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Impact of simultaneous stimulation of 5-lipoxygenase and myeloperoxidase in human neutrophils

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ABSTRACT

Human neutrophil 5-lipoxygenase (5-LOX) oxidizes arachidonic acid (AA) to 5*S*-hydro(pero)xy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-H(p)ETE) and leukotriene (LT)A_4, which is further converted to the chemoattractant LTB_4. These cells contain also the heme enzyme myeloperoxidase (MPO) producing several potent oxidants such as hypochlorous acid (HOCl). Previously, it was shown that MPO-metabolites influence 5-LOX product formation. Here, we addressed the question, whether a simultaneous activation of MPO and 5-LOX in neutrophils results in comparable changes of 5-LOX activity.

Human neutrophils were stimulated with H_2O_2 or phorbol 12-myristate 13-acetate (PMA) for MPO activation and subsequently treated with calcium ionophore A23187 inducing 5-LOX product formation on endogenous AA. Special attention was drawn to neutrophil vitality, formation of MPO-derived metabolites and redox status. The pre-stimulation with H_2O_2 resulted in a concentration-dependent increase in the ratio of 5-HETE to the sum of LTB_4+6-*trans*-LTB_4 in consequence of MPO activation. Thereby no impairment of cell vitality and only a slightly reduction of total glutathione level was observed. An influence of MPO on 5-LOX product formation could be suggested using an MPO inhibitor. In contrast, the pre-stimulation with PMA resulted in different changes of 5-LOX product formation leading to a reduced amount of 5-HETE unaffected by MPO inhibition. Furthermore, impaired cell vitality and diminished redox status was detected after PMA stimulation. Nevertheless, a MPO-induced diminution of LTB_4 was obvious. Further work is necessary to define the type of 5-LOX modification and investigate the effect of physiological MPO activators.

1. Introduction

The inflammatory response against infectious agents is characterized by a rapid recruitment of neutrophils from peripheral blood to the inflammatory loci driven by chemotactic agents like IL-8, fMet–Leu–Phe and others [1]. During this movement, neutrophils produce in a 5-lipoxygenase (5-LOX)-dependent reaction the potent autocrine chemotactic molecule leukotriene B_4 (LTB_4). Thereby, 5-LOX, a non-heme-iron dioxygenase, translocates to the nuclear envelope, interacts with 5-LOX activating protein (FLAP) and oxygenates arachidonic acid (AA), which is liberated by the action of cytosolic phospholipase A_2 ($cPLA_2$) [2]. The lipoxygenase reaction is initiated by abstraction of a hydrogen atom from C7 of AA by ferric 5-LOX [3] generating a radical, which migrates to the C5 position, where it reacts with dioxygen forming a peroxyl radical. Subsequently, the peroxyl anion is formed by interaction with ferrous 5-LOX yielding 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HpETE) after protonation [4]. Human 5-LOX also possesses a secondary leukotriene A_4 (LTA_4) synthase activity [5]. For this purpose a hydrogen atom at the C10 position of 5-HpETE is abstracted, followed by radical migration, rearrangement of double bonds and formation of an epoxide moiety [6]. Thereafter, unstable LTA_4 is hydrolyzed to leukotriene B_4 (LTB_4) by the action of LTA_4 hydrolase [7].

During respiratory burst in phagocytosing neutrophils a high proportion of consumed dioxygen (reported values of 28–72%) is converted into hypochlorous acid (HOCl) by the heme protein myeloperoxidase (MPO), which is present in azurophilic granules [8]. Upon reaction with H_2O_2, oxidized MPO can abstract two electrons from chloride generating HOCl [9]. However, the sole impact of HOCl on microbe killing is a matter of considerable debate [10,11]. When [$^{13}C_6$]-tyrosine labeled *Staphylococcus aureus* are phagocytosed from neutrophils, chlorinated bacterial proteins could be detected only after 5 min leading to microbe killing. But, the majority of chlorinated tyrosine residues, this means 94%, were detected in neutrophil

