British Journal of Pharmacology (1996) 119, 1438-1446 © 1996 Stockton Press All rights reserved 0007-1188/96 $12.00

Protection of low density lipoprotein oxidation at chemical and cellular level by the antioxidant drug dipyridamole

[1]L. Iuliano, A.R. Colavita, C. Camastra, V. Bello, C. Quintarelli, M. Alessandroni, *F. Piovella & F. Violi

Institute of Clinical Medicine I, University La Sapienza, 00185 Rome and *Institute of Clinical Medicine II (F.P.), 27100 Pavia, Italy

1 The oxidative modification of low density lipoprotein (LDL) is thought to be an important factor in the initiation and development of atherosclerosis. Natural and synthetic antioxidants have been shown to protect LDL from oxidation and to inhibit atherosclerosis development in animals. Synthetic antioxidants are currently being tested, by they are not necessarily safe for human use.

2 We have previously reported that dipyridamole, currently used in clinical practice, is a potent scavenger of free radicals. Thus, we tested whether dipyridamole could affect LDL oxidation at chemical and cellular level.

3 Chemically induced LDL oxidation was made by Cu(II), Cu(II) plus hydrogen peroxide or peroxyl radicals generated by thermolysis of 2,2'-azo-*bis*(2-amidino propane). Dipyridamole, (1-10 μM), inhibited LDL oxidation as monitored by diene formation, evolution of hydroperoxides and thiobarbituric acid reactive substances, apoprotein modification and by the fluorescence of *cis*-parinaric acid.

4 The physiological relevance of the antioxidant activity was validated by experiments at the cellular level where dipyridamole inhibited endothelial cell-mediated LDL oxidation, their degradation by monocytes, and cytotoxicity.

5 In comparison with ascorbic acid, α-tocopherol and probucol, dipyridamole was the more efficient antioxidant with the following order of activity: dipyridamole > probucol > ascorbic acid > α-tocopherol. The present study shows that dipyridamole inhibits oxidation of LDL at pharmacologically relevant concentrations. The inhibition of LDL oxidation is unequivocally confirmed by use of three different methods of chemical oxidation, by several methods of oxidation monitoring, and the pharmacological relevance is demonstrated by the superiority of dipyridamole over the naturally occurring antioxidants, ascorbic acid and α-tocopherol and the synthetic antioxidant probucol.

Keywords: Lipoproteins; atherosclerosis; free radicals; antioxidants; dipyridamole

Introduction

One of the most popular theories proposed for the biochemical mechanism by which macrophages become foam cells, the typical constituent of atherosclerotic lesions, is based on the oxidative modification of low density lipoproteins (Steinberg *et al.*, 1989). This theory is supported by much experimental evidence (Esterbauer *et al.*, 1992). Oxidized-LDL (ox-LDL) is accumulated in macrophages that express abundant scavenger receptors for modified LDL (Goldstein *et al.*, 1979). Among the different biological effects exerted by ox-LDL, the chemotactic and cytotoxic activities may induce the intimal accumulation of monocytes and smooth muscle cells (Quinn *et al.*, 1987; Autio *et al.*, 1990) and cause endothelial cell damage (Kuzuya *et al.*, 1991), which may contribute to the atherosclerosis process. Several lines of evidence suggest that LDL oxidation is likely to occur *in vivo*. Ox-LDL have been demonstrated inside atherosclerotic lesions in man and in LDL receptor-deficient rabbits (Haberland *et al.*, 1988; Yla-Herttuala *et al.*, 1989; Palinski *et al.*, 1989; Boyd *et al.*, 1989), and antibodies against malondialdehyde-LDL, that are independently correlated to atherosclerosis progression, have been reported to be present in human plasma (Salonen *et al.*, 1992). LDL susceptibility to oxidation *in vitro* is independently correlated to coronary atherosclerosis (Rengstrom *et al.*, 1992; Cominacini *et al.*, 1993). Evidence in support of the oxidized LDL hypothesis also comes from studies using antioxidants. LDL carries several antioxidants, such as tocopherols and carotenoids, which protect them from oxidation (Esterbauer *et al.*, 1992). Dietary supplying of vitamin E inhibits LDL oxidation *ex vivo* (Harats *et al.*, 1990; Dieber-Rotheneder *et al.*, 1991; Princen *et al.*, 1992; Jialal & Grundy, 1992; Reaven *et al.*, 1993) and prevents ox-LDL-mediated vascular injury (Belcher *et al.*, 1993). Also, synthetic antioxidants have been reported to possess antiatherosclerotic activity. Butylated hydroxytoluene inhibits accumulation of intimal smooth muscle cells and the development of intimal thickening after balloon injury of the aorta in cholesterol-fed rabbits (Freyschuss *et al.*, 1993). The potent synthetic antioxidant N,N'-diphenyl-phenylenediamine protects LDL from oxidation and delays the progression of atherosclerosis in cholesterol-fed rabbits (Sparrow *et al.*, 1992). Furthermore, probucol introduced as hypolipemic drug, is though to exert antiatherosclerotic effects through its antioxidant activity (Carew *et al.*, 1987; Chilsolm, 1991; Kuzuya & Kuzuya, 1993).

Previous studies from this and other laboratories have demonstrated that dipyridamole, used in clinical practice as an antithrombotic and vasodilator drug, possesses superoxide anion and hydroxyl radical scavenging activity and inhibits lipid peroxidation (Morisaki *et al.*, 1982; Iuliano *et al.*, 1989; 1992; Janero *et al.*, 1989; De La Cruz *et al.*, 1992). The antioxidant activity of the drug has been supported by electron spin resonance spectroscopy which detected a dipyridamole radical as a result of radical scavenging (Iuliano *et al.*, 1995), and unequivocally confirmed by the measurement of reaction constants for the reaction of dipyridamole with the hydroxyl (OH°) and peroxyl radicals (LOO°), highly oxidizing species playing a role in the initiation and propagation of the lipid peroxidation process. The measured $k_{(\text{dipyridamole} + \text{OH}^\circ)}$ of $\sim 10^{10}$ M^{-1} s^{-1} (Iuliano *et al.*, 1989) and $k_{(\text{dipyridamole} + \text{LOO}^\circ)}$ of

[1]Author for correspondence.

2×10^6 M^{-1} s^{-1} (Iuliano *et al.*, 1995) are of the same order of magnitude as the constants of well-known OH° scavengers and of the LOO° scavenger, vitamin E. Thus, we reasoned that dipyridamole would maintain its antioxidant activity in a more relevant physiological environment. LDL oxidation was chosen to address this question, and also because of the increasing evidence supporting the 'LDL hypothesis' and the potential antioxidant therapy of atherosclerosis.

In the present work, we demonstrate that dipyridamole is a potent antioxidant that very effectively prevents oxidation of LDL *in vitro*

Methods

Preparation of LDL

LDL (density 1.025–1.050 g ml^{-1}) was obtained from human plasma of healthy donors (age 24–55) by sequential flotation ultracentrifugation (Hatch & Lees, 1968), in the presence of EDTA to minimize oxidation. LDLs were collected by upward fractionation to minimize albumin contamination. The purity of LDL evaluated by agarose gel electrophoresis was >98%. Prior to oxidation of LDL, the purified lipoproteins were first desalted by Sephadex G25 chromatography, at 4°C, using phosphate buffer saline (PBS) containing 20 μM EDTA as exchanging buffer. Protein concentration determined by the bicinchoninic acid method (Smith *et al.*, 1985) was about 8 mg ml^{-1}. LDL was stored under N_2 at 4°C.

LDL oxidation by copper monitored by continuous u.v.-measurement of dienes

Copper-mediated LDL oxidation was monitored kinetically at 234 nm at 37°C in a PE-Lambda 2 instrument (Perkin Elmer Ltd., Beaconsfield, England) equipped with a Peltier thermostatted 6 positions cuvette holder. Oxidation was initiated by adding 5 μM Cu^{2+} to LDL (0.05 mg ml^{-1}) in PBS pH 7.4. Inhibitors were added to LDL as ethanolic solution and preincubated at 37°C for 30 min before starting the experiment.

