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Oxymetazoline Inhibits and Resolves Inflammatory Reactions in Human Neutrophils

Ingrid Beck-Speier1,*, Barbara Oswald1, Konrad L. Maier1, Erwin Karg1, and René Ramseger2

1Helmholtz-Center Munich, German Research Center for Environmental Health, Institute of Lung Biology and Disease, D-85758 Neuherberg/Munich, Germany
2Merck Selbstmedikation GmbH, D-64293 Darmstadt, Germany

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Abstract. The nasal decongestant oxymetazoline (OMZ) exhibits anti-oxidative and anti-inflammatory properties (I. Beck-Speier et al., J Pharmacol Exp Ther. 2006;316:842–851). In a follow up study, we hypothesized that OMZ generates pro-resolving lipoxins being paralleled by production of immune-modulating prostaglandin E2 (PGE2) and anti-inflammatory 15(S)-hydroxy-eicosatetraenoic acid [15(S)-HETE] and depletion of pro-inflammatory leukotriene B4 (LTB4). Human neutrophils (PMN) were chosen as the cellular system. The effect of OMZ on these parameters as well as on respiratory burst activity and oxidative stress marker 8-isoprostane was analyzed in unstimulated and co-stimulated PMN by ultrafine carbon particles (UCP) or opsonized zymosan (OZ), respectively. In unstimulated cells, OMZ induced formation of PGE2, 15(S)-HETE, and LXA4. The levels of LTB4 and 8-isoprostane were not affected, whereas respiratory burst activity was drastically inhibited. In UCP- and OZ-stimulated control cells, all parameters were elevated. Here, OMZ maintained the increased levels of PGE2, 15(S)-HETE, and LXA4, but substantially suppressed levels of LTB4 and 8-isoprostane and inhibited the respiratory burst activity. These findings suggest a switch from the pro-inflammatory eicosanoid class LTB4 to the pro-resolving LXA4. Since LXA4 is most relevant in returning inflamed tissue to homeostasis, OMZ is postulated to terminate rhinitis-related inflammation, thus contributing to shortening of disease duration.

Keywords: oxymetazoline, rhinitis, neutrophil, eicosanoid, lipoxin A4

Introduction

Upper respiratory tract infections (URTIs), which are mainly caused by rhinoviruses, lead to profound economic disprofit due to medical expenses and loss of productivity. Moreover complications of rhinovirus induced URTI, including otitis media (1) and sinusitis (2), as well as exacerbations of lower respiratory tract diseases, for example, asthma (3 – 9) and chronic obstructive pulmonary disease (COPD) (10 – 13), have an enormous impact upon health care.

The symptomatic therapy for an URTI, also known as the common cold, has been effectively carried out by application of the nasal decongestant oxymetazoline (OMZ) for nearly 50 years. OMZ is a sympathomimetic imidazoline derivate with direct alpha-adrenergic activity on vascular smooth muscle cells in the nasal mucosa, leading to vasoconstriction. The onset of action is within seconds (14) and lasts for up to twelve hours (15). In addition to its symptomatic effect, recent studies have also documented anti-oxidative (16, 17) and anti-inflammatory (16, 18) properties of OMZ.

Tüttenberg et al. (18) showed that the anti-inflammatory action of OMZ is partially mediated by the inhibition of pro-inflammatory cytokines (interleukin-1β, tumor necrosis factor-a, interleukin-6, and interleukin-8) as well as reduced T-cell stimulatory capacity of dendritic cells, resulting in a repressed stimulation of T cells. Westerveld et al. (17) showed that OMZ is a potent inhibitor of microsomal lipid peroxidation and a hydroxyl radical scavenger. Furthermore, we have recently reported that OMZ exhibits anti-oxidative and
anti-inflammatory properties in a cell-free system as well as in a cellular system consisting of canine alveolar macrophages (AM) (16). OMZ was found to clearly inhibit pro-inflammatory reactions such as the respiratory burst and the 5-lipoxygenase (5-LO) pathway with formation of leukotriene B4 (LTB4). Besides, this drug had the capability to induce the cyclooxygenase (COX) pathway for formation of immune-modulating prostaglandin E2 (PGE2) and the 15-lipoxygenase (15-LO) pathway for production of anti-inflammatory 15(S)-hydroxy-eicosatetraenoic acid [15(S)-HETE]. These protective processes are known to trigger pathways involving the resolution of inflammation. The resolution of inflammation is an active process with formation of so called “stop signals” such as the newly discovered anti-inflammatory and pro-resolving dual function mediators lipoxin A4 (LXA4) and lipoxin B4 (LXB4) (19).

