observed decrease in thymus gland weight.

SUMMARY

The addition of a NO-donating moiety onto conventional NSAIDs makes these drugs more effective and safer. A similar approach was followed to produce an NO-releasing glucocorticoid. The present study shows that in carrageenan-airpouch model, NO-releasing prednisolone derivative, NCX-1015, is a more potent anti-inflammatory drug than prednisolone. The NO-releasing moiety augments certain anti-inflammatory effects of NCX-1015; however, the molecular mechanisms through which NCX-1015 achieves these enhanced anti-inflammatory effects need to be explored.

Previous studies suggest that NCX-1015 may have less adverse effects on the bone compared to prednisolone. However, in the present study, we showed that NCX-1015 causes the atrophy of thymus gland and an increase in plasma glucose levels which could not be reversed by a lower dose. Therefore, more studies are needed to understand the systemic side effects of this drug.

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