Free Rad. Res., Vol. 29, pp. 377–387
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Carvedilol Inhibition of Lipid Peroxidation. A New Antioxidative Mechanism

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Accepted by Prof. J.M.C. Gutteridge

(Received 1 April 1998; In revised form 18 June 1998)

To define the molecular mechanism(s) of carvedilol inhibition of lipid peroxidation we have utilized model systems that allow us to study the different reactions involved in this complex process.

Carvedilol inhibits the peroxidation of sonicated phosphatidylcholine liposomes triggered by $FeCl_2$ addition whereas atenolol, pindolol and labetalol are ineffective. The inhibition proved not to be ascribable (a) to an effect on Fe^{2+} autoxidation and thus on the generation of oxygen derived radical initiators; (b) to the scavenging of the inorganic initiators $O_2^{\bullet-}$ and $^{\bullet}OH$; (c) to an effect on the reductive cleavage of organic hydroperoxides by $FeCl_2$; (d) to the scavenging of organic initiators. The observations that (a) carvedilol effectiveness is inversely proportional to the concentration of $FeCl_2$ and lipid hydroperoxides in the assay; (b) the drug prevents the onset of lipid peroxidation stimulated by $FeCl_3$ addition and; (c) it can form a complex with Fe^{3+}, suggest a molecular mechanism for carvedilol action. It may inhibit lipid peroxidation by binding the Fe^{3+} generated during the oxidation of Fe^{2+} by lipid hydroperoxides in the substrate. The lag time that carvedilol introduces in the peroxidative process would correspond to the time taken for carvedilol to be titrated by Fe^{3+}; when the drug is consumed the Fe^{3+} accumulates to reach the critical parameter that stimulates peroxidation. According to this molecular mechanism the antioxidant potency of carvedilol can be ascribed to its ability to bind a species, Fe^{3+}, that is a catalyst of the process and to its lipophilic nature that concentrates it in the membranes where Fe^{3+} is generated by a site specific mechanism.

Keywords: Carvedilol, lipid peroxidation, ferric ion, critical Fe^{2+}/Fe^{3+} ratio, phosphatidylcholine liposomes

Abbreviations: LOOH, lipid hydroperoxide; Mes, Morpholineethanesulphonic acid; Mops, Morpholinepropanesulphonic acid; NBT^{2+}, nitroblue tetrazolium; PC, phosphatidylcholine liposome

INTRODUCTION

Carvedilol, 1-[carbazolyl-(4)-oxy]-3-[(2-methoxyphenoxyethyl)-amino]-2-propanol, is a multiple action antihypertensive drug with non-selective β adrenoceptor and selectively α_1 adrenoceptor blocking activities.[1,2] The drug is clinically used as an antihypertensive and in the treatment of heart failure. It also possesses cardio- and vaso-protective[3–5] actions which are in part

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independent of α_1 and β blockade. This extraordinary cardioprotection seems to be partly mediated by the antioxidative action of carvedilol.[4]

The antioxidant properties of this molecule have been extensively investigated using physicochemical,[6–8] biochemical[9] and biological[5,7,8,10–14] assay systems in a variety of *in vitro* models, including isolated lipoproteins,[12,14] subcellular fractions[9] and cellular cultures.[7,8,10,11,13] Recently carvedilol was shown to decrease LDL oxidation *in vivo* in humans.[15]

Attempts have been made to define the molecular mechanisms responsible for the antioxidant activity of carvedilol. Previous findings implicate a direct ability of this drug to react with superoxide and hydroxyl radicals. The IC_{50} reported for scavenging these radicals[6] were much higher than those found for the inhibition of the secondary lipid peroxidation.[7] These reported discrepancies indicate that carvedilol may exert its effect by interfering with other reactions and/or reagents taking part in the complex processes leading to oxidative stress.

The awareness that peroxidation of either isolated[9] or integral[7,8] cell membranes appears to be a target of carvedilol action, prompted us to evaluate the possibility that carvedilol may directly affect this process. Therefore, we studied its effect on model liposomes and in experimental conditions, previously well characterized,[16–18] that are suitable not only to verify the hypothesis proposed but also to define the target and to elucidate the molecular mechanism of the antioxidant action of the studied molecule.

MATERIALS AND METHODS

Chemicals

Morpholineethanesulphonic acid (Mes), morpholinepropanesulphonic acid (Mops), bovine liver catalase, bovine erythrocyte CuZn superoxide dismutase, nitroblue tetrazolium, $FeCl_2$, $FeCl_3$, atenolol, labetalol, pindolol, α-tocopherol and all other chemicals, of the highest grade available, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Carvedilol was from Istituto Gentili (Pisa, Italy); egg phosphatidylcholine was from Lipid Products (Redhill, UK) and 1,10-phenanthroline was from Merck (Darmstadt, Germany). The thermolabile azocompound 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) was obtained from Polysciences Inc. (Warrington, PA, USA). *p*-Nitrosodimethylaniline was a generous gift of Prof. R. Stevanato. All reagents were prepared in Chelex resin-treated distilled water whose pH was brought to 6.5 with HCl.

