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SHORT COMMUNICATIONS

Propyl gallate as a hepatoprotector *in vitro* and *in vivo*

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Abstract—Recently, there has been renewed interest in propyl gallate, a preservative in foods and fuels. This compound, which exhibits antimicrobial activity, has been found to be toxicologically safe after almost 30 years of evaluation. In the present study, we examined whether propyl gallate is a hepatoprotective antioxidant, and investigated some of its bases of action vis-à-vis Trolox, a vitamin E analogue. In isolated rat hepatocytes, propyl gallate prolonged substantially cell survival against oxyradicals generated with xanthine oxidase-hypoxanthine. The protection was dose dependent and excelled that of Trolox, mannitol, or ascorbate, each at or near its optimum level in the same system. In rats undergoing an 80-min partial hepatic ischemia, infusion of propyl gallate at 20 μmol/kg body weight just before a 24- hr reperfusion salvaged the organ by 80.0 ± 11.5%, an extent comparable to that with Trolox. Mechanistically, we found that propyl gallate (a) protected hepatocytes against the cascade of oxyradicals produced by xanthine oxidase-hypoxanthine; (b) protected hepatocytes against superoxide radicals generated specifically by menadione; (c) protected the functionally important hepatic vascular endothelial cells more effectively than Trolox against xanthine oxidase-hypoxanthine, and (d) approximately halved the amount of lipid conjugated dienes (a more specific marker of oxyradical damage than malondialdehyde) formed in tissues after oxidant damage. Therefore, there are fundamental reasons why propyl gallate is an effective antioxidant-based hepatoprotector, both *in vitro* and *in vivo*.

Key words: propyl gallate; rat; liver; hepatocyte; oxyradical

PG* is a preservative used in oils, fats, and waxes [1]. It is a gallate ester that exhibits antimicrobial activity [2], and has been found to be a relatively safe compound after decades of toxicological evaluations [3]. As represented in Fig. 1, PG has a simple phenolic structure with three hydroxyl groups. However, PG had not been shown to be an antioxidant-based hepatoprotector *in vivo*. This verification is important because not all *antioxidants* that are effective *in vitro* are cytoprotective [4], and some cytoprotective antioxidants are cell-type [5] or species specific [4, 5]. In this work, we first examined if PG protects rat hepatocytes against oxidant damage and the post-ischemic rat liver from reperfusion injury. Second, we explored the mechanistic aspects of the action of PG, using Trolox (a vitamin E analogue with a molecular size similar to that of PG) [6, 7] as an arbitrary frame of reference.

Materials and Methods

Materials. Unless stated otherwise, all chemicals and enzymes used were purchased from the Sigma Chemical Co. (St. Louis, MO) and were of reagent grade. PG (3,4,5-trihydroxybenzoic acid propyl ester; Tenox PG; food grade antioxidant) was obtained from Eastman Chemicals Products, Inc., Kingsport, TN. TX (Trolox C; 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) was obtained from the Aldrich Chemical Co. (Milwaukee, WI).

Assay of the cytoprotective effect of PG in primary rat hepatocytes. Hepatocytes were prepared as described previously [7] and used in a morphometric assay of cell necrosis [6, 7]. The culture medium, in a dish (60 mm × 15 mm with 2-mm grids) containing 1.1 ± 0.03 × 10^5 cells, was replaced by 3 mL of 0.05 mM PBS (pH 7.4), to which XO and HP were added to final levels of 8.34 IU/L and 2 mM, respectively. When menadione was used as a superoxide radical generator [8], it was prepared in PBS as a stock solution of 1 mM. Under phase contrast microscopy, the cells exhibited symptoms of necrosis in 11–13 min with XO-HP and in 30–32 min with menadione, displaying shrunken cytoplasm and rupture of plasma membrane with extrusion of internal contents. The time for hepatocyte necrosis when an additive was present was measured in a blinded fashion in three to six replicates, and the mean value was compared with that of the control (minus additive). We previously validated the morphometric endpoint [9], using electron microscopy, trypan blue exclusion, ^{51}Cr-release and enzyme leakage.

Assay of the cytoprotective effect of PG in rat liver endothelial cells. Prepared from Sprague–Dawley rats by the method of Nagelkerke *et al.* [10], these cells were used for assaying the cytoprotective activity of PG versus Trolox by the same protocol that was described previously [11].

