

Oxymetazoline Inhibits Proinflammatory Reactions: Effect on Arachidonic Acid-Derived Metabolites

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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. The ability of oxymetazoline to model pro- and anti-inflammatory and oxidative stress responses was evaluated in cell-free systems, including 5-lipoxygenase (5-LO) as proinflammatory, 15-lipoxygenase (15-LO) as anti-inflammatory enzymes, and oxidation of methionine by agglomerates of ultrafine carbon particles (UCPs), indicating oxidative stress. In a cellular approach using canine alveolar macrophages (AMs), the impact of oxymetazoline on phospholipase A₂ (PLA₂) activity, respiratory burst and synthesis of prostaglandin E₂ (PGE₂), 15(S)-hydroxy-eicosatetraenoic acid (15-HETE), leukotriene B₄ (LTB₄), and 8-isoprostane was measured in the absence and presence of UCP or opsonized zymosan as particulate stimulants. In cell-free systems,

oxymetazoline (0.4–1 mM) inhibited 5-LO but not 15-LO activity and did not alter UCP-induced oxidation of methionine. In AMs, oxymetazoline induced PLA₂ activity and 15-HETE at 1 mM, enhanced PGE₂ at 0.1 mM, strongly inhibited LTB₄ and respiratory burst at 0.4/0.1 mM ($p < 0.05$), but did not affect 8-isoprostane formation. In contrast, oxymetazoline did not alter UCP-induced PLA₂ activity and PGE₂ and 15-HETE formation in AMs but inhibited UCP-induced LTB₄ 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 LTB₄ formation and respiratory burst at 0.1 mM ($p < 0.05$). In conclusion, in canine AMs, oxymetazoline suppressed proinflammatory reactions including 5-LO activity, LTB₄ formation, and respiratory burst and prevented particle-induced oxidative stress, whereas PLA₂ activity and synthesis of immune-modulating PGE₂ and 15-HETE were not affected.

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 rhinorrhea, nasal congestion, and sneezing, often accompanied by fever, a sore throat, and malaise. Approximately 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 experimen-

tally induced rhinovirus infections demonstrate that nasal secretions become enriched in proinflammatory mediators such as cytokines and arachidonic acid-derived lipid mediators including leukotrienes and prostaglandins (Gwaltney, 1995, 2002; Winther et al., 1998; Van Cauwenberge et al., 2000; Gentile and Skoner, 2001). 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 B₄ (LTB₄) is the most potent chemoattractant for neutrophils and might be responsible for the neutrophilic infiltrate in rhinitis patients (Denzlinger, 1996; Gentile and Skoner, 2001). LTB₄, together with cysteinyl leukotrienes, enhance mucus secretion (Gentile and Skoner, 2001). Levels of nasal cysteinyl leukotrienes such as leukotriene C₄ increase during an experimentally induced URTI and are temporally associated with the development of symptoms (Gentile et al., 2003). 5-Lipoxygenase (5-LO), the

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ABBREVIATIONS: URTI, upper respiratory tract infection; LTB₄, leukotriene B₄; 5-LO, 5-lipoxygenase; cPLA₂, cytosolic phospholipase A₂; COX, cyclooxygenase; PGE₂, prostaglandin E₂; 15-LO, 15-lipoxygenase; 15-HETE, 15(S)-hydroxy-eicosatetraenoic acid; UCP, ultrafine carbon particle; AM, alveolar macrophage; PBS, phosphate-buffered saline; CL, chemiluminescence.

initial enzyme for leukotriene synthesis, is induced in epithelial cells by respiratory syncytial virus infection and catalyzes formation of LTB₄ and cysteinyl leukotrienes (Behera et al., 1998).

