INFLUENCE OF CARVEDILOL ON SUPEROXIDE GENERATION AND ENZYME RELEASE FROM STIMULATED HUMAN NEUTROPHILS

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Activation of neutrophils induces generation of reactive oxygen species and release of granule enzymes, which not only participate in the bactericidal mechanisms of these cells, but also in possible tissue damage. We studied the effect of carvedilol (CARV) [0.1-100 µmol/l], an antihypertensive and cardiovascular drug with antioxidative properties, on superoxide generation (SO) and myeloperoxidase (MPO) release from isolated human neutrophils stimulated with fMLP, a specific receptor activator, or with PMA, a receptor bypassing stimulus. Unstimulated cells showed neither SO formation nor MPO release after preincubation with drug. CARV decreased fMLP and PMA stimulated MPO release and SO generation dose dependently. The inhibitory effect of CARV may attributed to non-specific action since its effect was not influenced by the type of stimulation. It might inhibit SO generation as well as MPO release either by membrane-operating stimulus (fMLP) or membrane bypassing activator (PMA).

INTRODUCTION

Carvedilol (CARV), [1-[carbazolyl-(4)-oxy]-3-[(2-methoxy-phenoxyethyl)amino]-2-propanol] is a multiple action antihypertensive drug with nonselective β -adrenergic and selective α -adrenergic blocking activity. CARV has been shown to possess antioxidant and free radical scavenging activities, which may contribute to its cardioprotective efficacy¹⁻⁴. The antioxidant activity of CARV has been evaluated and characterised in detail in a variety of *in vitro* test systems and in a broad spectrum of animal models of myocardial infarction^{1, 3-5}.

Neutrophils represent the most important source of free radicals and actively contribute to damage of the vessel wall in cardiac and cerebrovascular diseases⁶. Activation of neutrophils induces generation of reactive oxygen species and release of granule enzymes, which not only participate in the bactericidal mechanisms of these cells, but also in possible tissue damage⁷. Dandona *et al.*⁸ described the inhibitory effect of CARV on SO anion release from activated neutrophils *ex vivo*. CARV was also found to inhibit luminol-enhanced chemiluminescence of reactive oxygen species in blood cells *in vitro*⁹.

In this work we studied the effect of CARV on the first member of the chain of oxygen species – superoxide anion (SO) and on myeloperoxidase (MPO) release by human neutrophils stimulated with N-formyl-methionyl-leucyl-phenylalanine (fMLP), a specific receptor activator, or with phorbol-12-myristate-13-acetate (PMA), a receptor bypassing stimulus.

MATERIAL AND METHODS

Material

Carvedilol was supplied by La Roche (Mannheim); Dextran T500 (Pharmacia Fine Chemicals); Lymphoprep (Nycomed Pharma AS); PMA (phorbol-12-myristate-13-acetate) and fMLP (formyl-methionyl-leucyl-phenylalanine) from Sigma-Aldrich; cytochalasin B (Merck). All other chemicals used were of analytical grade.

The stock solution of carvedilol tartrate was diluted freshly before each experiment with deionised water to the desired concentrations.

Isolation of neutrophils

Neutrophils were isolated from blood of healthy volunteers into 3.8 % trisodium citrate dihydrate (9:1). After dextran sedimentation and centrifugation on Lymphoprep by modified Boyum's method¹⁰, neutrophils were resuspended in calcium-, magnesium-free phosphate buffer saline (PBS) solution (in mmol: 137 NaCl, 2.7 KCl, 8.1 Na₂HPO₄, 1.5 KH₂PO₄), pH 7.4 and washed once with PBS. For individual assays neutrophils were diluted as described below. The purity of isolated neutrophils was > 96 % (light microscopy).