Model of hepatic ischemia–reperfusion. An animal model involving the left lobe of rat liver was used as described previously [7]. Male Sprague–Dawley rats (0.3 to 0.4 kg) were deprived of food overnight, but water was given *ad lib.* Immediately before surgery, each rat was given gentamycin (3 mg/kg) intramuscularly, was anesthetized with enflurane (3.5% premixed with 1.5 to 2.0% enflurane mixed with O_2 at 0.2 L/min), and was treated with heparin (50 IU sodium heparin/kg body wt) intravenously. After a longitudinal midline incision of the abdomen, the liver was disconnected from the diaphragm and stomach. The vessels supplying the left lateral, median, and spigelian lobes were occluded for 80 min by clamping the left portal vein, left hepatic artery and left bile duct with a microaneurysm clamp. The right portal vein, right hepatic artery and right bile duct were left intact as an internal shunt. During

* Abbreviations: PG, propyl gallate; TX, Trolox; XO, xanthine oxidase; HP, hypoxanthine; and CD, conjugated dienes.

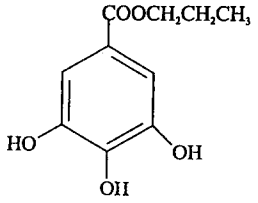

Fig. 1. Structure of propyl gallate (PG).

ischemia, 1 mL saline was given intravenously over 2–3 min to replace lost body fluid. Previous experiments had shown that an 80-min ischemia in this model produced an optimum percentage of liver necrosis without reaching the "point of no return" at which most cells are unsalvageable. At 45 sec before release of the occlusion, a 3-mL bolus of saline solution (placebo control) or saline solution with additive (PG or TX) was infused for 2 min through the penile vein. The abdomen was then closed. After 24-hr reperfusion, the animal was killed by aortic exsanguination. The liver was harvested for histochemical and biochemical analyses. Sham-operated rats were treated the same as the other rats, except that the blood supply to the liver was occluded for only 30 sec, followed by closure of the abdomen.

Estimation of extent of hepatic necrosis. The rat liver was sectioned transversely into five 1-cm thick slices and incubated, according to Lie *et al.* [12], in nitroblue tetrazolium for 20 min at 25°. No dye staining took place in the necrotic area, confirmed histologically by light and electron microscopy as done previously [7]. In the non-necrotic tissue, a deep-red stain developed. Computerized planimetry was done on both sides of each weighed slice to measure the total and necrotic area. From these data, the percent necrosis by weight was calculated according to Frederiks *et al.* [13], as detailed previously [14].

Quantitation of phospholipid CD. The method that we described previously [6, 7, 15, 16] was applied to phospholipid extracts of liver tissue, using [^{14}C]tetracyanoethylene as the reagent. As noted before [15, 16], CD has been termed the fingerprint signature of oxyradical damage, being a more selective marker than malondialdehyde of the phospholipid peroxidation product from oxidant impaired cellular membrane. The amount of CD was referenced against the nanomoles of phosphate in the phospholipid fraction of the rat liver extract. Usually 4–7 replicates were done at each permutation.

Statistical analysis. Unless otherwise stated, results were analyzed using ANOVA, as described previously [7].

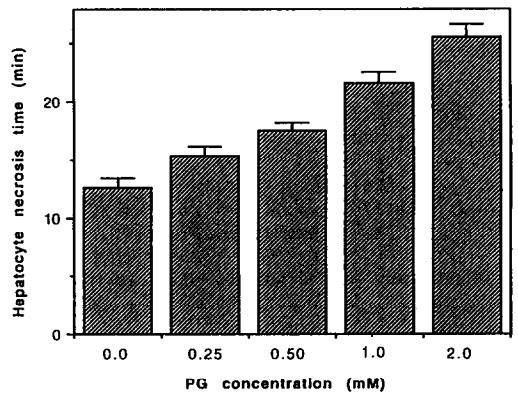

Fig. 2. Concentration dependence of the effect of PG on rat hepatocytes exposed to oxyradicals generated with xanthine oxidase (XO) and hypoxanthine (HP). The control refers to the time elapsed for necrosis of 10^5 cells exposed to free radicals without any added cytoprotective agent. Values are means ± SD of three to six replicates.

