INTRODUCTION

Monocytes, macrophages and granulocytes, often called professional phagocytes, are the part of innate immune system which plays a very important role in the initial response to infection. Their activation leads to an inducible nitric oxide synthase (iNOS) expression, myeloperoxidase release and reactive oxygen and nitrogen species generation (1, 2). Recent evidence suggests that the nitric oxide (NO) is one of the basic biological mediators which play essential bioregulatory roles in a wide range of processes critical to normal functions in the cardiovascular, nervous, and immune systems (3-7). Accordingly, factors which influence rates of NO generation by nitric oxide synthases are of significant interest.

The sympathetic modulation of the immune system which originates from the adrenergic signaling plays an important role in the down-regulation of inflammatory responses (8-10). Noradrenaline and adrenaline are able to directly suppress inflammation through activation of adrenergic receptors.
modulate immune responses via the adrenergic receptors expressed on immunologically active cells including neutrophils, lymphocytes, monocytes and macrophages (8-11).

The most common way of studying an immunomodulatory effect of adrenergic agonists or antagonists is to apply these drugs together with a known stimulator of immunologically active cells such as lipopolysaccharide (LPS). As demonstrated previously, the incubation of β-adrenergic agonists with immune cells results in the suppression of the LPS-evoked pro-inflamatory cytokine and NO production (8-11). Although, the exact mechanism is not yet fully understood many studies are focused on the immunomodulatory effect of the adrenergic agonists and antagonists which are widely used in the treatment of different diseases (12-16). This is also the case of carvedilol which is a combined β1-, β2-, and α1-adrenergic receptor's blocking agent mostly used in the treatment of arterial hypertension, cardiac arrhythmias and angina pectoris (17, 18).

The therapeutic effect of carvedilol is explained by its ability to block the β-adrenergic receptors and inhibit the binding of noradrenaline and adrenaline to adrenergic receptors. It is supposed that the part of the beneficial effect of carvedilol is associated with its antioxidant and anti-peroxidative properties. Moreover, it was also shown that carvedilol acts as a metal scavenger and can also protect mitochondria against the oxidative damage (19-21). However, there exists incomplete and contradictory information about the role of carvedilol in the regulation of immune cell functions, especially of the production of reactive nitrogen species by phagocytes (22, 23).

Therefore, the purpose of the present study was to provide new data about the involvement of carvedilol in the regulation of LPS-evoked NO production from macrophages. Another aim of this study was to describe the possible relations between the effects of carvedilol and endogenous adrenergic agonists (adrenaline, noradrenaline, and dopamine) and/or antagonists (prazosine, atenolol) on NO production and to find whether their potential influence can be mediated via the adrenergic receptors.

MATERIAL AND METHODS

Cell culture

Murine leukaemic macrophage cell line RAW 264.7 (ATCC, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% gentamycin (Sigma, USA). After reaching confluence, the cells were harvested and washed. Cell numbers and viability were determined by ATP test.

Experimental procedure

Three groups of chemical substances were tested: I) carvedilol (Zentiva, Czech Republic) at final concentrations of 1, 5, 10 and 25 µM (these concentrations are considered as optimal in various experimental models) (17, 19, 20) II) Adrenergic receptors agonists: adrenaline, noradrenaline and dopamine (Sigma, St. Louis, MO, USA) at a final concentration of 5 µM and III) ARs antagonists: prazosine and atenolol (AstraZeneca, England) at a final concentration of 25 µM.

The following experimental design was used: Aliquots of 1 ml of DMEM containing 2 x 10^6 cells were placed into 6-well plates and then cultured in an incubator (5% CO2 at 37°C) for 3 h. Then the tested compounds were added to wells. After 30 min of preincubation the cells were stimulated with 5 ng/ml of LPS (LPS from Escherichia coli serotype 0111:B4, Sigma, USA) and incubated for the next 24 h. Cells without LPS stimulation served as a negative control. After 24 h supernatants were harvested and the nitrite accumulation was determined. The cells were lysed and Western blot analysis of iNOS was performed.

Determination of nitrates

Supernatants (150 µl) were mixed with Griess reagent (Sigma, USA) in the ratio of 1:1 and incubated at room temperature for 15 min. The absorbance was measured at 550 nm using a microplate reader SPECTRA Rainbow (TECAN, Austria). Sodium nitrite was used as a standard. The detection limit of the assay was 0.2 µM NO2-.