Liposome Preparations

The standard sonicated egg yolk phosphatidylcholine liposomes (PC) were prepared as previously described.[16–18] The phospholipids (about 18 mg), dried under nitrogen, were added with 6 ml distilled water, vortex mixed for 10 min and stored at 4°C for 1 h. This suspension was subsequently sonicated with a titanium probe sonicator (Labsonic 2000) for different times to obtain liposomes with a different oxidation index.[19] The vesicle dispersion was then transferred to a small-volume extrusion apparatus (produced by Avestin, Ottawa, Canada) which allowed the extrusion of large unilamellar vesicles through standard 19 mm polycarbonate filters with 0.1 mm pore size. Usually samples were subjected to 37 passes through two filters mounted in tandem in the mini-extruder, as recommended by Hope *et al.*[20] This procedure makes possible the preparation of large unilamellar vesicles (LUVETs) with an average diameter close to the pore size. All extrusion procedures were conducted at 20°C. The multilamellar liposomes containing the azoderivative initiator of lipoperoxidation, AMVN, were prepared in a round bottom tube by adding in the

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following order: AMVN and, when present, carvedilol or α-tocopherol followed by PC.[21] After each addition the solvent was carefully removed with a stream of nitrogen in ice. The thin film obtained after evaporation was vortex mixed for 10 min with an aliquot of 5 mM Mes buffer pH 6.5, 0.1 mM EDTA in order to obtain 2 mM AMVN, 8 mM PC and the amount of anti-oxidant as defined in the legends to figures. Appropriate blank lacking the thermal initiator were also prepared. Lipid peroxidation was triggered by incubating the solutions in a water bath at the temperatures defined in the legends to the figures. The reaction vessels were kept under air in the dark. The phospholipid content of the liposome suspensions was determined by the method of Marinetti.[22]

Fe^{2+} Determination

Measurement of Fe^{2+} concentration was made by the *o*-phenanthroline method according to Mahler and Elowe.[23] The assays were conducted in 1 ml of the following media: Fe^{2+} autoxidation in 5 mM Mops buffer, pH 7.5; Fenton reaction in water; Fenton-like reaction and lipid peroxidation in 5 mM Mes buffer, pH 6.5. The reactions, initiated by $FeCl_2$ addition, were conducted at room temperature, stopped by the addition of 0.2 ml 25 mM 1,10-phenanthroline and A_{515} was immediately read. The concentrations of the components of the reaction mixtures and the incubation conditions are given in the figure legends.

Determination of Fe^{3+}/Carvedilol Complex

Evaluation of the formation of a Fe^{3+}/carvedilol complex was conducted by two methods. $FeCl_3$ (10 mM) in water was incubated with an equal volume of either ethanol or ethanol containing 10 mM carvedilol. After 30 min incubation the absorbance spectra of the solutions were recorded. The spectrum of the Fe^{3+}/carvedilol complex was obtained by the difference between the two spectra.

$FeCl_3$ (10 mM) in water was incubated with an equal volume of either water or ethanol or ethanol containing 10 mM carvedilol. After 30 min incubation, aliquots containing increasing amounts of $FeCl_3$ were added to the FOX2 reagent (100 μM xylenol orange, 90% v/v methanol, 25 mM H_2SO_4 final volume 1 ml).[24] The kinetics of the formation of the Fe^{3+}/xylenol orange complex was recorded.

$O_2^{\bullet-}$ Determination

The concentration of $O_2^{\bullet-}$ generated during the autoxidation of 100 μM $FeCl_2$ in 5 mM Mops buffer, pH 7.5 was measured by the reduction of 250 μM NBT^{2+}. The increase in absorbance at 560 nm associated with the reduction of NBT^{2+} by $O_2^{\bullet-}$ was used as an index of $O_2^{\bullet-}$ production. This reaction can be completely inhibited by superoxide dismutase (100 U/ml).

$^{\bullet}OH$ Determination

$^{\bullet}OH$ concentration was determined by its reaction with 45 μM *p*-nitrosodimethylaniline (PNDA).[25] The $^{\bullet}OH$ generation by the Fenton reaction ($FeCl_2$ 100 μM and H_2O_2 200 μM) in water was measured by the decrease in PNDA absorbance at 440 nm. The reaction had to be conducted in the absence of the Good type buffers as these molecules, because of their high rate constants for $^{\bullet}OH$ ($2–3 \times 10^9\ M^{-1}\ s^{-1}$)[26] and their standard concentration in the assay (5 mM), competed with the studied reaction. The reaction was completely inhibited by catalase (400 U/ml).

Oxygen Consumption

Lipid peroxidation was measured by monitoring oxygen consumption with a Clark-type electrode.

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Reaction mixtures containing 5 mM Mes buffer, pH 6.5 and the liposomes were continuously stirred in a 3 ml sealed chamber where the O_2 electrode was immersed via a sealed port. Additions were made via a resealable port on top of the chamber.