In view of the immune-modulating and anti-inflammatory properties of OMZ, we hypothesized that OMZ has the potential to induce the generation of lipoxins that in turn contribute to the resolution phase of inflammation. The resolution phase of inflammation is an important event to return the inflamed tissue to homeostasis and to complete resolution without leaving molecular signs or traces of the leukocyte battle (19). Otherwise chronic inflammatory processes may occur. Because infections by rhinoviruses induce inflammatory reactions with influx of polymorphonuclear neutrophils (PMN) into the nasal tissue (20, 21), these cells serve as a model in this study with OMZ. We suggest that OMZ is able to induce immune-modulating PGE2 and anti-inflammatory 15-HETE and to inhibit pro-inflammatory LTB4. Due to the activation and inhibition of these lipid mediators and their corresponding enzymes, a switch of eicosanoids may occur by OMZ, resulting in inhibition of 5-LO with LTB4 formation and in activation of PGE2 synthesis and 15(S)-HETE generation, leading to production of pro-resolving LXA4.

Materials and Methods

Materials
Phosphate-buffered saline (PBS) with and without Ca2+/Mg2+ was purchased from Biochrome (Berlin, Germany); luminol and zymosan were from Sigma (Deisenhofen, Germany).

Solutions of OMZ and suspensions of ultrafine carbon particles and opsonized zymosan OMZ (Merck, Darmstadt, Germany) was dissolved and diluted in PBS with Ca2+/Mg2+, pH 7, containing 0.1% glucose. Ultrafine carbon particles (UCP) were generated by spark discharging (22) and suspended in distilled water (32 μg/ml) by repeated vortexing and sonification (23). Opsonized zymosan was prepared from zymosan, purified by heating for 30 min at 90°C in 0.9% sterile NaCl solution, and incubated with fresh-frozen human serum in equal-volume portions for 30 min at room temperature according to Allen (24). The washed opsonized zymosan was suspended in PBS (pH 7) containing 0.1% glucose.

Isolation of PMN
PMN were isolated from citrate-anticoagulated venous blood of healthy human volunteers with Polymorphprep (Axis-Shield, Oslo, Norway) according to Beck-Speier et al. (25). Briefly, the isolated layer of PMN was washed with PBS (pH 7.4) without Ca2+/Mg2+ to remove the gradient medium. Residual erythrocytes were lysed by treating the cell pellet with distilled water for 30 s. Isotonicity was restored by adding the same volume of 1.8% (w/v) NaCl. The purified cells were resuspended in PBS (pH 7.4) with Ca2+/Mg2+ and 0.1% glucose. The viability of the cells was always over 95% as determined by trypan blue exclusion. The cells were identified by microscopic examination of cytospin preparations after May Grünwald Giemsa staining as >99% populations of PMN.

Viability of PMN with OMZ
To prove the effect of OMZ on the viability of the cells, PMN were incubated with various OMZ concentrations (0.1, 0.4, and 1 mM) in PBS (pH 7.4) with Ca2+/Mg2+ and 0.1% glucose in the absence of stimulants for 80 min at 37°C. After harvesting the cells by centrifugation (400 x g for 10 min at room temperature), the cells were resuspended in HEPES buffer, pH 7.4, containing 1 mM EDTA, and aliquots of the cell suspensions were used for determination of the viability by trypan blue exclusion.