Western blot analysis of iNOS

The cells were lysed on ice by 150 µl of lysis buffer. The protein concentrations in the cells lysates were determined using the detergent-compatible protein assay reagent (Bio-Rad Laboratories, USA) with bovine serum albumin (BSA) as a standard. The same amount of protein (50 µg) from each lysate was then subjected to SDS-polyacrylamide gel electrophoresis using 7.5% running gel described previously (24). After electrophoresis the proteins were transferred to immobilon polyvinylidene difluoride (Millipore, USA) membrane and then incubated with 5 % fat free milk in TRIS buffer-Tween 20 (TBS-T) at room temperature for 1 h, with a mouse iNOS-specific antibody (1/3000) (Anti-
iNOS/NOS Type II mAb, BIO-RAD, USA) for 24 h, and with horseradish peroxidase-labelled anti-mouse IgG antibody (1/2000) (ECL Anti-mouse IgG, Biosciences, USA) for 1 h. After each incubation, the membrane was washed three times with TBS-T for 15 min. The immunoreactive band was detected using an ECL detection reagent kit (BIO-RAD, USA) and exposed to radiographic film (AGFA, Belgium). Relative levels of proteins were quantified by scanning densitometry using the ImageJ™ program and the individual band density value was expressed in arbitrary units. The equal loading of proteins was verified by β-actin immunoblotting.

Detection of scavenging properties of drugs against NO

The potential ability of drugs to scavenge NO in chemical systems was tested by the electrochemical measurement of NO. This chemical system consisted of 10 ml of tested compounds which were permanently mixed during experiments. Temperature was kept at 37°C. Tested drugs were diluted in DMEM with 10% FBS to reach final concentrations 1, 5, 10 and 25 µM. DMEM with 10% FBS was used as a negative control and 10 µM quercetin (Sigma, St. Louis, MO, USA) which is a wine polyphenol with strong scavenging ability against NO in chemical system (24) was used as a positive control. The concentration of NO was measured electrochemically using three electrode system as described by Hrbac et al. (25). A porphyrinic microsensor working electrode, platinum wire counter electrode and a miniature saturated silver/silver chloride reference electrode were connected to the ISO-NO MARK II potentiostat (WPI, USA). According to the WPI manual, distilled water was saturated with NO (concentration after saturation 1.19 mM). The injection of the 5 µl NO-saturated water into the glass vial (final concentration of NO = 595 nM) caused the rapid increase (peak time = 15 ± 5 s) with a subsequent gradual decrease in NO induced signal until it reached the background current. In our experiments, the electrochemical signal was followed up for 250 s to obtain kinetic curves. The integral area under the resulting curve corresponded to the total amount of NO present in the glass vial and was used for the evaluation of scavenging properties of tested drugs.

ATP test of cell viability

The viability of cells was tested using the commercial ATP cellular kit (Biothema, Sweden) intended for the enumeration of viable cells by the quantification of cellular ATP. At the end of the incubation, the macrophages were lysed with 100 µl of somatic cell ATP releasing reagent (Sigma, USA) for 1 min. Then the ATP Reagent SL containing D-luciferin, luciferase and stabilizers were added to wells. The chemiluminescence measured with luminometer LM-01T (Imunotech, Czech Republic) showed that ATP concentration had not significantly changed in any of the experimental groups in comparison with the control cells. This indicates that none of the studied drugs was toxic for RAW 264.7 in applied concentrations (data not shown).

Data evaluation

The data were statistically analyzed by Student's two-tailed t test and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test using Statistica software (StatSoft, USA). All data are reported as means ± SD. A p value of less than 0.01 was considered significant.

RESULTS

Effect of carvedilol on NO production by macrophages stimulated by LPS

Carvedilol significantly decreased nitrite level in cell supernatants after 24 h with LPS. This effect was dependent on carvedilol concentration, the highest concentration of carvedilol (25 µM) reduced the nitrite accumulation by 30 % compared to the positive control value (see Fig. 1 - panel A).

The possibility that the decrease in nitrite concentration can be caused by scavenging of NO by carvedilol was tested using an electrochemical method. Time course of changes in current induced by injection of NO into the DMEM or DMEM with selected concentration of carvedilol is shown in Fig. 2. It is obvious that kinetics of signal was not influenced significantly by any of the carvedilol concentrations used. The absence of scavenging properties of carvedilol against NO was also verified by the calculation of integral area under these curves where the values for control, 1 µM, 5 µM, 10 µM and 25 µM carvedilol were 131 ± 14, 134 ± 13, 140 ± 2, 117 ± 13, 108 ± 10 pA*s*1000, respectively, without any significant difference. The ability of electrochemical system to detect NO scavenging properties of tested compounds was proved by using 10 µM quercetin. As it was expected quercetin caused a rapid decrease in NO induced signal (value of integral area for quercetin was 8 ± 1 pA*s*1000).