LDL oxidation by copper/hydrogen peroxide monitored by the thiobarbituric acid reaction and by iodometric measurement of hydroperoxides

Oxidation was made by exposing LDL (0.2 mg ml^{-1}) to 100 μM Cu^{2+} in 2 mM phosphate buffer pH 7.35 containing 40 μM hydrogen peroxide, at 37°C. At time intervals, aliquots of the reaction mixture were taken to measure the extent of lipid peroxidation evaluating the thiobarbituric acid (TBA) reactive substances and hydroperoxides. The TBA reaction was made essentially as previously described (Iuliano *et al.*, 1995). Briefly, to 100 μl of LDL solution was added 15 μl butylated hydroxytoluene (2% in ethanol, freshly prepared), 10 μl EDTA 50 mM and 1 ml TBA test solution (1% TBA w/v in 50 mM NaOH, 20% trichloracetic acid, 1:9). The solution was incubated in boiling water for 15 min and after cooling was read in a PE-LS50 instrument (Perkin Elmer Ltd., Beaconsfield, England); intrument settings: excitation wavelength 515 nm, emission wavelength 553 nm, and 10 nm slit width. The entity of oxidation was expressed as malondialdehyde equivalents (MDA) using as standard MDA obtained by acid hydrolysis of tetraethoxypropane (Bull & Marnett, 1985). Hydroperoxides (LOOH) were measured by the method of 'Cramer (1991)' on chloroform:methanol (2:1) extracts of 100 μl aliquots of the LDL solution diluted with 300 μl saline and acidified to pH 3.5 with citric acid; the triiodide ion was measured at 353 nm and conversions were based on the molar absorbance of 2.3×10^4 M^{-1} cm^{-1}.

LDL oxidation by peroxyl radicals monitored by cis-parinaric acid fluorescence

The fluorescent polyunsaturated fatty acid *cis*-parinaric acid (9,11,13,15-octadecatetraenoic acid) was used as a sensitive probe to monitor LDL oxidation (Laranjinha *et al.*, 1992). The fluorescent probe was incorporated into LDL by a 5 min incubation at 37°C (probe/LDL-protein ratio of 0.05 nmol μg^{-1}). The oxidation of labelled LDL, dissolved in PBS containing 50 μM EDTA (PBS-EDTA), was initiated by addition of 5 mM ABAP in stirred samples. Instrument settings: excitation wavelength 324 nm, emission wavelength 413 nm, and 3.5 nm slit width.

LDL oxidation by peroxyl radicals monitored by the iodometric measurement of hydroperoxides

Peroxyl radicals generated by thermolysis of ABAP (5 mM) (Iuliano *et al.*, 1989) were used to produce LDL oxidation. LDL (0.4 mg ml^{-1}), in PBS-EDTA, were incubated in thermostatted cuvettes with continuous stirring. Oxidation was carried out at 37°C for 4 h. At time intervals the extent of lipid peroxidation was measured by the TBA reaction and by the hydroperoxide assay.

Apo B100 changes during oxidation monitored by fluorescence spectroscopy and by agarose gel electrophoresis

Aproprotein associated fluorescence changes (Cominacini *et al.*, 1991; Esterbauer *et al.*, 1992) during oxidation were monitored kinetically in the fluorimeter equipped with a thermostatted cuvette holder and an external stirring device. Instrument settings: excitation wavelength 360 nm, emission wavelength 430 nm, and 5 nm slit width. LDL (0.3 mg ml^{-1}) oxidation by 200 μM copper was carried out in 2 mM phosphate buffer pH 7.4 in the presence of 50 μM hydrogen peroxide. Oxidation by peroxyl radicals was started by adding 20 mM ABAP to 0.6 mg ml^{-1} LDL in PBS-EDTA. The change in electric charge of the protein following oxidation was evaluated by agarose gel electrophoresis in comparison to MDA-derivatized LDL. Derivatization was made at 37°C for 60 min, by reacting LDL (0.2 mg ml^{-1}) with 200 mM MDA obtained by acid hydrolysis of tetraethoxypropane (Bull & Marnett, 1985). Free amino groups on LDL were titrated with TNBS (Habeeb, 1966) using valine as standard. Lipoprotein electrophoresis was performed in barbiturate buffer (50 mM 5,5-diethylbarbituric acid sodium salt, pH 8.6) on Paragon agarose gel blotters and stained with Sudan Black B. Samples under oxidation were placed on ice after addition of 10 mM EDTA to stop the reaction.

LDL oxidation by endothelial cells

Primary cultures of human umbilical vein endothelial cells were obtained from cord vein, after 15 min digestion by 0.2% collagenase solution (Jaffe *et al.*, 1973). Cells were plated into a 75 cm^2 tissue culture flask and allowed to grow to confluence in M199 containing 20% foetal calf serum, 10 u ml^{-1} penicillin, 10 μg ml^{-1} streptomycin and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Confluent human endothelial cell cultures in multiwell clusters (1.5×10^5 cells cm^{-2}) were washed three times with serum-free medium, supplemented with 5 μM $CuSO_4$, and incubated with LDL (0.5 mg ml^{-1}) in serum-free medium containing 1% human serum albumin. Before addition to endothelial cells LDL was loaded (30 min at 37°C) with vitamin E, dipyridamole or their vehicles (DMSO or ethanol respectively), and sterilized by passage through 0.22 μm Millipore filters. After 18 h incubation at 37°C the medium was aspirated, centrifuged to remove cell debris and processed for lipid peroxidation assay by the thiobarbituric acid reaction as described above.

Degradation of oxidized LDL by monocyte-macrophages

Human monocytes were obtained from the buffy coat of citrated, freshly donated blood. The buffy coat was diluted 1 : 1 with PBS and underlayered with Ficoll-Paque for separation of mononuclear cells by density gradient centrifugation. Mononuclear cells, washed and resuspended in RPMI 1640 medium, were seeded in a 24 wells plate, and incubated 2 h at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. After removing non adherent mononuclear cells, monocytes were resuspended and cultured in RPMI medium. Uptake and degradation of oxidized LDL was performed in competition with [^{125}I]acetyl-LDL essentially as described by Goldstein & Brown (1974). LDL was radiolabelled with ^{125}I using the iodine monochloride method (Bilheimer *et al.*, 1972). The final specific activity was 200 c.p.m. ng^{-1} protein. [^{125}I]LDL was acetylated by sequential addition of acetic anhydride (Frankel-Konrat, 1957). The extent of derivatization determined by TNBS reactivity was about 80%. After treatments LDL were freed from reactants by gel filtration with Sephadex G25.

Cytotoxicity assay

Epstein Barr virus transformed B cells (EBVB-cells) were used as target cells and maintained in culture in RPMI 1640 medium added with 10% FCS. Cytotoxicity was evaluated by ^{51}Cr release. EBVB-cells were loaded 2 h at 37°C with 0.1 mCi ^{51}Cr, washed three times to remove the unloaded radiotracer and resuspended in RPMI containing 0.1% FCS (100,000 cells/ml). After adding 90 μg ml^{-1} of the LDL preparation cells were incubated 4 h at 37°C. Cytotoxicity was evaluated by counting the radioactivity of supernatants in comparison to the total radioactivity of Triton X-100 lysed cells.

Materials

$CuSO_4$, hydrogen peroxide, ICI and tetraethoxypropane were purchased from Aldrich (Milwaukee, PA, U.S.A.); bicinchoninic acid from Pierce (Rockford, IL, U.S.A.); medium-M199, medium RPMI 1640 (RPMI), foetal calf serum (FCS), penicillin, streptomycin, L-glutamine were from Biochrom KG (Berlin, Germany); Paragon agarose gel precoated plates from Beckman (Fullerton, CA, U.S.A.); α-tocopherol, probucol, Sephadex G25, trinitrobenzene sulphonic acid (TNBS) and collagenase type IA were from Sigma (St. Louis, MO, U.S.A.); *cis*-parinaric acid from Molecular Probes (Eugene, OR, U.S.A.); 2,2′-azo-*bis*(2-amidino propane) hydrochloride (ABAP) was from Polysciences Inc. (Warrington, PA, U.S.A.). Carrier free Na^{125}I and Na^{51}Cr were from Amersham International Ltd. (Buckinghamshire, U.K.). Ascorbic acid and all other reagents were of the highest grade available from Merck (Darmstadt, Germany). Dipyridamole, vitamin E and probucol were dissolved in ethanol; ascorbic acid was dissolved immediately before use in metal-free distilled water (>18 Mohms). Vitamin E was dissolved in dimethylsulphoxide (DMSO) for experiments with endothelial cells. Ethanol and DMSO were present in the controls, apart from that of ascorbic acid, at the final concentration of 0.25%. Disposable sterile plastic ware for cell culture was from Costar (Cambridge, MA, U.S.A.). Water of high purity (>18 Mohms) was obtained by treating twice distilled water in a Milli-Q purifying system (Millipore, Bedford, MA, U.S.A.).