Superoxide determination

Suspension of neutrophils (10⁶ cells/1.5 ml PBS with 1.8 mmol/l CaCl₂ and 0.5 mmol/l MgCl₂) was preincubated for 5 min at 37 °C with carvedilol (0.1–100 µmol/l) and then stimulated with fMLP (0.1 µmol/l) for 3 min or with PMA (1 µmol/l) for 15 min at 37 °C. Controls were

included for the effect of each stimulus and of carvedilol on cytochrome c reduction. Superoxide formation was measured in isolated human neutrophils as superoxide dismutase inhibitable reduction of cytochrome c (ref.¹¹). The absorbance at 550 nm was measured using a spectrophotometer Hewlett Packard 8452 A.

Enzyme determination

For myeloperoxidase (MPO) determination, neutrophils were preincubated with cytochalasin B (5 µg/ml) at room temperature for 5 min. Subsequently the suspension of neutrophils (2×10^6 cells/ml PBS) was incubated with carvedilol (0.1–100 µmol/l) for 5 min at 37 °C and then stimulated with fMLP (0.1 µmol/l) for 3 min or with PMA (1 µmol/l) for 15 min at 37 °C. Controls for direct interaction of carvedilol and MPO were included. The activity of MPO was assayed in the supernatant after centrifugation $2000 \times g$ for 10 min at 4 °C by determining the oxidation of o-dianisidine in the presence of hydrogen peroxide in a Hewlett Packard 8452 A spectrophotometer at 463 nm¹².

Statistical analysis

All values are given as means $\pm$ 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 AND DISCUSSION

Superoxide (SO) radical generated during oxidative burst by neutrophil NADPH oxidase, a precursor of other reactive oxygen species used for host defense against bacterial and fungal pathogens, but they also inflict damage on adjacent tissue, and are thought to be of pathogenic significance in a large number of diseases¹³. Proteolytic enzymes released from activated neutrophils in the process of degranulation have been recognised as an important factor contributing to neutrophil-mediated tissue injury.

CARV is an antihypertensive agent with several mechanisms of antiarrhythmic and antiischaemic activities, underlying its cardio- and cerebroprotective^{14,15} as well as antioxidant properties. The antioxidant action may be relevant for preserving myocardial viability, particularly during ischaemia and reperfusion, conditions linked to the occurrence of oxidative stress^{1-3,16}. Ischaemia and reperfusion are potent stimuli for eliciting cardiomyocyte apoptosis, with neutrophils involved in the development of myocardial injury. CARV (at a dose range 1–10 nmol/l) significantly prevented activated neutrophil-induced cardiomyocyte apoptosis on cultured primary neonatal rat cardiomyocytes, possibly due to its antioxidant properties¹⁷. CARV inhibited oxidative stress in neutrophils and mononuclear cells in patients with hypertension¹⁸ and exerted a marked scavenger effect on free radical generation-induced red blood cell membrane damage¹⁹.

CARV inhibits luminol-enhanced chemiluminescence of reactive oxygen metabolites *in vitro*. Nosál *et al.*⁹ found

Table 1. Effect of carvedilol on fMLP (0.1 µmol/l, 3 min/ 37 °C) stimulated superoxide generation and myeloperoxidase release in human neutrophils

Carvedilol (µmol/l)	Superoxide generation (%)	Myeloperoxidase release (%)
0	100	100
0.1	95.39 $\pm$ 7.52	95.14 $\pm$ 5.70
1	96.32 $\pm$ 7.28	97.69 $\pm$ 5.30
10	89.03 $\pm$ 5.71	80.67 $\pm$ 6.00*
100	84.08 $\pm$ 2.99***	72.88 $\pm$ 9.30*

Values are expressed as percentage of control values obtained after stimulation with fMLP. Each value represents mean from 6–8 experiments $\pm$ SEM. *p < 0.05, ***p < 0.001 as compared to control values without carvedilol

Table 2. Effect of carvedilol on PMA (1 µmol/l, 15 min/ 37 °C) stimulated superoxide generation and myeloperoxidase release in human neutrophils

Carvedilol (µmol/l)	Superoxide generation (%)	Myeloperoxidase release (%)
0	100	100
0.1	99.65 $\pm$ 4.75	102.36 $\pm$ 3.35
1	93.17 $\pm$ 6.46	97.16 $\pm$ 53.41
10	87.53 $\pm$ 8.02	91.27 $\pm$ 5.56
100	70.24 $\pm$ 4.45***	62.04 $\pm$ 4.84***