Because 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 xylo-metazoline 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 and speculated that nasal decongestants might offer a new tool to reduce inflammatory mechanisms. Westerveld et al. (1995) also referred to antioxidant 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 proinflammatory reactions and prevents oxidative stress focusing on arachidonic acid-derived metabolites. We tested this hypothesis with both cell-free and cellular systems. Cytosolic phospholipase A₂ (cPLA₂) plays a central role in lipid mediator synthesis during inflammation by releasing arachidonic acid from membrane phospholipids. Arachidonic acid is further metabolized by cyclooxygenases (COXs) to immune-modulating prostaglandin E₂ (PGE₂) among other prostanoids, by 5-LO to proinflammatory LTB₄, 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., 1998), oxymetazoline's putative inhibitory effect on the activity of 5-LO was directly assessed in a cell-free system. In addition, 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 antioxidative potency to prevent the oxidation of methionine by agglomerates of ultrafine carbon particles (UCPs). 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 (Girotti et al., 2004; Beck-Speier et al., 2005). It is noteworthy that the tissue eicosanoid metabolism seems to be enhanced in upper airway diseases (Perez-Novo et al., 2005), and increased numbers of macrophages in the nasal mucosa during URTI (van Benten et al., 2001, 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 cPLA₂ activity and formation of PGE₂, 15-HETE, LTB₄, and 8-isoprostane. In addition, cPLA₂-dependent stimulation of respiratory burst activity was assessed to evaluate the microbicidal defense capacity of AMs.

Materials and Methods

Materials

Phosphate-buffered saline (PBS) with and without Ca²⁺/Mg²⁺ was purchased from Biochrome (Berlin, Germany), lucigenin and zymosan A were purchased from Sigma Chemie (Deisenhofen, Germany), and 5-LO and 15-LO were purchased from Cayman Chemical (Ann Arbor, MI).

Solutions of Oxymetazoline and Suspensions of Ultrafine Carbon Particles and Opsonized Zymosan

Oxymetazoline (Merck Biosciences, Darmstadt, Germany) was dissolved and diluted in PBS with Ca²⁺/Mg²⁺, 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 to 10 nm and a specific surface area of 750 ± 150 m²/g (*n* = 50). During aerosol generation, the primary particles aggregated to agglomerates of a size of about 70 nm. These agglomerates of UCPs 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 cm²/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 to 3 μm (Sherwood and Richardson, 1988; Dewitt et al., 2003). The zymosan A was purified by boiling for 30 min at 90°C 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 represents 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 m²/g) than UCP. The specific surface area is a decisive parameter for particles to elicit biological responses (Beck-Speier et al., 2005).

Cell-Free Systems with Oxymetazoline

Lipoxygenase Inhibitor Activity. The lipoxygenase inhibitor activity of oxymetazoline was determined by a lipoxygenase inhibitor screening assay (Cayman Chemical) 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 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.

Influence on the Oxidative Capacity of UCP. The influence of oxymetazoline on the oxidative capacity of UCP was studied by preincubating UCP (2 mg/ml H₂O) with various concentrations of oxymetazoline (0.1, 1, 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 preincubations were suspended in 1 ml H₂O and incubated in the presence of 100 μM methionine for 2 h at 25°C. Formation of methionine sulfoxide was measured fluorometrically after precolumn derivatization with *o*-phthalaldehyde and high-performance liquid

chromatography separation as described recently (Beck-Speier et al., 2005).

Alveolar Macrophages

Canine AMs were isolated by bronchoalveolar lavage of healthy beagle dogs, centrifuged at 400g for 20 min and resuspended in PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ 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: AMs (1×10^6 cells/ml) were incubated with various oxymetazoline concentrations in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$, pH 7, containing 0.1% glucose, for 80 min at 37°C; AMs (1×10^6 cells/ml) were preincubated with various oxymetazoline concentrations in PBS, pH 7, with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and 0.1% glucose for 20 min at 37°C, subsequently stimulated by UCP (32 $\mu\text{g}/\text{ml}$), and incubated for further 60 min at 37°C; and AMs (1×10^6 cells/ml) were preincubated with various oxymetazoline concentrations in PBS, pH 7, with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and 0.1% glucose for 20 min at 37°C, subsequently stimulated by opsonized zymosan (100 $\mu\text{g}/\text{ml}$), and incubated for further 60 min at 37°C. The incubation procedures were terminated by centrifugation at 400g 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 (3×15 s) and centrifuged at 10,000g for 15 min at 4°C. The supernatants were taken for determination of protein, cPLA₂ activity, and lipid mediators.

Cytosolic Phospholipase A₂ Activity of Alveolar Macrophages

The supernatants of the cell homogenates were analyzed for cPLA₂ activity by performing a cPLA₂ activity assay (Cayman Chemical) according to the instructions of the manufacturer. Protein was measured at 595 nm in a microtiter plate format by using 5 μl of homogenate and 200 μl of 1:5 diluted Biorad solution (Bio-Rad, 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 8-fold 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 10,000g 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 PGE₂, LTB₄, 15-HETE, and 8-isoprostane by their specific enzyme immunoassays (Cayman Chemical) 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 $\text{Ca}^{2+}/\text{Mg}^{2+}$, pH 7, containing 0.1% glucose and 0.8 mM lucigenin, for 10 min at 37°C in a chemiluminescence analyzer (Autumat LB 953; Berthold Technologies, Bad Wildbad, Germany). CL signals of AMs in the absence and presence of various oxymetazoline concentrations were recorded for 20 min at 37°C. Thereafter, 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 Student's *t* test (STAT-SAK, version 2.12, by G.E. Dallal, 1986; Malden, MA). 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.