Results

Effects of dipyridamole on copper-dependent LDL oxidation

LDL oxidation by copper can be followed at 234 nm directly in solution (Esterbauer *et al.*, 1992). The reaction kinetics of diene formation is composed of three phases (Figure 1, curve a). The first is the lag phase of very low oxidation rate due to counterbalance and consumption of endogenous antioxidants. The second phase corresponds to the maximal rate of oxidation and starts when the antioxidants are consumed. The third phase is the termination phase that is associated with a plateau in diene formation. Addition of antioxidants leads to a prolongation of the lag phase and this is also the case for dipyridamole (Figure 1, curves b, c). The inhibition period of diene formation in the presence of dipyridamole is concentration-dependent as shown by the plot of dipyridamole concentration vs. lag time (Figure 1, inset); it is interesting that the regression line fits with the zero inhibitor concentration value of lag time. The protective effect of dipyridamole is also confirmed in an amplified system of LDL oxidation driven by the combination of copper and hydrogen peroxide. Also in this system, in which a rapid and intense LDL oxidation is reached within 1 h, dipyridamole protects LDL from oxidation (Figure 2). Again the characteristic behaviour of antioxidants is demonstrated by the curvature of oxidation vs. time traces as dipyridamole induces a concentration-dependent lag phase (Figure 2). In the same set of experiments dipyridamole induces a more pronounced inhibition of LDL oxidation if this is measured in terms of

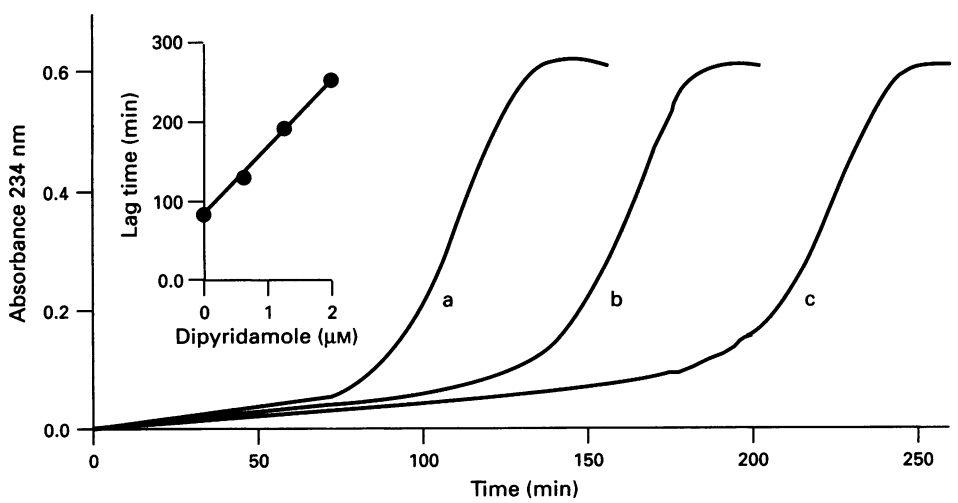

Figure 1 Effect of dipyridamole on copper-induced LDL oxidation monitored by diene evolution. (a) Control; dipyridamole 0.625 μM (b) and 1.25 μM (c). Inset: concentration-dependence of the inhibition period of diene formation. Each point represents the mean of three separate experiments with average variations <5%, error bars are not shown as they do not exceed the size of the symbols.

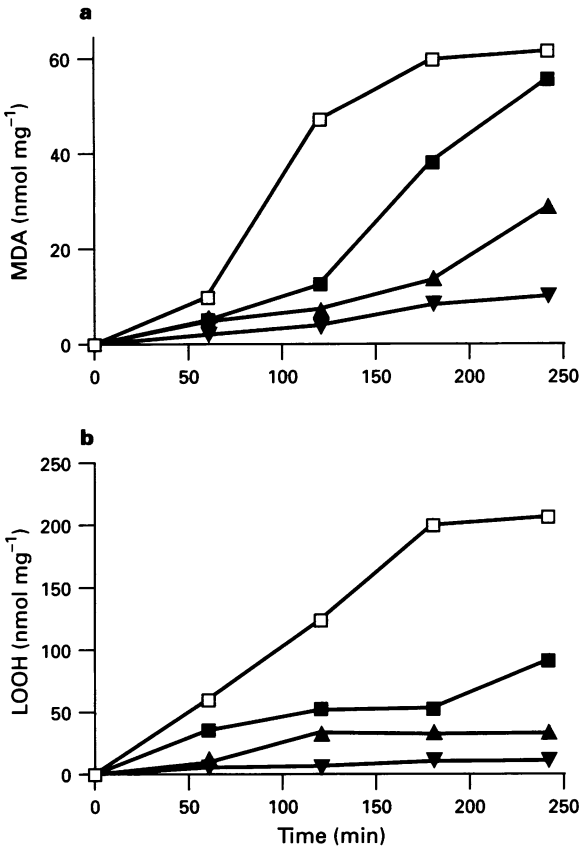

Figure 2 Effect of dipyridamole on LDL oxidation by Cu^{2+}/H_2O_2: LDL (0.2 mg ml^{-1}) in 2 mM phosphate buffer was oxidized by sequential addition of 40 μM H_2O_2 and 100 μM Cu^{2+}. Oxidation was monitored by the TBA reaction (a) and by the iodometry of hydroperoxides (b). Control (□); dipyridamole 2.5 μM (■), 5 μM (▲) and 7.5 μM (▼). Each point represents the mean of duplicate determinations; representative of at least three separate experiments with average variations <5%.

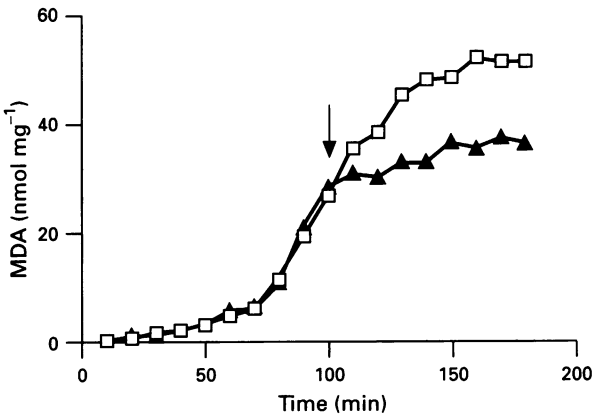

Figure 3 Inhibition of LDL oxidation by dipyridamole added (arrow) during the oxidation process, 100 min after the start with Cu^{2+}/H_2O_2. Control (□); dipyridamole 5 μM (▲). Representative experiment.