Abbreviations: MVP, 1-methyl-2-vinylpyridinium triflate; 4-ABAH, 4-aminobenzoic acid hydrazide; APF, aminophenyl fluorescein; JC-1, J-aggregate forming cationic dye

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proteins [12]. Furthermore, individuals with MPO deficiency, occurring with a relative high prevalence, showed only partially pathogen-dependent problems with severe infections indicating MPO-independent host defense mechanism that compensate in part their lack of MPO [13–15]. Therefore, the implication of HOCl in further biological aspects could be suggested. Typical reaction partners of HOCl are thiols, thioether and amino groups. This enables HOCl to oxidize amino acid residues such as cysteine, methionine, tyrosine, tryptophan, and lysine [16]. Interestingly, with respect to the above mentioned HOCl preference for neutrophil proteins, it could be shown that different granule proteins can be irreversible inactivated by HOCl or the MPO–H_2O_2–Cl^- system [17–19]. Additionally, HOCl can react with amines to produce chloramines or N-chlorinated derivatives with longer lifetime and membrane permeability [20]. Furthermore, HOCl can readily pass through membranes assuming MPO-dependent oxidative modification of cytosolic proteins [21,22].

Both neutrophil enzymes, 5-LOX and MPO, are sequentially activated during migration and in response to pathogen recognition, respectively. Here, the question arises whether HOCl, a potent amino acid-oxidizing agent, could affect the activity or product profile of 5-LOX. Previously, we demonstrated that MPO-derived oxidants exhibited a considerable impact on human recombinant 5-LOX, impairing the epoxidation of 5-HpETE, whereas the hydroperoxidation of arachidonic acid was unaffected [23]. Thereby, HOCl and HOBr, a further prominent hypohalous acid produced from peroxidases, increased the ratio of 5-H(p)ETE to 6-*trans*-LTB_4 in a concentration-dependent manner. Comparable results were obtained with the MPO–H_2O_2–Cl^-system, when glucose oxidase and glucose were applied as a source of H_2O_2. This was necessary due to a strong impairment of 5-LOX activity by H_2O_2. These results implicate, that MPO could terminate the 5-LOX dependent formation of LTB_4, raising the question of whether that could be of importance in human neutrophils.

The aim of this study was to assess the simultaneous activation of MPO and 5-LOX in human neutrophils by taking the activity and product profile of 5-LOX under consideration. Therefore, different MPO-stimulants were investigated. Analysis was performed by reverse-phase high-performance liquid chromatography (RP-HPLC) and flow cytometry. Further attention was paid on cell vitality, MPO activation and redox status of stimulated neutrophils. We could demonstrate MPO-induced changes of 5-LOX product formation in neutrophils after pre-stimulation with H_2O_2 or phorbol 12-myristate 13-acetate (PMA) followed by addition of the calcium ionophore A23187.

2. Material and methods

2.1. Material

The chemicals were obtained from the following sources: aminophenyl fluorescein (APF) and HPLC-standards 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HpETE), 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE), 5*S*,12*R*-dihydroxy-6*E*,8*E*,10*E*,14*Z*-eicosatetraenoic acid (5,12-DiHETE), 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE), LTB_4, prostaglandin B_2 (PGB_2) and AA were from Cayman Chemical (distributed by Biomol, Hamburg, Germany); HPLC solvents were from Carl Roth (Karlsruhe, Germany); glutathione colorimetric detection kit from Arbor Assays (Ann Arbor, Michigan, United States); biocoll (10 mM HEPES, 1.077 g/l, isotone) from Biochrom (Berlin, Germany) and all other chemicals including the JC-1 staining kit (CS0-390) were from Sigma (Taufkirchen, Germany).

Working solutions of H_2O_2 were prepared by dilution of the corresponding stock solutions. Their concentrations were determined spectrophotometrically using $\varepsilon_{240}=43.6\ M^{-1}\ cm^{-1}$ [24]. The buffer system hanks' balanced salt solution (HBSS) with and without Ca^{2+} for the resuspension of neutrophils was prepared daily and adjusted to pH 7.4.

