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No Effect of Carvedilol on Nitric Oxide Generation in Phagocytes but Modulation of Production of Superoxide Ions

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ABSTRACT. Since carvedilol has been claimed to possess antioxidative effects, this drug might affect functional responses, including nitric oxide (NO) generation, of polymorphonuclear neutrophils (PMN) and macrophages. When we assessed the effects of carvedilol on PMN responses *in vitro*, we observed that carvedilol dose dependently modulated generation of superoxide ions by NADPH oxidase when induced by the formylpeptide formyl-methionyl-leucyl-phenylalanine (fMLP) or the phorbol ester phorbol myristate acetate. This effect was not coupled to diminished phospholipase C activity. In contrast to the effect on NADPH oxidase, neither the fMLP-elicited NO generation by PMN nor the response of the murine macrophage cell line J774 to lipopolysaccharide was affected. There was no evidence from cell-free assay systems that carvedilol is a scavenger for superoxide ions or NO. Moreover, carvedilol did not affect other reactions dependent on NO, e.g. spontaneous or fMLP-stimulated PMN migration or lipoxin A_4-, fMLP-, or A23187-induced neutrophil cytotoxicity for human umbilical vein endothelial cells. Thus, these effects point to the possibility that carvedilol modulates the NADPH oxidase of PMN but leaves the nitric oxide synthase of phagocytes intact. BIOCHEM PHARMACOL **59**;8:1007–1013, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. neutrophils; nitric oxide; superoxide ions; chemotaxis; endothelial cells; carvedilol

Carvedilol is a vasodilating antihypertensive drug that selectively blocks α1-receptors and non selectively antagonizes β1- and β2-adrenoreceptors. Moreover, carvedilol and some of its hydroxylated metabolites were suggested to possess antioxidant properties and have a potential for myocardial and vascular protection [1–3]. Recently, carvedilol was reported to reduce the risk of death as well as the risk of hospitalization for cardiovascular causes in patients with heart failure [4, 5]. Consequently, interest has focused on mechanisms responsible for these beneficial clinical effects. Previous studies assessing the *in vitro* effects of carvedilol observed that the drug uniquely inhibited the generation of superoxide ions from PMN† with an apparent IC_{50} of 25–28 μM [6], as well as preventing peroxidation of low-density lipoprotein [7]. When endothelial cells were treated *in vitro* with carvedilol, they resisted injury induced by oxidants from xanthine–xanthine oxidase and by phorbol ester-stimulated PMN [8]. Finally, indirect evidence was presented that carvedilol may have an effect on chemically generated NO due to diminished superoxide ion generation [9].

The aim of this study was to assess, using highly sensitive *in vitro* assay systems [10–12], whether carvedilol affects the generation of NO in phagocytes in addition to being an inhibitor of superoxide ion production. Moreover, we sought to determine if an effect on NO generation translated into a well-characterized toxic effect of NO, namely the cytotoxic effect of neutrophils on endothelial cells [12–14]. We also assessed if the chemotaxis of neutrophils was modulated, since NO (as well as superoxide ions) may influence this response [15–19]. Finally, we analyzed a step in the response coupling for G-protein- and surface receptor-dependent agonists, namely phospholipase C-induced $[Ca^{2+}]_i$ transients, since a previous report suggested that carvedilol might influence such transients [1].

MATERIAL AND METHODS

Chemicals

Interleukin-1β and SOD were from Boehringer Mannheim GmBH. Endothelial growth factor was from Collaborative Research Inc. FBS, HBSS, and other tissue culture media and chemicals were from GIBCO. PBS and formaldehyde

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† *Abbreviations:* FBS, fetal bovine serum; fMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; HbO_2, oxyhemoglobin; HUVEC, human umbilical vein endothelial cells; L-NMMA, N^{122}-monomethyl-L-arginine; LXA_4, lipoxin A_4; NO, nitric oxide; NOS, nitric oxide synthase; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil granulocytes; SNAP, S-nitroso-*N*-acetyl-penicillamine; SOD, superoxide dismutase; and $[Ca^{2+}]$; intracellular free calcium concentration.