Evaluation of the Inhibitory 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. Figure 1 shows that oxymetazoline at concentrations from 0.4 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.

Evaluation of the Antioxidative Properties of Oxymetazoline. The effect of oxymetazoline on the oxidative capacity of UCP was studied by incubating the particles with various concentrations of oxymetazoline (0.1–10 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 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 lose 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 \pm S.D. resulting from three to four 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: activation of cPLA₂, including formation of PGE₂ and 15-HETE; and formation of LTB₄, CL as respiratory burst activity, and 8-isoprostane as marker for lipid peroxidation. These cellular responses were studied under three different conditions.

Oxymetazoline in the Absence of Stimulatory Agents. As shown in Fig. 2A, oxymetazoline exerted a mild but significant stimulatory effect on cPLA₂ activity and formation of 15-HETE at 1 mM concentration and on synthesis of PGE₂ at concentrations ranging from 0.1 to 1 mM. However, as demonstrated in Fig. 2B, LTB₄ formation was significantly inhibited by oxymetazoline at concentrations from 0.4 to 1 mM, and CL was decreased by oxymetazoline concentrations from 0.1 to 1 mM. Formation of 8-isoprostane was not altered by oxymetazoline (Fig. 2B). Cell viability

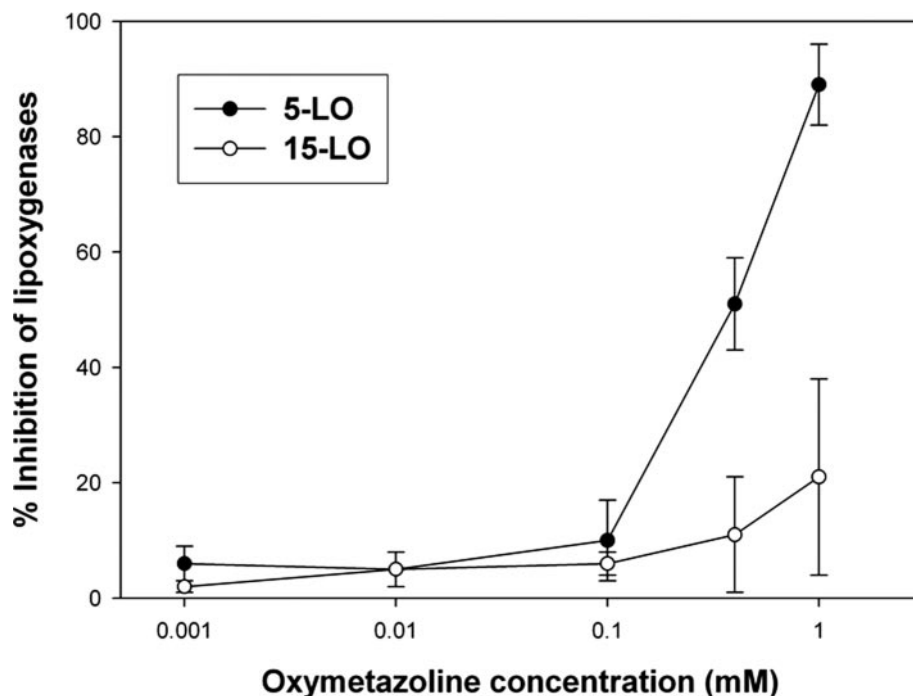


Fig. 1. Effect of oxymetazoline on inhibition of 5- and 15-lipoxygenase activity. In a cell-free system, concentrations of oxymetazoline ranging from 0.001 to 1 mM were added to 5-LO or 15-LO, respectively, and analyzed for their capacity to inhibit the activities of both enzymes. Data are given as mean \pm S.D. 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 $0.01113 \pm 0.00523 \mu\text{mol}/\text{min}/\text{ml}$ ($n = 4$) and for 15-LO was $0.0035 \pm 0.0025 \mu\text{mol}/\text{min}/\text{ml}$ ($n = 3$).

remained stable in the presence of oxymetazoline (mean \pm S.D. resulting from four different experiments): $93 \pm 1\%$ viability for control cells, $90 \pm 2\%$ for 0.001 mM oxymetazoline-treated cells, $91 \pm 3\%$ for 0.01 mM oxymetazoline-treated cells, $89 \pm 5\%$ for 0.1 mM oxymetazoline-treated cells, $89 \pm 4\%$ for 0.4 mM oxymetazoline-treated cells, and $85 \pm 6\%$ 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.

