ORIGINAL ARTICLE

Inhibition of superoxide generation and myeloperoxidase release by carvedilol after receptor and nonreceptor stimulation of human neutrophils

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Abstract

OBJECTIVES: To compare three stimuli which activate human neutrophils with different signal transduction mechanisms, in order to better localize the effect of the beta-adrenoceptor antagonist carvedilol (CARV) on superoxide generation ($O_2^{\bullet-}$) and myeloperoxidase release (MPO). The effect of CARV [0.1–100 μmol/l] on $O_2^{\bullet-}$ generation and MPO release from isolated human neutrophils was studied after specific receptor activator N-formyl-methionyl-leucyl-phenylalanine (fMLP) and nonreceptor phorbol-12-myristate-13-acetate (PMA) and calcium ionophor (A23187) stimuli.

METHODS: $O_2^{\bullet-}$ generation was measured as superoxide dismutase inhibitable reduction of cytochrome c and MPO release as the oxidation of o-dianisidine in the presence of hydrogen peroxide in a spectrophotometer Hewlet Packard 8452 A at respective 550 and 463 nm.

RESULTS: CARV had no effect on $O_2^{\bullet-}$ generation and MPO release in nonstimulated cells. In the concentration 10 and 100 μmol/l, it significantly decreased fMLP and PMA stimulated $O_2^{\bullet-}$ generation and MPO release. Incubation of neutrophils with CARV [100 μmol/l] caused significant inhibition of $O_2^{\bullet-}$ generation and MPO release induced by A23187.

Wortmannin, a specific inhibitor of 1-phosphatidylinositol-3-kinase, inhibited significantly only fMLP stimulated $O_2^{\bullet-}$ generation. CARV [100 μmol/l] with wortmannin [50 nmol/l] further decreased $O_2^{\bullet-}$ generation after the same stimulus.

CONCLUSION: CARV decreased $O_2^{\bullet-}$ generation and MPO release from isolated human neutrophils both by membrane-operating stimulus – fMLP and membrane bypassing activators – PMA and A 23187. This fact, together with effect the of wortmannin, indicates that the inhibition may be attributed to the non-specific action of CARV and its interference with phospholipase D signaling pathway, which plays only a minor role in proteinkinase C stimulated $O_2^{\bullet-}$ generation.

Abbreviations

A26187 - calcium ionophor
CARV - carvedilol
fMLP - N-formyl-methionyl-leucyl-phenylalanine
MPO - myeloperoxidase
PKC - proteinkinase C
PLD - phospholipase D
PMA - phorbol-12-myristate-13-acetate
ROS - reactive oxygen species
$O_2^{\bullet-}$ - superoxide
W - wortmannin

INTRODUCTION

Activation of neutrophils induces generation of reactive oxygen species (ROS) including $O_2^{\bullet-}$ generation and release of enzymes, which participate not only in the bactericidal mechanisms of these cells but also in possible inflammatory tissue damage (Karlsson &Dahlgren, 2002). Redundant generation of ROS and MPO release may influence an inflammatory response of the neutrophils, resulting in many serious diseases (Hare, 2004, Maes, 2007). Drugs developed to antagonize these oxidizing species and to decrease of enzymes release can prevent at least some of the damage of the tissues by these very reactive agents.

Carvedilol (CARV) is a non-selective beta-blocker, which has also alpha-blocking properties and antioxidant effects. In fact, its antioxidant activity synergizes with its anti-adrenergic effects and confers additional therapeutic advantages compared to classic beta-blockers (Carreira *et al*., 2006). Dandona *et al*. (2000) described the inhibitory effect of CARV on $O_2^{\bullet-}$ anion release from activated neutrophils *ex vivo*. CARV was also found to inhibit luminol-enhanced chemiluminescence of ROS in human whole blood *in vitro* (Nosáľ *et al*., 2005) and in rat neutrophils (Moravcová *et al*., 2007). It also decreased $O_2^{\bullet-}$ generation and MPO release from isolated human neutrophils activated by opsonized zymosan (Pečivová *et al*., 2007).

