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Title:

Oxymetazoline inhibits pro-inflammatory reactions: Effect on arachidonic acid-derived metabolites

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Abbreviations:

AMs: alveolar macrophages; UCP: ultrafine carbon particles; CL: chemiluminescence; $cPLA_2$: cytosolic phospholipase A_2; 5-LO: 5-lipoxygenase; 15-LO: 15-lipoxygenase; COX: cyclooxygenase; LTB_4: leukotriene B_4; PGE_2:

prostaglandin E_2; 15-HETE: 15-hydroxy-eicosatetraenoic acid; iNOS: inducible nitric oxide synthase; URTI: upper respiratory tract infection.

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Abstract

The nasal decongestant oxymetazoline effectively reduces rhinitis symptoms. We hypothesized that oxymetazoline affects arachidonic acid-derived metabolites concerning inflammatory and oxidative stress-dependent reactions. Oxymetazoline`s ability to model pro-, anti-inflammatory and oxidative stress responses was evaluated in cell-free systems including 5-lipoxygenase (5-LO) as pro-inflammatory, 15-lipoxygenase (15-LO) as anti-inflammatory enzymes and oxidation of methionine by agglomerates of ultrafine carbon particles (UCP) indicating oxidative stress. In a cellular approach using canine alveolar macrophages (AMs), the impact of oxymetazoline on phospholipase A_2 (PLA_2) activity, respiratory burst and synthesis of prostaglandin E_2 (PGE_2), 15-hydroxy-eicosatetraenoic acid (15-HETE), leukotriene B_4 (LTB_4) and 8-isoprostane was measured in the absence and presence of UCP or opsonized zymosan as particulate stimulants. In cell-free systems, oxymetazoline (0.4 mM to 1 mM) inhibited 5-LO but not 15-LO activity and did not alter UCP-induced oxidation of methionine. In AMs, oxymetazoline induced PLA_2 activity and 15-HETE at 1 mM, enhanced PGE_2 at 0.1 mM, strongly inhibited LTB_4 and respiratory burst at 0.4 mM/0.1 mM ($p<0.05$) but did not affect 8-isoprostane formation. In contrast, oxymetazoline did not alter UCP-induced PLA_2 activity, PGE_2 and 15-HETE formation in AMs, but inhibited UCP-induced LTB_4 formation and respiratory burst at 0.1 mM and 8-isoprostane formation at 0.001 mM ($p<0.05$). In opsonized zymosan-stimulated AMs, oxymetazoline inhibited LTB_4 formation and respiratory burst at 0.1 mM ($p<0.05$). In conclusion, in canine AMs oxymetazoline suppressed pro-inflammatory reactions including 5-LO activity, LTB_4 formation and respiratory burst and prevented particle-induced oxidative stress, whereas PLA_2 activity and synthesis of immune-modulating PGE_2 and 15-HETE were not affected.

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Introduction

An upper respiratory tract infection ("URTI"), also known as the "common cold", is the most frequent, acute infectious illness of humans. An URTI is characterized by rhinorrhoea, nasal congestion and sneezing often accompanied by fever, a sore throat and malaise. About 80% of the nasal infections are caused by viruses, the majority being rhinoviruses. Despite its frequent occurrence, there is still little known about the pathogenesis of URTI. Available data support the concept that the symptoms of an URTI are the result of the host's inflammatory immune response to the virus rather than a direct viral cytopathic effect, and that inflammatory mediators play an important role in the pathogenesis of URTI (Winther et al 1998; Van Cauwenberge et al. 2000; Gwaltney, 2002). Studies in both naturally and experimentally induced rhinovirus infections demonstrate that nasal secretions become enriched in pro-inflammatory mediators such as cytokines and arachidonic acid-derived lipid mediators including leukotrienes and prostaglandins (Gwaltney, 1995; Winther et al 1998; Van Cauwenberge et al. 2000; Gentile and Skoner, 2001; Gwaltney, 2002). This is usually accompanied by an infiltration of neutrophils in the nasal mucosa (Winther et al 1998; Van Cauwenberge et al. 2000). Among the lipid mediators, leukotriene B_4 (LTB_4) is the most potent chemoattractant for neutrophils and might be responsible for the neutrophilic infiltrate in rhinithis patients (Denzlinger, 1996; Gentile and Skoner, 2001). LTB_4 together with cysteinyl leukotrienes enhance mucus secretion (Gentile and Skoner, 2001). Levels of nasal cysteinyl leukotrienes such as leukotriene C_4 (LTC_4) increase during an experimentally induced URTI and are temporally associated with the development of symptoms (Gentile et al. 2003). 5-Lipoxygenase (5-LO) being the initial enzyme for leukotriene synthesis is induced in

epithelial cells by respiratory syncytial virus infection and catalyses formation of LTB_4 and cysteinyl leukotrienes (Behera et al. 1998).

Since nasal decongestants, e.g. oxymetazoline, effectively reduce rhinitis symptoms (e.g. obstruction, rhinorrhea,), a few studies also dealt with their possible anti-inflammatory activities. Bjerknes and Steinsvag (1993) reported that compounds such as oxymetazoline chloride and xylometazoline chloride inhibit human neutrophil functions including actin polymerization, phagocytosis and oxidative burst. Furthermore, Westerveld et al. (2000) showed that oxymetazoline strongly inhibits the expression of the inducible form of nitric oxide synthase (iNOS), and speculated that nasal decongestants might offer a new tool to reduce inflammatory mechanisms. Westerveld et al. (1995) also referred to anti-oxidant actions of oxymetazoline by showing that this compound is a potent inhibitor of microsomal lipid peroxidation and an excellent hydroxyl radical scavenger.

Based on these findings, we hypothesized that oxymetazoline inhibits pro-inflammatory reactions and prevents oxidative stress focusing on arachidonic acid-derived metabolites. We tested this hypothesis with both cell-free and cellular systems. Cytosolic phospholipase A_2 ($cPLA_2$) plays a central role in lipid mediator synthesis during inflammation by releasing arachidonic acid from membrane phospholipids. Arachidonic acid is further metabolized by cyclooxygenases (COX) to immune-modulating prostaglandin E_2 (PGE_2) among other prostanoids, by 5-lipoxygenase (5-LO) to pro-inflammatory LTB_4 and by 15-lipoxygenase (15-LO) to anti-inflammatory 15(S)-hydroxy eicosatetraenoic acid (15-HETE). Arachidonic acid can also be oxidized by free-radical-induced peroxidation to 8-isoprostane, a marker for oxidative stress in vivo (Roberts and Morrow 2000). Because 5-LO is involved in the pathogenesis of URTI (Behera et al. 1988), oxymetazoline's putative inhibitory

effect on the activity of 5-LO was directly assessed in a cell-free system. Additionally, 15-LO contributing to resolution of inflammation (Serhan et al. 2003) was also tested for its response to oxymetazoline. Another cell-free system covered oxymetazoline`s anti-oxidative potency to prevent the oxidation of methionine by agglomerates of ultrafine carbon particles (UCP). For the cellular system to study oxymetazoline's effect, alveolar macrophages (AMs) were selected which are competent immune cells with regard to eicosanoid metabolism (Denzlinger 1996). Particulate stimulants such as UCP and zymosan were recently shown to activate lipid mediator synthesis and to induce oxidative stress in macrophages (Beck-Speier et al. 2005; Girotti et al. 2004). Importantly, the tissue eicosanoid metabolism seems to be enhanced in upper airway diseases (Perez-Novo 2005) and increased numbers of macrophages in the nasal mucosa during URTI (van Benten et al. 2001 and 2005) might trigger this change. Therefore, canine AMs stimulated by UCP or opsonized zymosan were used as a model for activated lipid mediator synthesis and oxidative stress. They were analyzed for $cPLA_2$ activity and formation of PGE_2, 15-HETE, LTB_4 and 8-isoprostane. Additionally, $cPLA_2$-dependent stimulation of respiratory burst activity was assessed to evaluate the microbicidal defense capacity of AMs.