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were from Apoteksbolaget. Ninety-six-well microtiter plates (Immunolon™) were from Dynatech Laboratories, tissue culture plasticware was from Nunc, Percoll and Sephadex G25 from Pharmacia Fine Chemicals, and L-NMMA, SNAP, and fura-2/AM were from Calbiochem. All other chemicals were obtained from Sigma Chemical Co. Carvedilol was a gift from Dr. Ludvig Trang, Smith-Kline Beecham (Stockholm, Sweden); it was dissolved in ethanol just prior to use. Based on previous experiments on PMN superoxide ion production [6], we chose to treat cells for 10 min prior to stimulation.

Culture of HUVEC

Endothelial cells were obtained from human umbilical veins by treatment with 0.2% collagenase as described [12–14, 20]. When new monolayers were established after a maximum of 2 passages in culture wells, they were used. Monolayers were identified as homogenous HUVEC by using an indirect immunofluorescence staining for factor VIII-related antigen [12, 20, 21].

Neutrophil Preparations

PMN were obtained from healthy donors by a one-step discontinuous Percoll gradient centrifugation as described [12–14]. The purified neutrophils (>95% purity and viability) were resuspended in HBSS with or without 0.4% human serum albumin (as indicated).

J774 Murine Macrophages

J774 murine macrophages (a gift from Wellcome Research Laboratories, Beckenham, U.K.) were maintained in RMPI-1640 supplemented with 20% FBS, 100 units/mL of penicillin, 100 mg/mL of streptomycin, HEPES, sodium pyruvate, and non-essential amino acids. The cells were reseeded in fresh medium twice weekly. For experiments, 2×10^6 cells/well were washed twice in HBSS with 10% FBS (HBSS–FBS) at 37° and seeded in polystyrene 12-well culture plates. They were allowed to adhere for 30 min and non-adherent cells were removed by aspiration and washing with HBSS–FBS. Then, HBSS–FBS and 300 μM L-arginine or L-NMMA (as indicated) were added to yield 1 mL/well. The plates were inspected in an inverted microscope to confirm a confluent layer of adherent J744. Viability was confirmed by trypan blue exclusion on parallel samples treated in a similar manner.

In Vitro *PMN and Macrophage Assays*

The assays were chosen to reflect the various functional responses of PMN. We used stimuli that activate PMN by means of discrete surface receptors (the tripeptide fMLP and the lipoxygenase product LXA_4), by cytosolic receptors (the phorbol ester PMA), or via the activation of calcium membrane channels (the calcium ionophore A23187).

CYTOCHROME C REDUCTION. This was assessed as described previously [11, 22]. In brief, 1.25×10^9 PMN/L were suspended in HBSS. After treatment with carvedilol (or solvent) for 10 min, fMLP (at 0.1 or 1 μM) or PMA (at 1 μM) was added. Samples with and without SOD were always run simultaneously in a Perkin Elmer spectrophotometer at 37° until a plateau phase was reached. Cell-free O_2^- formation was assessed in an identical manner, but cells and stimuli were replaced with 10 μM hypoxanthine to which 0.02 U xanthine oxidase was added. The amount of SOD-inhibitable cytochrome *c* reduction was used as a measure of superoxide ion production using the coefficient 21.1 mM^{-1} cm^{-1}.

NO GENERATION OF HUMAN PMN. This was assessed by reduction of HbO_2 to methemoglobin, essentially as previously described [10, 11, 23]. Briefly, PMN in HBSS were treated with carvedilol or vehicle for 10 min at 37°, after which the cell suspension was incubated for another 30 min with either 100 μM L-arginine or 100 μM L-NMMA. SOD (30 μg/mL) and catalase (15 μg/mL) were then added. HbO_2, prepared as described [10, 11], stimuli, or corresponding volumes of HBSS were added immediately prior to analysis. The methemoglobin generation was followed spectrophotometrically at 401 versus 411 nm for 30 min at 37°, as indicated. All samples were assessed as the difference between samples with L-arginine and identical samples with L-NMMA instead of L-arginine. Thus, only the L-NMMA-inhibitable response was assessed and taken to represent the net amount of NO released from PMN. Cell-free NO formation was assessed by the same method, exchanging cells and stimuli, as well as the arginines, with SNAP (100 μM). The assay was run for 20 min when SNAP was used. An extinction coefficient of $\epsilon = 19.7$ mM^{-1} cm^{-1} was used to calculate the amount of NO produced [24].