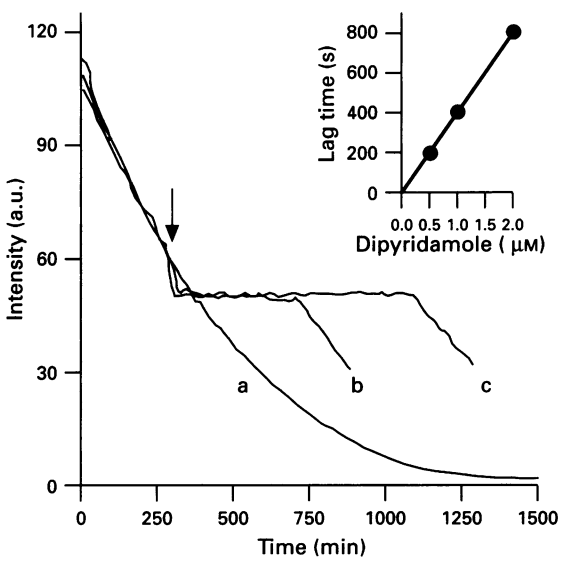

Figure 4 Monitoring of LDL oxidation by the quenching of *cis*-parinaric acid fluorescence. The loss of fluorescence of *cis*-parinaric acid upon oxidation of LDL by peroxyl radicals is represented in trace (a). Dipyridamole (b–c) added half way through the oxidation process causes suppression of fluorescence decay for a time proportional to the antioxidant concentration; trace (b), 0.5 μM, trace (c), 1 μM. Inset, concentration-dependence of the inhibition period of fluorescence quenching. Fluorescence expressed as arbitrary units (a.u.). Each point represents the mean of three separate experiments with average variations <5%.

hydroperoxide evolution: the lowest concentration of drug (2.5 μM) at 240 min inhibits only by 10% the generation of TBA-reactive substances, but blocks almost completely the hydroperoxide evolution. Dipyridamole added half-way through the reaction induces a sharp break of the oxidation curve (Figure 3), characteristic of highly efficient antioxidants that directly inhibit the chain propagation.

Effect of dipyridamole on peroxyl radical dependent LDL oxidation

To investigate further the activity of dipyridamole, LDL was oxidized in a metal independent way by peroxyl radicals. A controlled flux of peroxyl radicals was generated by thermolysis of ABAP, and oxidation was monitored by the fluorescence of the hydrophobic probe *cis*-parinaric acid pre-incorporated into LDL. This polyunsaturated fluorescent fatty acid has been used as a very sensitive probe to monitor the initial phases of lipid peroxidation (Laranjinha *et al.*, 1992). The four double bonds render the molecule very sensitive to oxidation that is associated with a loss of intrinsic probe fluorescence. Figure 4 shows the decrease in fluorescence emission of *cis*-parinaric acid incorporated into LDL after exposure to a flux of peroxyl radicals. Dipyridamole added half way through the oxidation reaction suppresses the fluorescence decay of *cis*-parinaric acid, for a time proportional to the antioxidant concentration (Figure 4, inset), after that fluorescence decay is resumed at the same rate as the control assay. A further demonstration of the protective action of dipyridamole in the peroxyl radical-mediated LDL oxidation is given by the experiments illustrated in Figure 5 that show the time course of MDA and hydroperoxides evolution. The rise in MDA during the oxidation of LDL induced by ABAP is kinetically different from that induced by the copper/H_2O_2 system. Comparable amounts of MDA equivalents are formed by copper/H_2O_2 three times faster than for ABAP. In contrast, hydroperoxide evolution is doubled in the ABAP-mediated oxidation. It should be remembered that the two oxidation methods have completely different characteristics: bolus oxidation of H_2O_2 causes an initial short lived burst in radical formation, whereas ABAP thermolysis produces a slow but steady flux of radicals.

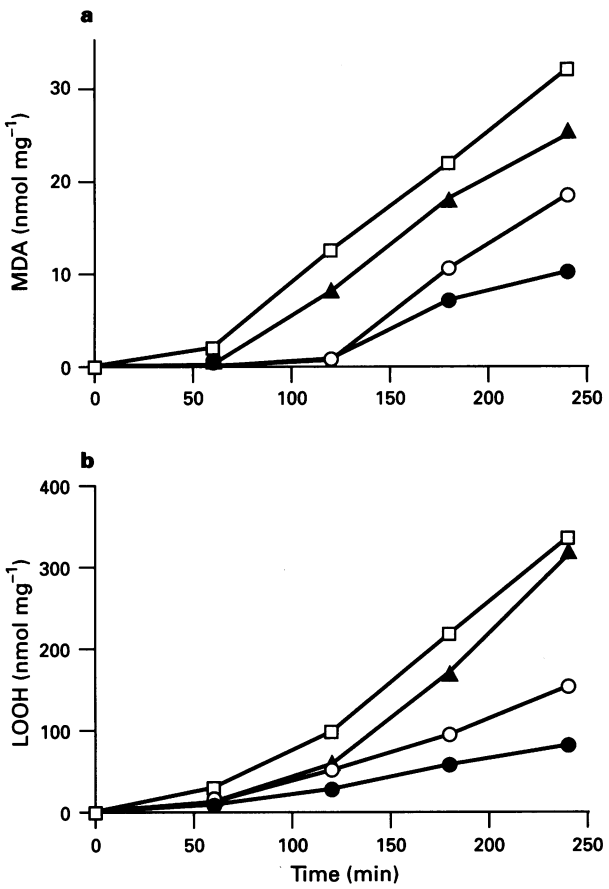

Figure 5 Effect of dipyridamole on peroxyl radical-dependent LDL oxidation. Oxidation of LDL induced by peroxyl radicals generated by thermolysis of ABAP (5 mM) was performed in stirred sample. At various time intervals lipid peroxidation products were measured by the TBA reaction (a) and by iodometry of hydroperoxides (b). Control (□); dipyridamole 5 μM (▲), 10 μM (○), and 20 μM (●). Each point represents the mean of duplicate determinations; representative of at least three separate experiments with average variations <5%.

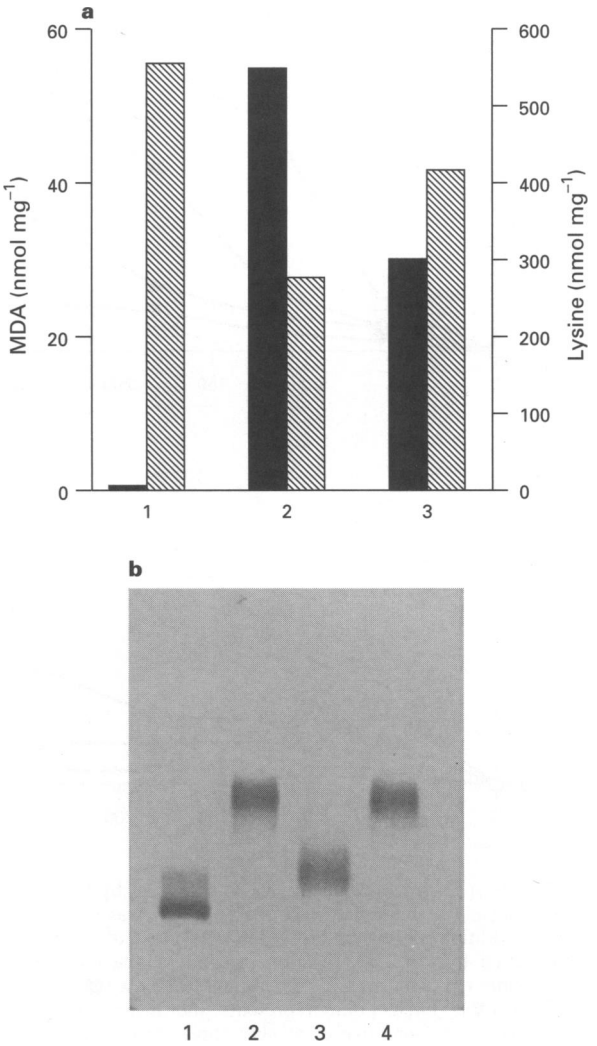

Figure 6 Effect of dipyridamole on electrophoretic changes of LDL after oxidation. Histogram (a), amount of MDA equivalents (solid columns) and the loss of TNBS titrable lysine residues (hatched columns) generated after oxidation of LDL by Cu^{2+}/H_2O_2. Electrophoretic changes are shown in (b): (1) native LDL; (2) ox-LDL; (3) LDL oxidized in the presence of 5 μM dipyridamole; (4) MDA-derivatized LDL.

