The cytoprotective effect of Trolox demonstrated with three types of human cells

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Trolox, a hydrophilic analogue of α-tocopherol, was reported to scavenge peroxyl radicals better than vitamin E in sodium dodecyl sulfate micelles and in liposomes. However, it was not known if Trolox protects human cells against oxyradical damage or if it acts as an antioxidant there. Here we demonstrate that Trolox prolonged substantially the survival of human ventricular myocytes and hepatocytes against oxyradicals generated with xanthine oxidase plus hypoxanthine, and prevented lysis of red cells exposed to an azo-initiator (2,2′-azo-bis(2-amidinopropane) HCl). Note that Trolox did not inhibit xanthine oxidase. In each cell type, the protection by Trolox was dose dependent and surpassed those given by such water-soluble antioxidants as ascorbic acid, superoxide dismutase, and (or) catalase, each examined at or near its optimal level in the same system. Using hepatocytes as a model, we further observed that Trolox reduced markedly the quantity of phospholipid conjugated dienes (a chemical imprint of oxyradical damage) in cells despite their exposure to oxyradicals. These data suggested that Trolox behaves as an antioxidant in cells as illustrated in hepatocytes.

Key words: oxyradicals, cytoprotection, hepatocytes, myocytes, erythrocytes.

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Le Trolox, un analogue hydrophile de l'α-tocophérol, est capable d'enlever les radicaux peroxyles mieux que la vitamine E dans les micelles de dodécylsulfate de sodium et dans les liposomes. Cependant, on ne sait pas si le Trolox protège les cellules humaines contre le dommage dû aux oxyradicaux ou s'il agit alors comme antioxydant. Nous démontrons ici que le Trolox prolonge de façon substantielle la survie des myocytes ventriculaires et des hépatocytes humains contre les oxyradicaux générés avec la xanthine oxydase plus l'hypoxanthine et qu'il empêche la lyse des érythrocytes exposés à un azo-initiateur (2,2′-azo-bis-(2-amidinopropane) HCl). Notons que le Trolox n'inhibe pas la xanthine oxydase. Dans chaque type de cellule, la protection accordée par le Trolox dépend de la dose et elle surpasse celle donnée par des antioxydants hydrosolubles tels que l'acide ascorbique, la superoxyde dismutase et (ou) la catalase, chacun étant examiné à ou près de son taux optimum dans le même système. Utilisant des hépatocytes comme modèle, nous avons observé que le Trolox réduit de façon marquée la quantité des diènes conjugués à des phospholipides (empreinte chimique du dommage causé par les oxyradicaux) dans les cellules en dépit de leur exposition aux oxyradicaux. Ces données suggèrent que le Trolox se comporte à la façon d'un antioxydant dans les cellules tel qu'illustré dans les hépatocytes.

Mots clés : oxyradicaux, cytoprotection, hépatocytes, myocytes, érythrocytes.

[Traduit par la revue]

ABBREVIATIONS: Trolox, Trolox C or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; SDS, sodium dodecyl sulfate; PBS, sodium phosphate buffer containing 0.9% saline; DMEM, Dulbecco's modified Eagle medium; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; BSS, balanced salt solution; BSA, bovine serum albumin; XOD, xanthine oxidase; AAPH, 2,2′-azo-bis(2-amidinopropane) HCl; TCNE, tetracyanoethylene; CD, conjugated dienes; SOD, superoxide dismutase; CAT, catalase.

