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Molecular Structure and Antioxidant Specificity of Purpurogallin in Three Types of Human Cardiovascular Cells

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ABSTRACT. Purpurogallin (PPG) is an active cytoprotector found in certain oak barks. We have shown that PPG prolongs the survival of cultured cardiocytes from rats and rabbits against different oxidants better than do antioxidants such as Trolox (a hydrophilic analogue of vitamin E) in a morphometric assay system. First, we verified by X-ray crystallography that PPG is a bicyclic molecule comprising a phenolic ring fused with a seven-membered ring in a highly planar conformation. In analogues of PPG wherein the two double bonds in the seven-membered ring of the parent molecule are saturated or where the four OH groups of the parent compound are substituted by four OCH_3 groups, the derivatives are less planar and less protective of the human cells than native PPG. Second, PPG in a concentration-dependent manner protected myocytes and endothelial cells of humans against oxyradicals generated with any one of the following oxyradical generators: (a) xanthine oxidase plus hypoxanthine, (b) menadione, or (c) paraquat. In each case, PPG was more cytoprotective than comparative antioxidants. Also, PPG protected erythrocytes against peroxyl radicals better than the two PPG derivatives mentioned. Third, the cytoprotective action of PPG detected *in vitro* was accompanied by declines of malondialdehyde. Finally, we observed that PPG chelated ferrous ions and, therefore, can suppress the formation of radicals in the Fenton reaction. Thus, PPG with its molecular architecture and presumably its affinity for ferrous ions protects multiple types of cardiovascular cells against oxyradicals. BIOCHEM PHARMACOL 52;7:1073–1080, 1996.

KEY WORDS. purpurogallin; molecular properties; human cardiovascular cells

It is recognized that oxidant damage of tissues plays an important pathophysiologic role in diverse disorders, including several in the cardiovascular system [1, 2]. We have been studying the bioactivity and plausible mechanisms of a number of cardioprotective antioxidants [3–9], of which PPG[‖] appears to be a particularly promising one. It is a flavinol being used to preserve foods and jet fluids [10], and was reported recently by Prasad *et al.* [11] to effectively scavenge oxyradicals generated by polymorphonuclear leukocytes.

In this work, our focus was 2-fold. First, we determined the molecular structure and geometry of PPG (and two of its analogues, Fig. 1) by X-ray crystallography. Second, we examined the antioxidant activity and specificity of PPG in protecting three types of human cells against three kinds of oxidants: (1) XO-HP; (2) menadione, a non-enzymic generator of superoxide radicals [12], and (3) paraquat, a hydroxyl and peroxyl radical generator [13]. The use of crystallography provided us with new insights into the structure of PPG, while the employment of human cardiovascular cells in our mechanistic studies offered greater clinical relevance than studies based mostly on animal cells. It should be noted that human ventricular myocytes and vascular endothelial cells were selected from the myocardium because they are intimately involved in myocardial infarction.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals used were reagent grade, and were from the Sigma Chemical Co. (St. Louis, MO). MPPG was purchased from the Aldrich Chemical Co. (Milwaukee, WI) while HPPG was a gift from Dr. J. Atkinson (Brock University). For the biochemical studies, PPG was dissolved freshly in 0.01 M sodium phosphate-buffer containing 0.9% NaCl (PBS), briefly sonicated (e.g. for 10–15 min), and adjusted to pH 7.4.

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‖ Abbreviations: PPG, purpurogallin; XO, xanthine oxidase; HP, hypoxanthine; MPPG, tetramethyl PPG; HPPG, hydrogenated PPG; MDA, malondialdehyde; and AAPH 2,2′-azo-bis(2-amidinopropane) dihydrochloride.

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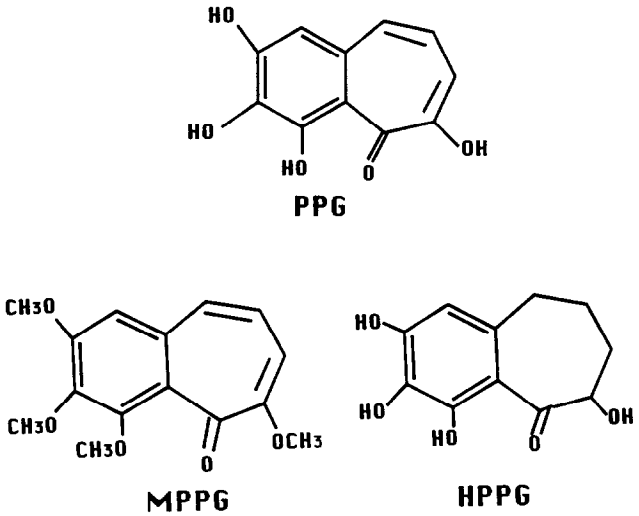

FIG. 1. Chemical structures of PPG and its derivatives.

