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Antioxidant and Prooxidant Effects of Phenolics on Pancreatic β-Cells in Vitro

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A number of natural phenolic compounds display antioxidant and cell protective effects in cell culture models, yet in some studies show prooxidant and cytotoxic effects. Pancreatic β-cells have been reported to exhibit particular sensitivity to oxidative stress, a factor that may contribute to the impaired β-cell function characteristic of diabetes. The aim of this study was to examine the potential of natural phenolics to protect cultured pancreatic β-cells (βTC1 and HIT) from H_2O_2 oxidative stress. Exposure of cells to H_2O_2 led to significant proliferation inhibition. Contrary to what one should expect, simultaneous exposure to H_2O_2 and the phenolics, quercetin (10−100 μM), catechin (50−500 μM), or ascorbic acid (100−1000 μM), led to amplification of proliferation inhibition. At higher concentrations, these compounds inhibited proliferation, even in the absence of added H_2O_2. This prooxidant effect is attributable to the generation of H_2O_2 through interaction of the added phenolic compounds with as yet undefined componenets of the culture media. On the other hand, inclusion of metmyoglobin (30 μM) in the culture medium significantly reduced the prooxidant impact of the phenolics. Under these conditions, quercetin and catechin significantly protected the cells against oxidative stress when these components were present during the stress period. Furthermore, significant cell protection was observed upon preincubation of cells with chrysin, quercetin, catechin, or caffeic acid (50 μM, each) prior to application of oxidative stress. It is concluded that provided artifactual prooxidant effects are avoided, preincubation of β-cells with relatively hydrophobic natural phenolics can confer protection against oxidative stress.

KEYWORDS: Pancreatic β-cells; phenolics; flavonoids; oxidative stress; antioxidants; H_2O_2; metmyoglobin; proliferation

INTRODUCTION

Polyphenolic metabolites of plants are a common component of human diet, being found in fruits, vegetables, and several derivatives such as wine, cider, and tea. They have been shown to act as strong antioxidants in various systems, exhibiting multiple biological actions (*1*, *2*). In tissue culture systems, dietary plant phenolics typically act as antioxidants, with cell-protective properties (*3*−*5*), but under some circumstances were found to be prooxidants and cytotoxic (*6*−*8*). Many studies of tumor cells have led to an emphasis of the antiproliferative effect of dietary polyphenols, raising the possibility that these compounds could contribute to the prevention and treatment of cancer (*9*−*11*). Although flavonoids and phenolic compounds are strong reducing agents, under in vitro conditions in the presence of metal ions such as copper or iron, they can act as prooxidants (*12*−*14*). Quercetin, one of the most thoroughly investigated plant flavonoids, was found in vitro to be converted to cytotoxic metabolites (*15*). Because phenolic compounds can react with other components of cell culture medium (such as metal ions), the overall effect on cells, whether prooxidative or antioxidative, is strongly dependent on the composition of the medium. As in vitro culture conditions are substantially different from the in vivo environment, an understanding of the impact of in vitro conditions is crucial to the validity of any extrapolations concerning possible effects of phenolics in vivo.

In the present study, we chose a tissue culture model of two mammalian pancreatic β-cell lines, because β-cells show pronounced sensitivity to oxidative stress (ref *16* and our unpublished findings). Moreover, reactive oxygen species may play a central role in β-cell death, leading to the development of type 1 diabetes (*17*−*19*), and a secondary pathogenic role in the development of type 2 diabetes. This effect was reduced by antioxidants (*20*, *21*). (For reviews see refs *22* and *23*.)

Hydrogen peroxide (H_2O_2) was found to produce multiple effects on β-cells such as activating K_{ij} channels, in association with metabolic inhibition and increased plasma membrane permeability (*24*), and interfering with glucose metabolism, which influence membrane potential and lead to inhibition of insulin secretion (*25*). H_2O_2 was also shown to activate heme proteins such as metmyoglobin to a catalytically active form—

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ferrylmyoglobin, which is able to cause membrane lipid peroxidation (*26*). Cell toxicity of activated metmyoglobin toward endothelial cells was recently demonstrated (*27*).

