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Modulation by Bicarbonate of the Protective Effects of Phenolic Antioxidants on Peroxynitrite-Mediated Cell Cytotoxicity

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ABSTRACT: Peroxynitrite ($ONOO^-$), a potent oxidant formed from the rapid reaction of nitrogen monoxide ($^{\bullet}NO$) with superoxide radical ($O_2^{\bullet-}$), is implicated in numerous pathologies including inflammation and age-related diseases (e.g., neurodegenerative disorders and cardiovascular disease). Therefore, there is an increasing interest to develop therapeutic peroxynitrite scavengers. Diet-derived phenolics have been suggested to be powerful peroxynitrite scavengers. However, peroxynitrite reactivity can be significantly modulated by physiological concentrations of carbon dioxide/bicarbonate (CO_2/HCO_3^-), an important buffer system in the body, due to the rapid reaction of $ONOO^-$ and CO_2/HCO_3^- to produce nitrosoperoxycarbonate ($ONOOCO_2^-$). Therefore, in order to examine the biological activity of phenolics (caffeic acid, catechin, epicatechin and quercetin) as potential peroxynitrite scavengers, the modulation of CO_2/HCO_3^- on the protective effects of phenolics on peroxynitrite-mediated chondrosarcoma cytotoxicity was assessed by using 4 model systems of peroxynitrite cytoxicity: crystal violet-staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction, cellular glutathione measurement and intracellular oxidant formation. All phenolics tested (caffeic acid, catechin, epicatechin and quercetin) significantly inhibited chondrosarcoma cytotoxity induced by peroxynitrite, although bicarbonate (25 mM) decreased their cytoprotective effects. This observation suggests that dietary phenolics were able to limit cell cytotoxicity. However, their activities could be substantially modified by physiological concentrations of bicarbonate. Hence, antioxidants for therapeutic use should be tested for peroxynitrite scavenging in the presence of bicarbonate for avoiding misleading results.

KEYWORDS: Bicarbonate, nitric oxide, phenolics, peroxynitrite, SW1353 cell cytotoxicity.

INTRODUCTION

Peroxynitrite (oxoperoxonitrate [1-]; $ONOO^-$) is a reactive nitrogen species (RNS) formed from one of the most significant and fastest reactions (diffusion-controlled) of nitric oxide ($^{\bullet}NO$) with superoxide radical ($O_2^{\bullet-}$)[1]; $^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$, $k = (4.3\text{-}19) \times 10^9\ M^{-1}s^{-1}$. Under physiological conditions, $ONOO^-$ has a half-life of under 1 second and it is converted to its protonated form peroxynitrous acid, ONOOH, which in turn decays to produce multiple reactive products with the reactivities similar to those of the nitryl cation (NO_2^+), nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl radical ($^{\bullet}OH$)[1]. It is suggested to be one of the potential biological species capable of oxidizing and nitrating lipids, proteins and DNA[2]. The reactions of peroxynitrite with protein tyrosine, DNA guanine and lipid have shown to produce cellular dysfunction and death and also been detected in pathologies such as inflammatory disorder[3]. Moreover, peroxynitrite is suggested to play a role in the modulation of cell signaling by altering activity of mitogen activated protein (MAP) kinases responsible for cell survival, death and proliferation[4]. Due to the evidence of peroxynitrite cytotoxicity and its formation at sites of pathologies, there has been a great attempt to study the roles of antioxidants in the inhibition of cellular damage induced by peroxynitrite. Several *in vitro* and *in vivo* studies have reported that phenolic compounds might play important roles in protecting against peroxynitrite-induced oxidative damage to biomolecules. However, the chemistry of peroxynitrite is complex as it can react with CO_2/HCO_3^- ($k = 3\text{-}5.8 \times 10^4\ M^{-1}s^{-1}$), one of the major blood buffers, to produce nitrosoperoxycarbonate ($ONOOCO_2^-$) at physiological conditions. $ONOOCO_2^-$ can undergo homolysis of the peroxide bond to yield the cage-escaped radicals nitrogen dioxide ($^{\bullet}NO_2$) and the carbonate radical anion ($CO_3^{\bullet-}$)[5]; $ONOO^- + CO_2 \Leftrightarrow [O{=}N\text{-}OOCO_2^-] \Leftrightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$, or rapidly decays to nitrate (NO_3^-) while regenerating carbon dioxide (CO_2); $[O_2N\text{-}O\text{-}CO_2^-] \rightarrow NO_3^- + CO_2$. Hence, one of the most biologically relevant reactions of peroxynitrite is that with CO_2, which is