Incubation of PMN with OMZ
The effect of OMZ on PMN was determined in the absence or presence of the co-stimulatory agents ultrafine elemental carbon particles (UCP) or opsonized zymosan (OZ). Both stimulants are particulate and are ingested by phagocytic cells, UCP via non-receptor-mediated phagocytosis and OZ via receptor-mediated phagocytosis (16). The following treatments were performed according to our previous study (16): i) to assess the effect of OMZ on unstimulated cells, PMN (0.5 x 10⁶ cells/0.5 ml) were incubated with various concentrations of OMZ (0.1, 0.4, and 1 mM, corresponding to 29.68, 118.72, and 296.8 μg/ml, respectively) in PBS, pH 7.4, with Ca2+/Mg2+ and 0.1% glucose for 80 min at 37°C; ii) to measure the effect of OMZ
on stimulated cells, PMN (0.5 × 10^6 cells/0.5 ml) were incubated in parallel incubations with various concentrations of OMZ (0.1, 0.4, and 1 mM) in PBS, pH 7.4, with Ca^{2+}/Mg^{2+} and 0.1% glucose for 20 min at 37°C and subsequently co-stimulated by UCP (32 μg/ml) or OZ (50 μg/ml) for an additional 60 min. After the incubation procedures, the cells were harvested by centrifugation at 400 × g for 10 min at room temperature. The cells were resuspended in cold HEPES buffer, pH 7.4, containing 1 mM EDTA; homogenized by sonification (3 times, 15 s each time) in ice; and centrifuged at 10,000 × g for 15 min at 4°C. Importantly, cells were kept on ice during preparation to avoid activation or degradation processes. The supernatants were taken for analysis of both protein and lipid mediators. Analysis of the lysate is a reliable tool to quantify the eicosanoid production that has to be favored over analysis of extracellular levels (26). The sensitivity of this intracellular approach is supported by normalization of eicosanoid levels to the protein content in the lysate. Analysis of the cellular eicosanoid content appears to be relevant with regard to the autocrine mechanisms of PGE_2, which might trigger both functional and metabolic parameters of PMN.

**Determination of protein**

Protein was measured at 595 nm in a microtiter plate format by using 5 μl of cell homogenate and 200 μl of 1:5 diluted Biorad solution (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

**Analysis of lipid mediators**

For analysis of lipid mediators, the supernatants of the cell homogenates were deproteinized by adding an 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 (16). 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(S)-HETE, 8-isoprostanе, and LXA_4 were measured by their specific enzyme immunoassays (Cayman Chemical) according to the instructions of the manufacturer.

**Respiratory burst activity**

The respiratory burst activity of human PMN was determined by luminol-dependent chemiluminescence (CL) according to Mishra et al. (27). Briefly, PMN (3 × 10^6 cells) were preincubated in 0.25 ml PBS, pH 7.4, containing Ca^{2+}/Mg^{2+}, 0.1% glucose, and 0.02 mM luminol for 10 min at 37°C in a chemiluminescence analyzer (Autoluminat LB 953; Berthold Technologies, Bad Wildbad, Germany). CL signals of PMN in the absence and presence of various OMZ concentrations were recorded for 20 min at 37°C. Thereafter, UCP or OZ, respectively, was added, and the CL signals of the cells were monitored for a further 20 min at 37°C.

**Statistical analyses**

Statistical significance was determined by analysis of variance and the two-sample Student’s t-test (STATSAK, version 2.12, by G.E. Dallal, 1986; Malden, MA, USA). Changes with P ≤ 0.05 were considered as significant.

**Results**

**Effect of OMZ on viability of human PMN**

The effect of OMZ on the viability of the cells was evaluated by trypan blue exclusion on a percentage basis. As shown in Fig. 1, OMZ did not affect the viability of PMN. The difference of dead and living cells between untreated PMN (0 mM) and OMZ-treated PMN (0.1, 0.4, and 1 mM) was not significant. These data indicate that OMZ exerts no toxic effect on PMN.

