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Morin: A wood pigment that protects three types of human cells in the cardiovascular system against oxyradical damage

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Abstract—Morin is a yellowish pigment extractable from the wood of *Chlorophora tinctoria*. In the present study, we have determined that morin protects three types of human cells—ventricular myocytes, saphenous vein endothelial cells, and erythrocytes—against damage by oxyradicals generated *in situ*. In myocytes and endothelial cells, morin prolonged substantially and in a concentration-dependent manner the survival of cells exposed to either xanthine oxidase-generated oxyradicals or superoxide radicals produced with menadione. Morin protected erythrocytes from lytic attack by peroxyl radicals generated with 2,2′-azo-bis (2-amidinopropane) dihydrochloride. In all three types of human cells, the protective effect of morin clearly excelled that displayed by Trolox (a vitamin E analog), ascorbate, or mannitol, which are water-soluble antioxidants of similar molecular size. Chemically, we verified that morin behaves as an antioxidant by diminishing markedly the amount of malondialdehyde (lipid peroxidation product) found in human cardiocytes despite their exposure to oxyradicals. In agreement with related reports, we also observed that morin is non-toxic in rats even when used at concentrations 2–3 orders of magnitude higher than those in our *in vitro* studies. Thus, morin acts as a broad-spectrum and non-toxic antioxidant.

Key words: morin; antioxidant; pigment; cardiovascular system; human cells

According to current thought, tissue damage by oxyradicals is an important pathophysiologic basis in diverse disorders, including several in the heart [1, 2]. Therefore, there have been searches for antioxidants that actively protect cells against oxyradical damage. In our laboratory, we have been studying the bioactivity and plausible mechanisms of a number of natural [3–5] and synthetic antioxidants [6–9] that protect various types of cells *in vitro* and *in vivo*.

In the present study, we investigated a novel and active cytoprotector from Brazilian wood. The substance, named morin or morin hydrate, is a yellowish pigment that has been used for spot testing of certain metal ions [1] (Fig. 1). Morin has been suspected of being a free radical scavenger [11], but little is known of its effect on cells. Here we report that morin effectively protected ventricular myocytes, vascular endothelial cells, and erythrocytes each exposed to oxyradical species generated *in situ*. There are three distinctive features in our study. First, we employed *human* rather than animal cells to ensure greater clinical relevance of our findings. Second, we used XO-HP* to produce the cascade of superoxide, hydrogen peroxide and hydroxyl radicals; to generate superoxide ions we employed menadione as the source [12], whereas to form peroxyl radicals we thermally activated an azo-initiator AAPH [13]. Third, we verified that morin behaves as a true antioxidant by showing that it reduced substantially the quantity of malondialdehyde detected in oxidant-exposed cells.

Materials and Methods

Materials. Unless otherwise stated, all chemicals used were reagent grade and were obtained from the Sigma Chemical Co. (St. Louis, MO). Morin hydrate was a product of the Aldrich Chemical Co. (Milwaukee, WI).

Preparation of cultured human cells. The detailed procedures employed have been documented elsewhere [9]. Human ventricular myocytes, cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, pH 7.4; were obtained from 5–10 mg left ventricular biopsies performed with the informed consent of the patients. 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, 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 medium 199 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]. For our studies, the cells were harvested from confluent cultures and incubated, at 37°, in 0.05 M sodium phosphate-buffered saline (PBS), pH 7.4, containing either 8.34 IU/L XO and 2 mM HP, or alternatively, 1.0 mM menadione as the oxyradical generator. The base for comparing control (no putative

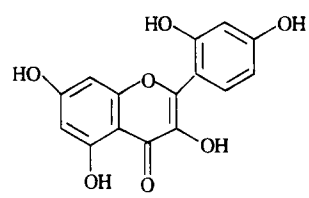

Fig. 1. Structure of morin or morin hydrate.

* Abbreviations: XO, xanthine oxidase; HP, hypoxanthine; AAPH, 2,2′-azo-bis(2-amidinopropane) dihydrochloride; and MDA, malondialdehyde.

cytoprotector) versus test (with a putative cytoprotector such as morin) 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 morin or its comparative antioxidant was added along with XO-HP or menadione 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, and by electron microscopy [14].