Methods

Materials

Phosphate buffered saline (PBS) with and without Ca^{2+}/Mg^{2+} was purchased from Biochrome (Berlin, Germany); lucigenin and zymosan A were from Sigma (Deisenhofen, Germany); 5-lipoxygnease (5-LO) and 15-lipoxygenase (15-LO) were from Cayman (Ann Arbor, USA).

Solutions of oxymetazoline and suspensions of ultrafine carbon particles and opsonized zymosan

Oxymetazoline (Merck, Darmstadt, Germany) was dissolved and diluted in PBS with Ca^{2+}/Mg^{2+}, pH 7, containing 0.1 % glucose.

Ultrafine carbon particles were generated by spark discharging according to Roth et al. (2004). The particles consisted of individual primary particles with a diameter of 5-10 nm and a specific surface area of 750 ± 150 m^2/g (n = 50). During aerosol generation the primary particles aggregated to agglomerates of a size of about 70 nm. These agglomerates of ultrafine carbon particles (UCP) were suspended in distilled water by repeated vortexing and sonification as described previously (Beck-Speier et al. 2005). In suspension, UCP formed even larger agglomerates with a size distribution of 70 % being < 100 nm and 30 % being > 100 nm (S. Takenaka, unpublished data). The specific surface area of these agglomerates is very similar to the sum of the surface areas of the primary particles. In the incubations, the cells were exposed to UCP at a mass concentration of 32 µg/ml which corresponded to a surface area of 240 cm^2/ml. This concentration of UCP was chosen to achieve optimal cellular responses of the arachidonic acid-derived metabolites and respiratory burst activity (Beck-Speier et al. 2005).

Opsonized zymosan was prepared from zymosan A with a diameter: of 2-3 µm (Sherwood and Richardson 1988; Dewitt et al., 2003). The zymosan A was purified by boiling for 30 min at 90C° in PBS, and incubated with fresh-frozen canine serum in equal volume portions for 30 min at room temperature according to Allen (1986). The opsonized zymosan was washed twice, suspended in PBS, pH 7, containing 0.1 % glucose, and aliquots were frozen until use. The cells were exposed to opsonized zymosan at 100 µg/ml, which represent a mass concentration to achieve optimal functional responses (Maier et al. 1992; Beck-Speier et al. 2005). In comparison with UCP, the zymosan particles with their larger diameter (2 - 3 µm) possess a smaller surface area per mass (estimated below 10 m^2/g) than UCP. The specific surface area is a decisive parameter for particles to elicit biologic responses (Beck-Speier et al. 2005).

Cell-free systems with oxymetazoline:

i) Lipoxygenase inhibitor activity

The lipoxygenase inhibitor activity of oxymetazoline was determined by a lipoxygenase inhibitor screening assay (Cayman, Ann Arbor, USA) in a cell-free system consisting of 5-LO with linoleic acid as substrate, or 15-LO with arachidonic acid as substrate, respectively. Oxymetazoline in concentrations ranging from 0.001 mM to 1 mM was added to 5-LO or 15-LO in the screening assay buffer, respectively, and the lipoxygenase inhibitor screening assay was immediately started by addition of the corresponding substrates and running for 5 min according to the instructions of the manufacturer.

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ii) Influence on the oxidative capacity of UCP

The influence of oxymetazoline on the oxidative capacity of UCP was studied by pre-incubating UCP (2 mg/ml H_2O) with various concentrations of oxymetazoline (0.1 mM, 1 mM and 10 mM) for 60 min at room temperature in parallel with the controls. To assay the oxidative capacity of UCP, aliquots of 50 µl (100 µg UCP) of particle suspension taken from the pre-incubations were suspended in 1 ml H_2O and incubated in the presence of 100 µM methionine for 2 h at 25°C. Formation of methionine sulfoxide was measured fluorometrically after pre-column derivatization with o-phthaldialdehyde and HPLC separation as described recently (Beck-Speier et al. 2005).

Alveolar macrophages

Canine AMs were isolated by bronchoalveolar lavage of healthy beagle dogs, centrifuged at 400 *g* for 20 min and resuspended in PBS without Ca^{2+}/Mg^{2+} as previously described by Beck-Speier et al. (2005).

Incubation of alveolar macrophages with oxymetazoline

To assess the effect of oxymetazoline on AMs in the absence and presence of UCP or opsonized zymosan as stimulatory agents, respectively, the following treatments were performed: i) AMs (1 x 10^6 cells/ml) were incubated with various oxymetazoline concentrations in PBS with Ca^{2+}/Mg^{2+}, pH 7, containing 0.1 % glucose, for 80 min at 37°C; ii) AMs (1 x 10^6 cells/ml) were pre-incubated with various oxymetazoline concentrations in PBS, pH 7, with Ca^{2+}/Mg^{2+} and 0.1 % glucose for 20 min at 37°C, subsequently stimulated by UCP (32 µg/ml) and incubated for further 60 min at 37°C; iii) AMs (1 x 10^6 cells/ml) were pre-incubated with various oxymetazoline

concentrations in PBS, pH 7, with Ca^{2+}/Mg^{2+} and 0.1 % glucose for 20 min at 37°C, subsequently stimulated by opsonized zymosan (100 µg/ml) and incubated for further 60 min at 37°C. The incubation procedures were terminated by centrifugation with 400 *g* for 10 min at room temperature. The cells were resuspended in Hepes buffer, pH 7.4, containing 1 mM EDTA. Aliquots were examined for cell viability as determined by trypan blue exclusion. The residual cells were homogenized by sonification (3 x 15 s) and centrifuged at 10,000 *g* for 15 min at 4°C. The supernatants were taken for determination of protein, $cPLA_2$ activity and lipid mediators.

Cytosolic phospholipase A_2 activity of alveolar macrophages

The supernatants of the cell homogenates were analyzed for $cPLA_2$ activity by performing a $cPLA_2$ activity assay (Cayman, Ann Arbor, USA) according to the instructions of the manufacturer. Protein was measured at 595 nm in a microtiter plate format by using 5 µl homogenate and 200 µl 1:5 diluted Biorad solution (Biorad, Munich, Germany) with bovine serum albumin as standard.

Lipid mediators of alveolar macrophages

For analysis of lipid mediators the supernatants of the cell homogenates were deproteinized by adding eightfold volume of 90 % methanol containing 0.5 mM EDTA and 1 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, pH 7,4 (Beck-Speier et al. 2005). These methanol suspensions were stored at -40°C for 24 h followed by two centrifugation steps at 10,000 *g* for 20 min at 4°C with a 24 h interval to remove the proteins. Aliquots of the obtained supernatants were dried in a vacuum centrifuge, dissolved in assay buffer and used for quantification of PGE_2, LTB_4, 15-HETE and 8-

isoprostane by their specific enzyme immunoassays (Cayman, Ann Arbor, USA) according to the instructions of the manufacturer.

