Effect of carvedilol on the production of reactive oxygen species by HL-60 cells

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Abstract

OBJECTIVES: The generation of reactive oxygen species (ROS) by phagocytes is one of the irreplaceable microbicidal tools of innate immunity. It has been reported in our previous studies that short-term treatment by carvedilol ex vivo inhibits ROS generation. The purpose of this study was to investigate the long-term effect of carvedilol on phagocytes.

METHODS: Human leukemia HL-60 cells differentiated into granulocyte-like cells were used as the model. Final concentrations of carvedilol were 0.1–100 μmol/l. The production of ROS by HL-60 cells was measured using luminol-enhanced chemiluminescence (CL).

RESULTS: Carvedilol in concentrations 0.1–10 μmol/l did not exhibit any toxic effect on cells (measured using bioluminescent bacteria Photorhabdus luminescens subsp. thracensis). One hour's treatment with 10 μmol/l carvedilol significantly decreased both spontaneous and activated CL of cells. Conversely, no inhibitory effects on CL were observed in 10 μmol/l carvedilol after 48 h incubation; lower concentrations of carvedilol even slightly increased the CL activity of HL-60 cells. A significant increase in spontaneous CL activity was detected in cells incubated with 10 μmol/l carvedilol in comparison with the control. Powerful antioxidative properties of carvedilol against peroxyl radical (ORAC assay) were proved. No scavenging of nitric oxide (electrochemical method) was observed.

CONCLUSIONS: Long-term influence of carvedilol can induce an increase in the generation of phagocyte-derived ROS and potentially also other inflammatory mediators. The increased ROS production is compensated for by antioxidative properties of carvedilol although the increased production of inflammatory mediators could affect the proper function of immune system.

ORIGINAL ARTICLE

Abbreviations

ATP - adenosine triphosphate
CaI - calcium ionophore A23187
CL - chemiluminescence
ORAC - oxygen radical absorbance capacity
OZP - opsonized zymosan
PMA - phorbol-12-myristate-13-acetate
RLU - relative light units
ROS - reactive oxygen species

INTRODUCTION

The production of reactive oxygen species (ROS) by phagocytic cells is an essential microbicidal mechanisms. Microbial invaders are phagocytosed and destroyed by ROS inside the phagosome. However, excessive or inappropriate ROS production by phagocytes is associated with various diseases, including cardiovascular diseases, neurodegenerative diseases and many others. Therefore an application of drugs with antioxidative and/or antiinflammatory effects may be considered potentially beneficial.

Carvedilol is a β-adrenoceptor antagonist with vasodilating antihypertensive and antioxidative properties. Carvedilol protects endothelial, neuronal and vascular smooth muscle cells against the effect of ROS. The antioxidative effect of carvedilol is ascribed to the direct scavenging of free radicals, the uncoupling of iron ions and the inhibition of oxidative burst of neutrophils (Cheng *et al*., 2001). The effect of carvedilol on ROS generation by neutrophils in whole blood and isolated neutrophils has already been described in our previous studies (Drabikova *et al*., 2006; Nosal *et al*., 2005; Pecivova *et al*., 2006). However, only the short-term effect of carvedilol (usually 1 h incubation) was analysed in these *ex vivo* and *in vitro* studies. In the present study we describe the long-term effect of carvedilol on the proliferation and respiratory burst of HL-60 cell line differentiated into neutrophil-like cells. New results describing antioxidative properties of carvedilol against peroxyl radical and nitric oxide are the further contribution of the study.

MATERIALS AND METHODS

Materials

Carvedilol was supplied by Zentiva (Czech Republic); luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Molecular Probes (USA); all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

HL-60 cells

Human leukemia HL-60 cells (ECAC, UK).) were grown and differentiated into the phenotype of granulocyte-like cells using all-*trans* retinoic acid as described previously (Soucek *et al*., 2006). For our experiments, cells were seeded at an initial density of $6x10^4$ cells/well in 96-well light-impermeable plates (Nunc, Denmark). Then the cells were incubated with carvedilol (final concentrations 0.1, 1, 10 and 100 μmol/l) for 24h, 48h and 72h. In another setting, cells were incubated 48h and carvedilol was added just before the measurement of oxidative burst.