NO GENERATION FROM CULTURED J744 MURINE MACROPHAGES. This was assessed colorimetrically with the Griess reagent [25]. Immediately after preparation of the adherent cells in the wells, carvedilol at 1, 5, 25, or 50 μM, or a corresponding volume of HBSS was added. Plates with adherent cells were placed in the incubator for 30 min. Plates without seeded cells but otherwise with corresponding medium and treatment were run in parallel for use as reference samples in the nitrite assay. Subsequently, lipopolysaccharide (1 μg/mL) was added and the plates were placed in the incubator for 19 hr (a duration selected after preliminary experiments over 4 to 48 hr and shown to give a stable and reproducible nitrite production). After this incubation period the medium was removed. Fresh HBSS–FBS was added to the wells and viability assessed by trypan blue exclusion. Assay of nitrite was performed on supernatants of the aspirated medium or reference samples (after centrifugation at $3000 \times g$ for 10 min at 4°) spectrophotometrically using Griess reagent at 550 nm.

^{51}Cr release cytotoxicity assay. The assay was performed as described previously [12–14, 26]. In brief, ^{51}Cr-labeled monolayers were covered with 1.25×10^6 PMN in HBSS with 1% FBS and after 30 min by the stimuli, i.e. A23187, fMLP, or LXA_4. We substituted PMA, used as stimulus in other experiments, with A23187 in the experiments described here, since the latter stimulus, which also acts via non-surface receptor pathways, gives a very reproducible and prominent cytotoxicity. Carvedilol was added together with the PMN or preincubated with the HUVEC monolayers for 30 min before PMN were added. After 4 hr of incubation at 37° supernatants were aspirated and centrifuged to pellet any HUVEC that may have detached but not lysed. HUVEC monolayers were then lysed. The radioactivity of supernatants, pellet fractions, or the lysed HUVEC was counted. Injury to the HUVEC was expressed as percent specific ^{51}Cr release [cf. 12–14, 26]. Less than 5% of the total radioactivity lost in the media was found in the pelleted fractions in almost all our assays, indicating that only minimal cell detachment occurred during cell incubation. The technical aspects of the cytotoxicity assay (e.g. incubation periods, temperatures, FBS concentrations, intra-assay variations, etc.) have been described [12–14, 26].

Chemotaxis. This was assessed with a modified Boyden chamber technique [27]. Cells, in HBSS supplemented with 0.4% human serum albumin, were allowed to migrate into 3-μm pore cellulose nitrate filters for 45 min at 37°. HBSS was used as control for spontaneous migration. Migration was assessed as the distance to leading front cells. The tripeptide fMLP (at 1, 10, or 100 nM, where 10 nM is the optimal concentration for a chemotactic response) served as the standard stimulus. Results are presented as net migration, i.e. the difference in distance migrated by stimulated and quiescent PMN.

Measurements of $[Ca^{2+}]_i$. These were calculated from the changes in fura-2 fluorescence as described [20, 21]. Neutrophils (5×10^6 cells/mL) in HBSS supplemented with 20 mM HEPES, pH 7.4, were incubated at 37° with 0.5 μM fura-2/AM for 30 min. Loaded cells were washed twice, reconstituted in HBSS. Subsequently, cells were warmed at 37° for 15 min and added to cuvettes in a spectrofluorometer. Fluorescence was excited at 340 nm and emitted light read at 510 nm. Measurements were made at 37° with continuous stirring of the cell suspension. After a stable baseline had been established, the stimulus was added and emitted light recorded. The calibration of the signal was performed by addition of EGTA, Tris buffer, Triton X-100, and $CaCl_2$, after which calculation of calcium concentrations was performed [21].