Respiratory burst activity of alveolar macrophages

The respiratory burst activity of AMs was determined by lucigenin-dependent chemiluminescence (CL) (Allen, 1986; Li et al. 1998). Canine AMs (1×10^5 cells/250 µl) were preincubated in PBS with Ca^{2+}/Mg^{2+}, pH 7, containing 0.1 % glucose and 0.8 mM lucigenin, for 10 min at 37°C in a chemiluminescence analyzer (Autolumat LB 953, Berthold, Wildbad, Germany). CL signals of AMs in the absence and presence of various oxymetazoline concentrations were recorded for 20 min at 37°C. Subsequently, UCP or opsonized zymosan, respectively, was added and the CL signals of the cells were monitored for further 20 min at 37°C.

Statistical analysis

Statistical significance was determined by analysis of variance and two-sample t-test (STAT-SAK, Version 2.12 by G.E. Dallal, 1986, Malden, MA, USA). Changes with $p \leq 0.05$ were considered as significant.

Results

Cell-free systems with oxymetazoline.

Cell-free systems were used as approach to assess the effect of oxymetazoline on enzyme activities involved in arachidonic acid metabolism and on oxidative potencies of UCP.

i) Evaluation of the inhibitor activity of oxymetazoline against 5-lipoxygenase and 15-lipoxygenase.

The inhibitory capacity of oxymetazoline against lipoxygenases was studied in a cell-free system consisting of oxymetazoline and 5-LO or 15-LO together with appropriate substrates, respectively. Fig. 1 shows that oxymetazoline at concentrations from 0.4 mM to 1 mM strongly inhibited the activity of 5-LO, whereas that of 15-LO was only marginally affected. A 50 % inhibition of 5-LO was achieved by 0.4 mM oxymetazoline.

ii) Evaluation of the anti-oxidative properties of oxymetazoline.

The effect of oxymetazoline on the oxidative capacity of UCP was studied by incubating the particles with various concentrations of oxymetazoline (0.1 mM to 10 mM) in a cell-free system. The oxidative potential of the particles was subsequently determined by their capacity to oxidize methionine to methionine sulfoxide. Oxymetazoline in concentrations between 0.1 mM to 10 mM did not reduce the oxidative capacity of UCP. In the absence of oxymetazoline, UCP (control) oxidized methionine resulting in formation of methionine sulfoxide of 11558 ± 1059 pmol/ml. In the presence of oxymetazoline, UCP did not loose this oxidative capacity: UCP treated with 0.1 mM oxymetazoline formed 11721 ± 995 pmol methionine sulfoxide/ml, UCP treated with 1 mM oxymetazoline formed 12981 ± 1320 pmol methionine sulfoxide/ml, and UCP treated with 10 mM oxymetazoline formed 11210 ±

940 pmol methionine sulfoxide/ml (mean ± SD resulting from 3 to 4 experiments with different particle preparations).

Cellular system with alveolar macrophages: Effect of oxymetazoline on generation of arachidonic acid-derived metabolites and respiratory burst activity.

The effect of oxymetazoline on AMs in the absence and presence of stimulators was studied in view of the following endpoints: a) activation of $cPLA_2$ including formation of PGE_2 and 15-HETE, and b) formation of LTB_4, CL as respiratory burst activity and 8-isoprostane as marker for lipid peroxidation. These cellular responses were studied under three different conditions.

i) Oxymetazoline in the absence of stimulatory agents:

As shown in Fig. 2a, oxymetazoline exerted a mild but significant stimulatory effect on $cPLA_2$ activity and formation of 15-HETE at 1 mM concentration, and on synthesis of PGE_2 at concentrations ranging from 0.1 mM to 1 mM. However, as demonstrated in Fig. 2b, LTB_4 formation was significantly inhibited by oxymetazoline at concentrations from 0.4 mM to 1 mM, and CL decreased by oxymetazoline concentrations from 0.1 mM to 1 mM. Formation of 8-isoprostane was not altered by oxymetazoline (Fig. 2b). Cell viability remained stable in the presence of oxymetazoline (mean ± SD resulting from four different experiments): 93 ± 1 % viability for control cells, 90 ± 2 % for 0.001 mM oxymetazoline-treated cells, 91 ± 3 % for 0.01 mM oxymetazoline-treated cells, 89 ± 5 % for 0.1 mM oxymetazoline-treated cells, 89 ± 4 % for 0.4 mM oxymetazoline-treated cells, and 85 ± 6 % for 1.0 mM oxymetazoline-treated cells. Similar findings were obtained when the cells were stimulated in the presence of oxymetazoline by UCP or opsonized zymosan (data not

shown) indicating that oxymetazoline in concentrations up to 1.0 mM did not remarkably impair cell viability.

ii) Oxymetazoline in the presence of ultrafine carbon particles:

To study the effect of oxymetazoline on UCP-stimulated AMs, a particle concentration of 32 µg/ml was selected to induce significant effects on arachidonic acid-derived metabolites and respiratory burst activity (Beck-Speier et al., 2005). As shown in Fig. 3a and b, UCP in the absence of oxymetazoline stimulated AMs for a strong increase in the levels of $cPLA_2$ activity, PGE_2, 15-HETE, LTB_4, CL and 8-isoprostane ($p < 0.01$). However, the presence of oxymetazoline exerted various effects on these UCP-induced responses. Oxymetazoline did not essentially change the UCP-induced levels of $cPLA_2$ activity and 15-HETE (Fig. 3a). The UCP-induced increase of PGE_2 formation was reduced by low oxymetazoline concentrations (0.001 mM and 0.01 mM) but not by higher oxymetazoline concentrations (0.1 mM and 1 mM). The UCP-induced levels of LTB_4 synthesis and CL were not affected by the low oxymetazoline concentrations (0.001 mM and 0.01 mM) but inhibited by higher oxymetazoline concentrations (Fig. 3b). The particle-induced formation of 8-isoprostane was strongly reduced by all concentrations of oxymetazoline (Fig. 3b).

iii) Oxymetazoline in the presence of opsonized zymosan:

To confirm the inhibitory effect of oxymetazoline on LTB_4 synthesis and CL, the cells were stimulated by opsonized zymosan in the absence and presence of oxymetazoline. Fig. 4 shows that AMs stimulated by opsonized zymosan in the absence of oxymetazoline exhibited elevated levels of LTB_4 formation and CL ($p < 0.001$). The amount of opsonized zymosan used in theses assays corresponded to the standard concentrations necessary to achieve optimal activation of AMs via the Fc- and complement-receptor mediated systems (Maier et al. 1992; Beck-Speier et

al. 2005). In the presence of oxymetazoline the levels of both parameters decreased drastically at higher oxymetazoline concentrations (0.1 mM to 1.0 mM).

Discussion

This study identifies oxymetazoline as a potent modulator of inflammatory reactions and inhibitor of oxidative stress concerning arachidonic acid-derived metabolites. Potential effects of oxymetazoline were tested in cell-free systems, (i) using the interference with 5-LO and 15-LO activities as pathways for pro- and anti-inflammatory responses, respectively, and (ii) using the oxidation of methionine by UCP to indicate oxidative stress. A cellular system with canine AMs stimulated by UCP or opsonized zymosan served as a model of inflammation and oxidative stress. Both particulate stimuli are phagocytosed by AMs, as we have shown recently for UCP (Beck-Speier et al. 2005) and as it is known for zymosan (Girotti et al. 2004). Phagocytosis is accompanied by respiratory burst (Root and Metcalf, 1977). According to our observation, opsonized zymosan elicits a much higher respiratory burst activity in AMs than non-opsonized UCP due to receptor-mediated versus non-receptor-mediated phagocytosis (Beck-Speier et al. 2005).