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Introduction

One of the physiologic roles of vitamin E is to prevent lipid peroxidation by oxyradicals, especially in cellular membranes (e.g., Burton *et al.* 1983; Cheeseman *et al.* 1986; Cross *et al.* 1987). An important document on the oxyradical scavenging effect of vitamin E *in vivo* was written by Marubayashi *et al.* (1986). They reported that rats preadministered with this chemical for days before surgically induced ischemia-reperfusion in the liver resulted in much less hepatic necrosis, lower level of tissue lipoperoxides, and better animal survival than in control rats that were not treated with the vitamin. However, the extreme lipophilicity and relatively slow cellular uptake of vitamin E (Ingold *et al.* 1987) severely limits its clinical usefulness, especially in emergency. Against that background, Trolox, a hydrophilic analogue of vitamin E (Fig. 1), appears to be a promising therapeutic alternative to α-tocopherol. Originally designed as an antioxidant for preserving food and fats (Scott *et al.* 1974), Trolox appears to penetrate biomembranes rapidly and was shown by Doba *et al.* (1985) to be an excellent protector of phospholipid bilayers against peroxidative attack. In an elegant study, Castle and Perkins (1986) estimated that Trolox traps peroxyl radicals from SDS micelles eight times better than vitamin E. Recently, we reported that Trolox was able to protect cultured canine myocytes against oxyradicals generated *in situ* (Mickle *et al.* 1989). Here, to bring more clinical relevance to our studies, we have examined if Trolox protects three distinct types of human cells, namely myocytes, hepatocytes, and erythrocytes, against artificially generated oxyradicals. Secondly, we also ascertained the plausibe mechanism of Trolox action in cells, using human hepatocytes as a model system.

Materials and methods

Unless otherwise stated, all chemicals and enzymes used were from Sigma Chemical Co. (St. Louis, MO).

Cell preparations

Myocytes were prepared by incubating 100–400 mg of freshly resected human ventricular myocardium with 5–10 mL of a solution of 0.1% collagenase and 0.2% trypsin in Ca^{2+}- and Mg^{2+}-free PBS (pH 7.4 ± 0.1) at 37°C with gentle shaking. After 15–20 min, the incubation mixture was decanted into a vial containing an equal volume of DMEM (Gibco) containing 10% fetal bovine serum, 100 μg penicillin/mL, and 100 μg streptomycin/mL. Undigested tissue was treated as above and all incubation mixtures were collected and centrifuged at 5000 × *g* for 15 min. The sedimented cells were suspended in fresh medium and counted in a Neubauer hemocytometer. Then the cells were incubated at 37°C under 5% CO_2 at a concentration of $6 \times 10^5 - 8 \times 10^5$. Following incubation for 1 h, the supernatant fluid was transferred to another culture dish. When the cells reached confluence they were separated by trypsin treatment as noted above. The cells were ready for experiment by 7–10 days after separation. All other details were as described previously for culturing canine myocytes (Mickle *et al.* 1989). The myocytes were identified microscopically by their characteristic morphologic appearance and by fluorescent staining with monoclonal antibodies specific for actin (Tsukada *et al.* 1987) and human ventricular myosin light chain 1 (Hoffman *et al.* 1988), respectively. Myocytes of the same generation and age were used to compare the effectiveness of Trolox against other oxyradical scavengers in preventing cellular necrosis from artificially generated free radicals. The criteria of myocyte necrosis include morphologic changes (e.g., sarcolemmal rupture and cytoplasmic shrinkage) and leakage of enzymes (e.g., LDH and AST) into the culture medium.

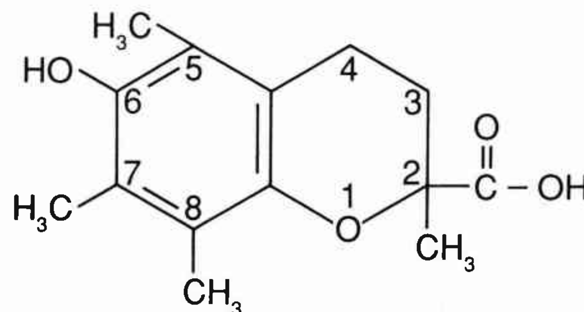

FIG. 1. Structure of Trolox or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