Effect of dipyridamole on the apo B100 changes of LDL associated with lipid oxidation

LDL oxidation is accompanied by a modification of the protein component characterized by an increased electronegativity attributed to derivatization of lysine residues by products of lipid peroxidation (Steinbrecher *et al.*, 1989). As shown in Figure 6, oxidation of LDL by Cu^{2+}/H_2O_2 causes a loss of titratable lysine residues and an increased electrophoretic mobility (Figure 6, column of sample 2 and lane 2 of the blot) similar to that of malondialdehyde-derivatized LDL (Figure 6, lane 4). In the presence of 5 μM dipyridamole, the inhibition of both lipid peroxidation and lysine derivatization (Figure 6, column of sample 3) is reflected to a reduced electrophoretic change (Figure 6, lane 3); higher dipyridamole concentrations that totally prevent lipid peroxidation cause a complete protection from lysine residue modification and electrophoretic alterations (data not shown). These results are in agreement with literature data demonstrating that products of fatty acid peroxidation are responsible for derivatization of apolipoprotein B (Steinbrecher *et al.*, 1989). Further support to the protective effect of dipyridamole towards LDL modifications during oxidation are given by fluorescence studies. Modification of apo B100 during LDL oxidation is also associated with a strong increase of protein instrinsic fluorescence at 360/430 nm excitation/emission wavelengths. Such changes can be followed kinetically and are shown in Figure 7. Dipyridamole dose-dependently prevents formation of the protein-bound fluorophore both in the copper- and in peroxyl radical-driven oxidation.

Experiments at cellular level

With the aim of substantiating whether the antioxidant drug is also acting under more physiological conditions, experiments at cellular level were undertaken using human endothelial cells to oxidize LDL. Dipyridamole successfully inhibited endothelial cell-mediated LDL oxidation in a dose-dependent fashion (Figure 8) and 50% inhibition was calculated to require about 2 μM. The concentrations of antioxidant and vehicle applied did not induce any toxic effect to endothelial cells: (i) they kept the characteristic cobblestone morphology; (ii) no detachment was observed after the incubation period; (iii) cells washed at the end of the incubation period were able to oxidize LDL again. In addition, in a series of parallel experiments the LDH release, as a parameter of cytoplasmic leakage, was measured (Iuliano *et al.*, 1994a) and no significant difference in LDH release was observed for treated and untreated samples (data not shown). By inhibiting the oxidative modification, dipyridamole decreased the scavenger receptor-mediated degradation of LDL as shown in Figure 9, where the effect of dipyridamole-protected-LDL can be compared to those of ox-

LDL, acetyl-LDL and native-LDL. In addition, dipyridamole prevented the toxic effect of oxidized LDL on target cells. As shown in Figure 10, an LDL preparation oxidized by peroxyl radicals carrying about 140 nmol LOOH mg^{-1} protein was able to induce significant ^{51}Cr release from the target cells. In contrast, similarly ox-LDL in the presence of dipyridamole, which reduced the level of hydroperoxides by 80%, did not induce appreciable ^{51}Cr release.

Comparison between dipyridamole, ascorbic acid, vitamin E and probucol

The protective activity of dipyridamole towards LDL oxidation was compared with the activity exerted by the synthetic antioxidant, probucol and the physiological antioxidants, ascorbic acid and vitamin E. Inhibitory activity was studied in the LDL oxidation by copper, copper/hydrogen peroxide and by endothelial cells. Cumulative data, shown in Table 1, are based on analysis of MDA and hydroperoxide formation and fluorescence of Apo B100. The order of increasing activity was dipyridamole > probucol > ascorbic acid > vitamin E. The magnitude of antioxidant activity of dipyridamole is >20 times higher than vitamin E. In the endothelial cell-mediated LDL oxidation, the IC_{50} was 2.1 and 20 μM for dipyridamole and vitamin E, respectively. Inhibition of MDA and hydro-

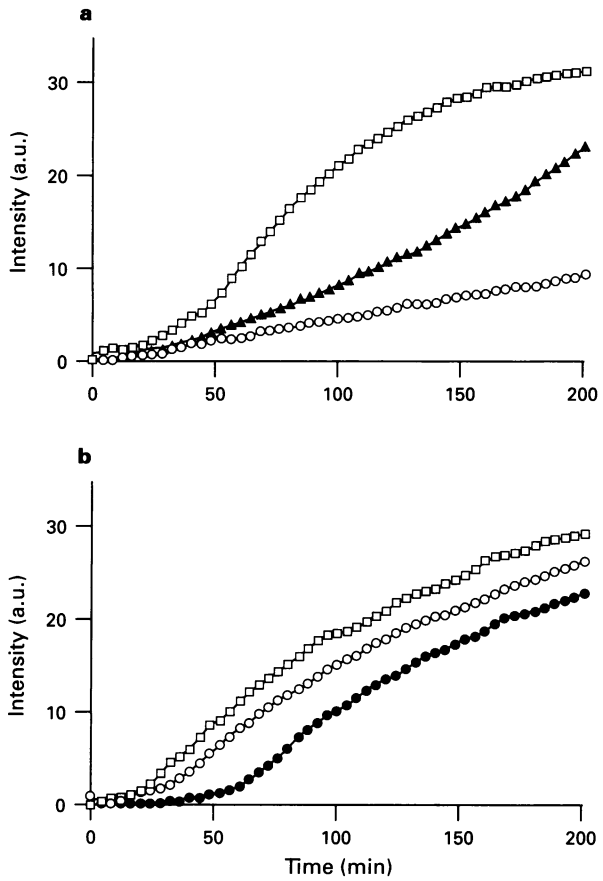

Figure 7 Inhibition of oxidation-mediated Apo B100 fluorescence changes by dipyridamole. Protein fluorescence at 430 nm (with 360 nm excitation) was monitored kinetically in samples incubated in the thermostatted cuvette, under stirring. (a) Cu(II)-dependent oxidation; (b) peroxyl radical-dependent oxidation, Control (□); dipyridamole 5 μM (▲), 10 μM (○), and 20 μM (●). Traces are displayed substracted from the background value. Fluorescence expressed as arbitrary units (a.u.). Representative of at least three separate experiments with average variations <5%.

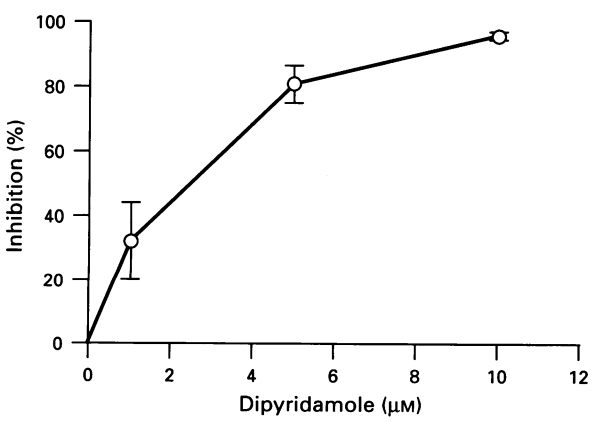

Figure 8 Concentration-dependent inhibition of endothelial cell-mediated oxidation by dipyridamole. Each data point represents mean ± s.d. of three separate experiments.

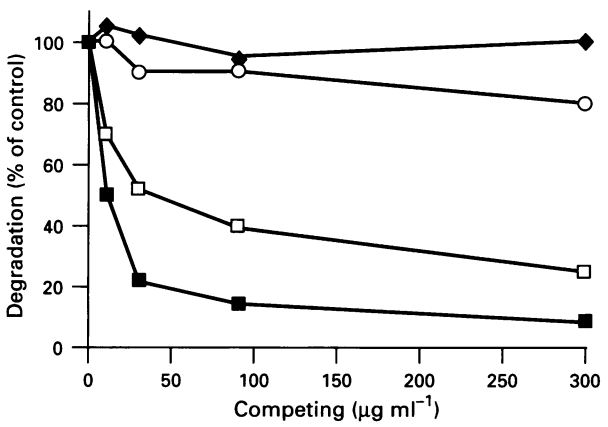

Figure 9 Competition of [^{125}I]LDL degradation by scavenger-receptor. Competition was performed using 5 $\mu g\,ml^{-1}$ of [^{125}I]acetyl-LDL and varying amounts of unlabelled LDL preparations. LDL was oxidized by Cu^{2+}/H_2O_2 in the absence (□) or presence of 10 μM dipyridamole (○). Native LDL (◆); acetyl-LDL (■). Each point is the mean of duplicate determinations. The 100% values of degradation of [^{125}I]acetyl-LDL in the absence of competitor was 2.1 $\mu g\,5h^{-1}\,mg^{-1}$ of protein.