Oxymetazoline in the Presence of Ultrafine Carbon Particles. To study the effect of oxymetazoline on UCP-stimulated AMs, a particle concentration of $32 \mu\text{g}/\text{ml}$ was selected to induce significant effects on arachidonic acid-derived metabolites and respiratory burst activity (Beck-Speier et al., 2005). As shown in Fig. 3, A and B, UCP in the absence of oxymetazoline stimulated AMs for a strong increase in the levels of cPLA₂ activity, PGE₂, 15-HETE, LTB₄, 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 cPLA₂ activity and 15-HETE (Fig. 3A). The UCP-induced increase of PGE₂ formation was reduced by low oxymetazoline concentrations (0.001 and 0.01 mM) but not by higher oxymetazoline concentrations (0.1 and 1 mM). The UCP-induced levels of LTB₄ synthesis and CL were not affected by the low oxymetazoline concentrations (0.001 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).

Oxymetazoline in the Presence of Opsonized Zymosan. To confirm the inhibitory effect of oxymetazoline on LTB₄ synthesis and CL, the cells were stimulated by opsonized zymosan in the absence and presence of oxymetazoline.

Figure 4 shows that AMs stimulated by opsonized zymosan in the absence of oxymetazoline exhibited elevated levels of LTB₄ formation and CL ($p < 0.001$). The amount of opsonized zymosan used in these 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 (0.1–1.0 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 using the interference with 5-LO and 15-LO activities as pathways for pro- and anti-inflammatory responses, respectively, and 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 nonopsonized UCP due to receptor-mediated versus nonreceptor-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. Likewise, oxymetazoline strongly inhibited the synthesis of proinflammatory