Human hepatocytes were isolated from healthy sections of freshly resected livers of donors with informed consent. Approximately 100 g of the tissue was transferred in ice-chilled saline to the culture room and perfused as described by Princen *et al.* (1986) with minor modifications (see below). The perfusion was done, first with 500 mL of 0.5 mM EGTA and 10 mM HEPES buffer solution (pH 7.6) containing 142 mM NaCl and 6.7 mM KCl via gravity drip at 30 mL/min, then with 150 mL of magnesium-free Hank's BSS containing 5 mM $CaCl_2$ and 0.05% collagenase according to Seglen (1973). The cells were harvested by dissociating the tissue in Hank's BSS containing 4 g% BSA and washed three times with Hank's BSS containing 2 g% BSA, centrifuged at 6 × *g* for 10 min, and plated in William's medium E with 10% fetal bovine serum containing insulin, dexamethasone, penicillin, and streptomycin according to Princen *et al.* (1986). The cells were cultured at 37°C under 5% CO_2 in a water-jacketed incubator (John's Scientific Inc.) for 18–20 h and identified to be hepatocytes by phase-contrast microscopy of their morphology and with electron microscopy of their subcellular structures. They were also identified by fluorescent staining with monoclonal antibodies specific to liver glutathione reductase isozymes (Rushmore *et al.* 1988). Cell death was indicated by such morphologic changes as nuclear shrinkage, rupture of cellular membranes with accompanying extrusion of cytoplasmic contents, and the invariable appearance of a "halo" within the cell. These observations were supported by electron microscopy (12 800 fold magnification) showing that necrotic hepatocytes had disruption of cellular and nuclear membranes, swollen and ruptured mitochondria containing electron-dense deposits, and disorganization of intracellular organelles. Concurrently, more than 95% of 10^5 cells had lost their ability to exclude trypan blue, exhibited maximal leakage of the activity of AST (Carey *et al.* 1989), and attained at least 50% specific release of externally supplemented ^{51}Cr into the culture medium, as described by Ota *et al.* (1988). Liver cells were harvested about 20–24 h after culture for our studies.

Human erythrocytes were collected from healthy donors and prepared for oxyradical studies according to Miki *et al.* (1987).

Free radical studies on cells

For cultured cells, these studies were done by removing the cell culture medium and adding to the cells 3 mL of 0.05 M PBS (pH 7.4) containing XOD at 50 IU/L for myocytes and 66.7 IU/L for hepatocytes and hypoxanthine (1 mM for myocytes and 2 mM for hepatocytes). It is important to note that each culture dish contained cells of the same generation and age, and that the cells were plated within a range of $1.10 \times 10^5 \pm 0.05 \times 10^5$ cells, over which the results of our study were verified, by prior experimentation, to be invariant with the relatively small fluctuations in cell numbers. The incubations were done at 37°C. All additives (i.e., Trolox or antioxidants) were supplemented to the cells immediately before adding XOD and hypoxanthine. Note that Trolox is poorly soluble above 1.8 mM. Accordingly, solutions containing higher concentrations of Trolox than 1.8 mM were prepared either by sonication and (or) by alkaline treatment (with, e.g., 100 μL of

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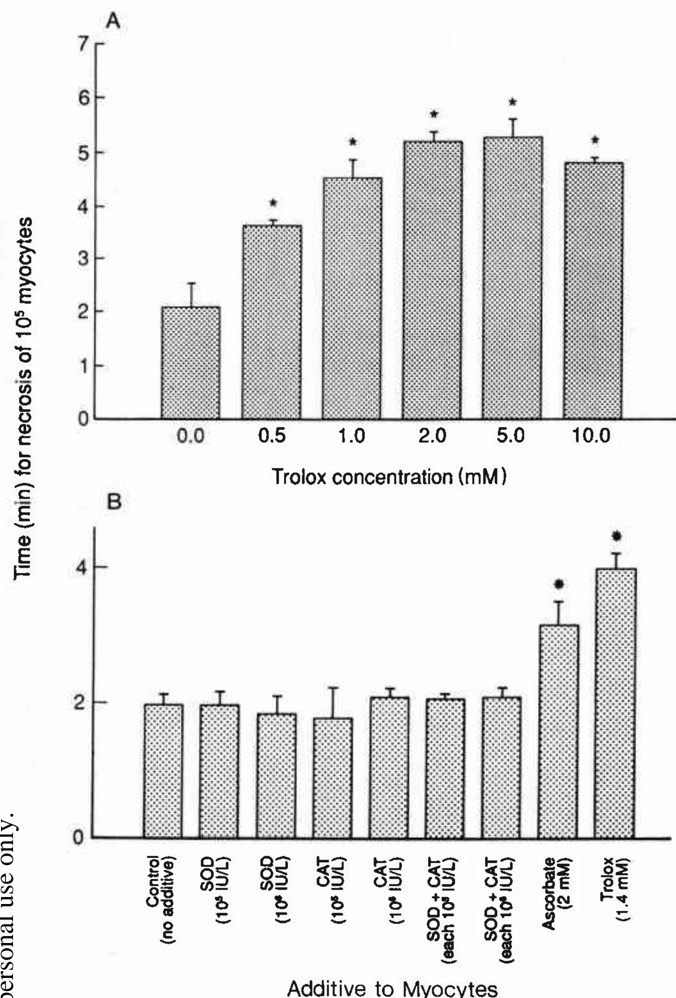