Assessment of viability. The viability of neutrophils and HUVEC prior to use or after separate incubations with the various inhibitors was determined by trypan blue exclusion, and that of EC by ^{51}Cr release. We could not demonstrate any decreased viability of any of the cell types under these experiment conditions. However, J774 cells reacted with detachment upon exposure to high concentrations of carvedilol, i.e. 25–50 μM, as described below.

Statistical Analysis

Data were analyzed using Student's two-tailed *t*-test for paired samples.

RESULTS

Cytochrome c Reduction

Firstly, we wanted to see if the previously published observations that carvedilol inhibited PMN superoxide generation [6] could be reproduced. PMN reacted to fMLP with an O_2^- generation, which was completed well within 10 min. With 0.1 μM fMLP, this amounted to 5.1 ± 0.3 nmol/10^6 PMN and with 1 μM to 7.0 ± 0.5 nmol/10^6 PMN. When carvedilol was added to the PMN prior to 1 μM fMLP, a low concentration of the drug (i.e. 1 μM) conferred a statistically significant enhancement of the O_2^- generation ($P = 0.0074$; N = 8), whereas higher concentrations gradually inhibited the response (Fig. 1a). Thus, carvedilol at 30–50 μM nearly blunted all O_2^- generation ($P = \leq 0.006$ N = 3–9). With fMLP at 0.1 μM, the enhancement at the low concentration did not reach statistical significance, but the inhibitions were all highly significant (Fig. 1b). With PMA at 1 μM as the stimulus, a non-significant enhancement of O_2^- generation was observed. Likewise, carvedilol conferred highly significant inhibitions at the same concentrations as when fMLP was used (Fig. 1c). The apparent IC_{50}s for fMLP at 0.1 and 1 μM were 21.4 and 23.3 μM, respectively, and for PMA 18.6 μM. In order to see whether carvedilol acted as a direct scavenger of released superoxide ions, we assessed generation of O_2^- from the hypoxanthine and xanthine oxidase reaction. Carvedilol (at 50 μM) did not affect that generation (data not shown).

NO Generation

Next, we sought to determine if the effect on NADPH oxidase translated into an effect on the NOS of PMN. When J744 macrophages were treated with carvedilol (at 0.5, 1, 5, 25, or 50 μM) for 30 min and then stimulated by lipopolysaccharide for 19 hr, the drug had no effect on NO generation when used at 0.5–5 μM (data not shown). At 25 or 50 μM, an inhibition was noted, but it was evident that the macrophages were detached from the surface of the wells, indicating a direct toxic effect of carvedilol. The generation of NO from human neutrophils was assessed in the presence of 1–50 μM carvedilol or vehicle alone by the L-NMMA-inhibitable HbO_2 to metHb (methemoglobin) oxidation method [10, 11, 23]. Spontaneous NO production from quiescent PMN over 30 min was unaffected by the presence of carvedilol (data not shown). When PMN were activated with fMLP (1 μM), they produced 0.64 ±