In the present study we compared three stimuli activating human neutrophils with different signal transduction mechanisms, in order to better localize the effect of the beta-adrenoceptor antagonist CARV on $O_2^{\bullet-}$ and MPO release. We studied the effect of CARV on $O_2^{\bullet-}$ and MPO release from isolated human neutrophils after specific receptor activator (fMLP) and non-receptor (PMA, A23187) stimuli.

MATERIALS AND METHODS

Material

Carvedilol was supplied by Zentiva (Czech Republic), Dextran T500 (Pharmacia Fine Chemicals), Lymphoprep (Nycomed Pharma AS), cytochalasin B (Merck). All other chemicals used were purchased from Sigma-Aldrich.

Isolation of neutrophils

Neutrophils were isolated from blood of healthy male volunteers into 3.8% trisodium citrate and erythrocytes were removed by dextran sedimentation and centrifugation on Lymphoprep by the modified Boyum's method. The final suspension contained more than 96% of viable cells, as evaluated by trypan blue exclusion (Drábiková *et al*., 2002). Neutrophils for $O_2^{\bullet-}$ and MPO determination were 1×10^6 and 2×10^6/sample, respectively.

Superoxide determination

Superoxide dismutase inhibitable reduction of cytochrome c was used to measure superoxide generation in isolated human neutrophils, as described by Pečivová *et al*. (2007). $O_2^{\bullet-}$ generation was determined by means of spectrophotometry (Hewlet Packard 8652 A) at 550 nm.

Myeloperoxidase release

Myeloperoxidase release was assayed by determinig the oxidation of o-dianisidine in the presence of hydrogen peroxide in a Hewlet Packard 8652 A spectrophotometer at 463 nm (Pečivová *et al*., 2006).

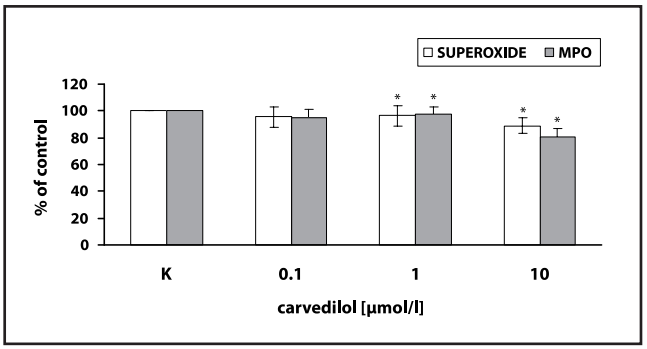

Figure 1. Effect of carvedilol on fMLP [0.1 μmol/l, 3 min/37 °C] versus control without drugs stimulated superoxide generation and myeloperoxidase release in human neutrophils. Results are mean ± SEM, n=6–8, *p<0.05 compared to control value without CARV.

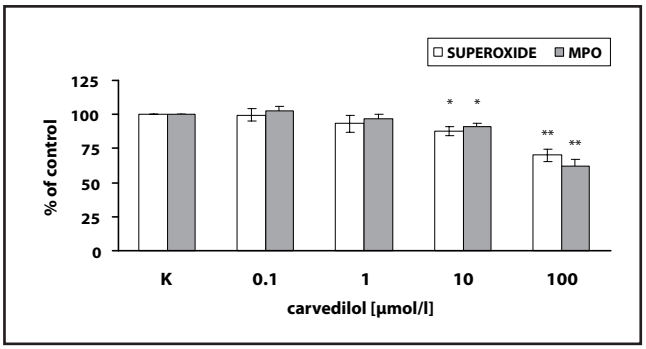

Figure 2. Effect of carvedilol on PMA [1 μmol/l, 15 min/37 °C] stimulated superoxide generation and myeloperoxidase release in human neutrophils. Results are mean ± SEM, n=6–8, *p<0.05 and **p<0.01 compared to control value without CARV.

Statistical analyses

All values are given as means of 6–8 experiments ± SEM. Statistical significance of differences between means was established by Student´s t-test and *p* values below 0.05 were considered statistically significant.