**Effect of OMZ on generation of eicosanoids and respiratory burst activity**

Human PMN were studied for the effect of OMZ in the absence and presence of co-stimulators. The following endpoints were analyzed: a) formation of PGE_2, 15(S)-HETE and LXA_4, and b) formation of 

![Fig. 1. Effect of OMZ on viability of human PMN. PMN were incubated with various OMZ concentrations (0.1, 0.4, and 1 mM) in PBS, pH 7.4, with Ca^{2+}/Mg^{2+} and 0.1% glucose in the absence of stimulants for 80 min at 37°C. Viability of cells was determined by trypan blue exclusion. Values are given as percentage of live or dead cells (mean ± S.D.) for n = 3 as the number of experiments.](image-url)
OMZ in the absence of co-stimulatory agents

At concentrations ranging from 0.1 to 1 mM, OMZ exerted a significant stimulatory effect on formation of PGE\(_2\), 15(S)-HETE, and LXA\(_4\) in unstimulated PMN (Fig. 2a), while formation of LTB\(_4\) and the oxidative stress marker 8-isoprostane were not affected by OMZ (Fig. 2b). In contrast, the respiratory burst activity was strongly inhibited by concentrations of 0.1 to 1 mM OMZ to below the baseline level (Fig. 2b).

OMZ in the presence of UCP

UCP were selected as a model stimulant. These particles are known to induce formation of PGE\(_2\), 15(S)-HETE, LXA\(_4\), LTB\(_4\), respiratory burst activity, and 8-isoprostane (23). As shown in Fig. 3, UCP applied in the absence of OMZ (UCP control) activated PMN for a significant increase in the level of each of these parameters (\(P<0.01\)) in comparison to the respective baseline level. OMZ exerted differential effects on the UCP-induced responses. OMZ did not substantially change the elevated UCP-stimulated levels of PGE\(_2\), 15(S)-HETE, and LXA\(_4\) (Fig. 3a). However, the increased levels of UCP-stimulated LTB\(_4\) and 8-isoprostane were drastically suppressed by concentrations of 0.4 to 1 mM OMZ, whereas respiratory burst activity was significantly inhibited by OMZ concentrations of 0.1 to 1 mM (Fig. 3b).

OMZ in the presence of OZ

In addition to the environmental co-stimulant UCP, which is non-physiologic, we also used OZ as a physiologic co-stimulant because of its opsonization with complement components. UCP and OZ are both particulate stimuli, which induce inflammatory and oxidative stress reactions (16). However, OZ with its opsonization elicited stronger biologic responses than UCP, especially concerning LTB\(_4\) and the respiratory burst activity. As shown in Fig. 4, OZ as control stimulant induced the formation of PGE\(_2\), 15(S)-HETE, LXA\(_4\), LTB\(_4\), and 8-isoprostane and activated respiratory burst activity. In contrast, the respiratory burst activity was strongly inhibited by concentrations of 0.1 to 1 mM OMZ (Fig. 4b).
activity ($P<0.01$). As already seen with UCP, OMZ exerted different effects on the OZ-induced responses. OMZ essentially did not change OZ-stimulated elevation of PGE$_2$, 15(S)-HETE, and LXA$_4$ levels (Fig. 4a). However, the increased levels of OZ-stimulated LTB$_4$, 8-isoprostane, and respiratory burst activity were drastically inhibited by OMZ nearly to baseline levels (Fig. 4b). LTB$_4$ levels and respiratory burst activity were reduced by OMZ concentrations of 0.4 and 1 mM, whereas the oxidative stress marker 8-isoprostane was inhibited by OMZ concentrations ranging from 0.1 to 1 mM.