X-ray Crystallographic Analysis of PPG

Thin reddish needles of PPG were obtained by evaporation from acetone, MPPG was crystallized from ethanol as thin colourless plates, and HPPG crystals were obtained from an acetone-benzene mixture (10:1) as thin yellowish plates. Space groups and cell parameters were determined from precession camera photographs. Refined cell parameters were obtained by diffractometer measurements of 12 high angle reflections ($40° < 2\Theta < 60°$) and application of the least squares method. Crystallographic data are given in Table 1. Crystals of dimensions 0.3 × 0.4 × 0.8 mm (PPG), 0.1 × 0.8 × 0.5 mm (MPPG), and 0.05 × 0.8 × 0.4 mm (HPPG) were chosen for data collection on a Picker FACS-1 diffractometer, using copper Kα radiation and a Θ:2Θ scan mode with a scan speed of 2°/min. Three standard reflections, monitored every 100 reflections, showed only random intensity variations within 5%. Totals of 1532 (PPG), 2385 (MPPG), and 1602 (HPPG) unique reflections were recorded ($3° < 2\Theta < 130°$), of which 1048 (PPG), 1521 (MPPG), and 609 (HPPG) were considered observed using ($F_o > 3\sigma Fo$) discrimination. The intensities were corrected for Lorentz and polarization factors. No absorption corrections were applied.

The crystal structures were determined by direct methods using the SHELXS-86 [14] program for PPG and MPPG and the SIR-88 program [15] package for HPPG. In each case the E-map revealed the positions of all nonhydrogen atoms in the structures. Structure refinement and difference electron density calculations revealed all hydrogen atoms and showed no significant residual electron density (maxima were $\pm 0.2\ \bar{e}/Å^3$ for PPG and MPPG and $\pm 0.3\ \bar{e}/Å^3$ for HPPG). For HPPG, the hydroxyl group hydrogens were allowed free movement during refinement; other hydrogen atoms were kept at appropriate distances from their carbon atoms by the refinement program. The final discrepancy factors converged at $R = 0.044$ (PPG) 0.061 (MPPG), and 0.065 (HPPG). Program SHELXL-93 [16] was used for all refinement calculations.

TABLE 1. X-ray crystallographic data

PPG	MPPG	HPPG
$P2_1/n$	$P2_1/c$	C2/c space group
a = 7.306(2)	a = 13.694(4)	a = 23.893(6) Å
b = 14.876(3)	b = 7.126(2)	b = 9.965(3) Å
c = 8.354(2)	c = 15.138(4)	c = 8.256(3) Å
beta = 103.86(3)	beta = 107.96(2)	beta = 93.61(5) deg.
V = 905	V = 1405	V = 1962 $Å^3$
Z = 4	Z = 4	Z = 8

Preparation of Cultured Cells

The detailed procedures employed have been documented elsewhere [9]. Myocytes from left ventricular 5–10 mg biopsies obtained with the informed consent of patients, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, pH 7.4. The myocytes were identified by morphological appearance and by fluorescent antibodies to both actin and to human ventricular myosin light chain 1, and by electron microscopy before free radical studies.

For vascular endothelial cells from humans, a segment of saphenous vein was coated with 0.1% collagenase. After 30 min of incubation at room temperature, endothelial cells were washed out and suspended in 199 medium supplemented with 20% fetal bovine serum, pH 7.4. The endothelial cells were identified by morphological appearance and by reaction with fluorescent antibody specific to factor VIII before free radical studies [4].

Morphometric Assay of Cytoprotective Effects

Free radical studies on cultured human ventricular myocytes and endothelial cells were performed in a morphometric assay according to Wu *et al.* [3, 6]. The cells were

TABLE 2. Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($Å^2 \times 10^3$)