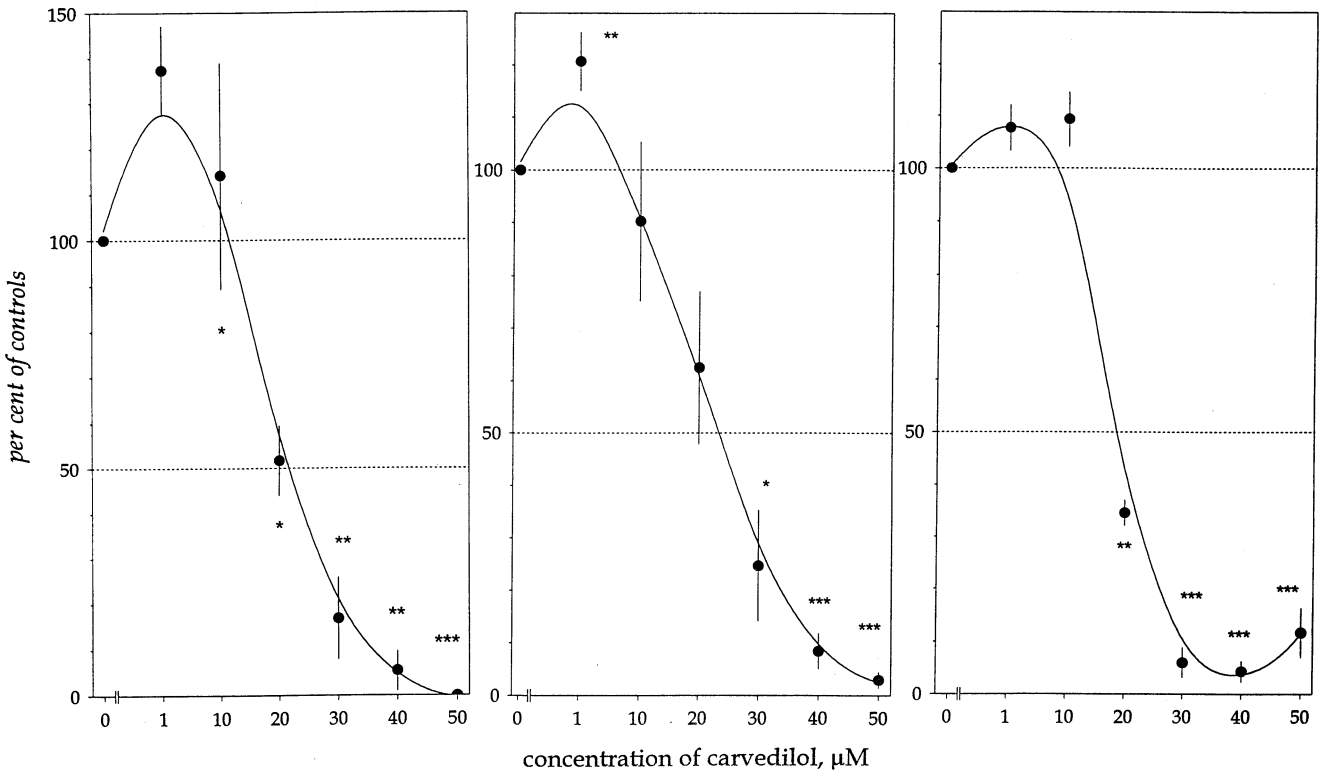

FIG. 1. Superoxide ion production in response to fMLP or PMA. The effect of carvedilol was assessed by the superoxide dismutase-inhibitable cytochrome c reduction method as detailed in the Methods section. Depicted is the effect of carvedilol on O_2^- generation induced by fMLP at 0.1 μM (a), by fMLP at 1 μM (b), and by PMA at 1 μM (c). The data are given as percentages of the simultaneously assessed controls (i.e. with vehicle but no active drug, designated as 100%). Mean and SEM values. * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

0.1 and 0.65 ± 0.03 μM NO over 30 min in the presence of vehicle or 50 μM carvedilol, respectively (mean ± SEM, N = 4, $P = 0.91$). Also, when PMN were activated with PMA (0.1 μM) over the same period of time, no significant differences between samples treated with vehicle alone or 50 μM carvedilol were noted, i.e. 0.62 ± 0.1 and 0.67 ± 0.1 μM NO, respectively (mean ± SEM, N = 4, $P = 0.44$). Finally, the effect of carvedilol as a scavenger of NO was assessed with the non-enzymatic release of NO from SNAP. In the absence of carvedilol, 100 μM SNAP released 5.9 ± 1.3 μM NO over 20 min. The corresponding release in the presence of 10 or 100 μM carvedilol was 5.8 ± 0.2 and 5.4 ± 0.1 μM, respectively (mean ± SEM, N = 4, $P \gg 0.05$).