FIG. 2. Trolox protection of human ventricular myocytes against oxyradical damage. (A) Variation of cell necrosis time with concentration of Trolox. Approximately $2.05 \times 10^5 \pm 0.06 \times 10^5$ myocytes of the same generation and age were incubated with 50 IU XOD/L, 1 mM hypoxanthine and Trolox at the concentrations indicated at 37°C. For the control without Trolox added, the mean time for necrosis of 95% of the cultured cells was 2 min. The single asterisk indicates a $p < 0.01$ for comparing with the control. At each concentration, the number of repeats was 7–10. (B) Protective effects of Trolox and several known antioxidants are compared. Each additive was examined at either one or more levels shown in our preliminary work to be at or near its optimal concentration range in the same cell system. In this particular myocyte preparation, the apparent optimum for Trolox was 1.4 mM (cf. the optimum of 1.75 ± 0.42 mM for multiple myocyte preparations over 2 years). Only results with Trolox and ascorbate were significantly different from that of the control ($p < 0.01$). At each permutation, $n = 7$.

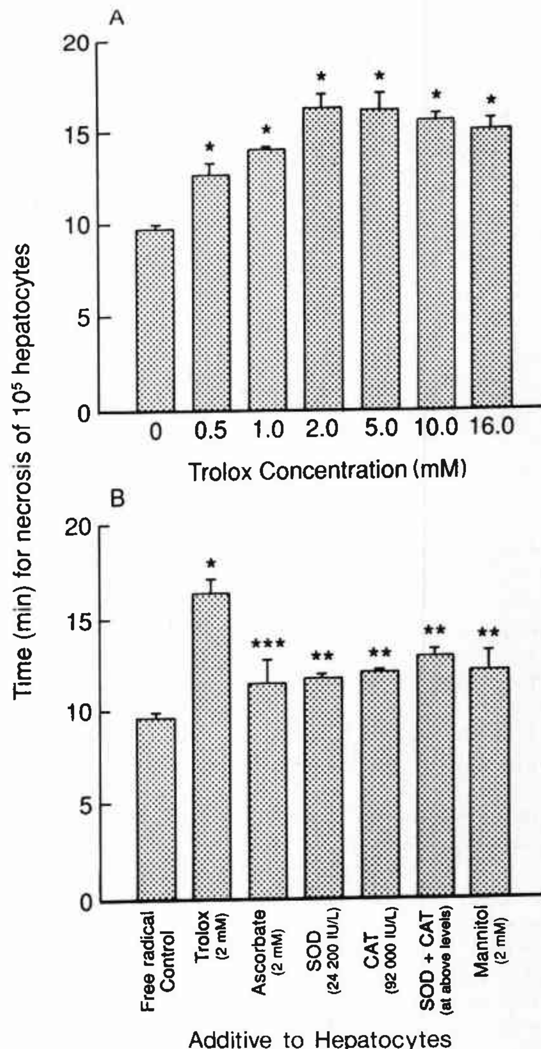

FIG. 3. Trolox protection of human hepatocytes against oxyradical damage. (A) Variation of cell necrosis time with the concentration of Trolox. The conditions were similar to those described for Fig. 1A, except that 66.7 XOD IU/L and 2 mM hypoxanthine were employed to generate oxyradicals. Note that the number of cells used in each condition was within the range of $1.05 \times 10^5 \pm 0.03 \times 10^5$, over which the necrosis time for the same oxyradical damage was invariant. This meant that it took ~10 min to achieve necrosis in 95% of 10^5 cultured hepatocytes when there was no cytoprotective agent added. The asterisk indicates $p < 0.001$. At each level of Trolox, $n = 5$–7 replicates. (B) Cytoprotective effect of Trolox and several water-soluble antioxidants are compared. As in Fig. 2B, the comparisons were made between Trolox and other additives at levels at or near their individual optima predetermined in this system. ***, $p < 0.01$; **, $p < 0.002$; *, $p < 0.001$. In each permutation, $n = 5$.