In the cell-free system, oxymetazoline strongly inhibited 5-LO activity with an effective concentration of 0.4 mM for 50 % inhibition but did not affect 15-LO activity (Fig. 1). This difference of oxymetazoline's influence on 5-LO and 15-LO was also found in the cellular system. Similarly, oxymetazoline strongly inhibited the synthesis of pro-inflammatory LTB_4 and respiratory burst activity in AMs, whereas $cPLA_2$ activity with production of PGE_2 and 15-HETE were enhanced (Fig. 2). In AMs stimulated by UCP, oxymetazoline (0.1 mM) did not alter UCP-induced $cPLA_2$ activity and formation of PGE_2 plus 15-HETE, but again inhibited UCP-induced LTB_4 formation and respiratory burst activity (Fig. 3). However, at lower oxymetazoline concentrations (0.001 mM and 0.01 mM), PGE_2 production of UCP-treated AMs was reduced. The underlying mechanism needs to be clarified. After stimulating AMs with

opsonized zymosan, oxymetazoline also inhibited the enhanced LTB_4 formation and respiratory burst activity (Fig. 4). Concerning the effective oxymetazoline concentration for 50 % inhibition of the 5-LO pathway with LTB_4 formation, the cellular system with 0.1 mM oxymetazoline responded even more sensitive as the cell-free system.

As reported recently, UCP showed a pronounced electron paramagnetic resonance (EPR) signal indicating unpaired electrons within the carbon matrix of the particles contributing to a highly reactive surface area (Beck-Speier et al. 2005). This EPR signal corresponded with a high oxidative capacity of UCP to oxidize methionine in a cell-free system and to induce oxidative stress in a cellular system with canine AMs, indicated by 8-isoprostane formation (Beck-Speier et al, 2005). Because oxymetazoline failed to reduce the oxidative potential of UCP in the cell-free system, we exclude a pronounced interaction of oxymetazoline with particle-associated radicals. However, in our cellular system, oxymetazoline did not induce 8-isoprostane formation by itself (Fig. 2b) but stopped very efficiently the particle-induced oxidative stress at the lowest concentration (0.001 mM) (Fig. 3b). This anti-oxidative and radical scavenger effect of oxymetazoline might result from its interference with the UCP-induced peroxidation of arachidonic acid.

Oxymetazoline was shown to reduce functions of human neutrophils including actin polymerization, phagocytosis and oxidative burst at concentrations of about 1 mM as described by Bjerknes and Steinsvag (1993). Furthermore, Westerveld et al. (2000) referred that 0. 3 mM oxymetazoline inhibited iNOS in a cellular system with rat alveolar macrophage cell line NR 8383, whereas the constitutive nitric oxide synthase was not affected. Oxymetazoline was also a potent inhibitor of lipid peroxidation and excellent hydroxyl radical scavenger (Westerveld et al. 1995). In a

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cell-free model of microsomal lipid peroxidation, consisting of Fe^{2+}/ascorbic acid and liver microsomes, oxymetazoline inhibited lipid peroxidation completely at concentrations between 0.015 mM to 0.02 mM (Westerveld et al. 1995). With regard to these earlier studies, it must be noted that relatively high concentrations of oxymetazoline (≥ 0.3 mM to reduce pro-inflammatory responses of neutrophils and macrophages, and 0.02 mM to inhibit lipid peroxidation) were necessary to induce the observed effects. In comparison to these findings, our cellular model with AMs was significantly more sensitive, because oxymetazoline concentrations as low as 0.1 mM suppressed pro-inflammatory reactions and as low as 0.001 mM inhibited UCP-induced lipid peroxidation. Nasal application of decongestants results in the development of a concentration gradient. Assuming a total nasal epithelial lining fluid volume of 800 µl/nostril (Kaulbach et al. 1993), oxymetazoline used in its current product concentration of 1.6 mM (nose sprays for adults and school children) at a dosage volume of 45 µl per puff will be diluted to form a concentration gradient that refers to levels of the active substance used in our experiments (estimated mean value ≈ 0.1 mM). Therefore, the obtained results are of relevance in situ.

Various studies demonstrated strong correlations between rises of inflammatory mediators, mainly cytokines and leukotrienes, and the expression of rhinitis symptoms (Gwaltney, 1995; Gwaltney 2002). Particularly leukotrienes such as LTB_4 and LTC_4, which rise in the nasal fluid of rhinitis patients are generated and released by virus infected cells of the respiratory tract (Ananaba et al. 1991; van Schaik et al. 1999; Gentile et al. 2001; Gentile et al., 2003). Leukotrienes such as LTD_4 applied directly to the nasal mucosa of noninfected individuals reproduced symptoms of nasal congestion and rhinorrhea (Bisgaard et al. 1986). Treatments with 5-LO enzyme inhibitors or cysteinyl leukotriene receptor antagonists have shown to

induce a significant clinical benefit since these compounds reduces nasal congestion in allergic rhinitis (Naclerio et al. 1991; Liu et al. 1998; Meltzer et al. 2000). Furthermore, oxidative stress also seems to play a pivotal role in the pathogenesis of viral respiratory infections, because reactive oxygen species like nitric oxide have been reported to increase in the exhaled air of patients with allergic rhinitis or URTI (Kharitonov et al. 1995; Martin et al. 1996). The data of our present study reveal oxymetazoline as potent inhibitor of inflammatory and oxidative stress-dependent reactions. This activity resembles the mechanisms described for nonsteroidal anti-inflammatory drugs (NSAIDS) that act by blocking cyclooxygenases (COX-1 and COX-2), thus inhibiting the conversion of arachidonic acid to prostanoids. Research done over the last few years suggest that drugs inhibiting both the COX enzymes and 5-LO might exert a potent anti-inflammatory effect (Pereg and Lishner 2005; Naveau 2005).

The reaction mechanism of oxymetazoline regarding arachidonic acid-derived metabolites is summarized in Fig. 5. Oxymetazoline interferes with AM membranes by activating $cPLA_2$ activity moderately to release slightly elevated levels of arachidonic acid from membrane phospholipids for downstream metabolism to immune-modulating PGE_2 by COX and anti-inflammatory acting 15-HETE by 15-LO. Because these pathways are not inhibited by oxymetazoline, we conclude that this compound does not affect specific pathways in modulation and resolution of inflammation. In contrast, oxymethazoline inhibits 5-LO resulting in reduced formation of pro-inflammatory LTB_4. Because 5-LO is the initial enzyme for leukotriene synthesis, oxymetazoline is probably able to inhibit also the production of the cysteinyl leukotrienes, which are responsible together with LTB_4 for the inflammatory reactions and symptoms occurring during URTI. In addition, oxymetazoline inhibits

respiratory burst activity leading to diminished microbial killing capacity of the cells thus intensifying its inhibitory effect on inflammatory reactions. These pro-inflammatory processes are also inhibited in the presence of environmental or physiologic stimuli such as UCP or opsonized zymosan. Furthermore, oxymetazoline suppresses UCP-induced formation of 8-isoprostane very effectively. Our previous findings, that UCP induced very similar responses of lipid mediators in canine AMs and human AMs (Beck-Speier et al. 2005), justifies the extrapolation of our data obtained with canine AMs to the human system. In consequence, oxymetazoline's anti-inflammatory and anti-oxidative action is offering additional benefits for the treatment of virus-induced upper respiratory tract inflammation and oxidative stress. The data shown here add significant new information for understanding oxymetazoline's way to modulate and reduce rhinitis symptoms.