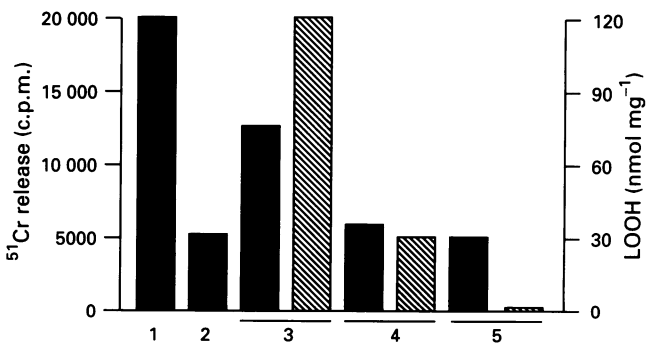

Figure 10 Cytotoxicity of oxidized LDL towards EBVB cells. EBVB cells loaded with ^{51}Cr (solid columns) were exposed to ox-LDL (3), LDL oxidized in the presence of 10 μM dipyridamole (4) or native LDL (5). Total radioactivity (Triton X-100 lysed cells) (1); background radioactivity of the supernatant (2). Oxidation of LDL was induced by 5 mM ABAP as described in the methods, the entity of oxidation in each LDL preparation is depicted by the hatched columns. Each column is the mean of duplicate determinations.

Table 1 Relative antioxidant activity of dipyridamole vs. the synthetic antioxidant probucol and the natural occurring antioxidants ascorbic acid and vitamin E

	Lipid peroxidation (¶) (Cu/H_2O_2)		*Apo B100 fluorescence (¶)*		*EC-mediated oxidation (‡)*
	MDA	*LOOH*	*Cu/H_2O_2*	*ABAP*	
Ascorbate	19.2	48.0	30.1	55.7	25.4
Dipyridamole	1.6	2.2	3.7	5.9	2.3
Probucol	20.7	14.0	18.5	16.2	9.6
Vitamin E	37.2	24.3	(§)	(§)	20.4

For each antioxidant data are obtained from concentration-dependent inhibition experiments. Data are shown as μM IC_{50} (concentration that gives 50% inhibition) calculated from the integral of the area under the time course profile (¶) or from the data points at the end of oxidation process (‡). (§) Very low, non-linear, inhibition.

peroxide formation required 10–20 times more vitamin E than dipyridamole, and vitamin E caused only minimal protection of Apo B100 oxidative alterations ($\approx$10% inhibition by 250 μM). Compared to ascorbic acid, the activity of dipyridamole is 10 times higher, apart from the diene system where the difference is reduced to a factor of 2.5. Finally, dipyridamole is more potent than probucol by a factor ranging from 3 to 12 times, depending on the system.

Dipyridamole partitioning into LDL

Dipyridamole interaction with the LDL is demonstrated by fluorescence measurements shown in Figure 11. In the presence of LDL the emission spectrum of dipyridamole in the region 400–600 nm is slightly blue-shifted with a large increase in maximal intensity, resembling the spectrum of dipyridamole in a polar organic solvent (Tabak & Borisevitch, 1992). This indicates the transfer from the aqueous solution to a non-aqueous medium. From the slope of the double reciprocal plot reported in the inset of Figure 11 a binding constant of 2.3×10^5 M^{-1} is calculated, indicating that reaction of dipyridamole with LDL is likely to take place at the level of the polar head groups of phospholipids without penetrating the LDL core. That dipyridamole is not associated with the LDL core, due to insolubility in saturated hydrocarbons, can be demonstrated by gel filtration chromatography with Sephadex that permits displacement of dipyridamole from LDL (data not shown). In addition, LDL incubated with dipyridamole and dialyzed by ultrafiltration are indistinguishable from control LDL in terms of oxidizability (data not shown).

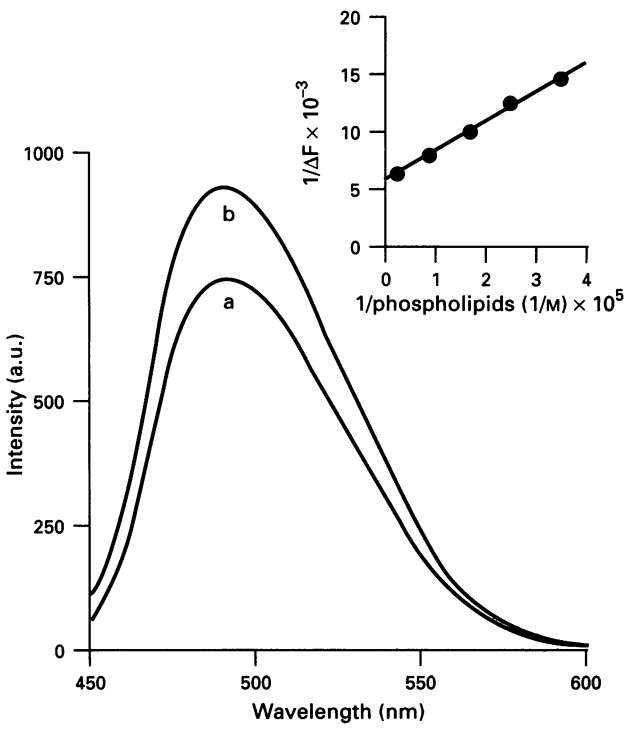

Figure 11 Reaction of dipyridamole with the LDL surface. The fluorescence emission spectrum, excited at 415 nm, of dipyridamole (a) is greatly affected by as little 8.8 μg ml^{-1} LDL (b), as there is a large increase in maximal intensity and a slight blue-shift (emission max shifts from 496 nm to 491 nm). Inset, double reciprocal plot showing the linear response of the fluorescence increment by the added LDL, calculation is based on the phospholipid mass (assuming an average molecular weight of 778) of LDL; $K_a = 2.3 \times 10^5$ M^{-1}.

Discussion

This work extends previously reported studies (Iuliano *et al.*, 1994b; Selley *et al.*, 1994) demonstrating that dipyridamole is a powerful inhibitor of LDL oxidation, induced chemically or by endothelial cells. LDL was oxidized chemically by copper ions in the absence or presence of hydrogen peroxide, or by a controlled flux of peroxyl radicals generated by an azo compound. In all cases, dipyridamole inhibited LDL oxidation with the characteristic induction of a concentration-dependent lag time, similar to classic antioxidants. The protective effect was verified by different methods: measurement of dienes, MDA, hydroperoxide, electrophoretic mobility, lysine titration, apoprotein fluorescence and quenching of *cis*-parinaric acid fluorescence, providing unequivocal evidence of the powerful antioxidant activity of the drug. Decisively, dipyridamole inhibited LDL oxidation at the cellular level. Endothelial cells have been proposed as one of the sources responsible for LDL modification *in vivo* (Steinberg *et al.*, 1989), by a free radical mediated mechanism. Inhibition of the oxidative modification of LDL is a crucial event in the suggested mechanism of atherosclerosis. Among several biological characteristic of ox-LDL is the uptake and degradation by the macrophage scavenger receptor, which causes the formation of foam cells, and cytoxicity to most cells (Cathcart *et al.*, 1989; Kuzuya *et al.*, 1991). By inhibiting LDL oxidation, dipyridamole prevented both LDL degradation by macrophages and LDL cytotoxicity, at concentrations (1–10 μM) within the range found in plasma after pharmacological doses (FitzGerald, 1987). To support further the physiological relevance of these results, dipyridamole was compared to ascorbic acid and vitamin E, which can be considered as reference antioxidants in biological systems, and with probucol. Vitamin E, a normal constituent of LDL, is generally thought to function as the major lipid-soluble antioxidant (Burton & Ingold, 1986), and ascorbic acid is considered the most important aqueous phase antioxidant in plasma. Probucol was selected because most research into LDL oxidation has concentrated on the protec-