**Discussion**

In a previous study we showed in an in vitro assay that OMZ inhibits the activity of 5-LO, which has pro-inflammatory properties. The activity of 15-LO, showing anti-inflammatory characteristics, is not inhibited. Using canine alveolar macrophages as a cellular model, we recently confirmed that OMZ inhibits the 5-LO pathway, causing a decreased production of LTB$_4$, and it induces the 15-LO pathway, resulting in an increase of 15(S)-HETE generation (16). The present study was designed to investigate the effect of OMZ on the eicosanoid metabolism in human PMN representing the major population of inflammatory cells in the virus-infected nasal tissue (20, 21). Based on our observation that OMZ activates the 15-LO pathway, we postulated that OMZ also induces formation of pro-resolving lipoxins, being the final metabolites of this pathway. These studies were performed both with unstimulated PMN and PMN stimulated by UCP or OZ to simulate inflammatory responses and oxidative stress. Our data demonstrate that OMZ inhibits respiratory burst activity very efficiently, while baseline levels of LTB$_4$ and 8-isoprostane used as an oxidative stress marker are not affected in unstimulated PMN. However, immune-modulating and anti-inflammatory responses, including the PGE$_2$ and 15(S)-HETE and the pro-resolving mediator LXA$_4$ production, are significantly increased. In UCP- or OZ-stimulated cells, OMZ
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strongly inhibits the LTB₄ and 8-isoprostane formation and the respiratory burst activity. The increased levels of PGE₂, 15(S)-HETE, and LXA₄ reached after stimulation were maintained in the presence of OMZ. We suppose that UCP- and OZ-stimulated cells are at their upper limit for producing PGE₂, 15(S)-HETE, and LXA₄, which does not allow a further increase by OMZ under these in vitro conditions. Similar responses regarding PGE₂, 15(S)-HETE, LTB₄, respiratory burst activity, and 8-isoprostane were already observed in canine AM (16), which react very similar to human AM (23). The effective concentrations of OMZ for inhibition of pro-inflammatory and oxidative stress reactions in PMN range between 0.1 and 1 mM. These OMZ concentrations have also been shown to be relevant in AM (16). In our previous study we estimated that OMZ in the current product concentration of 1.6 mM (nose sprays for adults and school children) at a dosage volume of 45 μl/puff (28) will be diluted to form a concentration gradient that is in the range of the levels of OMZ used in our experiments (estimated mean value: almost equal to 0.1 mM) (16). Therefore, our present results are of relevance in situ. However, it is still unknown by which receptor(s) OMZ modulates inflammation. Possibly OMZ exerts its effects on lipid mediators via α₁- and/or α₂-adrenoceptors since studies with stably transfected Chinese hamster ovary (CHO) cells have shown that OMZ behaves as a partial agonist of α₁A-adrenoceptors (29, 30), and this was also observed for α₂C-adrenoceptors (31). In addition, very low or not precisely measurable agonistic activity of OMZ has been observed in transfected CHO cells expressing α₁D- (29, 30) or α₂A- (32) adrenoceptors.

A viral infection induces a strong inflammation in the nasal tissue leading to an acute rhinitis. Inflammatory mediators play an important role in the pathogenesis of acute rhinitis (20, 21, 33). Nasal secretions become enriched with pro-inflammatory mediators, cytokines and eicosanoids (leukotrienes and prostaglandins), accompanied by an infiltration of PMN (20, 21, 34). LTB₄ is the most potent chemoattractant for PMN and therefore responsible for the neutrophilic infiltration in rhinitis-infected tissue (34, 35).