The aim of this study was to test the in vitro ability of dietary flavonoid antioxidants to protect pancreatic β-cells from oxidative stress, as reflected by measurement of rates of cell proliferation. These flavonoids themselves can produce paradoxical prooxidant effects. When this artifactual effect was eliminated, however, we were able to clearly demonstrate the ability of several natural hydrophobic phenolics to protect β-cells against oxidative stress.

MATERIALS AND METHODS

Tissue culture medium and serum were from Biological Industries Ltd. (Bet Haemek, Israel). Methanol (HPLC grade) was from J. T. Baker (Phillipsburg, NJ), and butylated hydroxytoluene (BHT), ferrous ammonium sulfate, xylenol orange, and triphenylphosphine (TPP) (all for FOX2 reagent) were from Sigma (St. Louis, MO). Thiobarbituric acid, glucose oxidase (GO), metmyoglobin (metMb, from horse skeletal muscle), catalase, [^{3}H]thymidine, quercetin, catechin, caffeic acid, gallic acid, and hesperetin were also from Sigma. Chrysin was from Fluka (Buchs, Switzerland). Hydrogen peroxide (30%), trichloroacetic acid (TCA), and (+)-ascorbic acid (As.A) were from Merck (Darmstadt, Germany). Ferric chloride was obtained from Riedel-de-Haen (Hannover, Germany).

Cell Culture. βTC1 cells (*28*) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal calf serum, streptomycin (100 μg/mL), and penicillin (100 IU/mL). HIT cells (*29*) were cultured in DMEM with 10% (v/v) fetal calf serum, streptomycin (100 μg/mL), and penicillin (100 IU/mL). Cells were seeded (15000 or 30000 cells/well) in 96-well plates (Falcon, Becton-Dickinson) 24 h before the addition of test compounds. GO, metMb, catalase, As.A, and gallic acid were all dissolved in phosphate-buffered saline (PBS). Quercetin, catechin, and chrysin were dissolved in PBS containing up to 0.5% dimethyl sulfoxide (DMSO). These concentrations of DMSO were found to have no effect on cell proliferation during a 20 h incubation (data not shown).

Oxidative stress was induced, in the presence or absence of antioxidants, by incubation for 20 h with GO (4×10^{-4} Sigma units), which generates H_2O_2, or by GO and metMb (30 μM) together, to activate the metMb to the ferryl form (*26*). In other experiments, phenolic antioxidants together with metMb were preincubated with the cultures for 24 h. Cultures were then washed three times with PBS, fresh medium was added, and cells were challenged with GO/metMb oxidative stress during 20 h. In all experiments, cell proliferation was determined by the [^{3}H]thymidine incorporation method (*30*), following the addition of [^{3}H]thymidine (1 μCi/well) to the plates 18 h before cell harvesting.

Lipid Peroxidation Assay. Lipid peroxidation was measured by thiobarbituric acid reactive substances assay (TBARS) (*31*), parallel to the proliferation assay on the same culture plates. Preliminary experiment showed that this did not affect any of the assays. The TBARS procedure was modified as follows: After 20 h of exposure to different treatments, samples of 100 μL were taken from the upper medium of the wells into clean 1.5 mL tubes, and 20 μL of TCA (60%) was added. Following vigorous mixing, the tubes were centrifuged for 7 min at 20000*g*. Supernatant samples (110 μL) were placed into clean 96-well plates, and 40 μL of TBARS reagent (1.3%, dissolved in NaOH 0.3%) was mixed with the samples in the wells. The plate was carefully wrapped with Saran (Ziploc, Indianapolis, IN), incubated for 20 min in a 90 °C bath, and then cooled on ice. The samples were spectral analyzed in the plate at 532 nm (OPTImax, Molecular Devices Corp., Sunnyvale, CA). Control samples were prepared the same but with 0.3% NaOH instead of TBARS reagent.

Generation of H_2O_2. Following the addition of phenolics, samples (50 μL) of culture medium were taken during the course of 120 min, and the H_2O_2 content was analyzed by the FOX2 assay (*32*), with the addition of a 1 min centrifugation (20000*g*) prior to the spectral analysis.