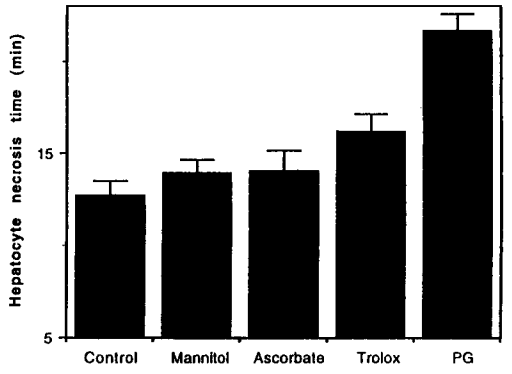

Fig. 3. Effect of PG versus those of other water-soluble antioxidants (each at a final concentration of 1 mM) on rat hepatocytes exposed to oxyradicals generated with XO and HP. Values are means ± SD of three replicates.

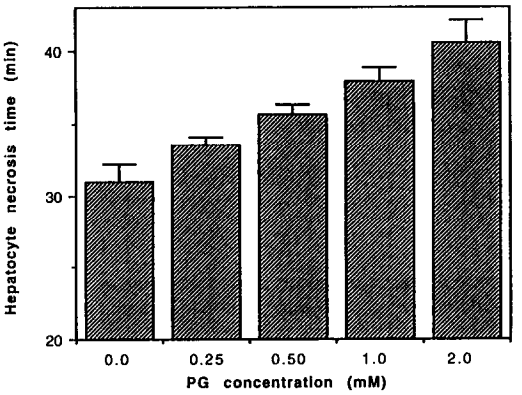

Fig. 4. Concentration dependence of the effect of PG on rat hepatocytes exposed to superoxide radicals generated with menadione. Cells were exposed to 1 mM menadione. All other details are given in the text. Values are means ± SD of three to six replicates.

Results

In vitro *protection of rat hepatocytes against oxyradicals generated with XO-HP.* Figure 2 shows that increasing the concentration of PG incubated with rat hepatocytes progressively prolonged the times elapsed for 95% necrosis of ~10^5 hepatocytes exposed to XO-HP. One-way ANOVA indicated a significant effect ($P < 0.001$) of PG level on the time required for cell necrosis. Indeed, over the range of PG concentrations, linear regression analysis supported a strong dependence of necrosis times with increasing PG levels, giving a $P < 0.004$ with a correlation coefficient of 0.978. Again, as noted previously [7], in the absence of

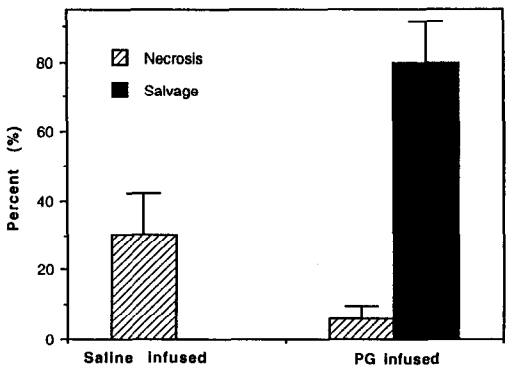

Fig. 5. Effect of PG (20 μmol/kg body wt) on the salvage of liver and on percent hepatic necrosis in a partial model of ischemia–reperfusion in rats. Organ salvage was calculated based on: (percent necrosis in control − percent necrosis with PG)/(percent necrosis in control) × 100%. Values are means ± SD of at least eight replicates.

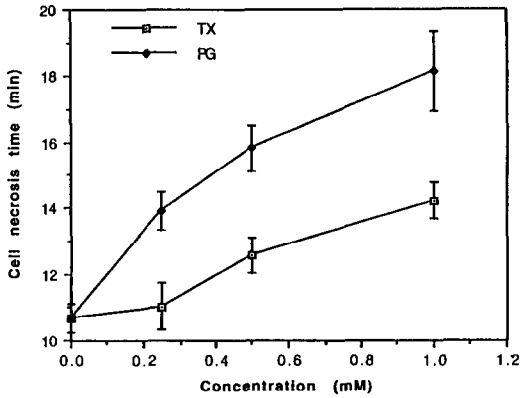

Fig. 6. Effects of PG and Trolox (TX) on the time of necrosis of rat liver endothelial cells in the presence of oxyradicals generated by XO-HP. Conditions of oxyradical damage were as described in the text. The averages ± SD of triplicate determinations are shown.