Cytotoxicity and Chemotaxis

In these experiments, we wanted to see if the absence of a drug effect on the NOS of PMN and the inhibitory effect on NADPH oxidase modulated two functional responses where these oxidants have been suggested to play roles [12–19]. When PMN were added to HUVEC in the absence of a stimulus, a spontaneous cytotoxicity of 1.2 ± 0.4% (N = 4) ensued. Stimulation of the PMN, added to the HUVEC, with LXA_4 (0.1 μM), fMLP (0.1 μM), or the calcium ionophore A23187 (2 μM) significantly increased the cytotoxicity; this is in line with previous reports, where the dose–response curves for these three agonists appear [12–14, 26]. The effect of these stimuli is primarily on the PMN. To assess the effect of carvedilol on PMN-induced endothelial injury, we added the drug to the system as described in Methods, i.e. either only to HUVEC for 30 min to see whether the drug would protect HUVEC from activated neutrophils, or together with PMN to inhibit or scavenge oxygen radicals produced upon activation of the neutrophils. Carvedilol at concentrations of 1, 3, and 10 μM was found to have no significant effect on LXA_4-, fMLP-, and A23187-induced cytotoxicity (neither stimulatory nor inhibitory) in any of these models (Table 1). The migration of quiescent PMN was not affected by 1, 5, or 25 μM carvedilol (Table 1 and data not shown). Upon stimulation by a low concentration of fMLP (1 nM), PMN migration increased to 52 ± 12 μm farther than for the quiescent cells. The corresponding figures for higher fMLP concentrations (10 or 100 nM) were +96 ± 17 μm and +48 ± 13 μm, respectively. These values are within the normal range in the laboratory. Treatment of PMN with carvedilol conferred no significant modulation of fMLP-induced chemotaxis (Table 1 and data not shown).

TABLE 1. Spontaneous migration and chemotaxis: cytotoxicity of HUVEC

Variable	Stimulus	Carvedilol 0 μM	Carvedilol 10 μM HUVEC†	Carvedilol 10 μM PMN‡	Carvedilol 25 μM
Spontaneous migration*	HBSS	42 ± 8			47 ± 1
Chemotaxis*	10 nM fMLP	+96 ± 17			+77 ± 1
Cytotoxicity of HUVEC	HBSS	1.2 ± 0.4	1.3 ± 0.5	1.2 ± 0.2	
	100 nM fMLP	4.4 ± 1.1	4.1 ± 1.2	4.4 ± 0.9	
	100 nm LXA_4	4.7 ± 0.8	4.5 ± 1.0	4.4 ± 0.8	
	2 μM A23187	17.8 ± 4.5	17.5 ± 5.3	17.4 ± 4.2	

No statistically significant differences were observed either for the carvedilol concentrations shown here or for the other concentrations tested (i.e. 1 and 5 μM for chemotaxis and 1 and 3 μM for cytotoxicity). Furthermore, no significant effect of carvedilol was noted for chemotaxis-induced fMLP at 1 or 100 nM. Values are means ± SE. The chemotaxis data are based on six separate experiments and the cytotoxicity data on four.

*The spontaneous migration data are given as the distances from the starting side of the filter to the leading front cells. The stimulated migration (i.e. chemotaxis) data are the net distances (in μm) migrated by stimulated PMNs (i.e. the distances for stimulated minus spontaneous migration).

†The cytotoxicity experiment was performed by pretreating HUVEC with carvedilol (or diluent only) for 30 min; subsequently PMN and stimuli were added.

‡In this assay, PMN and carvedilol (or diluent) were added to HUVEC, followed by the stimulus.

$[Ca^{2+}]_i$ Transients

Based on results from three separate experiments, we conclude that there was no effect of 1 or 50 μM carvedilol on resting or fMLP (at 100 nM)-induced $[Ca^{2+}]_i$ transients. The mean basal $[Ca^{2+}]_i$ levels were 50 ± 17, 50 ± 19, and 60 ± 1 nM Ca^{2+} for untreated PMN or those treated with 1 or 50 μM of the drug, respectively. The rise conferred by fMLP was +189 ± 25, +156 ± 23, and +175 ± 31 nM Ca^{2+}, respectively.

DISCUSSION

In previous studies [6, 7], carvedilol was reported to inhibit O_2^- with apparent IC_{50} values very close to those we show here. Thus, the O_2^--inhibiting capacity of carvedilol has been confirmed. The stimulatory effect of low, clinically relevant concentrations of carvedilol on O_2^--generation induced by fMLP is a novel finding. The mechanisms for this inhibition as well as for stimulation are not known. From the present results it can be concluded that there was no significant drug effect of the rapid rise in $[Ca^{2+}]_i$ transients generated by means of phospholipase C products. Further research is needed in order to map the drug effect to specific steps in the stimulus–response coupling of the PMN.