Data from our in vitro approach demonstrate that
OMZ inhibits the 5-LO–driven production of LTB₄ and the release of reactive oxygen species (ROS), which is paralleled by a substantial increase of PGE₂. Besides having some specific pro-inflammatory properties, PGE₂ was shown in the last decade to exhibit predominantly a regulatory function to control immune responses, for example, by attenuating pro-inflammatory reactions (36). One important target of this cyclooxygenase metabolite is the 5-LO–driven pathway, which is inhibited for leukotriene synthesis and translocation of 5-LO in stimulated PMN (37, 38). Furthermore, PGE₂ was found to up-regulate the expression of 15-LO in PMN (39), which activates the pathway for generation of 15(S)-HETE and lipoxins. Exposure of PMN to 15(S)-HETE also attenuates levels of LTB₄ substantially after stimulation with Ca²⁺-ionophore or the FMLP peptide (40). Up-regulation of 15-LO expression and release of 15(S)-HETE seem to be a further regulatory mechanism to cope with inflammation (41, 42). There is evidence that 15-LO is expressed in human nasal epithelium to mediate mucociliary differentiation (43). The expression of 15-LO in nasal epithelium may further support our findings about the effect of OMZ on 15(S)-HETE formation in PMN. 15(S)-HETE is the key metabolite for generation of lipoxins that also involves the action of 5-LO. Lipoxins are primarily engaged in resolution and termination of inflammatory events that return the inflamed tissue to homeostasis. Although PGE₂ initiates a number of responses relevant to inflammation, the central task of this metabolite is to trigger the “lipid mediator class switching” to signal the end of inflammation by activating the transcriptional regulation of 15-LO, resulting in production of LXA₄ (19, 39). Since OMZ induces formation of PGE₂ and 15(S)-HETE indicating an activation of 15-LO and reduces LTB₄ production indicating a reduction of 5-LO activity, OMZ is suggested to substantially support this switching of the eicosanoid class from LTB₄ to LXA₄. The importance of PGE₂ induced by OMZ during the inflammatory response in the nasal tissue is emphasized by the study of Han et al. (44), showing that the nasal patency is reduced when the COX activity is inhibited by non-steroidal anti-inflammatory drugs.

Moreover, the respiratory burst activity and the oxidative stress induced by UCP and OZ are inhibited by OMZ. The inhibition of both parameters might be due to the radical scavenger activity of OMZ as proposed by Westerveld et al. (17). OMZ contains a hydroxyl group on its phenyl ring that is able to easily provide a hydrogen atom for reaction with lipid peroxide–related radicals and other reactive oxygen species. Due to its radical scavenger activity, we suggest that OMZ also neutralizes radicals arising from the respiratory burst activity and abolishes UCP- and OZ-induced 8-isoprostane formation. This represents a protective mechanism avoiding excessive release of reactive
oxygen species as a tissue damaging principle.

The elevated levels of LXA₄ triggered by OMZ may also contribute to shortening the duration of the disease (15). In a prospective, multicenter, randomized, placebo-controlled, double-blind parallel group comparison study — OMZ vs. physiological saline solution — rhinitis duration was determined as primary endpoint in common-cold patients (268 Patients at 24 sites). Secondary endpoints were a noticeable relief of nasal congestion, runny nose, stuffy nose, sneezing, impairment of sense of smell and taste, and feeling unwell. The maximum treatment duration was 10 days [1 puff (45 μl of 1.6 mM/0.05% OMZ) in each nostril three times daily]. Symptoms improved significantly with OMZ versus the reference group treated with physiological saline solution as early as treatment day 2, and relief was maintained throughout the study period. Duration of rhinitis was significantly reduced by one-third (4 vs. 6 days) with OMZ.

In summary, we propose an OMZ-triggered signaling cascade in PMN as shown in Fig. 5. OMZ seems to be a leading signal that activates production of PGE₂, which then suppresses LTB₄ formation and activates the 15-LO pathway. In a concerted action with PGE₂, the pro-resolving lipid mediator LXA₄ stops further PMN influx, thus terminating the inflammation. Furthermore, LXA₄ stimulates monocyte recruitment for uptake of apoptotic PMN by macrophages (19). LXA₄ is most relevant for its capability to return the inflamed tissue to homeostasis (19). In this regard, the increase and maintenance of LXA₄ levels by OMZ may enhance the termination of rhinitis-related inflammation and thus contribute significantly to shortening the duration of the disease (15).

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