	x	y	z	U(eq)*
PPG				
C (1)	2058 (4)	3806 (2)	339 (3)	31 (1)
C (2)	1021 (4)	3373 (2)	-999 (3)	31 (1)
C (3)	395 (4)	3839 (2)	-2432 (3)	34 (1)
C (4)	875 (4)	4721 (2)	-2525 (3)	38 (1)
C (5)	1925 (4)	5200 (2)	-1180 (3)	35 (1)
C (6)	2212 (4)	6120 (2)	-1506 (3)	43 (1)
C (7)	3044 (4)	6786 (2)	-528 (3)	46 (1)
C (8)	3926 (4)	6754 (2)	1115 (3)	45 (1)
C (9)	4151 (4)	6047 (2)	2141 (3)	39 (1)
C (10)	3569 (4)	5112 (2)	1862 (3)	34 (1)
C (11)	2545 (3)	4736 (2)	340 (3)	31 (1)
O (1)	2597 (3)	3272 (1)	1661 (2)	43 (1)
O (2)	577 (3)	2484 (1)	-991 (2)	44 (1)
O (3)	-679 (3)	3425 (1)	-3754 (2)	45 (1)
O (9)	5052 (3)	6226 (1)	3699 (2)	50 (1)
O (10)	4019 (3)	4625 (1)	3111 (2)	46 (1)
MPPG				
C (1)	7877 (2)	118 (5)	9358 (2)	48 (1)
C (2)	8422 (2)	1052 (5)	8870 (2)	51 (1)
C (3)	8344 (2)	461 (5)	7964 (2)	53 (1)
C (4)	7644 (2)	-894 (5)	7547 (2)	56 (1)
C (5)	7004 (2)	-1743 (5)	8003 (2)	51 (1)
C (6)	6221 (3)	-2989 (5)	7449 (2)	62 (1)
C (7)	5361 (3)	-3582 (6)	7607 (2)	69 (1)
C (8)	5011 (3)	-3266 (5)	8387 (2)	66 (1)
C (9)	5555 (2)	-2667 (5)	9239 (2)	55 (1)
C (10)	6677 (2)	-2325 (5)	9548 (2)	54 (1)
C (11)	7160 (2)	-1307 (5)	8945 (2)	46 (1)
C (12)	8911 (3)	17 (6)	10917 (2)	70 (1)
C (13)	8592 (3)	4275 (6)	9096 (3)	77 (1)
C (14)	9102 (3)	496 (7)	6748 (3)	82 (1)
C (15)	4113 (3)	-2598 (7)	9801 (3)	82 (1)
O (1)	7978 (2)	680 (4)	10248 (1)	57 (1)
O (2)	9069 (2)	2503 (4)	9275 (2)	62 (1)
O (3)	8991 (2)	1329 (4)	7570 (2)	66 (1)
O (9)	5184 (2)	-2463 (4)	9968 (2)	68 (1)
O (10)	7175 (2)	-2814 (4)	10330 (2)	73 (1)
HPPG				
C (1)	3152 (2)	-415 (5)	1071 (8)	54 (2)
C (2)	3281 (2)	914 (5)	814 (8)	52 (2)
C (3)	3752 (2)	1244 (5)	32 (8)	55 (2)
C (4)	4093 (3)	246 (5)	-532 (8)	63 (2)
C (5)	3974 (2)	-1088 (5)	-306 (8)	60 (2)
C (6)	4361 (3)	-2135 (7)	-1009 (10)	98 (3)
C (7)	4628 (3)	-3077 (7)	109 (12)	135 (4)
C (8)	4239 (3)	-4039 (6)	929 (10)	89 (3)
C (9)	3659 (2)	-4029 (5)	310 (10)	69 (2)
C (10)	3330 (2)	-2821 (5)	875 (8)	52 (2)
C (11)	3495 (2)	-1452 (5)	545 (7)	50 (2)
O (1)	2692 (2)	-616 (4)	1887 (6)	68 (1)
O (2)	2967 (2)	1940 (3)	1378 (6)	72 (2)
O (3)	3887 (2)	2533 (4)	-294 (6)	73 (2)
O (9)	3395 (2)	-5226 (4)	895 (7)	92 (2)
O (10)	2905 (2)	-3072 (4)	1605 (6)	72 (2)

* U(eq) is defined as one-third of the trace of the orthogonalized Uij tensor.

subcultured from confluent cultures and incubated, at 37°, in 0.01 M sodium PBS, pH 7.4, containing either 8.34 IU/L XO and 2 mM HP, or, alternatively, 1.0 mM menadione or 100 mM paraquat as the oxyradical generator. The basis for comparing control (no putative cytoprotector) versus test (with putative cytoprotector such as PPG) was the time taken for necrosis of 95% of approximately 10^5 cells or, conversely, the survival time of 5% of cells in each culture dish. In the basic permutation, freshly solubilized PPG or its comparative antioxidant was added along with XO-HP, menadione, or paraquat to the cells and tested in randomized order in triplicates. Cell necrosis was observed by phase-contrast microscopy [6]. The necrotic endpoint was corroborated by >95% loss in the ability of the cultured cells to exclude trypan blue, by leakage of enzymes (e.g. lactate dehydrogenase and aspartate aminotransferase), and by electron microscopy [4].

Assay of Anti-peroxidative Activity Based on Red Cell Lysis

Blood from healthy donors was collected in heparinized tubes. Erythrocytes were separated by centrifugation from plasma and the buffy coat and were washed three times with 10 vol. of saline. During the last washing, the cells were centrifuged at 1000 *g* for 10 min to obtain a constantly packed cell preparation. The assay for hemolysis mediated by peroxyl radicals was done according to Mike *et al.* [17]. A 20% suspension of red cells in PBS was added to the same volume of 200 mM AAPH solution in PBS containing either PPG or its derivatives at different concentrations. The reaction mixture was shaken gently while being incubated at 37° for 180 min. After incubation, the reaction mixture was diluted with an appropriate volume of saline, and centrifuged at 1000 *g* for 10 min. Absorbance A of the supernatant at 540 nm was read. Similarly, the reaction mixture of each permutation was treated with distilled water to yield complete hemolysis, and absorbance B of the supernatant after centrifugation was measured at 540 nm. Percent inhibition of hemolysis observed with each level of antioxidant was calculated by the equation $(1 - A/B) \times 100\%$. Each comparative antioxidant was examined 7–10 times per concentration.