Conjugated Diene Content and Oxidation Index of Liposomes

The samples containing 100–150 μg phospholipid were extracted with 1 ml butan-1-ol. Phases were separated by centrifligation at 3000 rpm for 10 min and the 200–300 nm ultraviolet spectrum of the upper organic phase was recorded against appropriate blanks containing all reagents but liposomes. The content of conjugated dienes was determined by the increase in absorbance at 234 nm. The oxidation index of the liposomes was determined by the A_{233nm}/A_{215nm} ratio.[19]

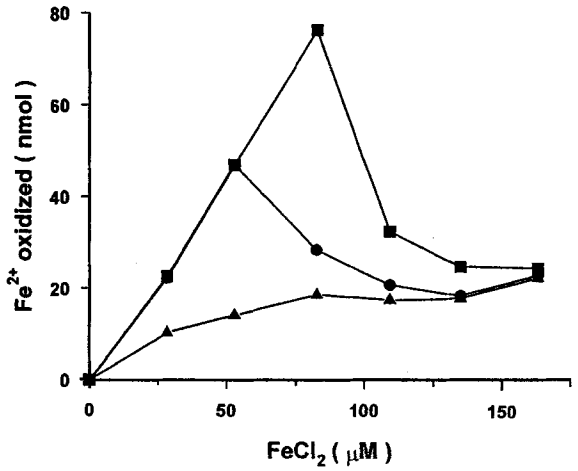

FIGURE 1 Effect of carvedilol on the peroxidation of PC liposomes. Increasing concentrations of $FeCl_2$ were incubated with PC liposomes (150 μg phospholipid), in 5 mM Mes buffer, pH 6.5 in the absence (■) and presence of either 50 μM carvedilol (▲), or the ethanol vehicle (final concentration in the assay 217 mM) of the drug (●). The Fe^{2+} oxidized by the liposomes after 10 min incubation was measured. Values plotted are means of triplicate assay with the same PC liposome preparation; SE never exceeded 5% of the mean. The results are representative of 10 experiments with different PC liposome preparations.

RESULTS AND DISCUSSION

The experimental model that we have utilized in this study of carvedilol inhibition of lipid peroxidation was previously well characterized[16–18] and consists of phosphatidylcholine liposomes whose peroxidation is triggered by $FeCl_2$ addition. Phosphatidylcholine liposomes prepared by sonolysis, when incubated with increasing concentrations of $FeCl_2$ in Mes buffer pH 6.5, oxidized the metal (Figure 1). The pattern of Fe^{2+} oxidation by PC liposomes after 10 min incubation was biphasic: as $FeCl_2$ concentration was increased, at first Fe^{2+} oxidation increased, reached a maximum and then decreased. The concentration at which Fe^{2+} oxidation is maximal is defined as critical Fe^{2+} concentration, $[Fe^{2+}]^*$. The kinetics of PC oxidation by increasing concentrations of $FeCl_2$, measured as formation of conjugated dienes, were rather complex (Figure 2A). At low $FeCl_2$ concentrations the time courses were hyperbolic whereas at intermediate $FeCl_2$ concentrations, an initial rapid but limited hyperbolic phase was followed by a sigmoid pattern. By further increasing $FeCl_2$ concentration, the onset of the sigmoid curve was delayed; at the highest $FeCl_2$ concentrations the sigmoid curve started beyond the studied length of the reaction and thus only a small and rapid formation of conjugated dienes was observed. These patterns are in agreement with the hypothesis that a "critical" Fe^{2+}/Fe^{3+} ratio controls the extent and the kinetics of lipid peroxidation. The Fe^{3+} is generated during the oxidation of the Fe^{2+} catalyst by the lipid hydroperoxides of the substrate. At Fe^{2+}/Fe^{3+} ratio lower than the critical one, the metal is mainly involved in the fast generation of inorganic (reaction 1) and organic (reaction 5) initiators supporting the propagation and chain-branching reactions (reactions 4, 6) of

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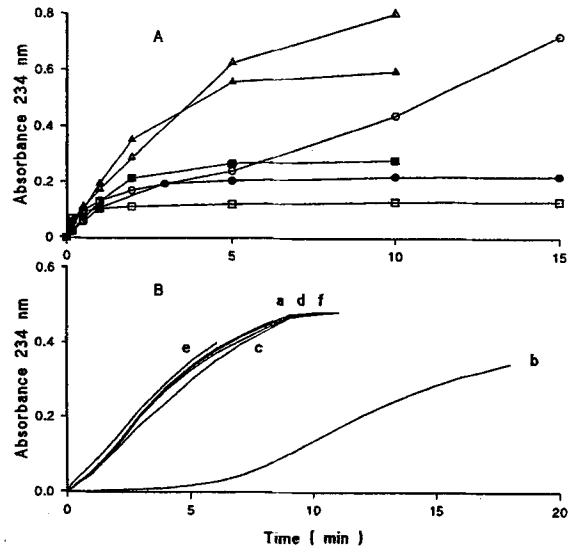