present at the relatively high concentration of 1.3-1.5 mM (in equilibrium with 24-25 mM bicarbonate, at pH 7.4) in blood plasma[6]. Such reactions can therefore proceed readily in physiological fluids and generate strong oxidants, $^{\bullet}NO_2$ and $CO_3^{\bullet-}$. The formation of the adduct, $ONOOCO_2^-$, and its derivatives are found to modulate the reactivity of peroxynitrite in several *in vitro* and cellular studies. Increasing evidence shows that many of the reactions of peroxynitrite with *in vivo* biomolecules are likely to be mediated by the reaction of peroxynitrite with CO_2/HCO_3^- rather than by peroxynitrite itself[7]. The formation of the reactive $O=N-OOCO_2^-$ could interfere with biological pathology mediated by peroxynitrite, thus redirecting the primary reactivity of peroxynitrite. For instance, the presence of CO_2/HCO_3^- could aggravate peroxynitrite-induced trypsin and dihydrorhodamine 123 oxidation, and tyrosine and guanine nitration[8,9]. However, its presence diminished peroxynitrite-dependent oxidation of low-density lipoprotein lipids[10].

Thus, the presence of bicarbonate could up- or down-regulate peroxynitrite-mediated biological events, and may either increase or decrease peroxynitrite toxicity. It is an open question of whether the presence of extracellular CO_2/HCO_3^- affects the diffusion of peroxynitrite to intracellular compartments and thus modulates peroxynitrite reactivity in mediating the damage of intracellular biomolecules and cell cytotoxicity.

Hence, this study aims to re-evaluate the protective effects of phenolic antioxidants on peroxynitrite-mediated cell cytotoxicity in the presence of bicarbonate at physiological conditions. Chondrocytic SW1353 cells were used in this study to assess the effect of peroxynitrite on cell cytoxicity and the cytoprotective effects of peroxynitrite scavengers since peroxynitrite is suggested to contribute to osteoarthritis and rheumatoid arthritis, which have been studied using the SW1353 chondrosarcoma cells as an appropriate model for primary chondrocytes[11]. Furthermore, the inhibitory effects of phenolics on peroxynitrite-mediated cell toxicity were examined in the presence and absence of physiological concentrations of bicarbonate using four types of cytotoxicity assays, namely crystal violet-staining (CVS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction, measurement of intracellular GSH, and detection of intracellular oxidants by 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA).

Materials and methods

Materials

SW1353 (human chondrosarcoma) cell lines were from American Type Culture Collection (ATCC, Rockville, Md, USA). Dulbecco's modified Eagle medium (DMEM) was obtained from Hyclone (Irvine, CA). Chemicals and reagents used were of the highest quality available and were purchased from Sigma-Aldrich (Singapore). The assays using microplate readers were done using a Spectra Max Gemini XS or a Spectra Max 190, both from Molecular Devices (Sunnyvale, CA, USA).

Cell cultures

The human chondrosarcoma cell line (SW1353, ATCC) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic antimycotic solution [1% penicillin (100 units/ml)-streptomycin (100 μg/ml)-amphotericin B (0.25 μg/ml)]. Cells were maintained at 37 °C in humidified air containing 5% CO_2 (P_{CO2} = 40 Torr) in a Forma Scientific CO_2 Water Jacketed incubator and cultured under physiological concentrations of bicarbonate (22 mM)-containing DMEM equilibrated with 5% CO_2 (1 mM) as a CO_2/bicarbonate buffer to control the pH of the medium (pH 7.4). Cells were passaged by trypsin-ethylenediaminetetraacetic acid (EDTA) treatment (10% in sterile phosphate buffer saline, PBS).