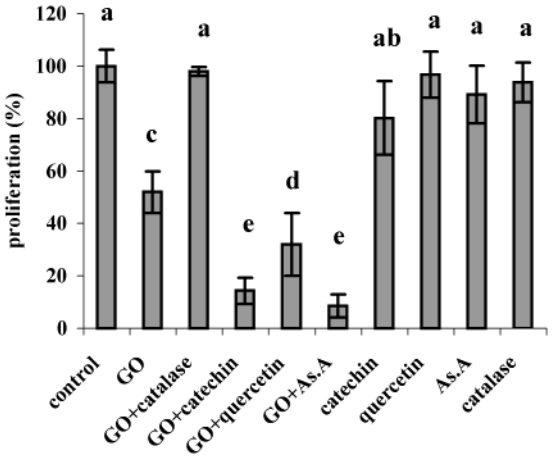

Figure 1. Effect of glucose oxidase and antioxidant treatment on βTC1 cell proliferation. Cells (30000 cells/well) were treated for 20 h, and [^{3}H]-thymidine incorporation was measured. Concentrations: 4×10^{-4} Sigma units of glucose oxidase, 0.8 Sigma unit of catalase, 125 μM catechin, 25 μM quercetin, 500 μM As.A. Data (mean ± SD) are expressed as a percentage of untreated control cells. $N = 6$–8. Different letters represent statistical significance ($p < 0.05$).

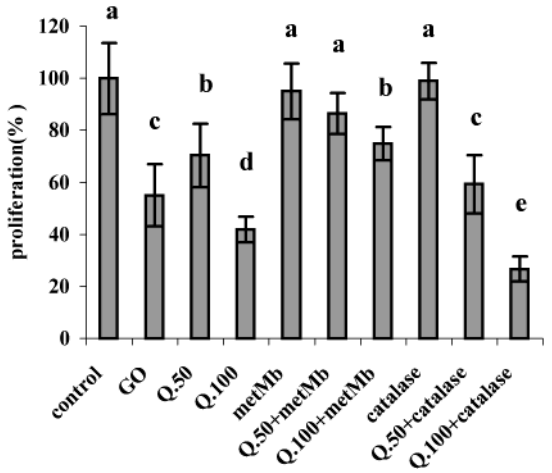

Figure 2. Effect of glucose oxidase or quercetin on HIT cell proliferation. Cells (30000 cell/well) were treated for 20 h with GO (4×10^{-4} Sigma units) or with quercetin (50 or 100 μM) in the presence or absence of metMb (30 μM) or catalase (0.8 Sigma unit), and [^{3}H]thymidine incorporation was measured. Data (mean ± SD) are expressed as a percentage of untreated control cells. $N = 6$–8. Different letters represent statistical significance ($p < 0.05$).

Determination of Iron. The concentration of chelatable iron ions in sera and media was determined according to the method of Carter et al. (*33*).

Statistical Analysis of Proliferation Assays. Results (mean ± SD) are expressed as a percentage of untreated control cells. Statistical significance was assayed using a one-way analysis of variance, following by a ranking procedure using the Student−Newman−Keuls test (SAS software, SAS Institute Inc., Cary, NC). Different letters represent statistical significance ($p < 0.05$).

RESULTS

βTC1 and HIT cell cultures were exposed to H_2O_2 oxidative stress, generated by incubation for 20 h with GO. This led to 50% reduction in cell proliferation, as determined by [^{3}H]-thymidine incorporation (**Figures 1** and **2**). The inclusion of catalase (0.8 Sigma unit) completely blocked the effect of GO, indicating that the reduction of cell proliferation was due to

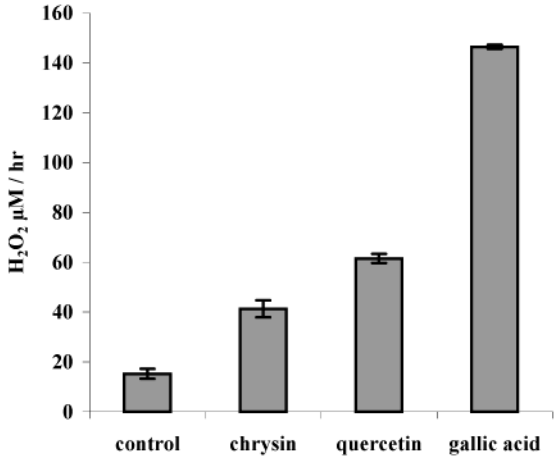

Figure 3. Generation of H_2O_2 in HIT cell medium. Culture medium was incubated at 37 °C with the phenolics (50 μM, each). H_2O_2 concentration was assayed by the FOX2 method. Data are mean ± SD. $N = 3$.