FIGURE 2 Effect of carvedilol on the kinetics of the peroxidation of PC liposomes. (A) PC liposomes (150 μg phospholipid) were incubated in 5 mM Mes buffer, pH 6.5 with 20 μM (•), 30 μM (■), 50 μM (▲), 75 μM (△), 100 μM (○), 125 μM (□) $FeCl_2$. The peroxidation was evaluated by measuring, at different times, the increase in A_{234}, indicative of the generation of conjugated dienes. Values plotted are means of triplicate assay with the same PC liposome preparation; SE never exceeded 5% of the mean. The results are representative of four experiments with different PC liposome preparations. (B) PC liposomes (250 μg phospholipid) were incubated in 5 mM Mes buffer, pH 6.5 with 30 μM $FeCl_2$ in the absence (line a) or presence of 10 μM carvedilol (line b); 10 μM atenolol (line c); 10 μM labetolol (line d); 10 μM pindolol (line e); the ethanol vehicle (final concentration in the assay 43 mM) of the drugs (line f). The peroxidation was evaluated by continuously monitoring the increase in A_{234}. The results are representative of four experiments with different PC liposome preparations.

lipid peroxidation.

$$nFe^{2+} + O_2 \rightarrow nFe^{3+} + \text{reduced } O_2\ (I^\bullet)$$
generation of O_2 derived $I^\bullet$ [1]

$$I^\bullet + LH \rightarrow IH + L^\bullet$$ initiation [2]

$$L^\bullet + O_2 \rightarrow LOO^\bullet$$ O_2 addition [3]

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet$$ propagation [4]

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + OH^- + LO^\bullet$$
generation of organic $I^\bullet$ [5]

$$LO^\bullet + LH \rightarrow LOH + L^\bullet$$ chain branching [6]

At a Fe^{2+}/Fe^{3+} ratio higher than the critical one the excess Fe^{2+} reduces the organic radicals generated (reaction 7) and exhibits dominant chain termination behavior until its concentration is lowered and the chain branching reactions are resumed: the higher the Fe^{2+}/Fe^{3+} ratio the longer is the latent period, the so called "lag time" required to start peroxidation.

$$Fe^{2+} + LOO^\bullet/LO^\bullet \rightarrow Fe^{3+} + LOOH/LOH$$
termination [7]

Carvedilol (50 μM) in ethanol inhibited Fe^{2+} oxidation by PC liposomes, in particular at the lowest $FeCl_2$ concentration tested (Figure 1). The ethanol vehicle was without effect in these conditions (Figure 1) indicating that the adrenergic blocker is the real inhibitor of peroxidation. Carvedilol (10 μM) affected the time course of the peroxidation of PC liposomes (oxidation index 0.21) catalyzed by $FeCl_2$ (30 μM) (Figure 2B). Equimolar amounts of the β blockers atenolol, pindolol and of the α,β blocker labetolol were ineffective (Figure 2B) indicating that the inhibition of the peroxidation is not a property of this class of drug but is specific for carvedilol. The major structural feature of carvedilol, distinguishing it from other tested drugs, is the carbazole portion. It has been shown that carbazole derivates possess scavenging properties and inhibit lipid peroxidation.[27]

To understand the molecular mechanism responsible for carvedilol action we studied its effect on several reactions stimulating the peroxidative pathway. At first, we evaluated the effect of carvedilol on initiator molecules. The drug (50 μM) did not scavenge $O_2^{\bullet-}$ generated by the spontaneous autoxidation of $FeCl_2$ (100 μM) that occurs in 5 mM Mops buffer, pH 7.5,[28] and under the same experimental conditions it did not influence the rate of Fe^{2+} autoxidation (results not shown). This result contrasts with a previous study where carvedilol was reported to scavenge $O_2^{\bullet-}$ generated during the metal catalyzed oxidation of dihydroxy-fumaric acid (DHF).[11] The reason for this discrepancy is at present not clear, although the influence of

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carvedilol on the rate of DHF oxidation was not assessed and may be responsible for the differences reported. We studied also the effect of carvedilol on •OH generated by the Fenton reaction; the drug (50 μM) did not inhibit Fe^{2+} oxidation in this experimental system (results not shown) but decreased •OH reaction with PNDA indicating its •OH scavenging ability, as already reported,[6] (Figure 3 line c). Although carvedilol proved to scavenge •OH, other results obtained in this study strongly challenge the hypothesis that its inhibition of lipid peroxidation is exerted by this molecular mechanism. In fact, the •OH scavenging ability of Mes buffer (Figure 3 line e), utilized in the lipid peroxidation studies, would hardly allow any •OH initiated lipid peroxidation to occur and, in any case, the inhibition contributed by micromolar carvedilol would be negligible. Besides, atenolol, which proved to be a better •OH scavenger than carvedilol (Figure 3 line d), is ineffective on lipid peroxidation (Figure 2B). These results indicate that carvedilol does not affect lipid peroxidation