Synthesis of peroxynitrite

Synthesis of peroxynitrite was essentially as previously described[11]. Briefly, an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with KNO_2 (0.6 M) on ice for one second and the reaction quenched with ice cold NaOH (1.2 M). Residual H_2O_2 was removed by mixing with granular MnO_2 pre-washed with NaOH (1.2 M). The stock solution was filtered and then frozen overnight (-20 °C) and the top layer of the solution collected for the experiment. Concentrations of stock peroxynitrite were re-determined before each experiment at 302 nm using a molar absorption coefficient of 1670 $cm^{-1}M^{-1}$. Concentrations of 200-250 mM were usually obtained. Once thawed, peroxynitrite solutions were kept on ice for no longer than 30 min before use.

Treatment of SW1353 cells with peroxynitrite and phenolics

Various stock concentrations of phenolics (caffeic acid, catechin, epicatechin and quercetin) were freshly prepared in phosphate buffer (0.25 M K_2HPO_4-KH_2PO_4 buffer, pH 7.4), except for quercetin which was dissolved in dimethyl sulfoxide (DMSO) such that the final concentration of which did not exceed 0.2 % (v/v) in culture medium). The final concentrations of test compounds were 15, 30, 60, 125, 250, 500 and 1000 μM. In all assays, SW1353 cells were washed and treated with different concentrations of phenolics with and

without the presence of 25 mM $NaHCO_3$ for 5 min prior to the addition of peroxynitrite (250 μM for MTT and DCFDA assay and 1 mM for crystal violet staining and GSH assay). Cell cytotoxicity was evaluated after treatments by visual observation using a microscope and by different assays used. Cells incubated with only phosphate buffer were calculated as a control and with DMSO (0.5% v/v) as a solvent control. DMSO alone did not have any effect on the parameters measured. The addition of peroxynitrite and 25 mM $NaHCO_3$ did not alter the pH of the reaction mixture; the pH measured after every experiment was 7.4-7.5. All control experiments showed that neither decomposed peroxynitrite nor 25 mM bicarbonate had any effect on cell viability.

Assay of crystal violet-staining (CVS)

Cell viability was determined using crystal violet-stained intact cell assay as previously described[12]. Cells were seeded in 96-well microplates and treated with phenolics at the indicated concentrations in the presence and absence of added bicarbonate. After the addition of peroxynitrite (1 mM), cells were washed with PBS twice and fixed with 50 μl of 0.2% (w/v) crystal violet solution. The attached cells were stained at room temperature for 15 min. Then the staining solution was discarded and the plates were rinsed with tap water. The CV stain was then eluted with 200 μl of 0.5% sodium dodecyl sulfate (SDS) in 50% ethanol and the plates were incubated for 60 min at 37 °C. The absorbance at 610 nm was determined spectrophotometrically using microplate reader.

Assay of MTT reduction

Cell mitochondrial and metabolic function was determined by measurement of MTT reduction[13]. After cells pre-incubated with phenolics were treated with peroxynitrite (250 μM) and then washed with PBS, 250 μl of culture medium containing MTT (0.5 mg/ml final concentrations) were added. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 1 h before addition 200 μl of DMSO to solubilize the formazan. The optical density was measured at 550 nm by microplate reader. MTT conversion was not affected by DMEM and phenolics tested.

Measurement of intracellular glutathione (GSH)

GSH contents were assayed as described[14], using the fluorescent reagent *o*-phthalaldehyde (OPA), which reacts specifically with GSH at pH 8. Cells (0.25×10^6 cells/well) in 24-well plate were pre-treated with phenolics at the final concentrations indicated for 10 min and then treated with peroxynitrite (1 mM) at pH 7.4 in the presence and absence of added bicarbonate. After lysing cells by 6.5% trichloroacetic acid, sample, phosphate/EDTA buffer and OPA (1 mg/ml in methanol) were added. Fluorescence was measured at 350 nm excitation and 420 nm emission using a spectrofluorometer. The GSH levels were calculated by comparing with standard curves using known concentrations of GSH. The results are expressed as nmol GSH/mg protein. The Lowry method was used to determine protein concentration. The reduction of the folin phenol reagent yielded a blue color read at 750 nm.