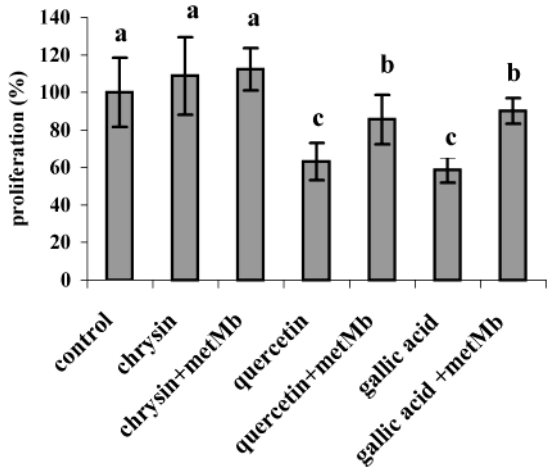

Figure 4. Effect of phenolics treatment on HIT cell proliferation. Cells (30000 cell/well) were treated for 20 h with chrysin, quercetin, or gallic acid (50 μM each), with or without metMb (30 μM), and [^{3}H]thymidine incorporation was measured. Data (mean ± SD) are expressed as a percentage of untreated control cells. $N = 6$–8. Different letters represent statistical significance ($p < 0.05$).

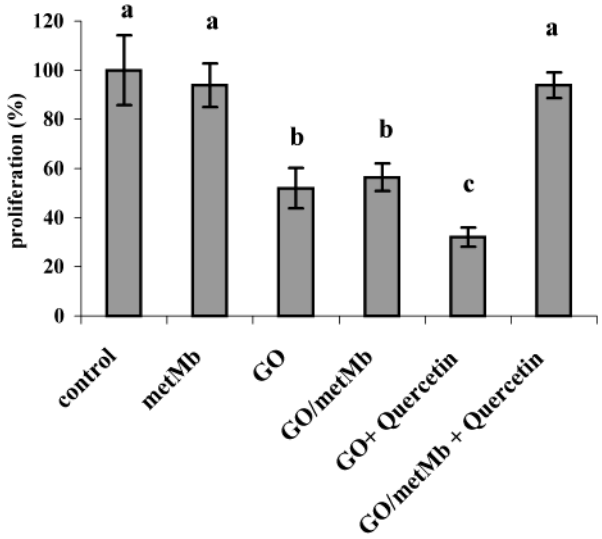

Figure 5. Effect of quercetin during H_2O_2 and H_2O_2-activated metMb stress on βTC1 cells. Cells (30000 cells/well) were treated for 20 h with GO (4×10^{-4} Sigma units) or GO plus metMb (30 μM), with or without quercetin (50 μM), and [^{3}H]thymidine incorporation was measured. Data (mean ± SD) are expressed as a percentage of untreated control cells. $N = 6$–8. Different letters represent statistical significance ($p < 0.05$).

H_2O_2 and not because of changes in glucose concentration resulting from GO activity. Surprisingly, addition of the antioxidants catechin (50−500 μM), quercetin (10−100 μM), and As.A (100−1000 μM) in the presence of GO did not have a protective effect, as might be expected from antioxidants. On the contrary, these antioxidants significantly amplified the GO inhibitory effect on the cells (**Figure 1**). Addition of lower doses of these antioxidants had no significant effect (not shown). Moreover, in the absence of added GO, these antioxidants themselves caused proliferation inhibition at higher concentrations [quercetin > 50 μM (**Figure 2**), catechin > 250 μM, and As.A > 800 μM (data not shown)]. To examine the possible involvement of iron ions in this process, we determined the amount of chelatable iron ions, in growth media of βTC1 and HIT cells and in two batches of fetal calf serum, and found them to contain 8 ± 0.4, 61 ± 6, 88 ± 8, and 119 ± 5 μM of "free" chelateable iron ions, respectively. These results led us to hypothesize that the proliferation inhibitory effect of flavonoids and other reducing compounds such as As.A on cell culture could be attributable to H_2O_2, generated through interaction of the reducing compound with components of the culture media, possibly iron ions. The inclusion of metMb with a flavonoid in the culture medium significantly reduced the proliferation inhibition of the flavonoid itself. Catalase was less efficient than metMb in preventing the inhibitory effect of quercetin on the cells (**Figure 2**).