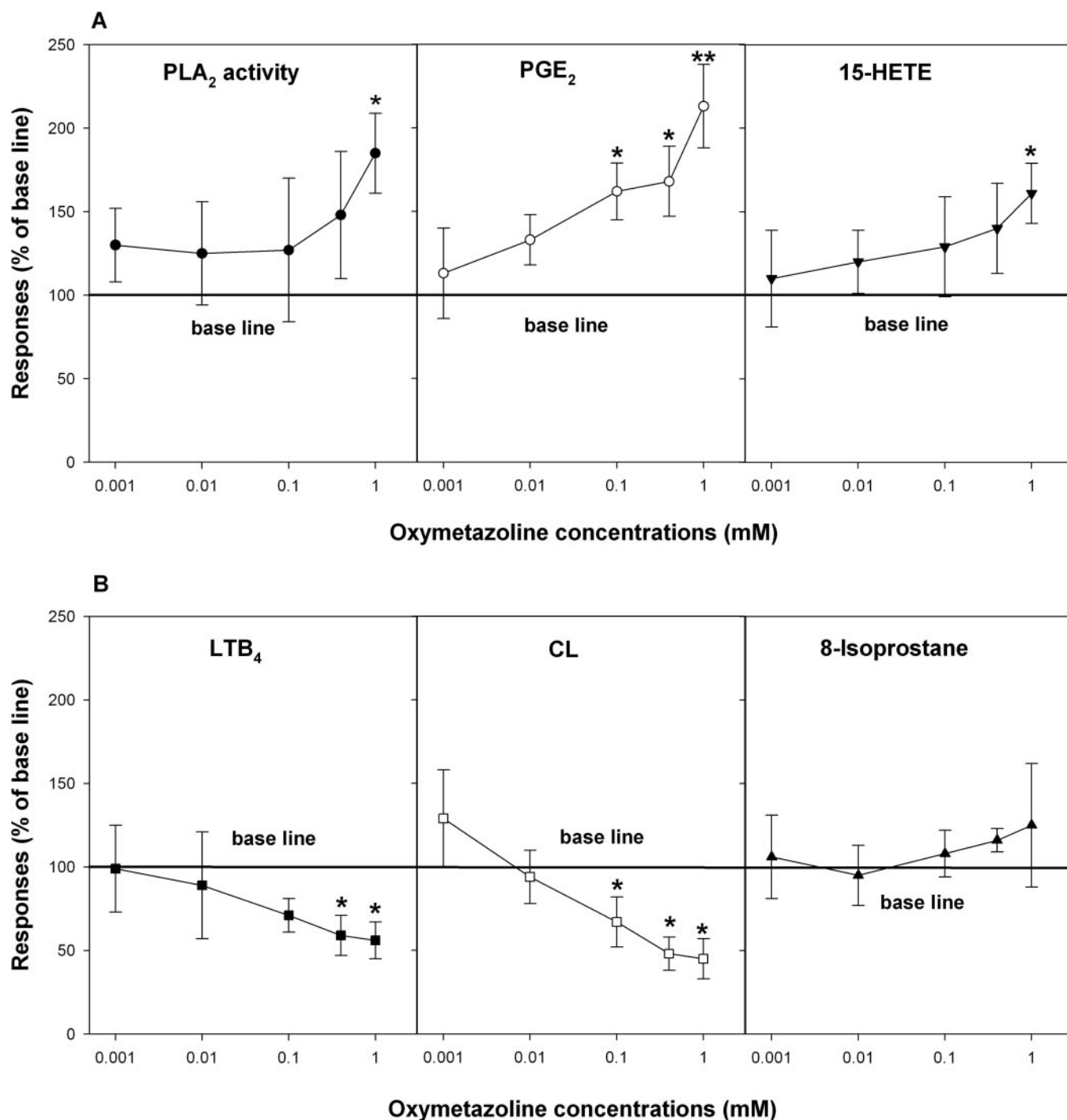


Fig. 2. Effect of oxymetazoline on cytosolic phospholipase A₂ activity, generation of lipid mediators, and respiratory burst activity in alveolar macrophages. Canine AMs were incubated with oxymetazoline in concentrations ranging from 0.001 to 1 mM. Baseline levels were obtained by parallel incubations of cells in the absence of oxymetazoline. The effect of oxymetazoline was measured for: A, cPLA₂ activity, formation of PGE₂, and 15-HETE; and B, generation of LTB₄, 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 \pm S.D.) for cPLA₂ activity, PGE₂, 15-HETE, LTB₄, CL, and 8-isoprostane are given as percentages of the corresponding baseline values. Baselines (100% values with $n = 4$) represent for cPLA₂ a specific activity of 17.12 ± 7.87 nM arachidonyl thio-PC/min/mg; for PGE₂, $11,047 \pm 3919$ pg/mg cellular protein; for 15-HETE, 8819 ± 2743 pg/mg cellular protein; for LTB₄, 1778 ± 526 pg/mg cellular protein; for respiratory burst activity, $38,598 \pm 8353$ CL counts during 20 min for 1×10^5 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 (*, $p < 0.05$; **, $p < 0.01$).

LTB₄ and respiratory burst activity in AMs, whereas cPLA₂ activity with production of PGE₂ and 15-HETE was enhanced (Fig. 2). In AMs stimulated by UCP, oxymetazoline (0.1 mM) did not alter UCP-induced cPLA₂ activity and for-

mation of PGE₂ plus 15-HETE but again inhibited UCP-induced LTB₄ formation and respiratory burst activity (Fig. 3). However, at lower oxymetazoline concentrations (0.001 and 0.01 mM), PGE₂ production of UCP-treated AMs was