Carvedilol toxicity

Toxicity was tested using bioluminescent bacteria *Photorhabdus luminescens* subsp. *thracensis* CCM 7295^T. Bacteria were cultivated in medium DSMZ 423. Bright bacterial cells collected from the culture in stationary phase ($2.4*10^8$ cells) were transferred to Hanks balanced salt solution with carvedilol and bacterial bioluminescence was measured using luminometer LM-01T (Immunotech, Czech Republic). The inhibition of bacterial bioluminescence was evaluated 5 minutes after measuring began.

ATP content in HL-60 cells

The long-term toxic effect of carvedilol on HL-60 cell proliferation was determined using the measurement of ATP content in HL-60 cells by the commercial ATP cellular kit (Biothema, Sweden). Chemiluminescence (CL) was determinated using luminometer Orion II (Berthold Detection Systems GmbH, Germany).

ROS generation by cells

The oxidative burst of cells was measured directly in the plates using the luminol-enhanced CL (luminometer LM-01T, Immunotech, Czech Republic). The oxidative burst was initiated by the injection of the following activators: calcium ionophore A23187 (CaI) – final concentration 0.5 μmol/l, phorbol-12-myristate-13-acetate (PMA) – 0.01 μmol/l or opsonized zymosan (OZP) – 0.1 g/l. Spontaneous CL (without activator) was also monitored. The mixtures were completed by luminol – 1 mmol/l. CL emission was measured during 1 h and integral values of of relative light units (RLU) were calculated.

Fluorimetric ORAC assay

Oxygen radical absorbance capacity (ORAC) measures the antioxidant scavenging activity against the peroxyl radical induced by 2,2'-azobis(2-amidinopropane) dihydrochloride at 37 °C. Fluorescein is used as a fluorescent probe. The loss of fluorescence is an indication of its reaction with the peroxyl radical. The protective effect of carvedilol is measured by assessing the area under the fluorescence decay curve as compared to that of a blank.

Scavenging properties of carvedilol against NO

Electrochemical analysis of NO concentration was used. A porphyrinic microelectrode was connected to the ISO-NO MARK II potentiostat (WPI, USA). The injection of the NO-saturated water into the glass vial (final concentration of NO=595 nM) caused the rapid increase with a subsequent gradual decrease of an

NO-induced signal until it reached the background current (for details see Hrbac *et al*., 2007). The scavenging property of the tested antioxidant is represented as a very rapid decrease in the NO-induced signal.

Statistical evaluation

Data are expressed as the mean ± standard error of the mean of 3 independent experiments. Results were analysed by Student's t-test using Statistica software (StatSoft, USA), *p* values below 0.05 (*) were considered statistically significant.

RESULTS

Toxicity of carvedilol

Carvedilol in 100 μmol/l concentration was found to inhibit the bioluminescence of *P. luminescens* to 58% of the control values after 5 min of incubation. No significant changes were detected after incubation with lower carvedilol concentrations.

The effect of carvedilol on HL-60 cell proliferation

The level of ATP in wells containing 100 μmol/l carvedilol was under the detection level after 24, 48 and 72 h incubation indicating the absence of viable cells. The proliferation of HL-60 cells was silenced after 48 h incubation with 10 μmol/l carvedilol (57% of control value). This ATP level corresponded to the ATP level of control cells without carvedilol after 24 h incubation. No significant effects on cell proliferation were observed in lower concentrations of carvedilol (data not shown).

Effect of carvedilol on oxidative burst of HL-60 cells

The production of ROS by HL-60 cells was tested 24 h, 48 h and 72 h after the addition of all-*trans* retinoic acid (the inductor of cell differentiation). While very low CL activity was observed after a 24 h period, the high and reliable CL signal was detected after 48 and 72 h incubation (data not shown). On the basis of these results, a 48 h incubation period was chosen for the next experiments.

Figure 1 demonstrates the dose-dependent effect of carvedilol on ROS production by activated HL-60 cells after 48 h incubation with all-*trans* retinoic acid; carvedilol was added just before the measurement of the oxidative burst. Carvedilol in 100 μmol/l concentration diminished CL induced with all activators used; 10 μmol/l carvedilol significantly decreased CL induced with A23187, and OZP. Spontaneous CL (without an activator) was also decreased after the treatment with both 100 μmol/l and 10 μmol/l carvedilol. Lower concentrations of carvedilol have no effect on the ROS generation.