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 Miki *et al.* [13] with minor modification [15]. A 20% suspension of red cells in PBS was added to the same volume of 200 mM AAPH solution in PBS containing either morin or another antioxidant 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. The absorbance (*A*) of the supernatant was measured at 540 nm. Similarly, the reaction mixture of each permutation was treated with

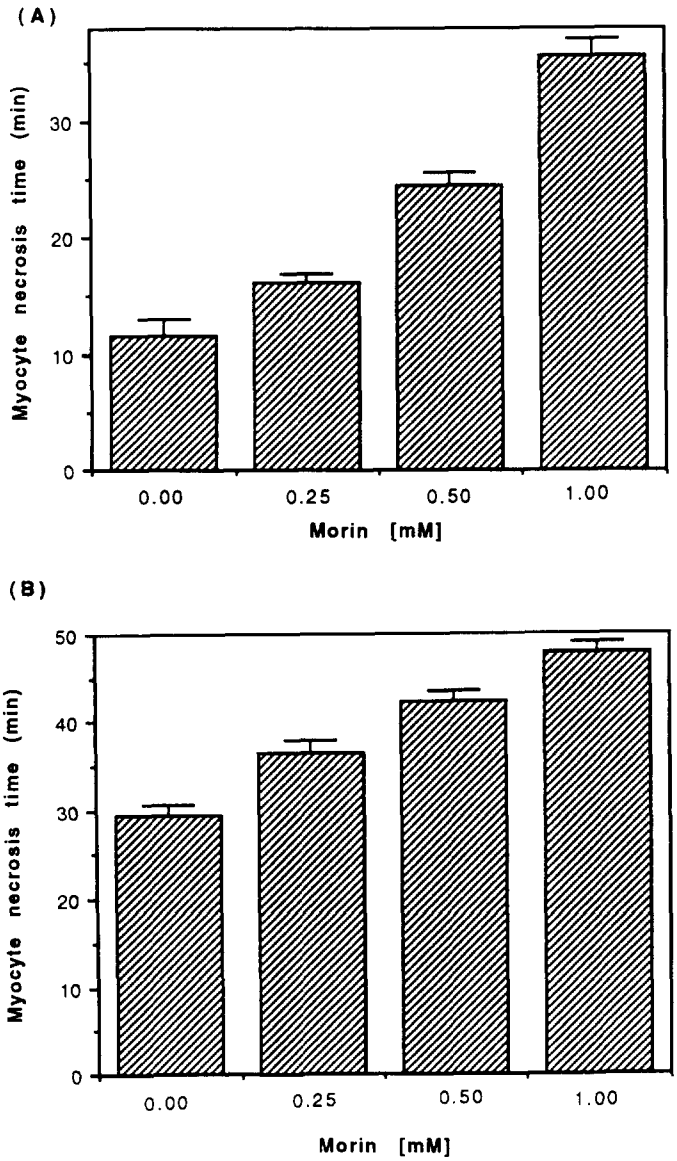

Fig. 2. (A) Protection by morin of human ventricular myocytes against oxyradicals generated with xanthine oxidase (XO)-hypoxanthine (HP). The first column indicates the results of incubating myocytes with XO-HP in the absence of morin. Values are means ± SD of 3–5 replicate incubations. All other details are given in the text. (B) Results with menadione replacing XO-HP as the source of oxyradicals.

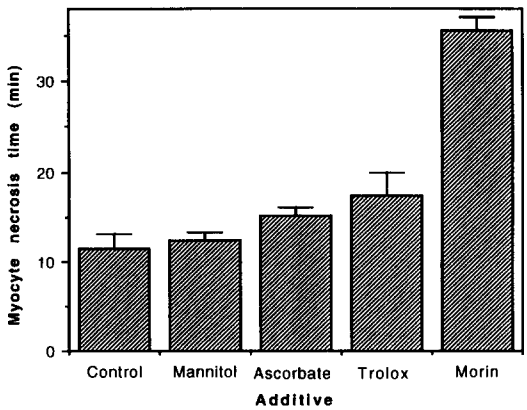

Fig. 3. Comparison of the protective effects of morin, Trolox, ascorbate, and mannitol, each at 1 mM, on human ventricular myocytes exposed to XO-HP. All other conditions are given in Materials and Methods. Values are means ± SD of 3–5 replicate incubations.

distilled water to yield complete hemolysis and the absorbance (B) of the supernatant after centrifugation was measured at 540 nm. The 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 at 5–10 times per concentration.

Malondialdehyde measurement based on thiobarbiturate assay. The method of Kirkland *et al.* [16] based on thiobarbiturate reaction was used for measuring the malondialdehyde in the cells. The average of several closely agreeing replicates was recorded.

Statistics. Either Student's t-test or the analysis of variance (ANOVA) method followed by Duncan's Multiple Range Test, where appropriate, was applied. All data are expressed as means ± SD. Significance was indicated by a $P < 0.05$.