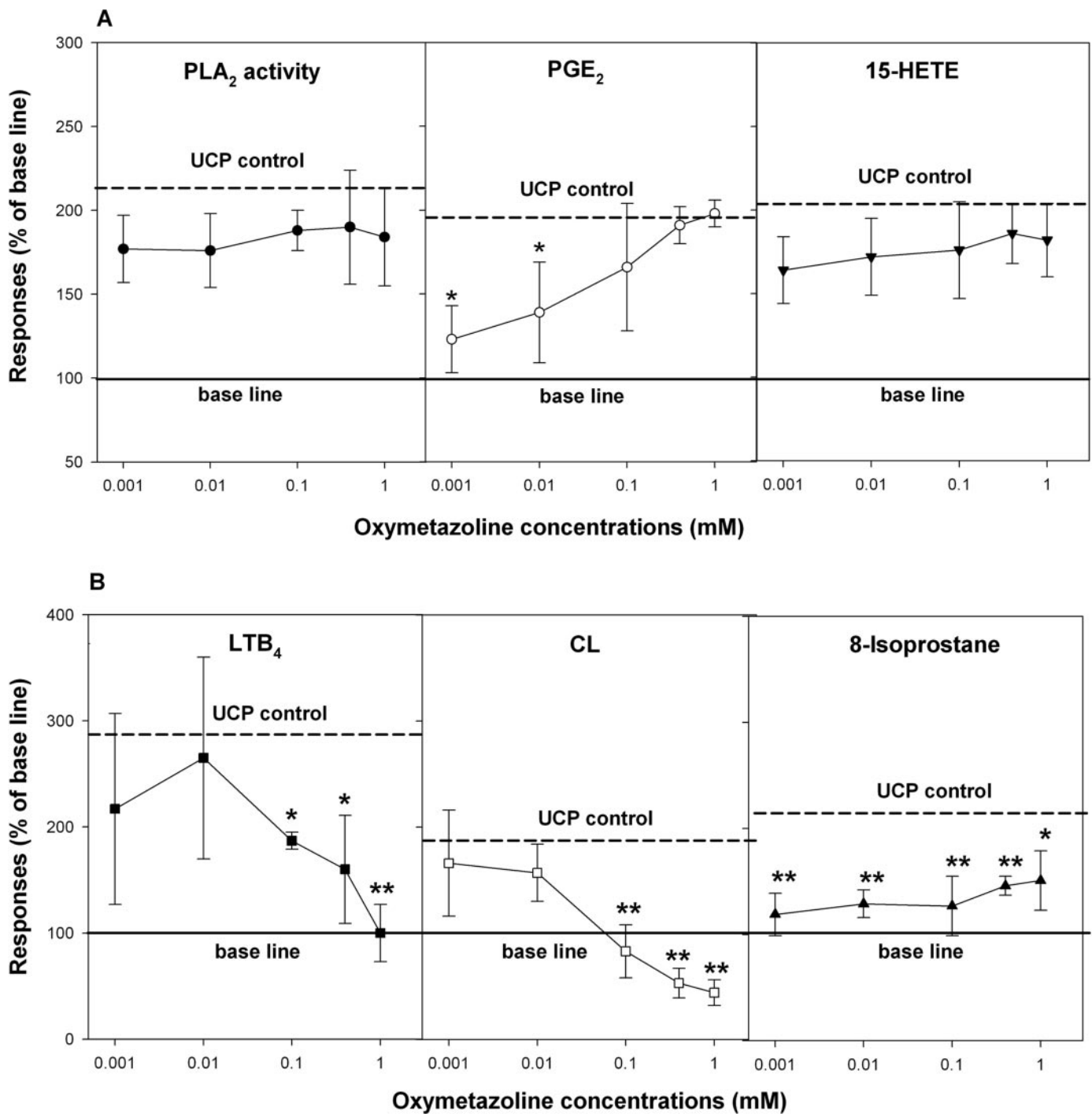


Fig. 3. Influence of oxymetazoline on ultrafine carbon particle-induced activation of cytosolic phospholipase A₂, synthesis of lipid mediators, and respiratory burst activity in alveolar macrophages. Canine AMs were preincubated in the absence and presence of oxymetazoline in concentrations ranging from 0.001 to 1 mM for 20 min and subsequently stimulated by UCPs for 60 min. Baseline levels were obtained by parallel incubations of cells in the absence of both UCP and oxymetazoline, whereas control levels for UCP were obtained in the absence of oxymetazoline. The effect of oxymetazoline was determined on: A, UCP-induced cPLA₂ activity, UCP-induced formation of PGE₂, and 15-HETE; and B, UCP-induced generation of LTB₄, UCP-induced 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 \pm S.D.) for cPLA₂ activity, PGE₂, 15-HETE, LTB₄, CL, and 8-isoprostane are given as percentages of the corresponding baseline values. Baselines (100% values with $n = 4$) are the same as described for Fig. 2. The UCP control levels in the absence of oxymetazoline were: $210 \pm 20\%$ for cPLA₂ activity, $195 \pm 11\%$ for PGE₂ formation, $204 \pm 34\%$ for 15-HETE formation, $288 \pm 74\%$ for LTB₄ formation, $182 \pm 48\%$ for CL, and $214 \pm 29\%$ for 8-isoprostane formation ($p < 0.01$ compared with baseline). Asterisks indicate significant increase or decrease of the parameters between UCP-induced values and the data obtained in the presence of oxymetazoline and UCP (*, $p < 0.05$; **, $p < 0.01$).