1 M NaOH), followed immediately by neutralization with acid (e.g., 1 M HCl) to pH 7.5 ± 0.1. With both hepatocytes and myocytes, the base for comparing the effect of different additives was the time taken by the above-defined XOD–hypoxanthine system to cause necrosis in 10^5 cells of the same generation within the same culture dish. With human erythrocytes, the free radicals were generated by thermal activation of the azo-initiator AAPH. The percent hemolysis and its inhibition by Trolox or other antioxidants were determined as described by Miki *et al.* (1987). In our study, Trolox and other cytoprotective agents were tested in a blinded fashion and in 3–10 replicates.

Quantitation of phospholipid conjugated dienes from tissues

The method of Waller and Recknagel (1977) was applied to phospholipid extracts of hepatocytes, using [^{14}C]TCNE. By this method, [^{14}C]TCNE undergoes a highly specific Diels–Alder condensation with unsaturated CD. In the actual experiment, $1.15 \times 10^5 \pm 0.06 \times 10^5$ cells in the culture dish were exposed to XOD and hypoxanthine as described above, but for 6 min. We selected this end point because our exploratory studies had indicated that, in the control with cells incubated with artificially generated oxyradicals, the CD formed were more reproducibly and quantitatively recovered at 6 min than at 8–10 min, even though the percentage of cells necrosed was 70–80% rather than 95–100% at

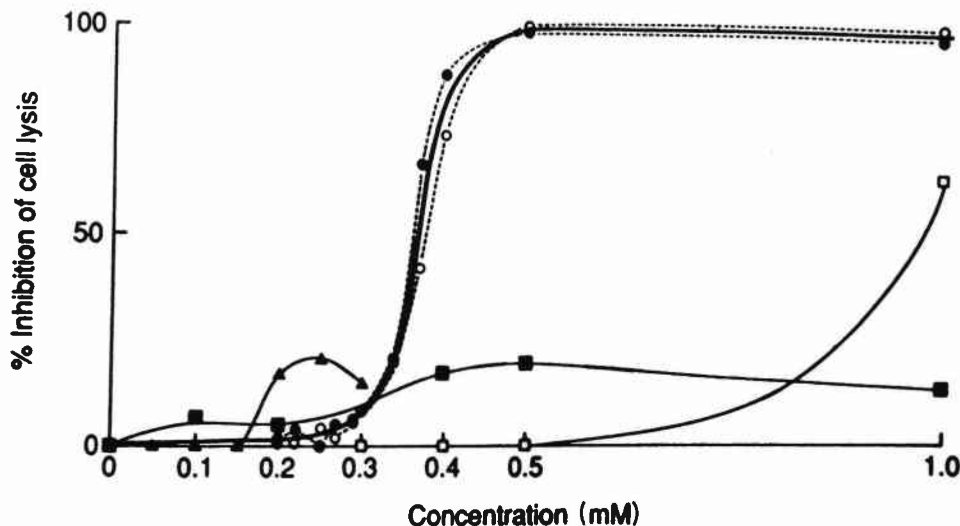

FIG. 4. Reduction of free radical-induced lysis of human erythrocytes by Trolox and other antioxidants. Red cells in suspension were exposed to free radicals generated by thermal activation of AAPH as described by Miki *et al.* (1987). The resulting lysis was measured by the amount of hemoglobin (based on its absorption at 541 nm) in the supernatant fluid after the cell debris and any residual unlysed cells had been spun down. The spectral absorption A for the control supernatant fluid after exposure to AAPH but without any additive and the absorption B for the permutation with additive supplemented along with AAPH were used in the equation $(1 - A/B) \times 100$ to give the percent inhibition of lysis (see vertical axis). The average of three closely agreeing replicates at each concentration per additive was shown in the graph. For the top three curves (for Trolox), the dotted lines with open or solid circles were results for red cells sampled from two unrelated donors, while the solid curve was the arithematic average of the two dotted curves. ○ and ●, Trolox; □, ascorbate; ■, L-cysteine; ▲, uric acid.