Determination of Chelation of Ferrous Ion with PPG

This was done according to Afanas'ev *et al.* [18]. PPG at 50 μM in PBS, pH 7.4, was mixed with an equimolar solution of $FeSO_4$ for 22 hr, at which time the presumptive iron–PPG complex became stable. The absorption spectra of PPG before and after complexation were recorded.

Statistics

The ANOVA method followed by Duncan's Multiple Range Test, where appropriate, was applied. All data are

expressed as mean ± SD. Significance was indicated by a $P < 0.05$.

RESULTS

The X-ray crystallographic structure of PPG is shown in Fig. 2, while those of MPPG and HPPG are given in Figs. 3 and 4, respectively; molecular coordinates are listed in Table 2. The PPG molecule is planar, with all atoms, including hydrogens, deviating insignificantly from the molecular plane (maximum deviation = 0.05 Å, mean atomic deviation = 0.03 Å). Bond lengths and angles indicate extensive π-electron delocalization over the molecule; planarity is also enhanced through intramolecular interactions between hydroxyl group hydrogens and carbonyl and hydroxyl oxygens. The bond lengths and angles in MPPG are similar to those in PPG, but despite having a delocalized electron

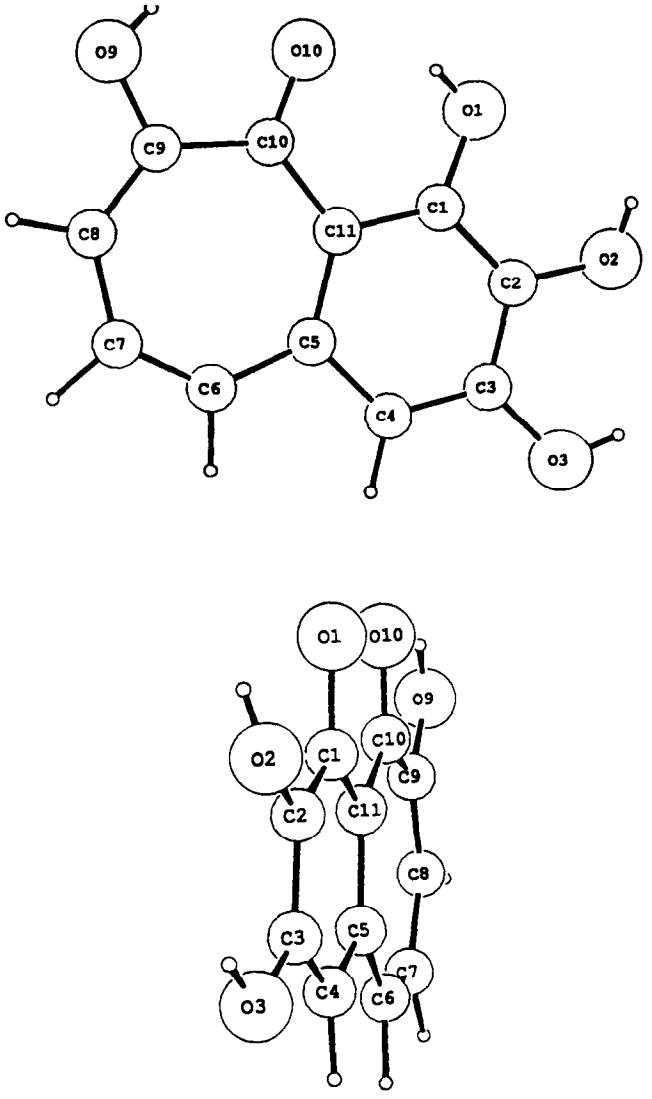

FIG. 2. Molecular structure of PPG. (Top) Perpendicular to the molecular plane. (Bottom) Parallel to the molecular plane.