RESULTS

Concerning $O_2^{\bullet-}$ generation and MPO release, preincubation with CARV [0.1–100 µmol/l] had no significant effect on unstimulated isolated human neutrophils. Figure 1 shows the effect of CARV on $O_2^{\bullet-}$ generation and MPO release from neutrophils stimulated by fMLP. CARV dose-dependently decreased $O_2^{\bullet-}$ generation and MPO release, however a significant decrease was recorded with CARV concentration of 10 and 100 µmol/l only. Incubation of neutrophils with CARV [10 and 100 µmol/l] caused significant inhibition of $O_2^{\bullet-}$ generation and MPO release induced by PMA (Figure 2). The effect of CARV on $O_2^{\bullet-}$ generation and MPO release in isolated neutrophils stimulated with A 23187 is given in Figure 3. CARV caused significant inhibition of both parameters only in 100 µmol/l concentration.

Since all stimuli studied are able to activate PLD, we used wortmannin, a specific inhibitor of 1-phosphatidylinositol-3-kinase, to see whether $O_2^{\bullet-}$ generation in stimulated neutrophils would be modulated by CARV. Wortmannin significantly inhibited only fMLP stimulated $O_2^{\bullet-}$ generation. CARV [100 µmol/l] with wortmannin [50 nmol/l] decreased $O_2^{\bullet-}$ generation after the same stimulus and enhanced the effect of wortmannin (Figure 4).

DISCUSSION

$O_2^{\bullet-}$ radicals, precursors of other ROS used for host defense against pathogens which may also inflict damage of adjacent tissues, are generated during oxidative burst by neutrophil NADPH oxidase. Together with proteolytic enzymes released from activated neutrophils in the process of degranulation have been recognized as an important factor contributing to netrophil-mediated injury (Babior, 2000).

We studied the effect of CARV [0.1–100 µmol/l] on $O_2^{\bullet-}$ generation and MPO release from isolated human neutrophils after specific receptor activator (fMLP) and nonreceptor (PMA, A23187) stimuli. We used three stimuli with different signal transduction mechanisms, to activate human neutrophils, in order to compare and better localize the effect of the beta-adrenoceptor antagonist CARV on human neutrophils.

All activators are able to stimulate neutrophils to generate $O_2^{\bullet-}$, including other ROS and MPO. The synthetic chemotactic tripeptide fMLP is a stimulus identical with natural bacterial polypeptide. Both are operating through membrane receptor with activation of phospholipase C. On the other hand PMA, a receptor bypassing stimulus, activates NADPH-oxidase via proteinkinase C (PKC) and calcium ionophor A 23187, bypasses G-protein-mediated signal transduction and promotes calcium movement across the plasma membrane. An increase in concentration of cytosolic free calcium is a powerful stimulus for neutrophils activation (Li *et al*., 2002).

Dandona *et al*. (2000) demonstrated that in patients who were given CARV, this drug significantly inhibited $O_2^{\bullet-}$ generation by neutrophils, as shown by chemiluminescence with fMLP stimulation. Åsbrink *et al*. (2000) observed that CARV dose dependently modulated $O_2^{\bullet-}$ generation by NADPH oxidase after stimulation by fMLP or PMA.

In our experimental conditions, CARV decreased $O_2^{\bullet-}$ formation similarly as recorded for PMA-stimulated neutrophils (Yue *et al*., 1992). CARV was shown to be a poor scavenger of $O_2^{\bullet-}$, as proven on cell-free system (Åsbrink *et al*., 2000, Nosáľ *et al*., 2005). In human neutrophils, CARV interfered *in vitro* and *ex vivo* both with ROS generation and with already generated ROS, suggesting not only its „preventive" but also its „therapeutic" effect (Drábiková *et al*., 2006).

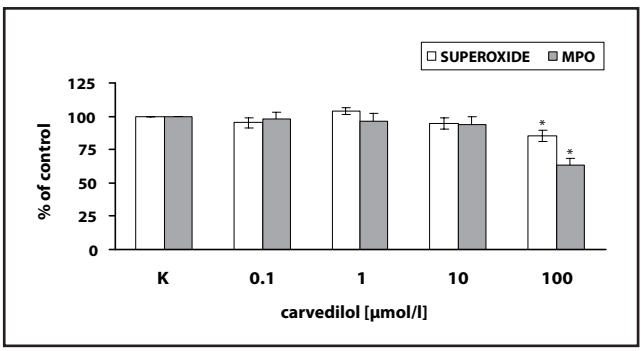

Figure 3. Effect of carvedilol on A 23187 [0.1 µmol/l, 15 min/37 °C] stimulated superoxide generation and myeloperoxidase release in human neutrophils. Results are mean ± SEM, n=6–8, **p*<0.05 compared to control value without CARV.