Measurement of cellular oxidant formation

2′, 7′-Dichlorofluorescein diacetate (DCFDA), a stable and non-fluorescent dye, is widely used to measure oxidative stress in cells[15]. Cells pre-incubated with media containing DCFDA (5 μM) for 1 h were washed with PBS and then treated with test compounds in 250 mM phosphate buffer with and without bicarbonate (25 mM final concentrations). After treatment with 1 mM peroxynitrite, the fluorescence was monitored on a spectrofluorometer immediately and 20 min after the treatment in Gemini plate reader with excitation wavelength 485 nm and emission wavelength 530 nm.

Statistical Analysis

All graphs are plotted with mean ± standard error of the mean (sem). In all cases the mean values were calculated from data taken from at least 6 separate experiments performed on separate days using freshly prepared reagents. Where significance testing was performed, an independent t-test (Student's; 2 populations) and an analysis of variance (ANOVA) were used; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Results

Peroxynitrite-induced cytotoxicity of SW1353 cells: the modulation by bicarbonate

The assays of CVS (Fig. 1A) and MTT reduction (Fig. 1B) showed that peroxynitrite (60-1000 μM) induced cytotoxicity of SW1353 cells in a concentration-dependent manner in the presence and absence of added bicarbonate. However, CVS assay (Table 1) shows that the presence of 25 mM bicarbonate significantly decreased cell cytotoxicity induced by peroxynitrite (1 mM) as cell viability increased from 51.8% ± 2.9 to 60.6% ± 3.9 ($p<0.05$) in the presence of 25 mM bicarbonate. Additionally, as indicated by MTT reduction assay, the presence of 25 mM bicarbonate markedly reduced cell cytotoxicity when cell were treated with 250 μM of peroxynitrite ($p<0.05$). Exposure of SW1353 cells to 250 μM peroxynitrite for 5 min yielded 30% ± 0.8 and 35.3% ± 1.4 MTT reduction in the absence and presence of bicarbonate, respectively

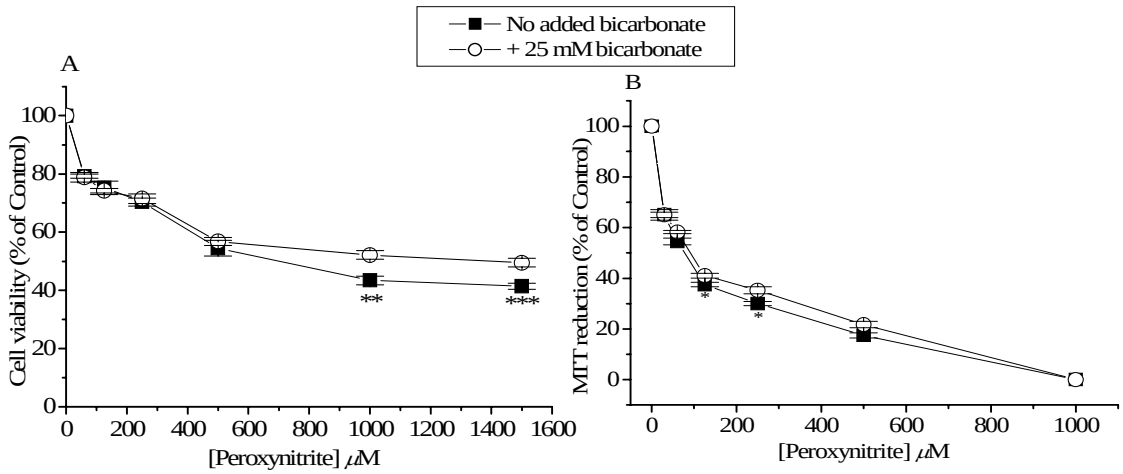

Fig 1. Concentration-dependence of peroxynitrite-mediated SW1353 cell cytotoxicity using crystal-violet staining (A) and MTT reduction assays (B): modulation by added bicarbonate. Cell viability indicated by CV-stained intact cells and MTT reduction was expressed as a percentage of untreated cells (100%). Values given are means ± sem for 6 determinations. Statistical significance was evaluated using analysis of variance. *p<0.05, **p<0.01, ***p<0.001, comparing no added bicarbonate with 25 mM bicarbonate.

Table 1. The effects of peroxynitrite-mediated SW1353 cell cytotoxicity determined by CV-staining and MTT reduction: modulation by added bicarbonate. Values given are means ± sem for 6 determinations.