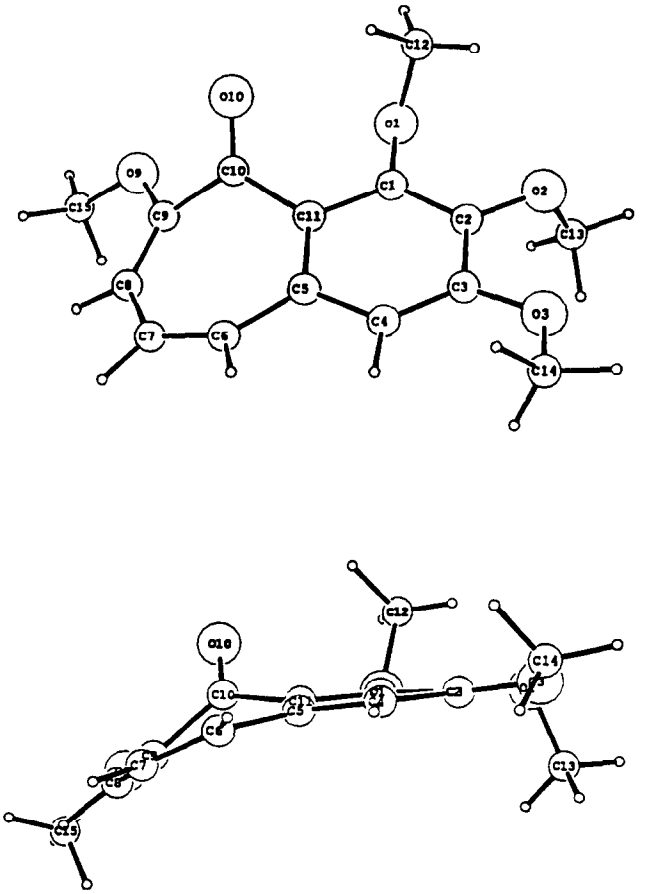

FIG. 3. Molecular structure of MPPG. (Top) Perpendicular to the molecular plane. (Bottom) Parallel to the molecular plane.

system, the molecule is not planar. The six-membered ring is planar, and six of the seven atoms of the other ring approach planarity, with C10 deviating by 0.56 Å from the best plane through the others. The angle between perpendiculars to the two planes is 18°. The lack of intramolecular hydrogen bonding likely contributes to the non-planarity of the molecule. In HPPG, the partial hydrogenation has disrupted the overall electron delocalization in the molecule; bond lengths C5–C6, C6–C7, C7–C8, and C8–C9 are about 0.1 Å longer in HPPG than in the other molecules, and the ring angles at C6, C7, C8, and C9 are about 12° smaller, reflecting the single bond character of that part of the molecule. The molecular conformation is such that five atoms of the seven-membered ring are coplanar with the six-membered ring (maximum deviation = 0.04 Å, mean atomic deviation = 0.02 Å), but C7 and C8 lie off that plane by 1.03 and 1.11 Å, respectively. An intramolecular hydrogen bonding network similar to that in PPG contributes to maintaining the planarity of the planar portion of the molecule.

Figure 5A summarizes results from incubating ~10^5 ventricular myocytes with 8.34 IU/L XO and 2 mM HP at 37°, and in the presence of increasing concentrations of PPG. With no PPG added (see first column) ~95% of the cells

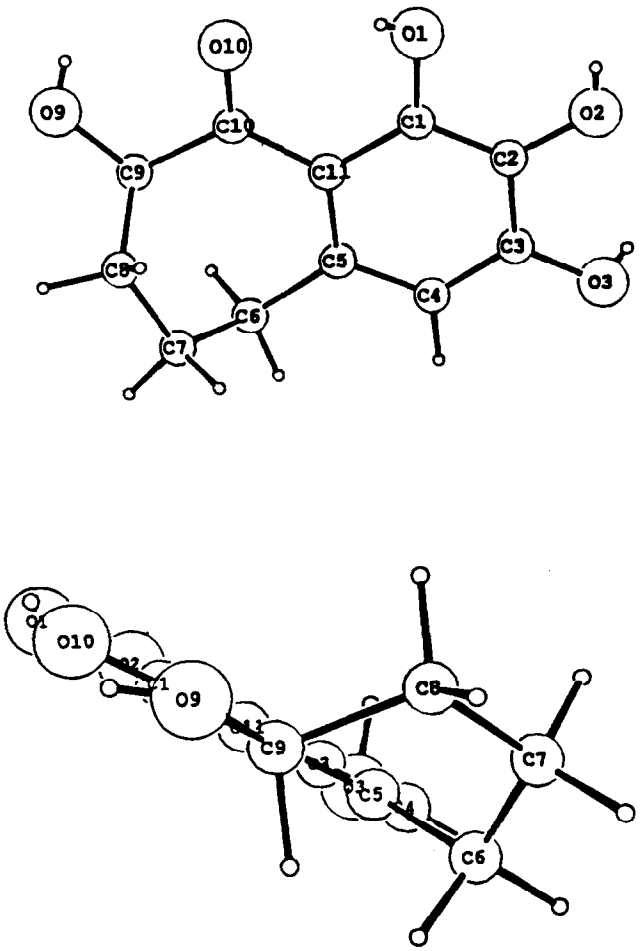

FIG. 4. Molecular structure of HPPG. (Top) Perpendicular to the molecular plane. (Bottom) Parallel to the molecular plane.

were necrosed in <10 min as judged with phase contrast microscopy and verified, as we had described previously [19], by electron microscopy, trypan blue exclusion, enzyme leakage, and ^{51}Cr release. The necrosis time was three or five times longer than that in the control at 0.5 and 1.0 mM, respectively. Analysis of data by one-way ANOVA revealed that there was a significant effect ($P < 0.01$) of PPG concentration on necrosis times. Note that, in the absence of XO and/or HP, the cells incubated with PBS alone survived for ~60 min. Figure 5B shows that PPG also prolonged the survival of ~10^5 myocytes when 1 mM menadione (a superoxide generator) was used in place of XO-HP as the source of oxyradical. Again, one-way ANOVA showed a significant relationship ($P < 0.05$) between the level of PPG and cell necrosis times.