To further examine the hypothesis, we incubated several phenolic compounds (50 μM, each) with culture medium (in the absence of cells) at 37 °C and monitored the production of H_2O_2 over 2 h (**Figure 3**). In a parallel proliferation assay, HIT cells were exposed for 20 h to the same phenolics at the same concentration, with and without metMb (30 μM). The flavonoid chrysin, which generated a minor amount of H_2O_2 in the cell medium (**Figure 3**), did not show significant inhibitory effect on cell proliferation (**Figure 4**). However, quercetin and gallic acid caused higher production of H_2O_2 (**Figure 3**) and significant proliferation inhibition, which was reduced by the inclusion of metMb (**Figure 4**). Further investigation of the H_2O_2 generation phenomenon and the possible role of iron was published elsewhere (*34*).

To eliminate H_2O_2 generation in the medium due to the interaction of the phenolics with the culture media, cells were exposed to a combination of GO/metMb. Metmyoglobin rapidly reacts with H_2O_2 and is transformed to the active ferryl form, which is able to cause lipid peroxidation on membranes (*35*), leading to a shift of oxidative stress from hydroxyl radicals to ferryl myoglobin. Quercetin (50 μM) was able to efficiently act as an antioxidant against the H_2O_2/metMb oxidative stress when all of the components were introduced to the cells together (**Figure 5**). Catechin (100 μM) reduced the proliferation inhibition of H_2O_2/metMb stress on HIT cells by 19% (calculated from the untreated control, not shown). The proliferation data, as resulted from the H_2O_2/metMb stress and the antioxidants protection, were inversely associated with the lipid peroxidation findings, measured by TBARS assay (**Figure 6**).

To examine the ability of phenolic antioxidant to protect cells from H_2O_2/metMb oxidative stress (**Figure 7**), HIT cells were preincubated with phenolic antioxidants (50 μM, each) and Mb (30 μM) for 24 h, washed extensively, and then challenged with GO/metMb for 20 h. The addition of Mb during the preincubation period prevented potential oxidative stress from artifactual

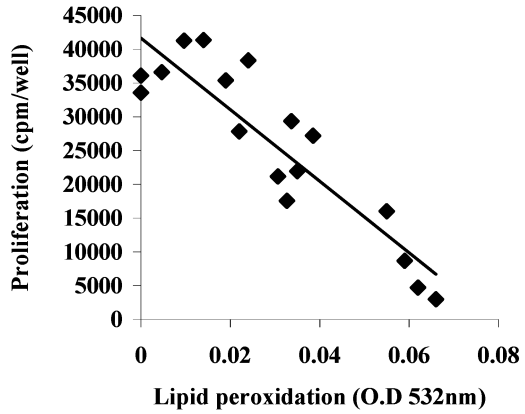

Figure 6. Inverse association between HIT and βTC1 cells proliferation and lipid peroxidation. Cells (15000 cells/well) were treated for 20 h with GO (4×10^{-4} Sigma units) plus metMb (30 μM), with or without quercetin (50 and 100 μM). Proliferation was assayed by [^{3}H]thymidine incorporation. Data are means ($N = 24$). Lipid peroxidation was measured by TBARS. Data are means ($N = 8$). $R = -0.90$, $p < 0.001$.