reduced. The underlying mechanism needs to be clarified. After stimulating AMs with opsonized zymosan, oxymetazoline also inhibited the enhanced LTB₄ formation and respiratory burst activity (Fig. 4). Concerning the effective

oxymetazoline concentration for 50% inhibition of the 5-LO pathway with LTB₄ formation, the cellular system with 0.1 mM oxymetazoline responded even more sensitive as the cell-free system.

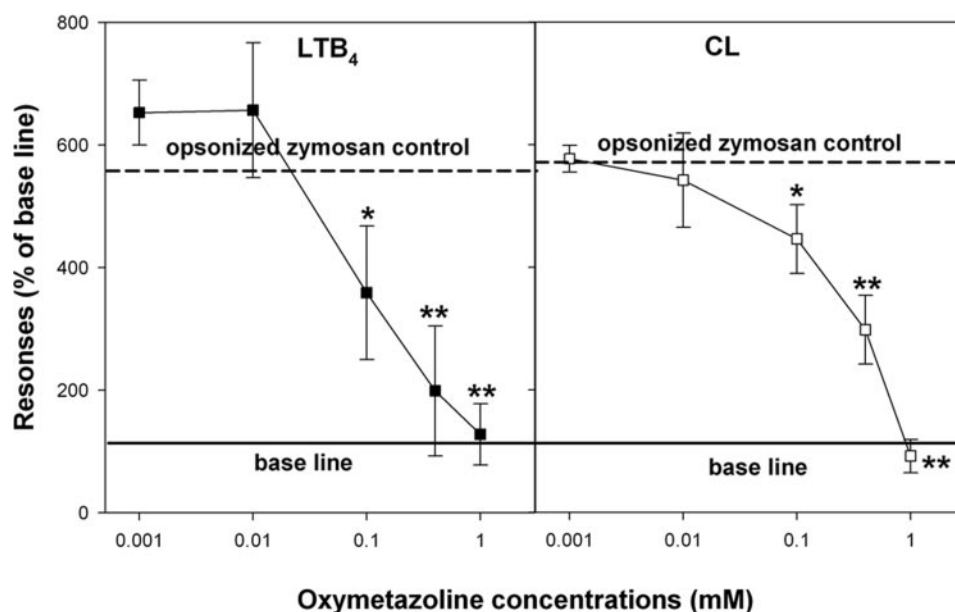


Fig. 4. Effect of oxymetazoline on opsonized zymosan-stimulated formation of leukotriene B₄ and respiratory burst activity in alveolar macrophages. Canine AMs were preincubated in the absence and presence of oxymetazoline for 20 min and subsequently stimulated by opsonized zymosan for 60 min. Baseline levels were obtained by parallel incubations of cells in the absence of both opsonized zymosan and oxymetazoline, whereas control levels for opsonized zymosan were obtained in the absence of oxymetazoline. The effect of oxymetazoline was determined on the opsonized zymosan-induced generation of LTB₄ and CL as respiratory burst activity. The number of experiments performed with AMs of different dogs was $n = 4$ for all parameters. The data (mean \pm S.D.) for LTB₄ and CL are given as percentages of the corresponding baseline values. Baselines (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: $558 \pm 97\%$ for LTB₄ formation and $569 \pm 79\%$ for CL ($p < 0.001$ compared with baseline). 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 (*, $p < 0.05$; **, $p < 0.01$).

As reported recently, UCP showed a pronounced electron paramagnetic resonance signal indicating unpaired electrons within the carbon matrix of the particles contributing to a highly reactive surface area (Beck-Speier et al., 2005). This electron paramagnetic resonance 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 antioxidative 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 inducible nitric oxide synthase 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 cell-free model of microsomal lipid peroxidation, consisting of Fe²⁺/ascorbic acid and liver microsomes, oxymetazoline inhibited lipid peroxidation completely at concentrations between 0.015 and 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 proinflammatory responses of neutrophils and macrophages and 0.02 mM to inhibit lipid peroxidation) were necessary to induce the observed effects. In comparison with these findings, our cellular model with AMs was significantly more sensitive because oxymetazoline concentrations as low as 0.1 mM suppressed proinflammatory 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 μ l/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 μ l/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, 2002). Particularly leukotrienes such as LTB₄ and leukotriene C₄, which rise in the nasal fluid of rhinitis patients, are generated and released by virus-infected cells of the respiratory tract (Ananaba and Anderson, 1991; van Schaik et al., 1999; Gentile and Skoner, 2001; Gentile et al., 2003). Leukotrienes such as LTD₄ 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 been shown to induce a significant clinical benefit because these compounds reduces nasal congestion in allergic rhinitis (Naclerio et al., 1991; Liu et al., 1998; Meltzer et al., 2000). Furthermore,