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Legends for Figures

Figure 1: Effect of oxymetazoline on inhibition of 5- and 15-lipoxygenase activity.

In a cell-free system concentrations of oxymetazoline ranging from 0.001 mM to 1 mM were added to 5-lipoxygenase (5-LO) or 15- lipoxygenase (15-LO), respectively, and analyzed for their capacity to inhibit the activities of both enzymes. Data are given as mean ± SD. Number of experiments performed with different solutions of oxymetazoline was n = 4 for 5-LO and n = 3 for 15-LO. Initial activity of 5-LO was 0.01113 ± 0.00523 µMol/min/ml (n = 4) and for 15-LO was 0.0035 ± 0.0025 µMol/min/ml (n = 3).

Figure 2: Effect of oxymetazoline on cytosolic phospholipase A_2 activity, generation of lipid mediators and respiratory burst activity in alveolar macrophages.

Canine alveolar macrophages (AMs) were incubated with oxymetazoline in concentrations ranging from 0.001 mM to 1 mM. Baseline levels were obtained by parallel incubations of cells in the absence of oxymetazoline. The effect of oxymetazoline was measured for a) cytosolic phospholipase A_2 ($cPLA_2$) activity, formation of prostaglandin E_2 (PGE_2) and 15-hydroxy-eicosatetraenoic acid (15-HETE), and b) generation of leukotriene B_4 (LTB_4), chemiluminescence (CL) as indicator of respiratory burst activity, and formation of 8-isoprostane as marker for oxidative stress. The number of experiments performed with AMs of different dogs was n = 4 for all parameters. The data (mean ± SD) for $cPLA_2$ activity, PGE_2, 15-HETE, LTB_4, CL and 8-isoprostane are given as percentages of the corresponding base line values. Base lines (100 % values with n = 4) represent for $cPLA_2$ a specific activity of 17,12 ± 7,87 nM arachidonyl thio-PC /min/mg, for PGE_2 11047 ± 3919 pg/mg cellular protein, for 15-HETE 8819 ± 2743 pg/mg cellular protein, for LTB_4

1778 ± 526 pg/mg cellular protein, for respiratory burst activity 38598 ± 8353 CL-counts during 20 min for 1 x 10^5 cells, and for 8-isoprostane 328± 163 pg/mg cellular protein. Asterisks indicate significant increase or decrease of the parameters between control values and values obtained in the presence of oxymetazoline (*p< 0.05; **p< 0.01).

Figure 3: Influence of oxymetazoline on ultrafine carbon particles-induced activation of cytosolic phospholipase A_2, synthesis of lipid mediators and respiratory burst activity in alveolar macrophages.

Canine alveolar macrophages (AMs) were pre-incubated in the absence and presence of oxymetazoline in concentrations ranging from 0.001 mM to 1 mM for 20 min and subsequently stimulated by ultrafine carbon particles (UCP) for 60 min. Baseline levels were obtained by parallel incubations of cells in the absence of both UCP and oxymetazolin, while control levels for UCP were obtained in the absence of oxymetazoline. The effect of oxymetazoline was determined on: a) UCP-induced cytosolic phospholipase A_2 ($cPLA_2$) activity, UCP-induced formation of prostaglandin E_2 (PGE_2) and 15-hydroxy-eicosatetraenoic acid (15-HETE), and b) UCP-induced generation of leukotriene B_4 (LTB_4), UCP-induced chemiluminescence (CL) and UCP-induced formation of 8-isoprostane. The number of experiments performed with AMs of different dogs was n = 4 for all parameters. The data (mean ± SD) for $cPLA_2$ activity, PGE_2, 15-HETE, LTB_4, CL and 8-isoprostane are given as percentages of the corresponding base lines values. Base lines (100 % values with n = 4) are the same as described for Fig. 2. The UCP control levels in the absence of oxymetazoline were: 210 ± 20 % for $cPLA_2$ activity, 195 ± 11 % for PGE_2 formation, 204 ± 34 % for 15-HETE formation, 288 ± 74 % for LTB_4 formation, 182 ± 48 % for

CL, and 214 ± 29 % for 8-isoprostane formation ($p < 0.01$ compared to base line). Asterisks indicate significant increase or decrease of the parameters between UCP-induced values and the data obtained in the presence of oxymetazoline and UCP (*$p< 0.05$; **$p< 0.01$).

Figure 4: Effect of oxymetazoline on opsonized zymosan-stimulated formation of leukotriene B_4 and respiratory burst activity in alveolar macrophages.

Canine alveolar macrophages (AMs) were pre-incubated in the absence and presence of oxymetazoline for 20 min and subsequently stimulated by opsonized zymosan for 60 min. Baseline levels were obtained by parallel incubations of cells in the absence of both opsonized zymosan and oxymetazolin, while control levels for opsonized zymosan were obtained in absence of oxymetazoline. The effect of oxymetazoline was determined on the opsonized zymosan-induced generation of leukotriene B_4 (LTB_4) and chemiluminescence (CL) as respiratory burst activity. The number of experiments performed with AMs of different dogs was n = 4 for all parameters. The data (mean ± SD) for LTB_4 and CL are given as percentages of the corresponding base line values. Base lines (100 % values with n = 4) are the same as described for Fig. 2. The opsonized zymosan-induced control levels in the absence of oxymetazoline were: 558 ±97% for LTB_4 formation, and 569 ± 79 % for CL ($p < 0.001$ compared to base line). Asterisks indicate significant decrease of the parameters between values stimulated by opsonized zymosan and the data obtained in the presence of oxymetazoline and opsonized zymosan (*$p< 0.05$; **$p< 0.01$).

Figure 5: Reaction mechanism of oxymetazoline on activation of cytosolic phospholipase A_2 and generation of lipid mediators.

Oxymetazoline (OMZ) interferes with alveolar macrophages to activate $cPLA_2$, leading to a release of arachidonic acid from membrane phospholipids. Arachidonic acid is further metabolized to immune-modulating PGE_2 by cyclooxygenase (COX) and to anti-inflammatory acting 15-HETE by 15-lipoxygenase (15-LO). These pathways are not inhibited by oxymetazoline, suggesting that this substance does not affect specific events in modulation and resolution of inflammation. PGE_2 is known as immune-modulating mediator with anti-inflammatory properties because of suppression of LTB_4 synthesis and expression of pro-inflammatory cytokines, upregulation of anti-inflammatory acting interleukin-10 and induction of subsequent pathways for resolution of inflammation (Göggel et al. 2002; Vancheri et al. 2004; Levy et al. 2001). 15-HETE acts anti-inflammatory because of inhibition of 5-LO and leukotriene synthesis (Vanderhoek et al. 1980). In contrary to PGE_2 and 15-HETE, oxymethazoline inhibits 5-lipoxygenase (5-LO), the initial enzyme of leukotriene synthesis, resulting in reduction of metabolizing arachidonic acid to pro-inflammatory LTB_4 and probably also to the cysteinyl leukotrienes, which are both responsible for inflammation and symptoms of upper respiratroy tract infections. In addition, oxymetazoline inhibits respiratory burst NADPH oxidase activity leading to diminished microbial killing capacity of the cells thus intensifying its inhibitory effect on pro-inflammatory reactions. In the presence of environmental or physiologic stimuli such as ultrafine carbon particles (UCP) or opsonized zymosan, oxymetazoline also inhibits these pro-inflammatory responses, thus indicating its anti-inflammatory properties. Furthermore, oxymetazoline prevents particle-induced formation of 8-isoprostane, that results from the oxidative capacity of environmental particles and indicates oxidative stress. This capacity of oxymetazoline is due to its anti-oxidative and radical scavenger properties.