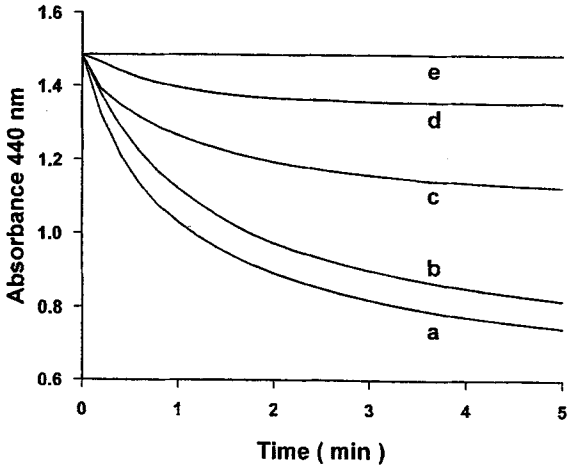

FIGURE 3 Effect of carvedilol on the reaction of PNDA with •OH generated by the Fenton reaction. PNDA (45 μM) was incubated in water with 100 μM $FeCl_2$ and 200 μM H_2O_2. PNDA trapping of •OH was determined by continuously monitoring the decrease in A_{440} in the absence (line a) or presence of 50 μM carvedilol (line c), 50 μM atenolol (line d), the ethanol vehicle (final concentration in the assay 217 mM) of the drugs (line b) and 5 mM Mes buffer (line e). The results are representative of four experiments.

by interfering with the primary initiation of this process, the so called "LOOH-independent initiation", which is mainly sustained by oxygen derived radicals.

We verified the possibility that the drug may affect the "LOOH-dependent initiation" interfering with the generation of the organic initiators by reaction 5. As a model system we studied the kinetics of a Fenton-like reaction. Carvedilol (50 μM) did not affect the rate of $FeCl_2$ (100 μM) oxidation by cumene hydroperoxide (50 μM) and thus the generation of alkoxyl radicals (results not shown). The possibility that carvedilol may scavenge the organic radicals generated during lipid peroxidation, i.e. act as a chain breaking antioxidant, was also studied. For comparison, we tested in the same experimental system also the well known chain breaking antioxidant α-tocopherol. The peroxidation of PC liposomes was initiated by the azocompound AMVN (2 mM)[21] and typical traces of either oxygen uptake or conjugated diene generation are reported in Figures 4A and B. Vesicles were oxidized at a constant rate without any noticeable lag period. In the presence of the chain breaking antioxidant α-tocopherol (3 and 6 μM) a clear induction period occurred and, after all the antioxidant had been depleted, the reactions proceeded at the same rate observed in its absence, as expected. The results obtained in the presence of 155 and 168 μM carvedilol Figures 4A and B respectively) showed that the peroxidation rate, although decreased with respect to the control experiments, was faster than that in the presence of α-tocopherol. Moreover, the amount of α,β blocker required to observe a significant decrease in the peroxidation rate was so high as to prevent detection of the induction period. These results indicate that carvedilol, as already suggested by Yue and coworkers[7,8] can scavenge organic radicals; however its chain breaking antioxidant activity appears to be so modest as not to justify its effectiveness in inhibiting the Fe^{2+} catalyzed LOOH-dependent lipid peroxidation.

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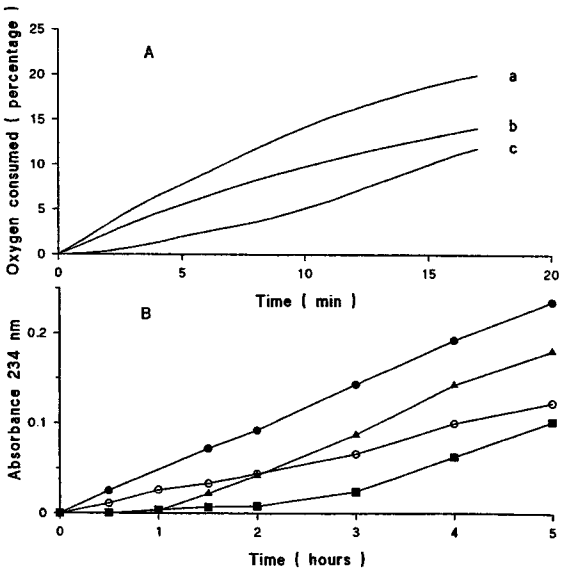

FIGURE 4 Effect of carvedilol on the peroxidation of PC peroxidation initiated by AMVN. PC liposomes (8 mM phospholipid) containing 2 mM AMVN were prepared by vortex mixing for 10 min in 5 mM Mes buffer, pH 6.5, 0.1 mM EDTA. (A) Peroxidation was evaluated by oxygen consumption. The PC liposome suspension was incubated at 50°C in the absence (line a) or presence of 155 μM carvedilol (line b), 3 μM α-tocopherol (line c). The results are representative of three experiments with different PC liposome preparations. (B) Peroxidation was evaluated by the conjugated diene formation. The PC liposome suspension was incubated at 41°C in the absence (•) or presence of 168 μM carvedilol (○), 3 μM α-tocopherol (▲), 6 μM α-tocopherol (■). At the stated time 50 μl of the suspension were added to 950 μl ethanol and the absorbance at 234 nm was measured against a suitable T_0 blank. Values plotted are means of triplicate assay with the same PC liposome preparation; SE never exceeded 5% of the mean. The results are representative of four experiments with different PC liposome preparations.