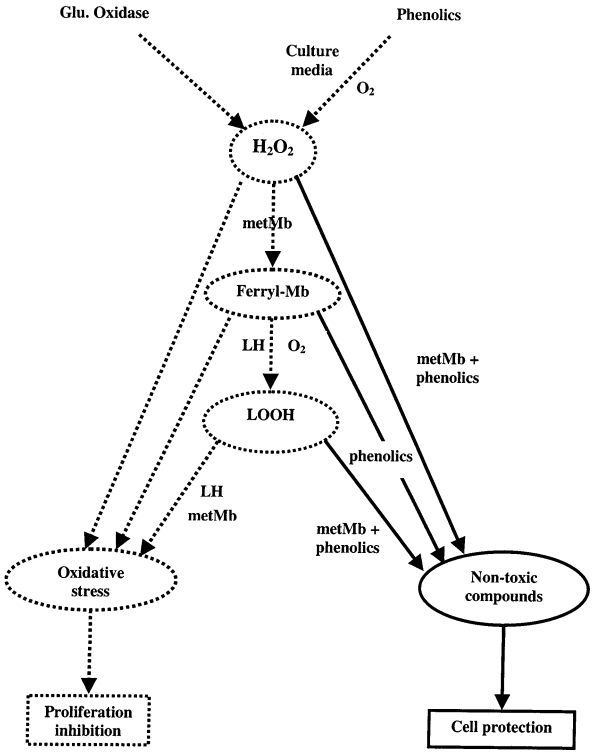

Figure 7. In vitro effect of phenolics in H_2O_2 and H_2O_2-activated metmyoglobin oxidative stress systems: (- - -) oxidative pathway; (—) cell protective pathway.

generation of H_2O_2. Under these conditions, chrysin, quercetin, catechin, and caffeic acid were able to protect the cells significantly (**Table 1**).

Table 1. Effect of Preincubation with Phenolics Followed by Ferryl Stress on HIT Cells Proliferation[a]

treatment	proliferation (%)
control	100 ± 8 a
DMSO 0.5%/metMb	99 ± 9 a
GO/metMb	66 ± 14 b
chrysin/metMb + GO/metMb	109 ± 21 a
quercetin/metMb + GO/metMb	86 ± 10 a
caffeic acid/metMb + GO/metMb	88 ± 9 a
catechin/metMb + GO/metMb	84 ± 11 a
hesperetin/metMb + GO/metMb	57 ± 8 b
gallic acid/metMb + GO/metMb	65 ± 8 b

[a] Cells (30000 cells/well) were incubated with the phenolics (50 μM) for 24 h, washed three times with PBS, and challenged with GO (4×10^{-4} Sigma units) plus metMb (30 μM) in fresh medium for 20 h. Proliferation was assayed by [^{3}H]thymidine incorporation. Data (mean ± SD) are expressed as a percentage of untreated control cells. $N = 6$–8. Different letters represent statistical significance ($p < 0.05$).

DISCUSSION

Flavonoids and phenolics from dietary sources, especially from tea, have been investigated in many cell culture models (for reviews see refs *36* and *37*). Much of the resulting data in the literature can be divided into two main groups: data showing cell protective effects of phenolics against various oxidative stresses (*4*, *38*) and data, mainly with tumor cells, demonstrating proliferation inhibition and cell death induced by phenolics (*9*, *10*). This apparent discrepancy is not yet resolved.

In this study, we report that in cell culture systems, the behavior of flavonoid or phenolic acid whether as antioxidant and cell protective or as prooxidant and cell proliferation inhibitor is dependent on the nature of the oxidative stress.

Hydrogen peroxide is a well-documented cause of oxidative stress to various cells (*39*) and to pancreatic β-cells in particular (*22*). In cultures supplemented with GO, which produces H_2O_2, quercetin, catechin, and ascorbic acid amplified the inhibitory effect of GO on cell proliferation in a synergistic manner. We suggest that this effect can be explained by interaction of the H_2O_2 with iron ions in the culture media, which are subsequently reduced by the supplemented phenolic compounds creating a redox cycle and generating the cytotoxic hydroxyl radical (*40*). The observed concentrations of chelateable iron ions in the culture media are consistent with such a mechanism, which was further investigated (*34*).