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Figure 1

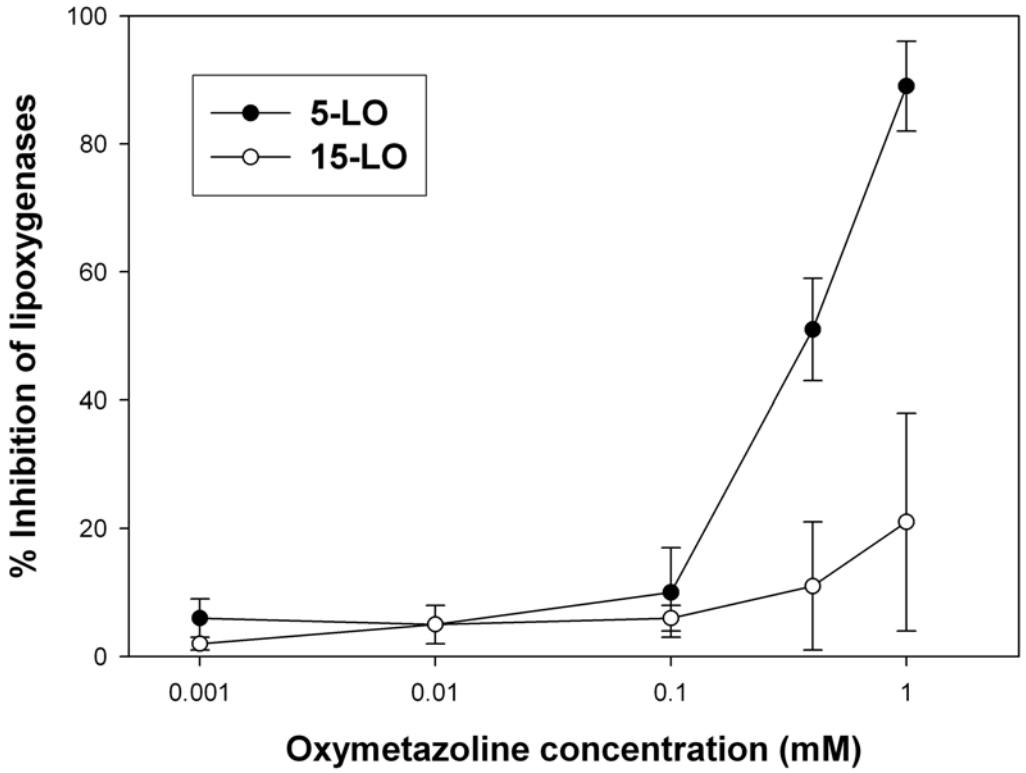
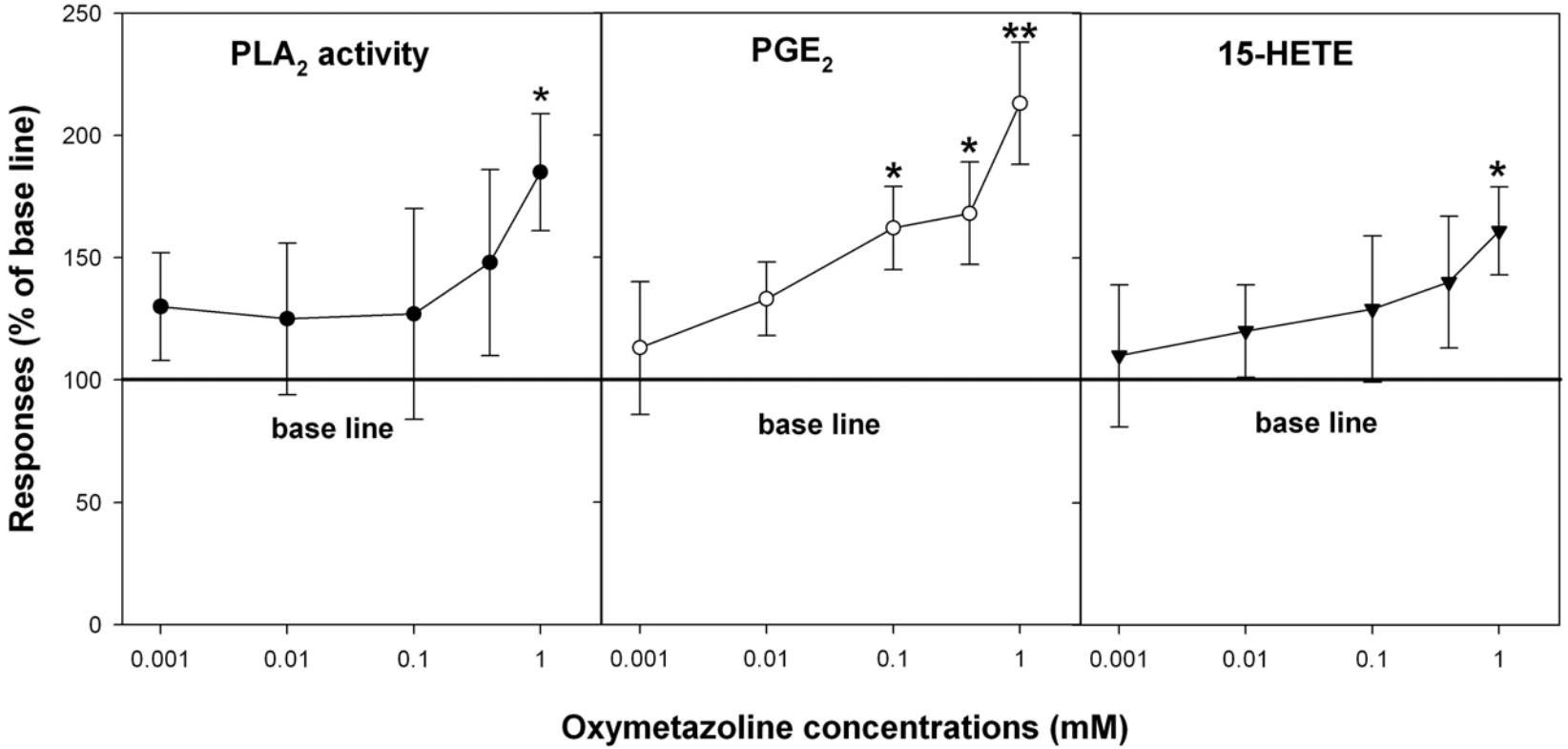