tion by this synthetic compound. In all instances, dipyridamole produced the most potent inhibition of LDL oxidation. The LDL oxidation is a complex mechanism involving initiator radicals generated by Fenton-like chemistry, and propagating carbon-oxygen radicals, like peroxyl radicals. The high efficiency of dipyridamole can be attributed to scavenging of O_2^- (Iuliano *et al.*, 1989), OH° (Iuliano *et al.*, 1992) and peroxyl radicals (Iuliano *et al.*, 1995). Thus, dipyridamole should operate at the two main levels, the initiation and propagation, of the lipid peroxidation. In addition the superiority of dipyridamole can be explained in terms of partitioning in the LDL-lipids/aqueous phase and by better accessibility to sites of free radical attack. Dipyridamole, as demonstrated in this study, strongly associates with LDL phospholipids (binding constant: 2.3×10^5 M^{-1}), and is sufficiently water soluble to be able to intercept both the oxidation initiating radicals coming from the aqueous phase and the lipid radicals generated during the chain reaction. In contrast, probucol and vitamin E, being located entirely within the lipoprotein (Burton & Ingold, 1986; McLean & Hagaman, 1989), do not effectively intercept radicals formed initially in the aqueous phase (Iuliano *et al.*, 1995). In contrast, ascorbic acid does not have access to radicals formed in the lipid phase. The low protection by vitamin E, reported in the present paper, is in agreement with Stocker's suggestion that vitamin E is not an efficient antioxidant without a suitable water-soluble reductant, and in particular conditions it is actually a prooxidant (Bowry & Stocker, 1993). Higher antioxidant activity of dipyridamole towards vitamin E has been reported for systems of arachidonic acid micellae (Iuliano *et al.*, 1995) and for liver membranes (Iuliano *et al.*, 1992). In the literature there are no data available comparing dipyridamole with probucol and ascorbic acid.

Taken together these data show that dipyridamole is a powerful inhibitor of LDL oxidation *in vitro* and suggest evaluation of this activity *in vivo*. Several studies have demonstrated that antioxidants inhibit the atherosclerosis process in animal models. Probucol, originally used in human subjects as a hypolipaemic drug is thought to be antiatherogenic due to its antioxidant activity (Carew *et al.*, 1987; Steinberg *et al.*, 1989). Other suggested antioxidants are currently being tested (Rice-Evans & Diplock, 1993) but such compounds are not necessarily safe: the potent synthetic antioxidant N,N'-diphenyl-phenylenediamine is mutagenic and is not suitable for studies in human subjects (Sparrow *et al.*, 1992).

Dipyridamole has been used for a long time as an antithrombotic drug, on the basis of its antiplatelet activity (FitzGerald, 1987). However, a recent metanalysis clearly indicated that dipyridamole does not affect the occurrence of acute cardiovascular events in patients at risk (APT collaboration, 1988). On the other hand, dipyridamole has been proved to delay the progression of peripheral occlusive arterial disease (Hess *et al.*, 1985). This prospective arteriographic study demonstrated that the combination of dipyridamole and aspirin lowered progression of the disease more than aspirin alone, and the effects were attributed to platelet inhibition.

In conclusion, results reported in this paper extend data presented in previous papers (Morisaki *et al.*, 1982; Iuliano *et al.*, 1989; 1992; 1994b; 1995; Janero *et al.*, 1989; De La Cruz *et al.*, 1992; Selley *et al.*, 1994) documenting the antioxidant activity of dipyridamole at the physiological level and provide elements to plan *in vivo* studies to assess the potential usefulness of dipyridamole as an antioxidant agent in the prevention of atherosclerosis progression.

We are indebted with C. Piccheri for fruitful collaboration. This work was supported by CNR (grant to L.I. No. 06152, 95.02298.04). Some of this work was presented at The Clinical Research Meeting held in Baltimore, April 29–May 2 1994, and published in abstract from (*Clin. Res.*, (1994), **42**, 178A).

References

APT COLLABORATION. (1988). Secondary prevention of vascular disease by prolonged antiplatelet treatment. *Br. Med. J.*, **296**, 320–331.

AUTIO, I., JAAKKOLA, O., SOLAKIVI, T. & NIKKARI, T. (1990). Oxidized low density lipoprotein is chemotactic for arterial smooth muscle cells in culture. *FEBS Lett.*, **227**, 247–249.

BELCHER, J.D., BALLA, J., JACOBS, D.R., GROSS, M., JACOB, H. & VERCELLOTTI, G.M. (1993). Vitamin E, LDL, and endothelium. Brief oral vitamin E supplementation prevents oxidized LDL-mediated vascular injury in vitro. *Arterioscler. Thromb.*, **13**, 1779–1789.

BILHEIMER, D.W., EISENBERG, S. & LEVY, R.I. (1972). The metabolism of very low density lipoproteins. *Biochim. Biophys. Acta*, **260**, 212–221.

BOWRY, V.W. & STOCKER, R. (1993). Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J. Am. Chem. Soc.*, **115**, 6029–6044.

BOYD, H.C., GROWN, A.M., WOLFBAUER, G. & CHAIT, A. (1989). Direct evidence for a protein recognized by a monoclonal antibody against oxidatively modified LDL in atherosclerotic lesions from Watanabe heritable hyperlipidemic rabbits. *Am. J. Pathol.*, **135**, 815–825.

BULL, A.W. & MARNETT, L.J. (1985). Determination of malondialdehyde by ion-pairing high performance liquid chromatography. *Anal. Biochem.*, **149**, 284–290.

BURTON, G.W. & INGOLD, K.U. (1986). Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.*, **19**, 194–201.

CAREW, T.E., SCHWNKE, D.C. & STEIMBERG, D. (1987). Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7725–7729.

CATHCART, M.K., MCNALLY, A.K., MOREL, D.W. & CHISOLM, G.M. (1989). Superoxide anion participation in human monocyte-mediated oxidation of low-density lipoprotein and conversion of low-density lipioprotein to a cytotoxin. *J. Immunol.*, **142**, 1963–1969.

CHILSOLM, G.M. (1991). Antioxidants and atherosclerosis: a current assessment. *Clin. Cardiol.*, **14**, I-25–3Q.

COMINACINI, L., GARBIN, U., DAVOLI, A., MICCIOLO, R., BOSELLO, O., GAVIRAGHI, G., SCURO, L.A. & PASTORINO, M. (1991). A simple test for predisposition to LDL oxidation based on the fluorescence development during copper-catalyzed oxidative modification. *J. Lipid Res.*, **32**, 349–358.

COMINACINI, L., GARBIN, U., PASTORINO, A.M., DAVOLI, A., CAMPAGNOLA, M., DE SANTIS, A., PASINI, C., FACCINI, G.B., TREVISAN, M.T., BERTOZZO, L., PASINI, F. & LO CASCIO, V. (1993). Predisposition of LDL oxidation in patients with and without angiographically established coronary artery disease. *Atherosclerosis*, **99**, 63–70.

CRAMER, G.L., MILLER, J.F., PENDLETON, R.B. & LANDS, W.E.M. (1991). Iodometric measurement of lipid hydroperoxides in human plasma. *Anal. Biochem.*, **193**, 204–211.

DE LA CRUZ, J.P., CARRASCO T., ORTEGA, G. & SANCHEZ DE LA CUESTA, F. (1992). Inhibition of ferrous-induced lipid peroxidation by pyrimido-pyrimidine derivatives in human liver membranes. *Lipids*, **27**, 192–194.

DIEBER-ROTHENEDER M., PUHL, H., WAEG, G., STRIEGEL, G. & ESTERBAUER, H. (1991). Effect of oral supplementation with D-alpha-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. *J. Lipid. Res.*, **32**, 1325–1332.

ESTERBAUER, H., GEBICKI, J., PUHL, H. & JURGENS, G. (1992). The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Rad. Biol. Med.*, **13**, 341–390.

FITZGERALD, G.A. (1987). Dipyridamole. *New Engl. J. Med.*, **316**, 1247–1257.

FRANKEL-CONRAT, H. (1957). Methods for investigating the essential groups for enzyme activity. *Methods Enzymol.*, **4**, 247–269.

FREYSCHUSS, A., STIKO-RAHM, A., SWEDENBORG, J., HENRIKSSON, P., BJORKHEM, I., BERGLUND, L. & NILSSON, J. (1993). Antioxidant treatment inhibits the development of intimal thickening after balloon injury of the aorta in hypercholesterolemic rabbits. *J. Clin. Invest.*, **91**, 1282–1288.