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these times, respectively. Where Trolox was added, its final concentration was 2 mM. After 6 min of incubation, allopurinol (an inhibitor of XOD) was added to the cutlure dish in all permutations to reach 2 mM. The cells were frozen in liquid nitrogen and freeze-dried immediately. The lyophilate was resuspended in saline, homogenized in ice-chilled chloroform–methanol, and further processed for diene measurement as described by Waller and Racknagel (1977). Preliminary work showed that Trolox (2–8 mM) had no effect on the quantitation of CD here (N. Hashimoto, unpublished results).

Statistical analysis

This analysis was done using the Student's t-test. Unless otherwise stated, all data were expressed as means ± SD. In all comparisons with the control, statistical significance of data was indicated by a p value < 0.05.

Results

Trolox protection of human myocytes against oxyradicals

Figure 2A shows a typical response of human myocytes to Trolox despite the presence of oxyradicals. Clearly, Trolox prolonged significantly the survival of cells relative to the control without Trolox added. Also, the prolongation of cell survival increased with Trolox concentration, with an apparent optimum at ~2 mM Trolox. From similar experiments conducted on multiple batches of myocytes over 2 years, this optimum was observed to average at 1.75 ± 0.42 mM Trolox. In control experiments, we observed that myocytes which were incubated alone in buffer, with hypoxanthine (1 mM), or with XOD (50 IU/L) did not exhibit any evidence of necrosis until after 40–60 min. Figure 2B compares the protective effect of 1.4 mM Trolox against those afforded by a number of well-known water-soluble antioxidants. The other water-soluble antioxidants studied were each examined at concentration(s) at or near its optimal level in the same system (data not shown). Apart from ascorbate, the antioxidant enzymes SOD and CAT, either alone or together, did not reveal significant protection for the myocytes against oxyradicals generated here.

Trolox protection of human hepatocytes against oxyradicals

Figure 3A illustrates the dose-dependent effect of Trolox on liver cells. As in myocytes, the maximum cytoprotective effect for Trolox in human hepatocytes was at or near 2 mM. Figure 3B shows that all other water-soluble antioxidants tested, including ascorbate, were less protective of the cells than Trolox at 2 mM, even though their normal effects on cell survival were statistically different from the control (without any additive). Again, in control experiments, there was no discernible morphologic or biochemical evidence of cell necrosis for at least 30 min when the hepatocytes were incubated with either buffer, XOD, or hypoxanthine.

Possible direct effect of Trolox on XOD reaction

To examine this possibility, we measured directly the XOD reaction with hypoxanthine as substrate at concentrations as used in our free radical studies. However, the assay was performed in a YSI oxygen electrode according to instructions of its manufacturer rather than in a spectrophotometer, because the enzyme product (urate) overlaps spectrally with Trolox. To start the reaction, XOD was added to hypoxanthine either with or without Trolox present. After a brief incubation period of 30 s, the rate of oxygen consumption at 37°C became linear for at least 3 min and was monitored during this period in triplicates per permutation. Based on either the initial velocity or extent of oxygen consumption in 3 min, we observed that Trolox over a range of concentrations including 2 mM did not inhibit the XOD reaction. Thus, the net consumption of oxygen during the first 3 min of XOD catalysis, which encompasses the initial and highly linear phase of the reaction, was 80.0 ± 1.5, 80.3 ± 0.58, and 80.0 ± 0.68% for 0.5, 1, and 2 mM of Trolox, respectively (for n = 3 in each case), which did not differ signifi-

cantly from that of 76.8 ± 1.4% for the control (i.e., enzymic reaction without Trolox added, for $n = 3$). In contrast, allopurinol (a potent XOD inhibitor) at either 1 or 2 mM completely inactivated XOD under otherwise identical conditions.