Figure 2



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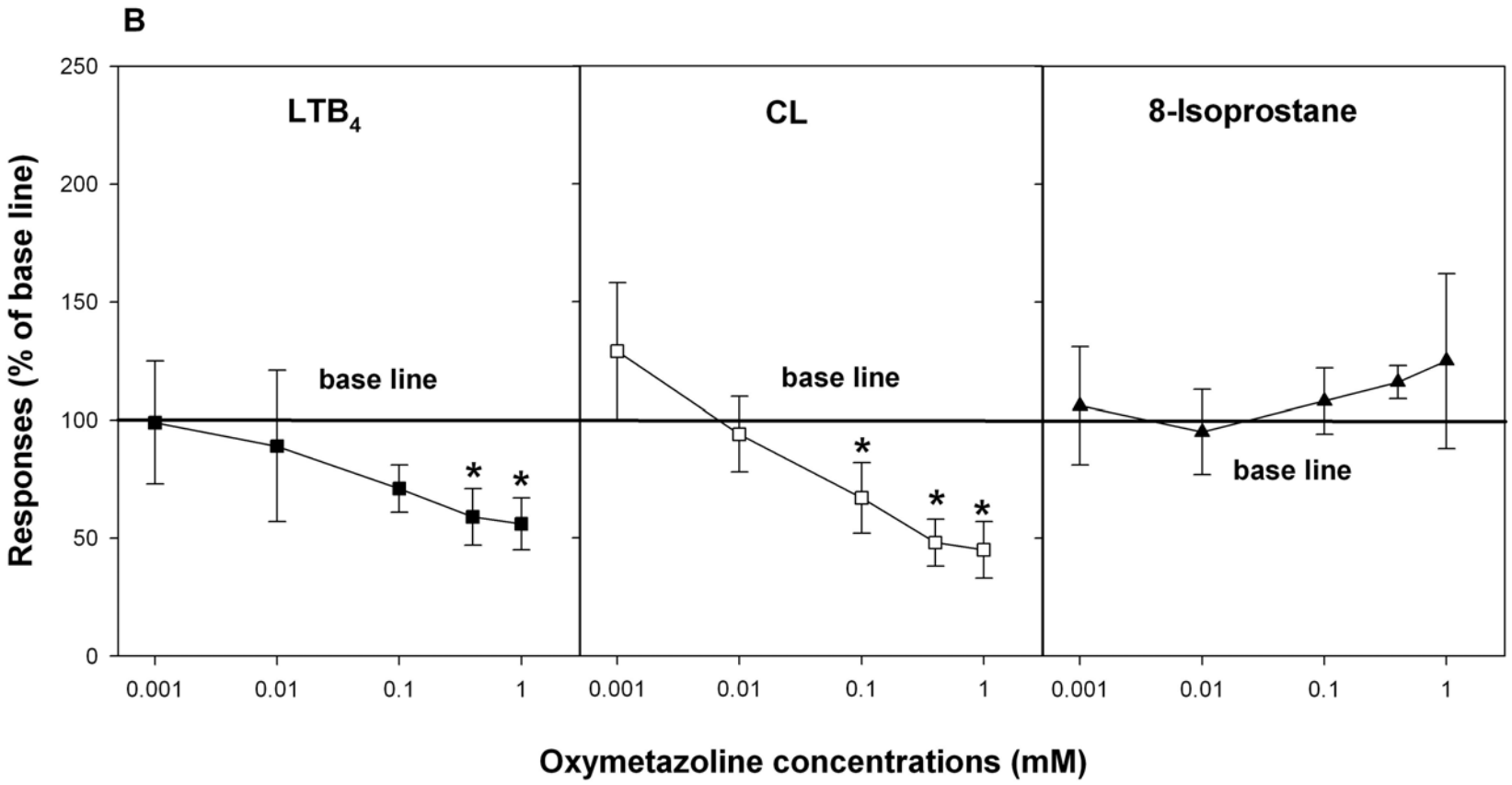
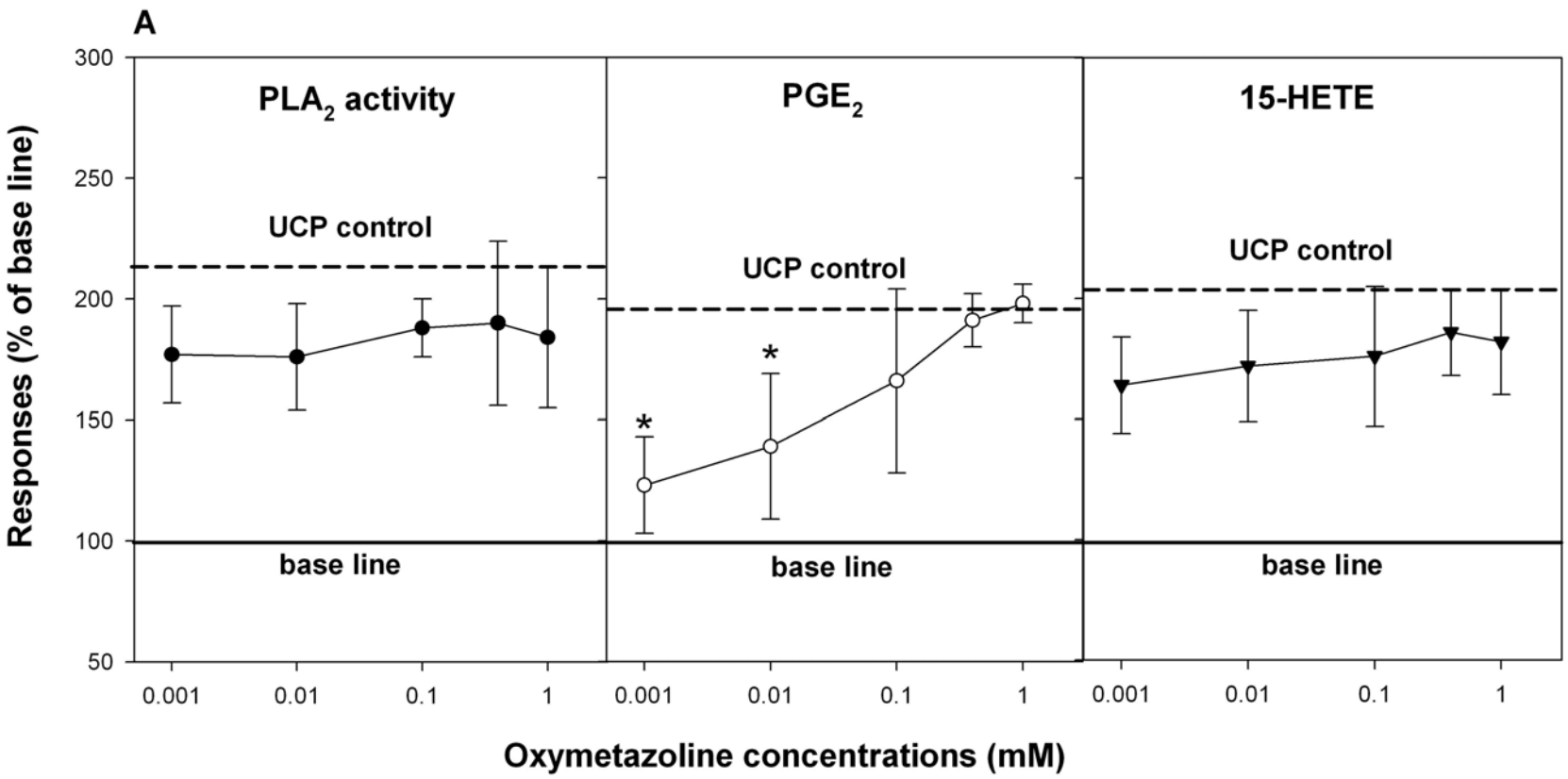