XO and/or HP, the cells remained viable for at least 60 min. Figure 3 illustrates that PG also protected hepatocytes against oxyradicals generated with XO-HP better than other water-soluble antioxidants such as TX, mannitol, and ascorbate (each at 1 mM).

In vitro *protection of rat hepatocytes against superoxide radicals generated with menadione.* Figure 4 demonstrates that PG also prolonged the survival of hepatocytes when 1 mM menadione was used in place of XO-HP as the oxyradical source. Again, one-way ANOVA indicated a significant relationship ($P < 0.005$) between the level of PG and cell necrosis times.

Animal model study. Figure 5 shows that a single infusion of PG (20 μmol/kg body wt) into the rat liver just after an 80-min hepatic ischemia but before 24-hr reperfusion produced a mean liver necrosis that was 80% less than that in the saline-infused (placebo) control ($P < 0.001$). This mean hepatic salvage is comparable to that given by Trolox in the same animal model [7]. In six sham-operated animals, there was negligible hepatic necrosis.

Protection of hepatic endothelial cells. When PG and TX were added separately to endothelial cells cultured from rat liver (Fig. 6), it was observed that PG was substantially more effective than TX in prolonging survival of these endothelial cells against XO–HP-generated oxyradical damage.

Chemical evidence of antioxidant action. As we reported previously with Trolox [7], PG at 1.0 mM was also able to approximately halve the total amount of phospholipid CD found in rat liver tissue during the first half-hour after reperfusion following ischemia (Hashimoto N, Sugiyama H, and Wu T-W, unpublished results).

Discussion

Lepran and Lefer [17] reported that infusion of PG into post-ischemic cat hearts at doses that do not exert hemodynamic effects prevents leakage into blood of such ischemic damage markers as creatinine kinase and myocardial amino-nitrogens. They speculated, among other possibilities, that the apparent cardioprotective effect of PG may be related to its ability to inhibit lipoxygenase. However, the authors did not verify that PG acts truly as an antioxidant *in vivo* or that it was this activity that actually protected the cat heart from post-ischemic damage.

In the present study, we demonstrated that propyl gallate is a more effective hepatocellular protector than several known water-soluble antioxidants in prolonging survival of cultured hepatocytes. We have also shown that PG is almost as effective as TX in salvaging the post-ischemic and reperfused rat liver.

Mechanistically, we illustrated that PG, which is several-fold more water soluble than Trolox, has a higher dynamic range of protection of hepatocytes than TX. As an antioxidant, PG scavenges not only the cascade (e.g. superoxide, hydrogen peroxide and hydroxyl) of oxyradicals formed enzymatically from XO [18], but also the superoxide radical generated non-enzymatically by menadione [8]. The presence of antioxidant activity in PG, as in TX [6, 19, 20], was also independently corroborated by the substantial decline in the tissue level of CD in rat livers treated with PG in our ischemia–reperfusion model.

A most important clinical finding here is that PG is also a better antioxidant protector of vascular endothelial cells than TX. Endothelial cells are increasingly recognized to be a key target of oxidant generation and attack in diverse tissues [21]. Viewed in this light, it is likely that PG may exert a broader or even systemic influence in the vasculature and/or on circulation. Indeed, we have witnessed a superior effect of PG over TX in several other types of vascular endothelial cells.

Younes *et al.* [22] reported that PG reduces the levels of hepatic enzymes (e.g. cytosolic lactate dehydrogenase and alanine aminotransferase) detected in the perfusate after 30-min hypoxia followed by 1-hr reoxygenation in isolated perfused rat liver. From these data they inferred that PG protects the liver from hypoxia–reoxygenation injury, which was presumed to be oxyradical-based. We have demonstrated in the present study that PG protects liver cells from damage by oxyradicals both *in vitro* and *in vivo*. It may be worth noting that PG is an amphipathic molecule [1], i.e. it is both hydrophilic and lipophilic. As such, and based on the elegant review of Weglicki *et al.* [23], the interplay between these attributes of PG can be expected to determine the distribution and action of PG in blood versus cell membrane. The consequences of this partition can be substantial and will be studied further.

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