Some of the phenolics themselves (in the absence of cells or GO) caused significant H_2O_2 generation following incubation in the culture media. This could be the cause for the inhibition of cell proliferation by antioxidants such as gallic acid (50 μM), quercetin (50 μM), catechin (>250 μM), and As.A (>800 μM). Previous studies have also shown similar dose-dependent proliferation inhibition by phenolics and As.A with other cell lines (*6*, *10*, *41*), but they have not ruled out the possibility of indirect H_2O_2 formation in the culture medium. Our former (*34*) and recent results place in question the findings of other in vitro studies of the effects of phenolics on cell cultures, where the option of artifactual H_2O_2 generation was not considered (for example, refs *42*−*44*). Consistent with this idea, chrysin (50 μM), which did not cause rapid generation of H_2O_2 in the culture media, did not inhibit cell proliferation. Moreover, metMb (30 μM), which interacts with H_2O_2 and reduces its level (*26*), significantly decreased the proliferation inhibitory effect of gallic acid, quercetin, and catechin. Interestingly, catalase, although it prevented the GO inhibitory effect on cells, was not as efficient as metMb in preventing the effect of quercetin. Quercetin, a hydrophobic flavonoid, may become concentrated at the cell membrane (*45*, *46*), whereas catalase [isoelectric point = 5.4 (*47*)], may have a low effective concentration at membranes due to its negative charge at the pH of culture medium (*48*). Production of H_2O_2 by phenolics in culture models was demonstrated also by Yang et al. (*6*), who studied the effect of tea phenolics, and more recently by Long et al. (*49*). In contrast to our results, other studies (*38*, *50*) found that flavonoids reduced H_2O_2 oxidative stress in cell culture, but cells were only preincubated with the flavonoids rather than

having the H_2O_2 and the flavonoids introduced to the cells at the same time.

In our culture model, phenolic compounds that were prooxidants in an H_2O_2 oxidative stress system functioned as efficient antioxidants in an H_2O_2/metMb system. Heme protein such as metmyoglobin, in the presence of low levels of H_2O_2, can be converted to the active ferryl form, which reduces H_2O_2 levels but may cause lipid peroxidation (*40*), as was demonstrated by the TBARS results. We have found in membranal models that flavonoids are strong antioxidants and can protect against this lipid peroxidation (*51*, *52*). In these models, the combination of active metmyoglobin and a reducing phenolic compound seems to act together like a peroxidase. The present work demonstrates this protective combination in a cell culture model as well. The effects of phenolics in both of the oxidative systems are summarized in **Figure 6**.

We have also examined the impact of preincubation of cells with the phenolics (50 μM each) and metMb (30 μM) on the resistance to H_2O_2/metMb challenge. Under these conditions, chrysin, quercetin, catechin, and caffeic acid protected the cells significantly. Similar effects of caffeic acid on U937 human monocytes were reported by Nardini et al. (*5*). Gallic acid and hesperitin had no significant protecting effect. Thus, only the more hydrophobic antioxidants were protective in preincubation conditions. Previous studies on the structure−activity relationship of flavonoids attributed higher reducing potential and enhanced antioxidant activity to the number of hydroxyl substituents, mainly on the B-ring of the flavonoid (*53*, *54*). Chrysin, the most hydrophobic compound in our experiments, although possessing a lower reducing power than gallic acid (*54*) and lacking a hydroxyl group on the B-ring, conferred better protection to the cells. Hydrophobic character was also found by Belinky et al. (*55*) to be essential to isoflavan antioxidant activity in $CuSO_4$-induced low-density lipoprotein oxidation. Thus, it seems that in a cellular system, effective antioxidant activity requires not only the ability to donate electrons but also the ability to reach the specific target site.

In summary, the effect of dietary phenolics in a cell culture model is strongly dependent on the composition of the growth medium. The inclusion of metMb in the model effectively eliminates cell culture artifacts and enables measurement of the antioxidant activity of phenolic compounds against oxidative stress. Under our oxidative stress conditions, several natural flavonoids demonstrate a powerful protective effect on pancreatic β-cells in vitro. Future studies should examine whether such protection against oxidative stress can be conferred also in vivo, with a view to reducing the pathophysiological changes associated with diabetes.

ABBREVIATIONS USED

GO, glucose oxidase; MetMb, metmyoglobin; As.A, ascorbic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid.

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