Trolox protection of human erythrocytes

When erythrocytes were exposed to oxyradicals generated with AAPH, Trolox again emerged as the best of the compounds testes in protecting the cells against lysis. Figure 4 shows typical profiles depicting the percentage inhibition of red cell lysis over a range of concentrations of each compound tested. The sigmoidal profiles of Trolox protection were shown in dotted lines for samples of red cells provided by two unrelated donors, with a solid curve representing the mean. A convenient index for comparing the relative efficacies of different cytoprotective agents here is the concentration of each agent that gives 50% inhibition of cell lysis (I_{50}). For Trolox, the I_{50} was 0.34 mM, while that for ascorbic acid was 0.95 mM. L-Cysteine and uric acid, two other known water-soluble antioxidants (Cross *et al.* 1987), did not even produce 50% inhibition of cell lysis under our experimental conditions. Not shown in Figure 4 was the observation that SOD (at as high a level as 24 200 IU/L), CAT (92 000 IU/L), or both together at these levels failed to elicit >30% inhibition of cell lysis (relative to the control without enzymes added) under the same conditions.

Formation of conjugated dienes in hepatocytes with and without Trolox present

To gain possible insights into the mechanism of Trolox action in cells, we also quantitated the CD, which have been called the fingerprint signatures of free radical damage (Romaschin *et al.* 1987), in cultured hepatocytes before and after 6 min of exposure to oxyradicals. The reason for choosing a 6-min incubation time was given in Materials and methods. Figure 5 shows no significant change in the background CD level before and after incubation, either for cells in buffered saline (the control) or for cells treated with Trolox (at 2 mM) in saline. However, in cells that were exposed to oxyradicals for 6 min and in which 70–80% of the 10^5 cells exhibited morphologic and biochemical evidence of necrosis, there was four- to five-fold higher CD formed than in the control. Finally, when Trolox was present during the presumptive oxyradical attack on hepatocytes, the CD level at 6 min was markedly (~40%) lower than that exposed to free radical attack without Trolox being present.

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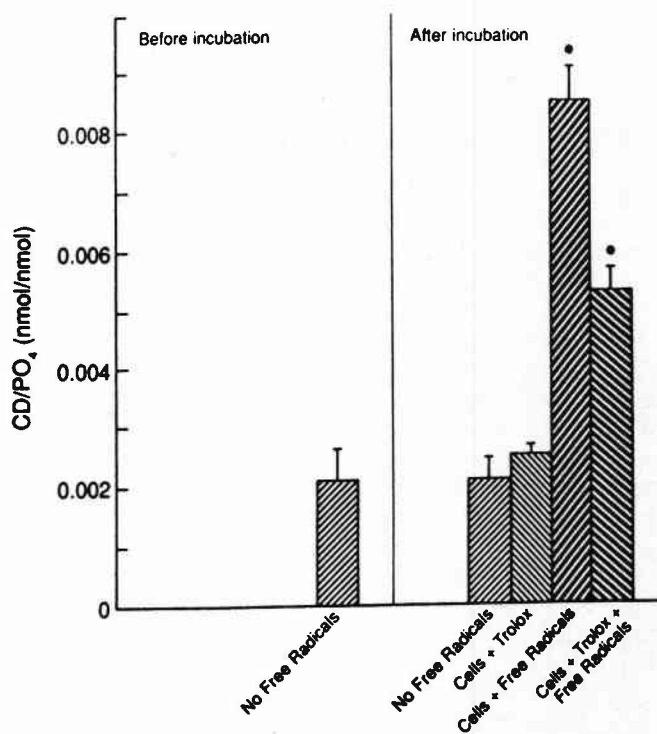

FIG. 5. Quantitation of conjugated dienes in human hepatocytes before and after 6 min of incubation of the cells with XOD and hypoxanthine. Details of the experiment were given in the text. Note that Trolox (at 2 mM in the incubation mixture) was added ~30 s after XOD plus hypoxanthine and that the lyophilate of frozen cells was washed at least five times with double-distilled water before processing of diene measurement. Triplicates were done at each permutation. The vertical axis represents the amount of phospholipid-conjugated dienes (CD) referenced against the amount of phosphate (PO_4). An asterisk indicates a $p < 0.01$ for the result of each permutation relative of the control.