Values are expressed as percentage of control values obtained after stimulation with PMA. Each value represents mean from 6–9 experiments $\pm$ SEM. ***p < 0.001 as compared to control value without carvedilol

that in the cell-free system CARV dose-dependently decreased chemiluminescence in the following range of order of radicals: hydroxyl radicals > hydrogen peroxide > superoxide radical. CARV also dose-dependently inhibited chemiluminescence of isolated human neutrophils in the ranging order of stimuli: A 23187 > OpZ > fMLP. Dandona *et al.*⁸ demonstrated that CARV in patients who were given this drug significantly inhibited SO generation by neutrophils and mononuclear cells *via* chemiluminescence with fMLP stimulation.

Åsbrink *et al.*²⁰ observed that CARV dose dependently modulated generation of SO ions by NADPH oxidase when induced by fMLP or PMA.

In the present work, we studied the effect of CARV on SO generation and MPO release by human neutrophils stimulated with fMLP, a specific receptor activator, or with PMA, a receptor bypassing stimulus. In every experiment we used parallel incubation of unstimulated controls. These cells showed neither SO formation nor enzyme release after preincubation with CARV. Table 1 illustrates the effect of CARV (0.1, 1, 10 and 100 µmol/l) on fMLP activated SO generation in isolated human neutrophils. CARV inhibited dose-dependently SO generation. In the concentration 100 µmol/l, CARV significantly inhibited SO generation to 84.08 ± 2.99 %.

CARV suppressed superoxide generation of human neutrophils stimulated with PMA, as determined by MCLA-dependent chemiluminescence²¹. The ability of CARV to prevent cell damage and death of activated neutrophils may be due to its property to scavenge SO ions and to inhibit the production of SO radicals by neutrophils. In a concentration-dependent manner, CARV was demonstrated to inhibit the production and generation of SO ions by neutrophils, based on direct measurement of SO release from PMA activated human neutrophil, or its generation during autooxidation of dihydroxyfumaric acid^{2,3}.

In our experimental conditions, we observed that the effect of CARV (0.1, 1, 10 and 100 µmol/l) on PMA activated SO generation in isolated human neutrophils was dose-dependent (Table 2). CARV in the concentration 100 µmol/l significantly decreased SO generation to 70.21 ± 4.45 %.

The haeme-containing enzyme myeloperoxidase (MPO) is a major constituent of the azurophilic cytoplasmic granules of neutrophils, which is gradually released either intracellularly into the formed phagocytes or extracellularly upon stimulation of cells. Together with the membraneous NADPH oxidase, MPO is involved in the formation of reactive oxygen species and oxidation of biological material. CARV inhibited MPO activity of the cell-free system in a concentration-dependent manner⁹. CARV treatment reduced MPO levels at both the area-at-risk and the necrotic zone in the ischaemic myocardium²².

In our experiments, CARV (0.1, 1, 10 and 100 µmol/l) dose-dependently decreased MPO release from isolated human neutrophils. Incubation of neutrophils with CARV (10 and 100 µmol/l) caused significant inhibition of 80.69 ± 6.00 % and 72.88 ± 9.30 % in MPO release induced by fMLP (Table 1). In the case of PMA stimulated human neutrophils we observed an inhibitory effect of CARV on MPO release in the concentration 1, 10 and 100 µmol/l, in concentration-dependent manner. In the concentration 100 µmol/l CARV significantly inhibited MPO release to 62.04 ± 4.84 % (Table 2).

Our results showing that CARV inhibited SO generation as well as MPO release both by membrane-operating stimulus (fMLP) and by or membrane bypassing activator

(PMA) lead to the conclusion that the inhibitory effect may be attributed to its non-specific action.

Inhibition of SO generation by CARV in stimulated human neutrophils is not only able to reduce the toxicity caused by SO itself, but also, even more importantly, to prevent the formation of higher reactive and toxic hydroxyl radical, and thus to protect against further damage induced by the oxygen-radical chain reactions.

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