The assumption that carvedilol reduces nitrite accumulation via the suppressive effect on iNOS
Fig. 1. Effect of carvedilol on LPS-stimulated NO production by RAW 264.7 cells. Carvedilol was injected into cell suspension 30 min before the LPS treatment and subsequent 24 h incubation. Panel A: Nitrite accumulation in cell supernatants. Data are expressed as mean ± S.D. of eight separated measurements performed in duplicates. *** indicates significance at level p < 0.001 when compared with positive control. Panel B: iNOS protein expression in the cell lysates. An equal loading of proteins was verified by β-actin immunoblotting. Data are expressed as mean ± S.D. of five separated measurements. ** indicates significance at level p < 0.01 when compared with positive control.

Fig. 2. Time course of changes in NO concentration induced by injection of distilled water saturated by NO into the DMEM + 10% FBS (control sample) and DMEM + 10% FBS with different concentrations of carvedilol. Quercetin in DMEM + 10% FBS was used as a positive control (inserted figure). Each curve is constructed from 50 points, each point representing the mean value from three independent experiments.

Fig. 3. The combined effect of carvedilol and adrenergic blockers on LPS-stimulated NO production. Atenolol and prazosine were injected 30 min before the carvedilol and 60 min before the LPS treatment and subsequent 24 h incubation. Panel A: Nitrite accumulation in cell supernatants. Data are expressed as mean ± S.D. of eight separated measurements performed in duplicates. *** indicates significance at level p < 0.001 when compared with positive control. Panel B: iNOS protein expression in the cell lysates. Equal loading of proteins was verified by β-actin immunoblotting. Data are expressed as mean ± S.D. of five separated measurements. ** indicates significance at level p < 0.01 when compared with positive control.
Protein expression was tested in further experiments using Western blot analysis. iNOS protein expression was inhibited by carvedilol in all of the concentrations used (Fig. 1 - panel B).

**Combined effect of carvedilol and adrenergic blockers on NO production by macrophages stimulated by LPS**

Prazosine (the selective blocker of α1-adrenergic receptors) and atenolol (blocker of β-adrenergic receptors) were used to investigate whether the suppressive effect of carvedilol on iNOS expression and subsequent NO production was caused by the binding of carvedilol to adrenergic receptors. Both prazosine and atenolol (25 µM), in the individual application, had no scavenging properties against NO and did not affect the nitrite accumulation and iNOS protein expression after LPS stimulation (data not shown). After the evidence that prazosine and atenolol are suitable blockers for our experiments, these adrenergic antagonists were added to cell suspension 30 min prior to carvedilol administration and LPS-stimulation. As shown in Fig. 3 the blockade of adrenergic receptors with adrenergic antagonists abolished the inhibitory effect of 5, and 10 µM carvedilol on iNOS protein expression and nitrite accumulation while the inhibitory effect of 25 µM carvedilol remained unchanged. It can be concluded that carvedilol in concentrations 10 µM and lower inhibited iNOS protein expression and NO production via binding to adrenergic receptors. Henceforward 10 µM carvedilol was chosen for further experiments to prevent non-specific (non-receptor mediated) effect of 25 µM carvedilol.

**Effect of carvedilol on NO production: comparison with adrenergic agonists**

To investigate whether the suppressive effect of carvedilol can be caused by its ability to interact with the adrenergic receptors, its effect on macrophage NO production was compared with adrenergic agonists - adrenalin, noradrenaline and dopamine. The administration of 5 µM adrenergic agonists (concentration chosen according to literature data (26)) 30 min prior to LPS injection caused the reduction of nitrite concentration (see Fig. 4). In combination with carvedilol (10 µM) dopamine was the most effective blocker of iNOS protein expression. None of the agonists can scavenge NO according to our electrochemical measurements (data not shown). As demonstrated for carvedilol, also adrenalin, noradrenaline and dopamine caused...
the decrease of NO production and iNOS protein expression in LPS-treated macrophages (Fig. 5).

When the cells were treated with prazosine and atenolol 30 min before agonist-treatment and 60 min before LPS-stimulation, the inhibitory effect of adrenergic agonists on nitrite accumulation and iNOS expression was completely abolished confirming the involvement of adrenergic receptors (data not shown).

There was no additive effect of carvedilol and adrenaline or noradrenaline on determined parameters. However, the significant inhibition of nitrite accumulation and iNOS expression was detected when the combination of carvedilol with dopamine was used (Fig. 4, 5).

**DISCUSSION**

In this study, the effect of carvedilol on LPS-treated RAW 264.7 cells was investigated. It was demonstrated that carvedilol inhibits LPS-induced NO production and iNOS protein expression in macrophages and that these effects are mediated, at least in part, by the interaction of carvedilol with adrenergic receptors.