Assay	Cell viability (% of control)		p-value
	No added bicarbonate	25 mM bicarbonate	
CVS			
1 mM peroxynitrite	51.8 ± 2.9	60.6 ± 3.9	<0.05
MTT			
250 μM peroxynitrite	30 ± 0.8	35.3 ± 1.4	<0.05

(Table 1).

As depletion of GSH is found in apoptotic cells, evaluation of GSH contents could determine cell cytotoxicity[16]. Exposure of SW1353 cells to 1 mM peroxynitrite resulted in intracellular GSH depletion, from 100% to 47.1% ± 2.1 and 38.4% ± 3.6, p<0.001, in the absence and presence of bicarbonate, respectively (Table 2). The results indicate that the presence of physiological concentrations of bicarbonate significantly promoted the depletion of GSH mediated by peroxynitrite.

Additionally, DCFDA was used as a fluorescent probe for detecting intracellular redox status in cell since oxidation of the probe in cellular systems reflects the rate of production and reactivity of oxidants[15]. SW1353 cells exposed to peroxynitrite (250 μM) exhibited a significant increase in DCF fluorescence in the presence and absence of added bicarbonate. The increased fluorescence was probably due to the induction of intracellular oxidants produced by peroxynitrite, indicating intracellular oxidative stress. The addition of 250 μM and 1 mM peroxynitrite to cells resulted in a concentration-dependent increase in intracellular fluorescence (Fig. 2) to the levels of 3.8 ± 0.3 RFU and 2 ± 0.3 RFU, p<0.001 when 250 μM peroxynitrite was added and 13.5 ± 1.3 RFU and 5.9 ± 0.5 RFU, p<0.05 when 1 mM peroxynitrite was added, in the absence and presence of 25 mM bicarbonate, respectively. The presence of physiological concentrations of bicarbonate significantly reduced intracellular oxidant formation induced by peroxynitrite.

Table 2. Effects of peroxynitrite-mediated intracellular GSH depletion in SW1353 cells: modulation by 25 mM bicarbonate. Cellular GSH levels were related to the protein concentration in each sample and expressed as a percentage of untreated cells (100%). Values given are means ± sem for 6 determinations.

Treatment of cells	Intracellular GSH			
	nmol/mg	p-value	(% Of control)	p-value
Untreated cells	11.8 ± 2.45		100	
1 mM peroxynitrite	5 ± 0.89	< 0.01	47.1 ± 2.1	< 0.001
1 mM peroxynitrite + 25 mM bicarbonate	4 ± 0.85		38.4 ± 3.6	

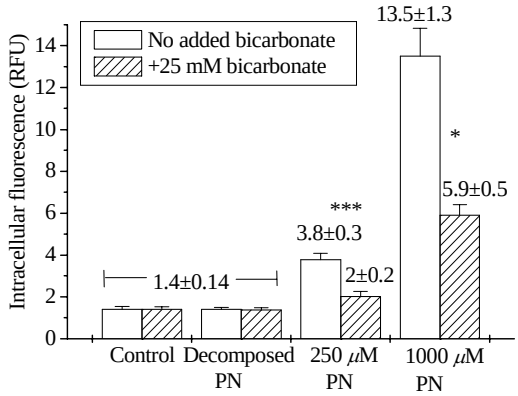

Fig 2. Effects of peroxynitrite (PN)-mediated intracellular oxidant formation in SW1353 cells: modulation by 25 mM bicarbonate. DCF fluorescence was measured as described in the materials and methods section. Data are expressed as relative fluorescence units. Values given are means ± sem for 6 determinations. Statistical significance was evaluated using analysis of variance. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, comparing no added bicarbonate with 25 mM bicarbonate.

The inhibitory effects of phenolics on peroxynitrite-induced cytotoxicity of SW1353 cells: the modulation by bicarbonate

Assay of CV-stained intact cells

Pre-treatment of SW1353 cells with caffeic acid, catechin, epicatechin and quercetin (15-500 μM) prior to the addition of peroxynitrite (1 mM) attenuated cell cytotoxicity in a concentration-dependent manner in the presence and absence of added bicarbonate (Fig. 3). In comparing the effects of phenolics in the presence and absence of 25 mM bicarbonate, the zero point on each plot in Fig. 3 indicates an addition of 1 mM peroxynitrite without test compounds in the presence or absence of added bicarbonate, respectively. Higher concentrations of the phenolics were needed in the presence of 25 mM bicarbonate to reach a degree of inhibition similar to that in the absence of added bicarbonate. The cytoprotective effects of all phenolics tested at inhibiting peroxynitrite-induced cell cytotxicity was substantially reduced, as indicated by