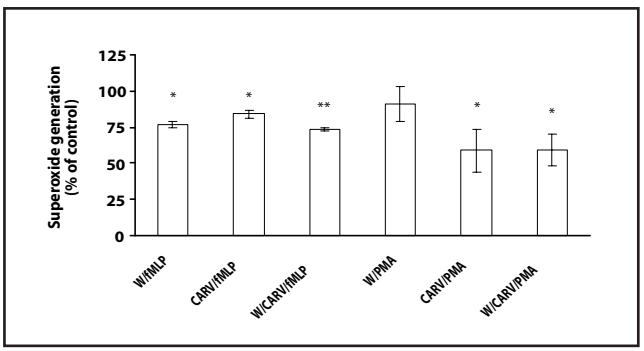

Figure 4. Effect of wortmannin [50 nmol/l] and/or carvedilol [100 µmol/l] on superoxide generation in fMLP and PMA stimulated human neutrophils. Results are mean ± SEM, n=6–8, **p*<0.05 compared to control value without CARV.

CARV as a drug with high lipid solubility may be likely to accumulate in the lipid layer of membranes, resulting in alteration of their physical properties. Thus CARV may affect the activity of enzymes playing an important role in ROS generation after PMA activation of neutrophils: PKC, NADPH oxidase and MPO (Butler *et al*., 2006, Nosáľ *et al*., 2005).

CARV was shown to be a direct dose-dependent scavenger of hydroxyl radical, hydrogen peroxide and $O_2^{\bullet-}$ radicals and to affect dose-dependently the chemiluminescence of rat neutrophils by receptor and nonreceptor activators (Moravcová *et al*., 2007). This indicates that CARV interferes with already generated ROS rather than with specific receptors for opsonins or chemotactic peptides localized in the plasma membrane or with intracellular calcium mobilisation and PKC activation (Nosáľ *et al*., 2005).

In our experiments $O_2^{\bullet-}$ generation stimulated by fMLP was significantly decreased by wortmannin, an inhibitor of phospholipase D (PLD) signal transduction, and by CARV. On adding them together, CARV pronounced the effect of wortmannin, decreasing $O_2^{\bullet-}$ generation by further 10%. Our results with wortmannin inhibition demonstrated that PLD signaling pathway plays a role in fMLP stimulated $O_2^{\bullet-}$ generation.

MPO is a major constituent of the azurophilic granules of neutrophils. MPO, the most abundant enzyme in neutrophils, is a major NO scavenger and marker of oxidative stress (Brennan & Hazen, 2003). Together with the membrane NADPH oxidase, MPO is involved in the formation of ROS and oxidation of biological material. CARV inhibited MPO activity of the cell-free system in a concentration-dependent manner (Nosáľ *et al*., 2005). Our results showed that CARV decrased MPO release from human neutrophils stimulated by fMLP, PMA and A 23187.

In our experiments, the inhibitory effect of CARV was not dependent on the type of stimulus. CARV was found to inhibit $O_2^{\bullet-}$ generation and MPO release both by membrane-operating stimulus (fMLP) and by membrane bypassing activators (PMA, A 23187). The findings lead to the conclusion that the inhibitory effect could be attributed to the non-specific membrane effect of CARV and its interference with PLD signaling pathway, potentially on the level of lipid second messengers, such as 1,2-diacylglycerol (DAG). Physicochemical properties of CARV and its antiplatelet activity support this conclusion (Petríková *et al*., 2002). CARV, similarly to other lipophilic beta-adrenoceptor/bloking drugs, interferes with membrane structure, influencing predominantly phospholipid metabolism (Petríková *et al*., 2002, Nosáľ *et al*., 2005). Inhibition of $O_2^{\bullet-}$ generation and MPO release from stimulated human neutrophils by CARV may also increase protection against further damage induced by oxygen-radical chain reactions.

ACKNOWLEDGEMENTS

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