GOLDSTEIN, J.L. & BROWN, M.S. (1974). Binding and degradation of low density lipoproteins by cultured human fibroblasts. *J. Biol. Chem.*, **249**, 5153–5162.

GOLDSTEIN, J.L., HO, J.K., BASU, S.K. & BROWN, M.S. (1979). Binding sites on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 333–337.

HABEEB, A.F.S.A. (1966). Determination of free amino groups in proteins by trinitrobenzene sulphonic acid. *Anal. Biochem.*, **14**, 328–336.

HABERLAND, M.E., FONG, D. & CHENG, L. (1988). Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science*, **241**, 215–218.

HARATS, D., BEN-NAIM, M., DABACH, Y., HOLLANDER, G., HAVIVI, E., STEIN, O. & STEIN, Y. (1990). Effect of vitamin C and E supplementation on susceptibility of plasma lipoproteins to peroxidation induced by acute smoking. *Atherosclerosis*, **85**, 47–54.

HATCH, F.T. & LEES, R.S. (1968). Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.*, **6**, 1–68.

HESS, H., MIETASCHK, A. & DEICHSEL, G. (1985). Drug-induced inhibition of platelet function delays progression of peripheral occlusive arterial disease. A prospective double-blind arteriographically controlled trial. *Lancet*, **i**, 415–419.

IULIANO, L., PEDERSEN, J.Z., PRATICÒ, D., ROTILIO, G. & VIOLI, F. (1994a). Role of hydroxyl radicals in the activation of human platelets. *Eur. J. Biochem.*, **221**, 695–704.

IULIANO, L., PEDERSEN, J.Z., ROTILIO, G., FERRO, D. & VIOLI, F. (1995). A potent chain breaking activity of the cardiovascular drug dipyridamole. *Free Rad. Biol. Med.*, **18**, 239–247.

IULIANO, L., PRATICÒ, D., GHISELLI, A., BONAVITA, M.S. & VIOLI, F. (1992). Reaction of dipyridamole with the hydroxyl radical. *Lipids*, **27**, 349–353.

IULIANO, L., QUINTARELLI, C., ALESSANDRONI, M., ALESSANDRI, C., COLAVITA, A.R. & VIOLI, F. (1994b). Dipyridamole is a powerful antioxidant that inhibits LDL oxidation by endothelial cells. *Clin. Res.*, **42**, 178A.

IULIANO, L., VIOLI, F., GHISELLI, A., ALESSANDRI, C. & BALSANO, F. (1989). Dipyridamole inhibits lipid peroxidation and scavenges oxygen free radicals. *Lipids*, **24**, 430–433.

JAFFE, E.A., NACHMAN, R.L., BECKER, C.G. & MINICK, C.R. (1973). Culture of human endothelial cells derived from human umbilical veins. *J. Clin. Invest.*, **27**, 2745–2756.

JANERO, D.R., BURGHARDT, B., LOPEZ, R. & CARDELL, M. (1989). Influence of cardioprotective cyclooxygenase and lipooxygenase inhibitors on peroxidative injury to myocardial-membrane phospholipid. *Biochem. Pharmacol.*, **38**, 4381–4386.

JIALAL, I. & GRUNDY, S.M. (1992). Effect of dietary supplementation with alpha-tocopherol on the oxidative modification of low density lipoprotein. *J. Lipid. Res.*, **33**, 899–906.

KUZUYA, M. & KUZUYA, F. (1993). Probucol as antioxidant and antiatherogenic drug. *Free Rad. Biol. Med.*, **14**, 66–77.

KUZUYA, M., NAITO, M., FUNAKI, C., HAYASHI, T., ASAI, K. & KUZUYA, F. (1991). Lipid peroxide and transition metals are required for the toxicity of oxidized low density lipoprotein to cultured endothelial cells. *Biochim. Biophys. Acta*, **1096**, 155–161.

LARANJINHA, J.A.N., ALMEIDA, L.M. & MADEIRA, V.M.C. (1992). Lipid peroxidation and its inhibition in low density lipoproteins, quenching of *cis*-parinaric acid fluorescence. *Arch. Biochem. Biophys.*, **297**, 147–154.

MCLEAN, L.R. & HAGAMAN, K.A. (1989). Effect of probucol on the physical properties of low-density lipoproteins oxidized by copper. *Biochemistry*, **28**, 321–327.

MORISAKI, N., SLITTS, J.M., BARTELS-TOMEI, L., MILIO, G.E., PANGANAMALA, R.V. & CORNWELL, D.G. (1982). Dipyridamole, an antioxidant that promotes the proliferation of aorta smooth muscle cells. *Artery*, **11**, 88–107.

PALINSKI, W., ROSENFELD, M.E., YLA-HERTTUALA, S., GURTENER, G.C., SOCHER, S.S., BUTLER, S.W., PARTHASARATHY, S., CAREW, T.E., STEINBERG, D. & WITZTUM, J.L. (1989). Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 372–1376.

PRINCEN, H.M.G., VAN POPPEL, G., VOGELEZANG, C., BUYTENHEK, R. & KOK, F.J. (1992). Supplementation with vitamin E but not β-caroten in vivo protects low density lipoprotein from lipid peroxidation in vitro, effect of cigarette smoking. *Arterioscler. Thromb.*, **12**, 554–562.

QUINN, M.T., PARTHASARATHY, S., FONG, L.G. & STEINBERG, D. (1987). Oxidatively modified low density lipoproteins, a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 2995–2998.

REAVEN, P.D., KHOUW, A., BELTZ, W.F., PARTHASARATHY, S. & WITZTUM, J.I. (1993). Effect of dietary antioxidant combinations in humans. *Arterioscler. Thromb.*, **13**, 590–600.

REGNSTROM, J., NILSSON, J., TORNVALL, P., LANDOU, C. & HAMSTEN, A. (1992). Susceptibility to low density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet*, **339**, 1183–1186.

RICE-EVANS, C.A. & DIPLOCK, A.T. (1993). Current status of antioxidant therapy. *Free Rad. Biol. Med.*, **15**, 77–96.

SALONEN, J.T., YLÄ-HERTTUALA, S., YAMAMOTO, R., BUTLER, S., KORPELA, H., SALONEN, R., NYYSSÖNEN, K., PALINSKI, W. & WITZTUM, J.L. (1992). Autoantibody against oxidized LDL and progression of carotid atherosclerosis. *Lancet*, **339**, 883–887.

SELLEY, M.L., CZETI, A.L., MCGUINESS, J.A. & ARDLIE, N.G. (1994). Dipyridamole inhibits the oxidative modification of low density lipoprotein. *Atherosclerosis*, **111**, 91–97.

SMITH, P.K., KROHN, R.I., HERMANSON, G.T., MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENT, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, **150**, 76–85.

SPARROW, C.P., DOEBBER, T.W., OLSZEWSKI, J., WU, M.S., VENTRE, J., STEVENS, K.A. & CHAO, Y. (1992). Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant N′,N′-diphenyl-phenylenediamine. *J. Clin. Invest.*, **89**, 1885–1891.

STEINBERG, D., PARTHASARATHY, C., CAREW, T.E., KHOO, J.C. & WITZUM, J.L. (1989). Beyond cholesterol Modifications of low density lipoproteins that increases its atherogenicity. *New Engl. J. Med.*, **320**, 915–919.

STEINBRECHER, U.P., LOUGHEEED, M., KWAN, W.-C. & DIRKS, M. (1989). Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivativization of apolipoprotein B by products of fatty acid peroxidation. *J. Biol. Chem.*, **264**, 15216–15223.

TABAK, M. & BORISEVITCH, I.E. (1992). Interaction of dipyridamole with micelles of lysophosphatidylcholine and with bovine serum albumin, fluorescence studies. *Biochim. Biophys. Acta*, **1116**, 241–249.

YLA-HERTTUALA, S., PALINSKI, W., ROSENFELD, M.E., PARTHASARATHY, S., CAREW, T.E., BUTLER, S., WITZTUM, J.L. & STEIMBERG, D. (1989). Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.*, **84**, 1086–1095.

(Received May 30, 1996
Revised July 1, 1996
Accepted July 19, 1996)