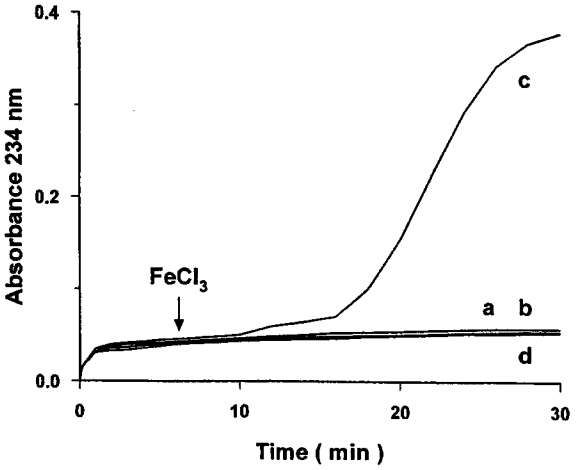

FIGURE 5 Effect of carvedilol on the stimulation of lipid peroxidation by $FeCl_3$. PC liposomes (100 μg phospholipid) were incubated in 5 mM Mes buffer, pH 6.5 with 125 μM $FeCl_2$ in the absence (line a) and presence of 10 μM carvedilol in ethanol (final concentration in the assay 43 mM) (line b). At the time stated 20 μM $FeCl_3$ was added to reaction mixtures where 10 μM carvedilol was absent (line c) or present (line d). Lipid peroxidation was evaluated by continuously monitoring the generation of conjugated dienes at 234 nm. The results are representative of three experiments with different PC liposome preparations.

We evaluated the possibility that carvedilol affects the LOOH-dependent lipid peroxidation by interacting with Fe^{3+}, the other product of reaction 5. It is well known that Fe^{3+} stimulates lipid peroxidation.[29–32] To verify whether carvedilol could counteract this stimulation we studied its effect on the peroxidation catalyzed by $FeCl_2$ concentrations higher than $[Fe^{2+}]^*$. At 125 μM $FeCl_2$, very low amounts of conjugated dienes were formed (Figure 5 line a) as the termination phase of lipid peroxidation was prevailing.[18] In this experimental condition carvedilol addition did not affect the phenomenon observed (Figure 5 line b). A delayed addition of $FeCl_3$ (30 μM) to the reaction mixture containing an inhibitory $FeCl_2$ concentration triggered the generation of conjugated dienes (Figure 5 line c). The stimulatory effect exerted by $FeCl_3$ addition[18] in this experimental condition was inhibited by the presence of carvedilol (10 μM) in the reaction mixture (Figure 5 line d). These data indicate that carvedilol prevents exogenously added $FeCl_3$ from exerting its stimulatory effect. To understand the molecular mechanism of such an action we studied the possible formation of a Fe^{3+}/carvedilol complex by two experimental methods. First, we evaluated whether carvedilol was able to compete with the ferric sensitive dye xylenol orange for Fe^{3+}. $FeCl_3$ (100 μM $FeCl_3$ in water) was rapidly bound by the dye and a colored complex was formed (Figure 6A line a).[24] By contrast, the rate of $FeCl_3$ complexation by the dye was greatly

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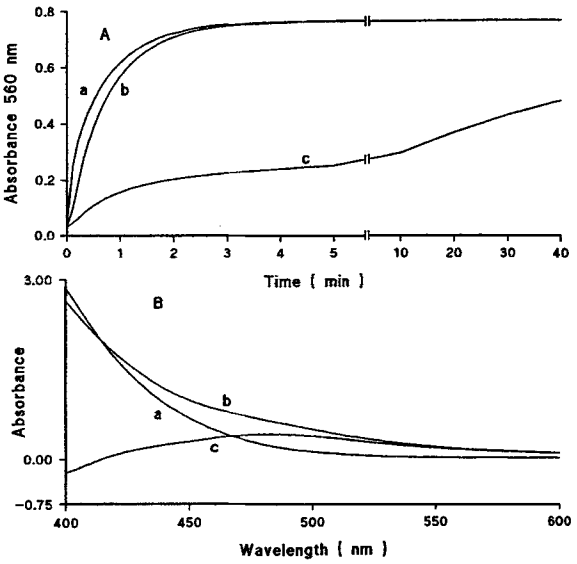