CL of activated HL-60 cells after 48 h incubation with carvedilol is shown in Figure 2. 100 μmol/l carvedilol diminished CL induced with all activators used. In contrast with short-term incubation, no inhibitory effects were observed in 10 μmol/l carvedilol; lower concentrations of carvedilol even slightly increased the CL activity of HL-60 cells. Interestingly, significant increase in spontaneous CL activity was detected also in cells incubated with 10 μmol/l carvedilol in comparison with the control (1545 ± 48 RLU and 1029 ± 152 RLU, resp.).

Antioxidative properties of carvedilol

ORAC assay demonstrated very powerful antioxidative properties of carvedilol (see Figure 3). Carvedilol significantly prevented the loss of fluorescence starting from 1 μmol/l concentration. The scavenging of nitric oxide, which can also contribute to the CL signal, was not observed (data not shown).

DISCUSSION

Phagocytes produce highly reactive oxygen metabolites known to participate in the most efficient microbicidal mechanisms. Phagocytes thus represent the front-line defence cells in protecting the organism against infec-

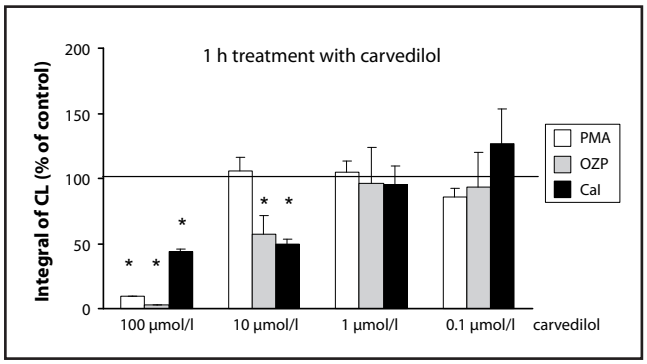

Figure 1. Short-term effect of carvedilol on the chemiluminescence (CL) of HL-60 cells. Cells were cultivated for 48 h in medium, then activators (OZP, PMA, Cal) and carvedilol were added and CL was measured continuously for 1 h. The asterisk (*) marks a significant difference ($p<0.05$) when compared with control cells.

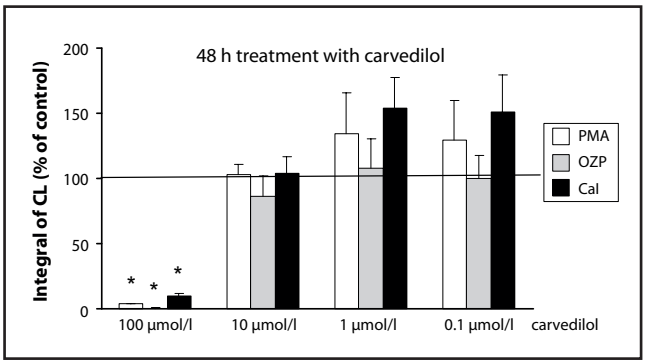

Figure 2. Long-term effect of carvedilol on the chemiluminescence (CL) of HL-60 cells. Cells were cultivated for 48 h in medium containing carvedilol, then activators (OZP, PMA, Cal) were added and CL was measured continuously for 1 h. The asterisk (*) marks a significant difference ($p<0.05$) when compared with control cells.

tion and play an irreplaceable role in the proper performance of the immune system. On the other hand, excessive ROS production can also damage the body`s own cells and tissues and contribute to the development of a number of serious diseases (Lojek *et al*., 2002). All these reasons make the analysis of the metabolic activity of phagocytes important in clinical practice as well as in the development and testing of pharmaceuticals and in biomedical research (Jancinova *et al*., 2006; Nosal *et al*., 2006). Among the several existing methods, recording the phagocyte CL response, first reported by (Allen *et al*., 1972), is the most convenient method to measure the cells' oxidative burst.