Results

Effect of morin in protecting human ventricular myocytes against oxyradicals generated with XO-HP. Figure 2A summarizes the results from incubating ~10^5 ventricular myocytes with 8.34 IU XO/L and 2 mM HP at 37°, in the

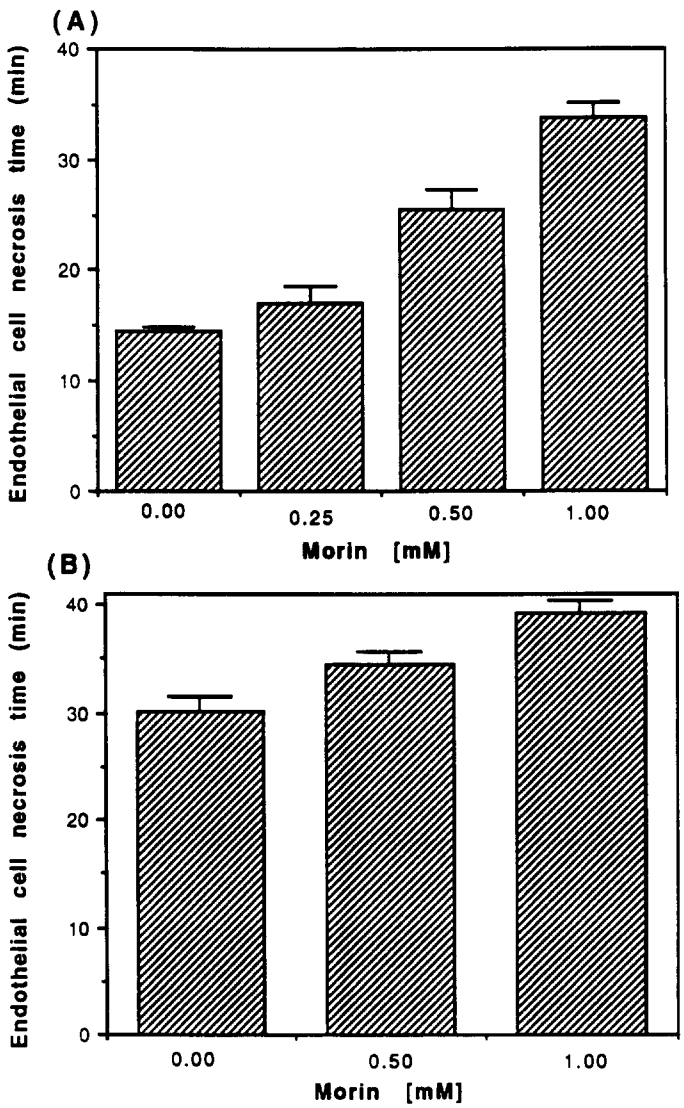

Fig. 4. Effect of morin in protecting human saphenous vein endothelial cells against oxyradicals generated with (A) XO-HP or (B) menadione. All other details are given in the text. Values are means ± SD of 3–5 replicate incubations.

absence and in the presence of increasing concentrations of morin. With no morin added (see first column), ~95% of the cells were necrosed in 11–12 min as judged with phase contrast microscopy and verified, as described previously [14], by electron microscopy, trypan blue exclusion, enzyme leakage, and ^{51}Cr release. The necrosis time was doubled at 0.5 mM and tripled at 1.0 mM. One-way ANOVA revealed that there was a significant effect ($P < 0.0001$) of morin concentration on necrosis times. Note that in the absence of XO and/or HP the cells incubated with PBS alone survived for ~60 min.

Effect of morin against menadione damage in human ventricular myocytes. Figure 2B shows that morin also prolonged the survival of ~10^5 myocytes when 1 mM menadione was used in place of XO-HP as the oxyradical source. Again, one-way ANOVA indicated a significant relationship ($P < 0.001$) between the level of morin and cell necrosis times.

Comparative protective effects of morin, Trolox, ascorbate, and mannitol (each at 1.0 mM) on human ventricular myocytes exposed to XO-HP or menadione. Figure 3 illustrates that, under otherwise identical conditions, morin prolonged survival of the myocytes more extensively than Trolox, ascorbate or mannitol. Similarly, morin excelled over the same comparators in protecting myocytes against menadione (data not shown).

Effect of morin in protecting human saphenous vein endothelial cells against oxidant injury. Figure 4 illustrates the corresponding cytoprotective effects of morin 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 results presented in Fig. 5 demonstrate that morin was also a better protector of endothelial cells than Trolox, ascorbate, and mannitol against oxyradicals generated with XO-HP ($P < 0.01$).

Protection of human erythrocytes by morin against peroxyl radical attack. Figure 6 shows that morin inhibited the lysis of human red cells due to a thermally activated azo-initiator (AAPH) more effectively than several other antioxidants. Thus, the IC_{50} (the average concentration of an additive that inhibits 50% cell lysis) for morin was 0.35 mM, substantially smaller than the IC_{50} values of 0.57, 0.65, and >1.0 mM for Trolox, ascorbate and mannitol, respectively, each tested with the same preparation of blood and reagents ($P < 0.001$).