FIGURE 6 Fe^{3+}/carvedilol complex. (A) Rate of formation of a Fe^{3+}/xylenol orange complex in the presence of carvedilol. Reactions, containing 100 μM xylenol orange, 90% methanol, 25 mM H_2SO_4 in a final volume of 1 ml were started by adding $FeCl_3$ at a final concentration of 100 μM (line a), 100 μM $FeCl_3$ and 217 mM ethanol (line b), 100 μM $FeCl_3$, 217 mM ethanol and 100 μM carvedilol (line c). Fe^{3+}, ethanol and carvedilol solutions were equilibrated at room temperature for 30 min. The results are representative of five experiments. (B) Absorbance spectrum of the Fe^{3+}/carvedilol complex. Equal volumes of 10 mM $FeCl_3$ and either ethanol (line a) or 10 mM carvedilol in ethanol (line b) were mixed and the spectra of the complexes formed were recorded after 30 min incubation at room temperature. The spectrum of the Fe^{3+}/carvedilol complex (line c) was obtained by subtracting a line from line b. The results are representative of five experiments.

decreased when carvedilol (1:1 $FeCl_3$/carvedilol) was present in the sample (Figure 6A line c). The decreased rate of formation of the Fe^{3+}/xylenol orange was hardly ascribable to the ethanol vehicle of the drug (Figure 6A line b). We, then, incubated an aqueous solution of $FeCl_3$ (10 mM) for 30 min with an equal volume of carvedilol (10 mM in ethanol); a complex was formed whose absorbance spectrum (Figure 6B line b) differs from that of an equal amount of $FeCl_3$ in 1:1 water/ethanol (Figure 6B line a); the carvedilol/Fe^{3+} spectrum has a maximum at 480 nm (Figure 6B line c). These data strongly suggest that carvedilol forms a complex with Fe^{3+}.

Recent results we have obtained demonstrated[18] that the parameter that controls the kinetics of the LOOH-dependent lipid peroxidation is the Fe^{3+} endogenously generated by the reductive cleavage of lipid hydroperoxides by Fe^{2+}; in particular, a critical Fe^{2+}/Fe^{3+} ratio must be reached to shift Fe^{2+} from serving as stoichiometric reactant in the termination reaction to being the repeatedly cycled catalyst of the propagation and chain branching reactions. In the different experimental conditions the concentration of Fe^{3+} generated, and thus the Fe^{2+}/Fe^{3+} ratio, is mainly controlled by the $FeCl_2$ added, the lipid hydroperoxides within the substrate and the incubation time. When the Fe^{2+}/Fe^{3+} ratio is lower than the critical one, Fe^{2+} ions are prooxidants at all concentrations and at any time of incubation. When the ratio is higher than the critical value, a lag phase associated to the prevailing of the termination reactions is observed; the length of the lag phase depends on the time occurring to approach the permissive Fe^{2+}/Fe^{3+} ratio. In this light, to further support the hypothesis that carvedilol affects the Fe^{2+} catalyzed LOOH-dependent lipid peroxidation by stoichiometrically interacting with the Fe^{3+} endogenously generated, we investigated the effect of the drug concentration on the kinetics of peroxidation in experimental conditions where Fe^{3+} is exerting its stimulation, i.e. where the propagation reactions are prevailing. We selected two experimental conditions where the amount and the kinetics of the generation of Fe^{3+} differ. In the first experiment (Figure 7) we tested three $FeCl_2$ concentrations (10, 25 and 50 μM) catalyzing the oxidation of PC liposomes with a 0.32 oxidation index. In each case carvedilol affected the kinetics of lipid peroxidation; by increasing the concentration of the drug a longer lasting latent period developed. However, in the presence of the lower $FeCl_2$ concentrations, the effectiveness of carvedilol was higher, i.e. the same concentration of the drug exerted a greater inhibition (compare lines f). In a second experiment (Figure 8) we

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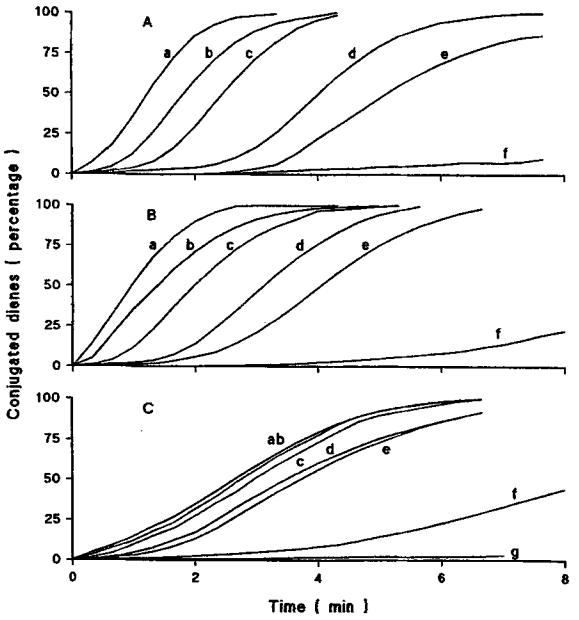

FIGURE 7 Effect of $FeCl_2$ concentration on carvedilol inhibition of lipid peroxidation. PC liposomes (0.32 oxidation index) were incubated with $FeCl_2$ 10 μM (panel A), 25 μM (panel B), 50 μM (panel C) in 5 mM Mes buffer, pH 6.5. The experiments were conducted in the absence (line a) or presence of carvedilol 1 μM (line b), 2.5 μM (line c), 5 μM (line d), 10 μM (line e), 25 μM (line f) and 50 μM (line g). The peroxidation was evaluated by measuring the increase in A_{234}, indicative of the generation of conjugated dienes. The maximal A_{234} in the three experimental conditions was 0.29, 0.43 and 0.56. The results are representative of three experiments with different PC liposome preparations.