The effect of carvedilol on the generation of oxygen radicals was limited to superoxide ions. When two assays for NO generation were employed, we could not detect any effect of carvedilol. Thus, neither the NOS of human PMN (which is not yet sufficiently characterized as to being constitutively or inducibly expressed) nor the lipopolysaccharide- or cytokine-inducible NOS of murine macrophages was affected by carvedilol. This finding has several implications. Firstly, it points to an effect of carvedilol on NADPH oxidase or steps in the stimulus–response coupling immediately prior to activation of that enzyme but distal to $[Ca^{2+}]_i$ transients. This hypothesis gains support from the finding of parallel inhibitory effects on responses to fMLP and PMA, which activate NADPH oxidase by separate pathways, as well as the absence of a superoxide-scavenging effect (as assessed in the xanthine–xanthine oxidase system). Secondly, the protective effect of carvedilol in animal or human *in vivo* systems is possibly not dependent on effects on NOS [1–5, 8, 9]. This conclusion is based on the finding that the NO-detecting system used here, the oxyhemoglobin method, is extremely sensitive and specific. It can detect nanomolar concentrations of NO and corresponds very well to data generated by a specific NO-sensitive electrode [11]. The Griess method, which measures NO indirectly by assessing nitrite, is convenient for detection of the massive generation (in the micromolar range) by cytokine-stimulated murine macrophages. Yet, results with the Griess method corresponded well to those generated by means of the oxyhemoglobin method.

Given the absence of an effect of carvedilol on NOS and the modulatory effect on NADPH oxidase, we wondered if that translated into effects in *in vitro* assay systems, where NO as well as superoxide ions have been demonstrated to exert significant effects [12–19, 26]. For this purpose, we used chemotaxis and PMN-dependent cytotoxicity for human endothelial cells. In the first system (chemotaxis), NO as well as superoxide or related oxidants can interact with cell responsiveness or chemoattractant stability [15–19]. In the second, where HUVEC are exposed to neutrophils stimulated by means of various agonists, the ensuing cytotoxicity has been shown to be abrogated by various inhibitors of the NOS system as well as by a scavenger of extracellular NO (and also by SOD and catalase) [12–14, 26]. In line with the key role played by NO in PMN reactivity in these two assays, carvedilol did not affect the responses. The results also suggest that the modulatory effect of carvedilol on superoxide generation did not translate into biological effects in these two systems. One might speculate that the reason for this might be that NO is the

major determinant in a balance system between superoxide ions and NO, one that is specific for any given cellular function or organ. This balance might also differ with the *in vitro* and *in vivo* conditions. It should be emphasized that neutrophil-mediated endothelial cytotoxicity is a complex process dependent not only on released toxic metabolites, but also on proteolytic enzymes, adhesion molecules, and released nitric oxide, since NO generated in the system is directly cytotoxic and inhibition of NOS abrogates HUVEC cytotoxicity. Therefore, the fact that carvedilol inhibits O_2-generation by stimulated neutrophils does not necessarily mean that PMN-mediated endothelial injury is reduced. Moreover, our assay system differs considerably from those used in other reports [8, 9] in terms of the ability of carvedilol to abrogate oxidant-induced endothelial cytotoxicity; those studies used bovine cells, no protein in the medium, and chemically induced oxygen radicals, where iron and xanthine–xanthine oxidase were added to endothelial cells. The possibility that these chemicals may induce or facilitate cell injury needs further study.

Thus, the modulatory effects on superoxide ion generation in PMN (as shown here and in [6, 7]) might explain why carvedilol was reported to prevent monocyte adhesion to low-density-protein-enhanced adhesion to endothelial cells [28] and inhibit apoptosis in myocardial cells [2]. Most importantly, since carvedilol did not show any effect on generation of the vasodilatory molecule NO, this may favor a cardioprotective role for the drug.

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