Panels C and D of Fig. 5 illustrate the corresponding cytoprotective effects of PPG on human endothelial cells when the latter were exposed to either XO-HP or menadione alone. Again, the protective effect appeared striking and concentration dependent.

The top panel of Fig. 6 illustrates that, under otherwise identical conditions, PPG prolonged survival of the myocytes more extensively than did Trolox, ascorbate, and mannitol. Similarly, PPG excelled when compared with the identical compounds as above (including our two PPG analogues) in protecting myocytes against menadione as it did against XO-HP (data not shown). The bottom panel of Fig. 6, depicts that PPG was also a better protector of endothelial cells than Trolox, or SOD ± catalase against oxyradicals generated with XO-HP ($P < 0.01$).

Figure 7 demonstrates that PPG protected human ventricular myocytes against paraquat. Note that this oxidant has been reported to produce peroxide and hydroxyl radicals [13].

Figure 8 shows that PPG inhibited the lysis of human red cells due to a thermally activated azo-initiator more effectively than did its derivatives. Thus, the IC_{50} (the average concentration of an additive that inhibits 50% cell lysis) for PPG was 0.1 mM, substantially smaller than that of 0.2 and 0.35 mM for MPPG and HPPG tested under the same conditions.

Figure 9 strongly suggests that PPG chelates ferrous ions. The mixture of PPG + Fe^{2+} showed a considerable absorbance at longer wavelength, whereas PPG demonstrated very little absorbance and Fe^{2+} did not show such absorption at all. Therefore, it can be inferred that PPG forms a complex with Fe^{2+}. This complex is stable over 22 hr as the absorption spectrum showed little change after that time. Note that the absorption shown in this figure was obtained after 22 hr of incubation.

DISCUSSION

In this study, we have shown that PPG is a broad-spectrum antioxidant that protects three distinct types of cells in the human cardiovascular system. The cytoprotective activity of PPG is especially noteworthy in the vascular endothelium for three reasons. First, it is rich in XO [20]. Second, many known antioxidants (e.g. Trolox) protect endothelial cells poorly against XO-generated oxyradicals when compared with PPG [4]. Third, the endothelium is an early target of tissue damage as in organ rejection [1, 2]. In view of these factors, the beneficial effect of PPG on endothelium commands special significance.

Our data using menadione (a superoxide radical generator) [12] have established that both ventricular myocytes and vascular endothelial cells are independently sensitive to superoxide anion and that PPG can counter such effects more effectively than can several other antioxidants such as Trolox, ascorbate, mannitol, and exogenously added SOD ± catalase. Likewise, we have presented data suggesting that PPG can overcome the damaging effect of hydroxyl/peroxyl radicals generated by paraquat on cardiocytes as illustrated with myocytes *in vitro*. There may be other causes for this effect which remain to be identified. The use of a well-validated red cell system [17] has also allowed us to demonstrate that PPG protects the red cell membrane more effectively than its derivatives against peroxyl radicals. We