The results presented showed the dose-dependent effect of carvedilol on CL of neutrophil-like HL-60 cells evoked by receptor-mediated stimulus (OZP) and two receptor-bypassing stimuli (CaI, PMA). Carvedilol, a potent scavenger, may act at two sites. First, due to carbazole moiety in the drug molecule carvedilol acts predominantly extracellularly (Feuerstein *et al*., 1997). Second, being highly liposoluble, carvedilol is distributed in cells and tissues (Varin *et al*., 1986). Very efficient antioxidative properties of carvedilol against peroxyl radical were proved in our study using the fluorimetric method. It corresponds with the previous results where the scavenging of hydrogen peroxide, superoxide anion and hydroxyl radical by 10 μmol/l carvedilol was described. CL activity of neutrophils (and also HL-60 cells) also depends on the activity of myeloperoxidase (Lilius and Nuutila 2006). According to our previous results, carvedilol decreases myeloperoxidase release and activity (Nosal *et al*., 2005; Pecivova *et al*., 2006). On the other hand we showed that carvedilol does not scavenge nitric oxide which also contributes to the total CL activity of cells.

The short-term incubation of cells with 10 μmol/l carvedilol (nontoxic concentration) significantly decreased CL induced with CaI (receptor-bypassing stimulus) and OZP (receptor-operated stimulus). This indicates that carvedilol interferes with pathways leading to the production of inflammatory mediators or reacts with already generated free oxygen radicals rather than with specific receptors for OZP localised at a plasma membrane (Nosal *et al*., 2005).

In contrast with short-term incubation, no inhibitory effects were observed in cells treated with 10 μmol/l carvedilol for 48 h. Lower concentrations of carvedilol even slightly increased the CL activity of HL-60 cells. According to the measurement of the ATP level, the proliferation of cells was silenced by 10 μmol/l carvedilol and only 57% of cells were presented in the reaction mixture in comparison with untreated control after 48 h incubation. It means in fact, that the generation of ROS by these cells was about 30–40% higher in comparison with controls. This difference is even more pronounced when one takes account of the antioxidative properties of carvedilol and its inhibitory effect on myeloperoxidase activity. Our results correspond with the study of (Yang *et al*., 2004). These authors concluded that carvedilol might mediate its therapeutic effects through differentially regulating cytokine production (e.g. upregulated IL-12, downregulated IFN-γ) from activated mononuclear cells.

The stimulatory effect of carvedilol observed in our study is very important especially as it applies to spontaneous ROS generation. It can be suggested that the long-term influence of 10 μmol/l carvedilol itself can induce an increased generation of phagocyted-derived ROS, and potentially other inflammatory mediators as well. The increased production of ROS is compensated for by antioxidative properties of carvedilol but the increased production of inflammatory mediators could affect the proper functioning of the immune system.

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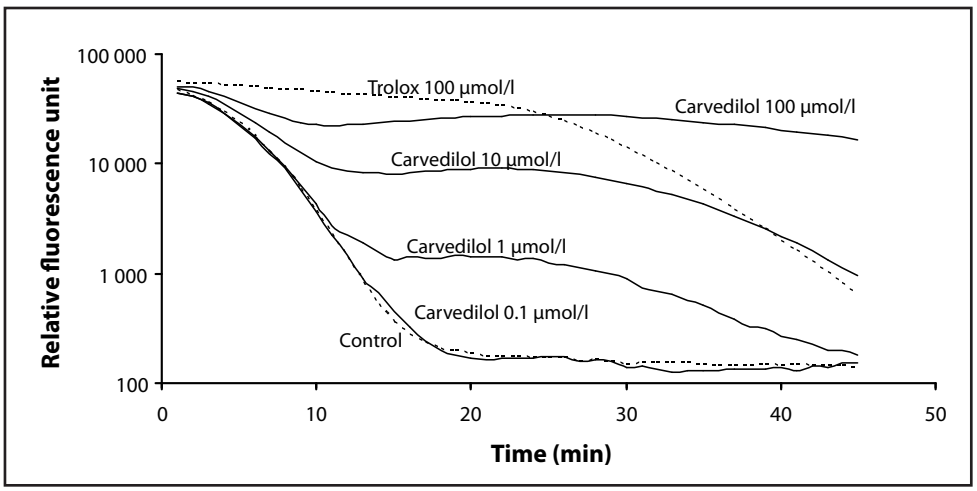

Figure 3. Antioxidant scavenging activity of carvedilol against peroxyl radical measured using ORAC assay

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