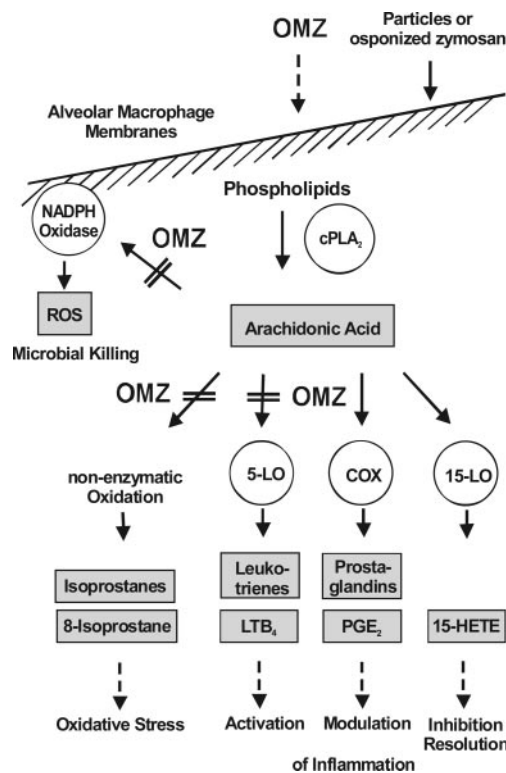


Fig. 5. Reaction mechanism of oxymetazoline on activation of cytosolic phospholipase A₂ and generation of lipid mediators. Oxymetazoline (OMZ) interferes with alveolar macrophages to activate cPLA₂, leading to a release of arachidonic acid from membrane phospholipids. Arachidonic acid is further metabolized to immune-modulating PGE₂ by COX and to anti-inflammatory acting 15-HETE by 15-LO. These pathways are not inhibited by oxymetazoline, suggesting that this substance does not affect specific events in modulation and resolution of inflammation. PGE₂ is known as an immune-modulating mediator with anti-inflammatory properties because of suppression of LTB₄ synthesis and expression of proinflammatory cytokines, up-regulation of anti-inflammatory-acting interleukin-10, and induction of subsequent pathways for resolution of inflammation (Levy et al., 2001; Göggel et al., 2002; Vancheri et al., 2004). 15-HETE acts as an anti-inflammatory mediator because of inhibition of 5-LO and leukotriene synthesis (Vanderhoek et al., 1980). In contrast to PGE₂ and 15-HETE, oxymetazoline inhibits 5-LO, the initial enzyme of leukotriene synthesis, resulting in reduction of metabolizing arachidonic acid to proinflammatory LTB₄ and probably also to the cysteinyl leukotrienes, which are both responsible for inflammation and symptoms of upper respiratory 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 proinflammatory reactions. In the presence of environmental or physiologic stimuli such as UCPs or opsonized zymosan, oxymetazoline also inhibits these proinflammatory 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 antioxidative and radical scavenger properties.

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 non-steroidal anti-inflammatory drugs that act by blocking COX-1 and COX-2, thus inhibiting the conversion of arachidonic acid to prostanoids. Research done over the last few

years suggests that drugs inhibiting both the COX enzymes and 5-LO might exert a potent anti-inflammatory effect (Naveau, 2005; Pereg and Lishner, 2005).

The reaction mechanism of oxymetazoline regarding arachidonic acid-derived metabolites is summarized in Fig. 5. Oxymetazoline interferes with AM membranes by activating cPLA₂ activity moderately to release slightly elevated levels of arachidonic acid from membrane phospholipids for downstream metabolism to immune-modulating PGE₂ 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, oxymetazoline inhibits 5-LO, resulting in reduced formation of proinflammatory LTB₄. 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 LTB₄ 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 proinflammatory 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. As a 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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