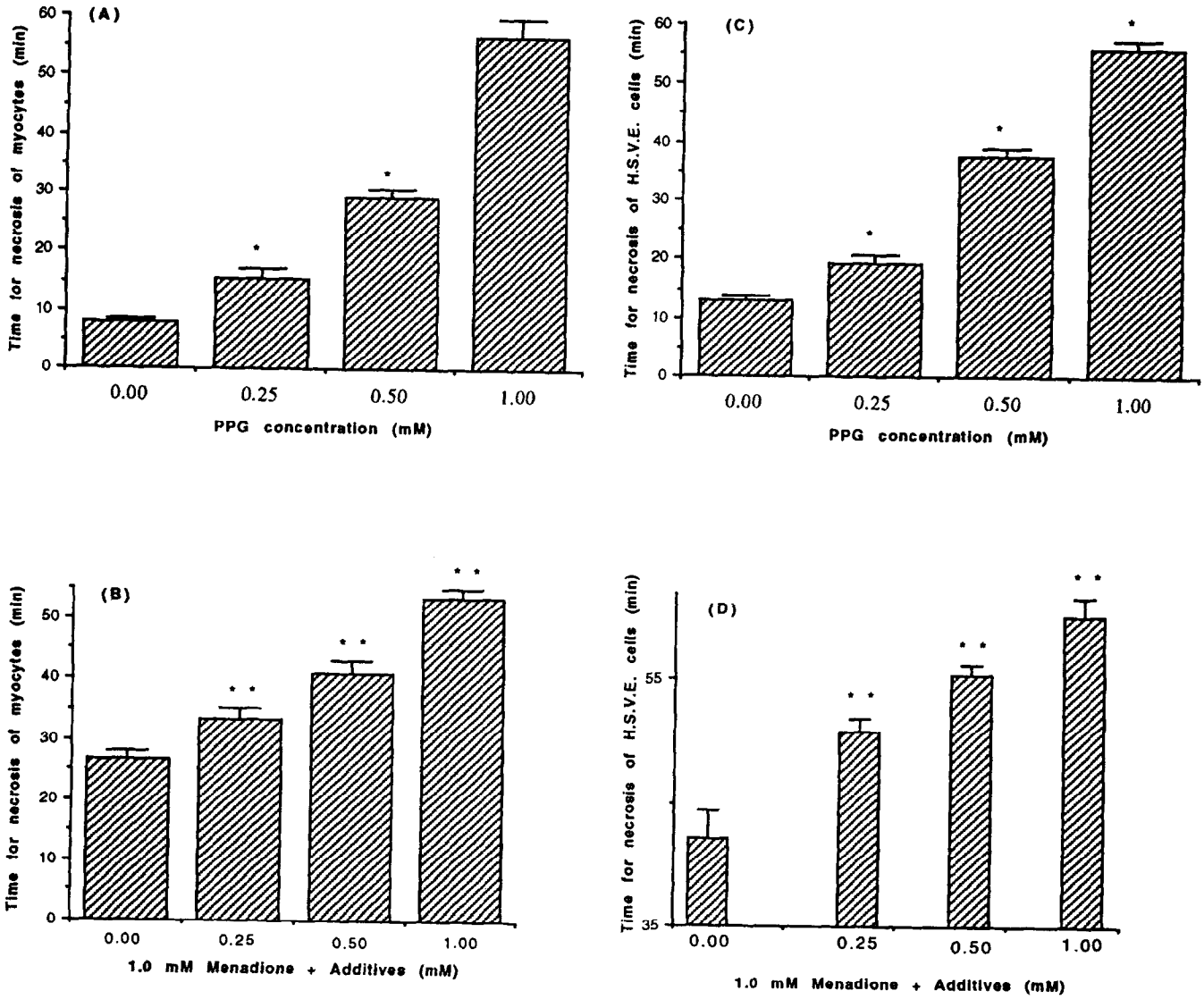

FIG. 5. (A) Protection of human ventricular myocytes by PPG against oxyradicals generated with XO-HP. The first column indicates the results of incubating myocytes with XO-HP without PPG present. (B) Results with menadione replacing XO-HP as the source of oxyradicals. (C and D) Effect of PPG in protecting human saphenous vein endothelial (H.S. V.E.) cells against oxyradicals generated with (C) XO-HP or (D) menadione. Key: (*) $P < 0.01$, and (**) $P < 0.05$. Values are the means ± SD of 3–5 replicate incubations. All other details are given in the text.

have also shown that PPG chelates ferrous ion. In this way, PPG can suppress the formation of the hydroxyl radical in the Fenton reaction, which is more toxic than superoxide anion and hydrogen peroxide.

Lipid peroxidation has been implicated in oxyradical-induced injuries to cells and tissues [18]. MDA is the most commonly used marker of this process. We have observed that 1 mM PPG reduced ~60% MDA formed in endothelial cells after a 1 to 2-min exposure to the XO/HP-produced radicals (data not shown). These data support the role of PPG as a free radical scavenger.

The X-ray crystallographic structure determinations of PPG, MPPG, and HPPG have given insights into the molecular bases for their relative efficacies as antioxidants. PPG is a planar molecule with extensive electron delocalization; intramolecular hydrogen bonding (Fig. 2) contributes to the maintenance of the planar structure. Interaction with oxyradicals either *in vitro* and/or *in vivo* may result in abstraction of a hydrogen atom from PPG by the oxyradical, a mechanism thought to be common to many antioxidants [18]. The resulting unpaired electron on PPG can be stabilized by reconjugation of the delocalized ring system. MPPG is a less effective antioxidant; methylation of the hydroxy groups prevents intramolecular H-bonding and molecular planarity (Fig. 3), and decreases the number of possible resonance structures for stabilizing an unpaired electron over the entire molecule. Similarly, examination of the HPPG structure (Fig. 4) shows that partial saturation of the molecule destroys molecular planarity and limits its electron delocalization, which correlates with its di-