Discussion

In the first part of this paper, we documented that Trolox protects three distinct types of human cells against free radical damage. In each cell type, we further delineated that the protection of Trolox is dose dependent and exceeds those given by a number of known water-soluble antioxidants. Although we had shown Trolox to protect canine myocytes against similar free radical attack (Mickle *et al.* 1989), there has been no evidence of a dose effect for Trolox in any cell (human or otherwise) and no rigorous comparison of its efficacy vis-à-vis that of other antioxidant(s) in the cellular milieu.

The fact that the optimal dose of Trolox in both myocytes and hepatocytes is ~1.8 mM could be coincidental or may be related to fact that Trolox is also maximally soluble in that neighbourhood. Note, however, that hepatocytes appeared to require 5–10 times longer exposure to a higher dose of oxyradicals (being generated with ~1.3 times the level of XOD and 2 times the hypoxanthine concentration needed) to cause necrosis in a similar number of myocytes. This may stem, among others, from the fact that the liver is better fortified with endogenous antioxidants than the heart (Cross *et al.* 1987). Therefore, myocytes may be more sensitive than hepatocytes to oxyradical damage. Indeed, Doroshow and colleagues (1980) had estimated that heart muscle contains only 27% of the SOD activity and <1.3% of the CAT activity found in the liver tissue. The ability of Trolox to prevent lysis of the erythrocyte membrane by free radicals further highlights the protective effect of Trolox on a type of cell that is known to differ from myocytes and hepatocytes in structure (e.g., the absence of a nucleus), in function (e.g., gaseous transport versus detoxification and glycogen storage in hepatocytes or contractility in myocytes), and in endogenous antioxidant defenses (the erythrocytes being enriched in ascorbate, catalase, and glutathione). It is possible that Trolox is a general cytoprotective agent, but this is still conjectural. It is also possible that Trolox may protect certain subtypes of cells better than others within the same tissue or organ, but this needs to be confirmed.

In the second part of this work, we explored the mechanism of Trolox action in cells. Using human hepatocytes as a model, we demonstrated a strong circumstantial link between the level of CD: the putative chemical imprint of

oxyradical damage in human hepatocytes (Romaschin *et al.* 1987) and the necrosis of cells induced by oxyradicals. This was further reinforced by the observation that Trolox reduced markedly the level of dienes detected, while prolonging hepatocyte survival despite the presence of highly reactive oxyradicals. Further experiments are in progress to better define the detailed kinetics of formation of CD and its modulation by Trolox. It may be worth noting that presumptive products of oxyradicals (including CD) are likely unstable and transient in nature, as was also suggested by our data in Fig. 5.

Although these observations confirmed the cytoprotective action of Trolox, they did not, *per se*, distinguish between the possibilities that Trolox might act as an inhibitor of XOD (as is the case for allopurinol) and (or) as a scavenger of the oxyradicals formed. Theoretically, either mechanism could have led to the same overall result. In this connection, our data from measuring XOD activity under conditions closely mimicking those in our hepatocyte culture are highly germane. There, we have illustrated that Trolox does not affect the XOD reaction, either in rate or extent. Taken together, these results suggest strongly that the behaviour of Trolox as exemplified in human hepatocytes is consistent with its being an antioxidant. We are isolating and characterizing a presumptive oxidation product of Trolox from the incubation mixture represented by the last column in Fig. 5. This may allow us to better elucidate the chemical fate of Trolox in the milieu of the cultured liver cell.

In summary, our observations have established Trolox to be a relatively effective cytoprotective agent in multiple types of human cells, each with its own unique structural and functional characteristics. We have also quantitated directly, for the first time, the CD (a marker of oxyradical damage in human hepatocytes) and illustrated that Trolox can, in its plausible role of an antioxidant, reduce the level of dienes while prolonging cell survival.

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