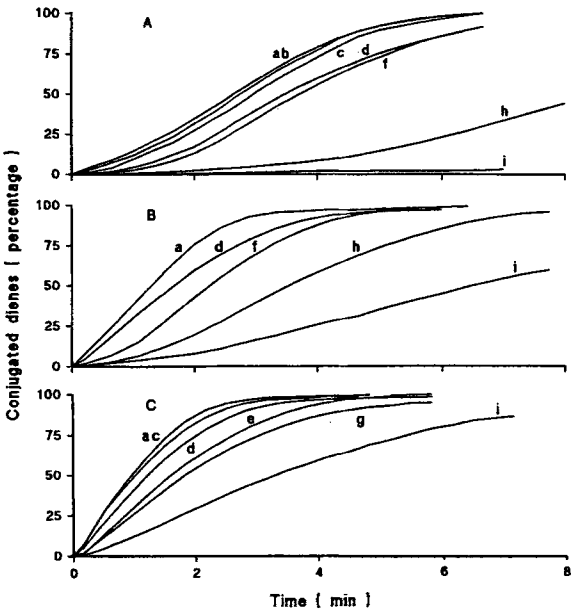

FIGURE 8 Effect of the oxidation index of PC liposomes on carvedilol inhibition of lipid peroxidation. $FeCl_2$ 50 μM was incubated with PC liposomes 0.32 oxidation index (panel A), 0.4 oxidation index (panel B), 0.68 oxidation index (panel C) in 5 mM Mes buffer, pH 6.5. The experiments were conducted in the absence (line a) or presence of carvedilol 1 μM (line b), 2.5 μM (line c), 5 μM (line d), 7.5 μM (line e), 10 μM (line f), 15 μM (line g), 25 μM (line h) and 50 μM (line i). The peroxidation was evaluated by measuring the increase in A_{234}, indicative of the generation of conjugated dienes. The maximal A_{234} in the three experimental conditions was 0.56, 0.60 and 0.64. The results are representative of three experiments with different PC liposome preparations.

evaluated the effect of carvedilol concentration on the peroxidation, by 50 μM $FeCl_2$, of PC liposomes with different oxidation indices (0.32, 0.4, 0.68). From the comparison of the results obtained, it is evident that the effectiveness of carvedilol is higher when PC liposomes with the lower oxidation index are peroxidized (compare lines i). The fact that the drug is more effective when lower concentrations of $FeCl_2$ and lipid hydroperoxides are reacting, is in agreement with the hypothesis that the drug stoichiometrically interacts with the Fe^{3+} endogenously generated. In the presence of carvedilol, in fact, the Fe^{3+} may be chelated by the drug and the attainment of the critical ratio delayed; the lag period would last until the drug has been titrated and enough Fe^{3+} has been accumulated to obtain the critical Fe^{2+}/Fe^{3+} ratio; carvedilol would cause a longer latent period when the amount and the rate of Fe^{3+} generation is lower.

The molecular model hypothesized to explain carvedilol action allows us to better understand the reason for its potency. Since *in vivo* the concentration of "free" Fe^{2+} and lipid hydroperoxides are very low, due to complexation to transport and storage molecules,[33] and to the action of specific hydroperoxide-removing enzymes,[35] the concentration of Fe^{3+} generated by their reaction is modest. For this reason carvedilol

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and other molecules that exert an antioxidant effect by binding Fe^{3+} would be expected to be active at very low concentrations. Besides, the generation of Fe^{3+} occurs by a site specific mechanism which localizes it in proximity of the membrane where the lipid hydroperoxides are embedded. The lipophilic nature of carvedilol, which accumulates in a lipid environment[36] would further favor its possibility to interact with Fe^{3+}, and thus its antioxidative properties.

Our data confirm the work of many groups who described the inhibition of lipid peroxidation by carvedilol. However, our experimental conditions have allowed us to define that the major molecular mechanism of carvedilol action is not to scavenge primary inorganic and secondary organic initiators as does α-tocopherol. Carvedilol appears to bind the Fe^{3+} catalyst of the LOOH-dependent lipid peroxidation which is produced in close proximity to the lipophilic environment where the drug is located. To this location and to the catalytic role of Fe^{3+} may be ascribed the effectiveness of carvedilol as antioxidant. The molecular mechanism proposed, and the increasing evidences that free radical injuries are often catalyzed by transition metals, in particular by iron,[37,38] suggest a wider therapeutic role for this molecule.

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Acknowledgements

This work was supported by a grant from the Istituto Gentili, Pisa, Italy. PNDA used in the present study was kindly provided by Prof R. Stevanato, Università di Venezia, Italy.

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