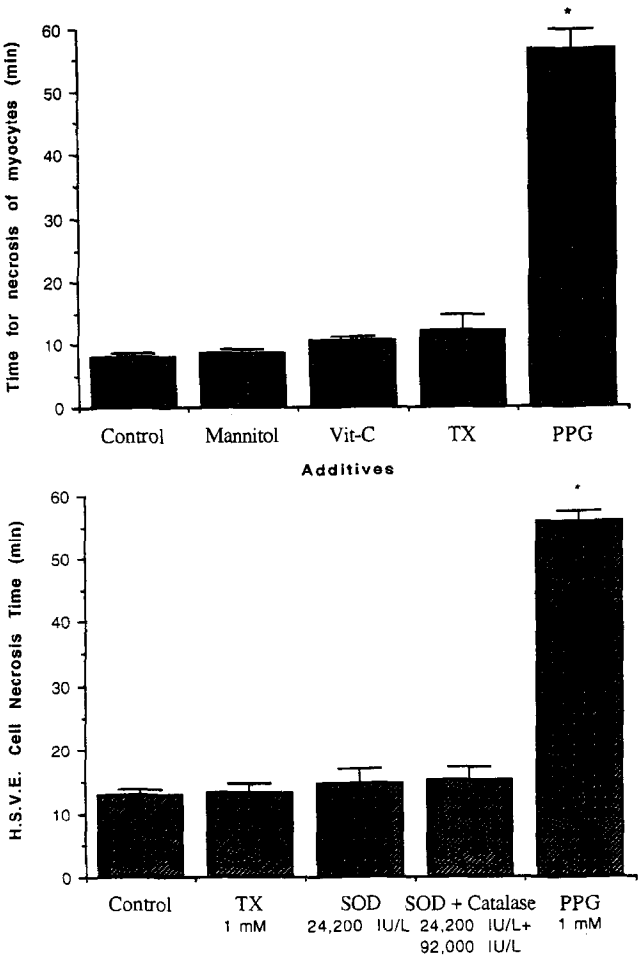

FIG. 6. (Top) Comparison of the protective effects of PPG, Trolox, ascorbate, and mannitol on myocytes exposed to XO-HP. Each protector was used at 1 mM. The first column indicates the results of incubating myocytes with XO-HP without PPG present. (Bottom) Comparison of the protective effects of PPG, Trolox, and SOD ± catalase on human saphenous vein endothelial (H.S.V.E.) cells against damage inflicted by XO-HP Key: (*) $P < 0.01$. All values are means ± SD of 3–5 replicate incubations.

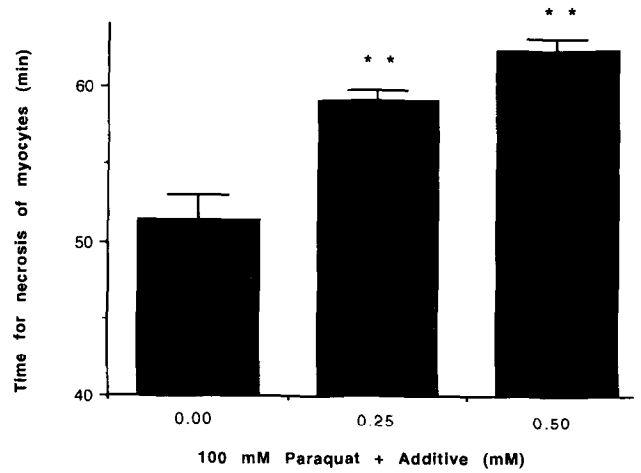

FIG. 7. Effect of PPG in protecting human myocytes against free radicals generated with 100 mM paraquat. Key: (**) $P < 0.05$. Values are means ± SD of 3 replicates.

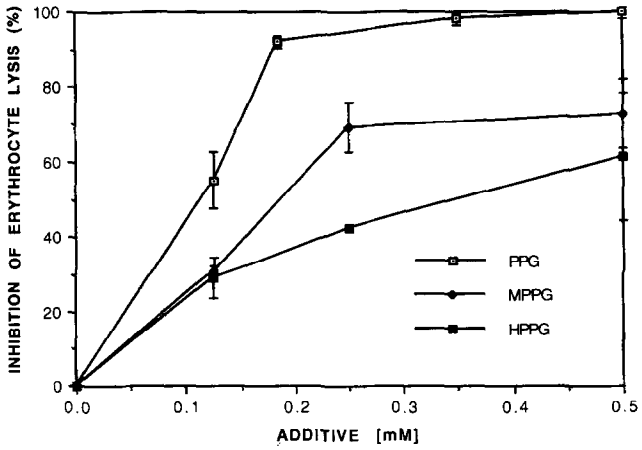

FIG. 8. Comparison of the protective effects of PPG, MPPG, and HPPG in inhibiting red cell lysis by AAPH. See text for experimental details. Each point is the mean ± SD of 7–10 replicates.

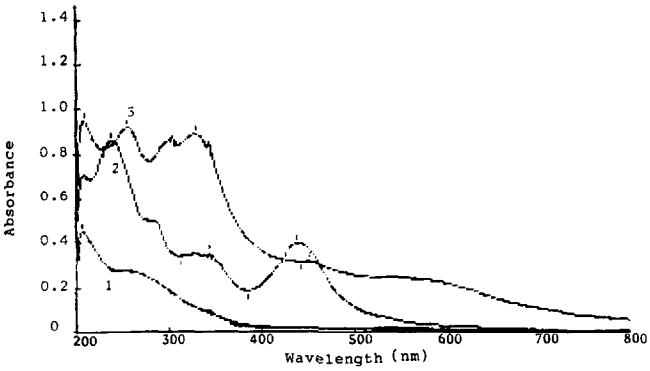

FIG. 9. Illustration of three absorption spectra against 6 mM PBS. Curve 1 is the absorption spectrum of Fe^{2+} (50 μM), curve 2 is that of PPG (50 μM), and curve 3 is that of an equal molar mixture of PPG + Fe^{2+} (50 μM each).

minished antioxidant activity. Further studies of the molecular structures and activities of related analogues *in vitro* and *in vivo*, along with considerations such as solubilities and chelating properties, may yield additional insights in the future.

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