MDA levels in cells exposed to oxyradicals with or without morin present. When either human ventricular myocytes or vascular endothelial cells were exposed to oxyradicals, MDA was detected reproducibly in the cells. However, when morin was added to cells as a putative antioxidant protector, the MDA measured was reduced, and the reduction paralleled in magnitude the amount of morin added initially. Thus, for example, at 1 mM morin, the MDA detected in the endothelial cells diminished by ~40% relative to that in the control without morin.

Discussion

In this study we have presented direct evidence that morin is a broad-spectrum antioxidant that effectively protects three distinct types of cells in the human cardiovascular system. The cytoprotective activity of morin may be especially important in the vascular endothelium because it is here that most of the cardiac XO in the human cardiovascular system is concentrated [17]. It may be worth noting that XO generates a cascade of oxyradicals including the superoxide, hydrogen peroxide, and hydroxyl radicals.

In this work, we have used menadione (a superoxide radical generator [12]) to establish, for the first time, that both ventricular myocytes and vascular endothelial cells are independently sensitive to superoxide anion and that morin can counter such effects more effectively than several other antioxidants such as Trolox, ascorbate and mannitol.

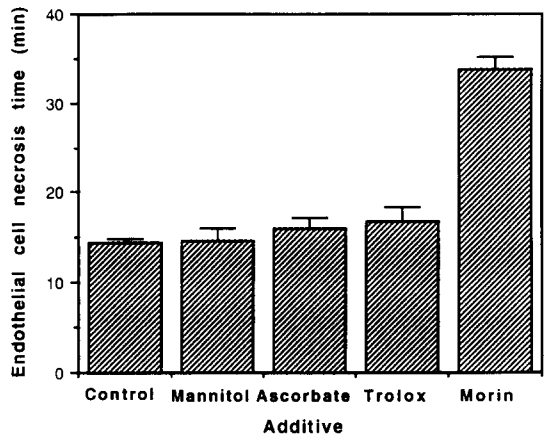

Fig. 5. Comparison of the protective effects of morin, Trolox, ascorbate and mannitol (each at 1 mM) on human saphenous vein endothelial cells against damage inflicted by XO-HP. Values are means ± SD of 3–5 replicate incubations.

The use of a well-validated red cell system [13, 15] has also allowed us to demonstrate that morin protects human red cells against peroxyl radicals better than various antioxidants do.

Although cytotoxicity is not the primary concern here, the fact that morin at or near 1 mM protected three types of cells effectively raises the question of the *in vivo* tolerance to this agent at that dose. In a preliminary study of six Sprague–Dawley rats each infused with 1 mM morin, we observed no toxic side-effects in the heart and liver (based on histopathology, assays of serum alanine and aspartate transaminases, transmission electron microscopy) 24 hr after infusion (Wu *et al.*, unpublished data). This finding is complementary to two other reports. First, Yugarani *et al.* [18] reported that Wistar rats fed a diet supplemented with 100 mg morin/rat/day for 4, 7 and even 10 weeks exhibited no toxic symptoms. Second, Havsteen [19] reported that the IC_{50} of flavonols, including morin, in rats ranged between 2 and 10 g/animal. Note that in these studies, the concentrations employed to demonstrate the

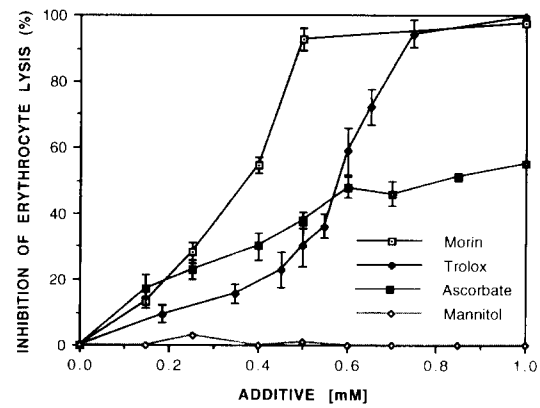

Fig. 6. Comparison of the protective effects of morin, Trolox, ascorbate, and mannitol in inhibiting lysis of human erythrocytes by AAPH. Each point is the average of 8–10 replicates, with the standard deviation indicated by a vertical bar.

effect of, or *in vivo* tolerance to, morin were 2–3 orders of magnitude higher than the ones we used in our *in vitro* and *in vivo* studies. From these data, we infer that morin is not a toxic antioxidant under the conditions specified.

In summary, our data have shown that morin is an unusual wood-derived antioxidant that protects three distinct types of cells in the human vascular system against a broad range of oxyradicals, and more effectively than Trolox (a vitamin E analogue), ascorbate and mannitol. We are conducting further mechanistic studies into the action of morin *in vitro* and *in vivo*.

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