Figure 3



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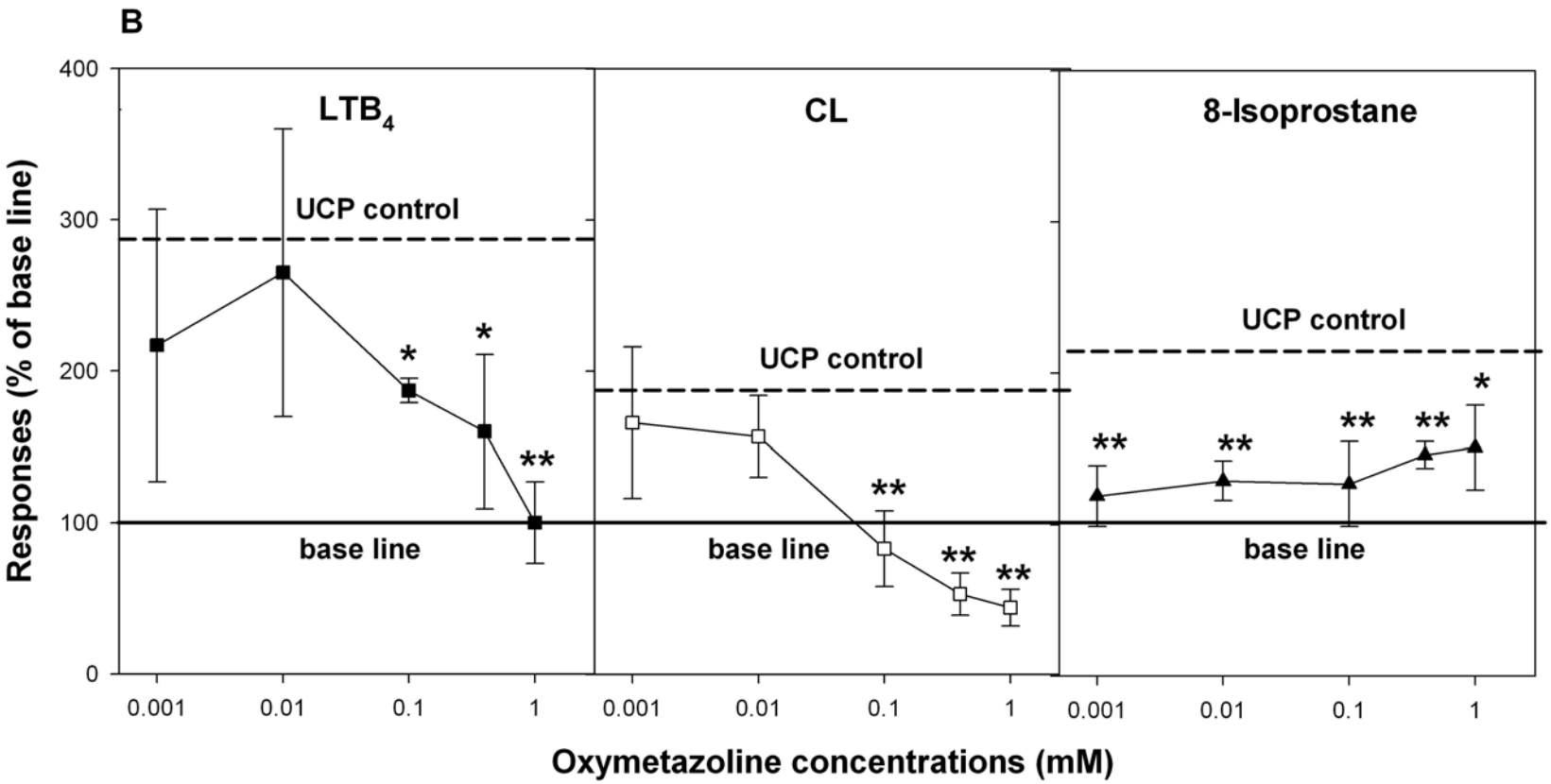

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Figure 4

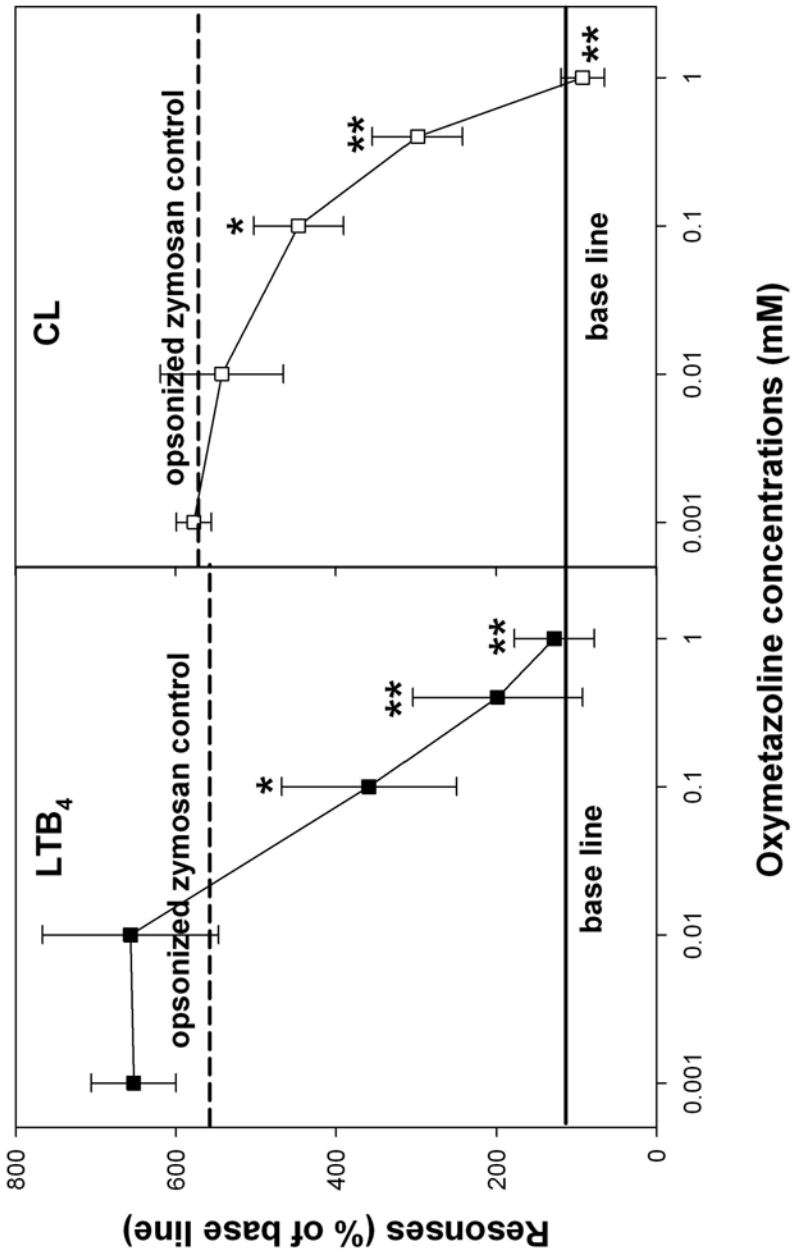

Figure 5