Carvedilol is a highly lipophilic drug and confers unique therapeutic advantages in comparison to classic β-blockers by antioxidant activity which synergizes with its non-selective β- and α-blocking activities (17, 27). We have only partial information about the effect of carvedilol on immune cells, and the mechanism of its antioxidant, antiproliferative and anti-inflammatory ability is still not well understood (17-21). It was recently shown that carvedilol might differentially regulate cytokine production from activated mononuclear cells (28). Carvedilol is able to inhibit reactive oxygen species generation by leukocytes (2). It decreases phagocyte degranulation and the amount of free myeloperoxidase (1), which at the sites of inflammation may function as a catalytic sink for NO (30). With respect to these facts, we supposed that the pathways which led into the NO production from macrophages might be another target of carvedilol and we suggested that this can be one of the mechanisms responsible for its anti-inflammatory action.

One of the most important findings of this study is that carvedilol in the concentration dependent manner (1-25 µM) inhibits NO production from LPS-stimulated macrophages. Since carvedilol is described as a potent antioxidant and a free radical scavenger in recent studies (19, 22), one of the possible explanations for its inhibitory effect on the total amount of NO produced by stimulated macrophages could be its scavenging activity against NO. Nevertheless, none of the carvedilol concentrations was able to scavenge NO according to our electrochemical measurements. Therefore, we conclude that the decrease of NO production in LPS-stimulated cells must have been caused by another mechanism.

As a further step towards characterizing the pathway for the inhibition of NO production by carvedilol, we showed for the first time that prazosine and atenolol, the α- and β-adrenergic antagonists, were able to block the inhibitory effect of carvedilol in concentrations 1, 5 and 10 µM, while the inhibitory effect of 25 µM carvedilol remained unchanged. From this data we can supposed that the effect of carvedilol in the concentration 10 µM and lower could be partially mediated by interfering with adrenergic receptors.

A further important finding of this paper is that noradrenaline and dopamine analogous to adrenaline, as already documented by Sigola and Zinyama (9), significantly decreased LPS-stimulated NO production in macrophages via their binding to the adrenergic receptors. Our findings accord well with recently published studies which showed that α- and β-adrenergic agonists have the immunosuppressive ability against the inflammatory mediators, such as TNF-α (9, 11-15). It has been recognized that these anti-inflammatory characteristics are related to the activation of β-adrenergic receptors and to the generation of cAMP by these agents (11, 31-34). Moreover, it was found by several authors (9, 11-15) that α- and β-adrenergic agonists can indirectly down-regulate the activation of the transcription factor, NF-kappaB (which is up-regulated by LPS), which can consequently result in the reduction of the production of monocyte- and macrophage-derived mediators.

In comparison with adrenergic agonists, the effect of carvedilol, an adrenergic antagonist, on stimulated phagocytes seems to be more complex. Carvedilol is capable of binding to the β-receptor’s blocking site and effectively prevent their activation by agonists (17). However, in the light of our results with prazosine and atenolol, our data suggests that carvedilol in concentrations of 1, 5 and 10 µM mediate its effect on LPS-treated macrophages via the modulation of the adrenergic receptors activity. We assume that carvedilol, after it binds to the β-adrenergic receptors blocking site, is able to induce the partial activation of β-adrenergic receptors which are presented on the surface of macrophages (35) or to affect other mechanisms which are associated with the regulation of iNOS protein expression and activity. However, other explanations exist which can be connected with its unique properties such as the ability to decrease phagocyte degranulation (1), reduce the level of lipid
peroxidation (36) and reduce total cellular oxidative stress in congestive heart failure (37).

Our experiments further showed the absence of the additive effects of carvedilol with adrenaline or noradrenaline. On the other hand, dopamine in combination with carvedilol caused the pronounced inhibition of iNOS protein expression and NO production. This was probably caused by the fact that dopamine can interact with both dopamine and adrenergic receptors which are present on the surface of the macrophages (38) and trigger the signal transduction via the activation of both receptors. But because the adrenergic antagonists, prazosine and atenolol were able to prevent the dopamine-induced suppression of NO production, we suggest that dopamine mediates its suppressive action on NO production by RAW 264.7 through the activation of adrenergic receptors. Our results with dopamine accord well with recent studies which discovered that dopamine suppresses the production of several proinflammatory cytokines (IL-12, TNF-α, IL-6) via the β-adrenergic receptor mediated mechanism (16, 39).

In conclusion, we demonstrated that adrenergic antagonist carvedilol is able to suppress the production of NO in LPS-stimulated macrophages analogically to adrenaline, noradrenaline and dopamine. Further, we demonstrated that carvedilol and adrenergic agonists participate in the regulation of the NO production in response to LPS via the adrenergic receptors. We suggest that this can be one of the mechanisms responsible for the anti-inflammatory action of carvedilol. In addition, our data demonstrates that not only the adrenergic agonists but also some of the adrenergic antagonists may play an important role in the regulation of inflammatory responses in stimulated macrophages.

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