2.2. Isolation of neutrophil granulocytes

Neutrophils were isolated from heparinized peripheral human blood (10 U/ml), obtained from healthy volunteers after written consent, by dextran-enhanced sedimentation in the presence of 2% dextran for an hour, followed by a density centrifugation (biocoll 1.077 g/l, 20 °C, 400 *g*, 20 min). Remaining erythrocytes in the cell pellet were lysed by addition of one part distilled H_2O and incubation for 30 s. Then, five parts of HBSS without Ca^{2+} were added and after centrifugation this procedure was repeated. Neutrophils with a purity as well as vitality higher than 90% were isolated.

2.3. Determination of 5-LOX products from neutrophils by RP-HPLC

Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}) were incubated in the presence or absence of the MPO inhibitor 4-aminobenzoic acid hydrazide (4-ABAH) with a final concentration of 2.5 mM for 15 min followed by stimulation with PMA or H_2O_2 for 20 min. During the last 5 min Ca^{2+} ionophore A23187 was added. All incubation steps took place at 37 °C in a final volume of 1 ml. Control experiments without the addition of inhibitor or stimulant were incubated for comparable duration. Afterwards, cell stimulation was stopped by addition of 1.01 ml ice-cold methanol containing 200 ng PGB_2 as internal standard. Samples were acidified with 30 µl of 1 N HCl, mixed for 30 s and incubated at 4 °C for 30 min. The precipitate was separated by centrifugation (4 °C, 10,000 *g*, 10 min) and supernatants were applied to C_{18} solid-phase extraction columns (100 mg, Discovery DSC-18 SPE Tube, Sigma, Taufkirchen, Germany) preconditioned with 1 ml methanol and 1 ml H_2O. The columns were washed with 1 ml H_2O and 1 ml H_2O/methanol (75/25, v/v). 5-LOX metabolites were eluted with 300 µl methanol. Extraction was performed by centrifugation (10 *g*, 4 °C). The samples were analyzed by RP-HPLC after maximal storage at −80 °C for 24 h. Here, a C_{18} column (Supelcosil LC-18-DB, 25 cm × 4.6 mm i.d., 5 µm) was used with an isocratic eluent consisting of acetonitrile/H_2O/acetic acid (60/40/0.2, v/v/v) and a flow rate of 1 ml/min. The eluate was monitored at 234 nm (quantification of conjugated dienes) and 270 nm (quantification of conjugated trienes). The HPLC device consisted of a Shimadzu liquid chromatographic system equipped with a Shimadzu LC-10ATvp isocratic solvent delivery system, Shimadzu SPD-10Avp dual wavelength absorbance detector, Shimadzu CTO-10ASvp column oven (35 °C) and Rheodyne injector with 20 µl loop volume. Major lipoxygenase products (5-H(p)ETE, LTB_4 and 6-*trans*-LTB_4) were quantified using a calibration curve (13-point calibration) of the appropriate synthetic standards. Both forms of the non-enzymatic hydrolysis product 6-*trans*-LTB_4 (5*S*,12*S*/*R*-DiHETE) eluted at the same retention time and were measured as one product. LTB_4 was quantified using the 6-*trans*-LTB_4 calibration curve. Within each experiment, samples were referenced to the internal standard PGB_2.

2.4. Determination of cell vitality using JC-1

Cell vitality of neutrophils were assessed using the lipophilic, cationic dye JC-1 (J-aggregate forming cationic dye: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide). In vital cells JC-1 accumulates in mitochondrial matrix forming red fluorescent JC-1 aggregates. Upon changes of the mitochondrial electrochemical potential for example through the initiation of apoptosis, the JC-1 dye dispersed throughout the entire cell preventing JC-1 aggregate formation and leading to a shift to green fluorescence of JC-1 monomers. The cell vitality is defined as percentage of live, vital cells in the whole neutrophil population with intact membrane potential as assessed by red fluorescent JC-1 aggregates. For JC-1 staining 1×10^6