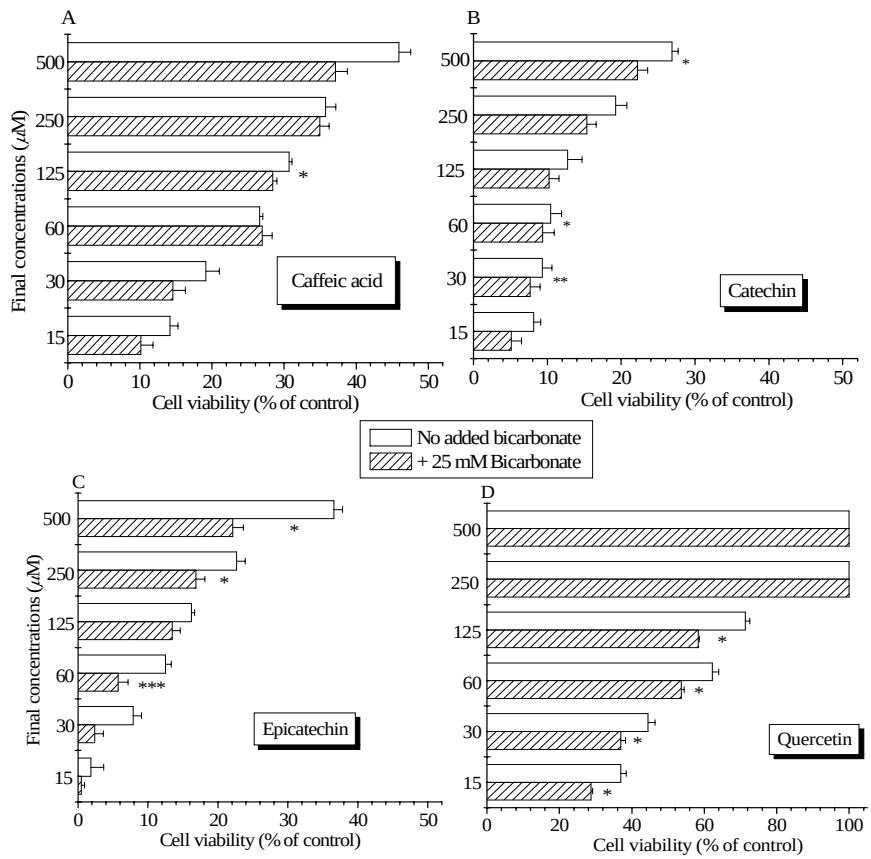

Fig 3. Cytoprotective effects of phenolics [caffeic acid (A), catechin (B), epicatechin (C) and quercetin (D)] on peroxynitrite-mediated SW1353 cell cytotoxicity determined by crystal-violet staining: modulation by added bicarbonate. Cell viability indicated by CV-stained intact cells was expressed as a percentage of cells treated with peroxynitrite in the in the presence or absence of bicarbonate (100%). Values given are means ± sem for 6 determinations. Statistical significance was evaluated using analysis of variance. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, comparing no added bicarbonate with 25 mM bicarbonate.

the fact that concentrations of test phenolics to achieve 30% inhibition of cell cytoxicity increased (epicatechin; > 500 μM, $p<0.01$, caffeic acid; 158.4 ± 18 μM, $p<0.05$, quercetin; 17.7 ± 1 μM, $p<0.01$ as compared to no bicarbonate addition).

Assay of MTT reduction

Caffeic acid, catechin, epicatechin and quercetin exhibited cytoprotective effects as pre-treatment of SW1353 cells with the phenolics tested attenuated loss of MTT reduction induced by peroxynitrite in a concentration-dependent manner in the presence and absence of added bicarbonate (Fig. 4). As observed with CVS assay, the abilities of all phenolics tested were reduced in the presence of bicarbonate as higher concentrations of the phenolics tested (caffeic acid; 820.3 ± 22.9 μM, $p<0.01$, epicatechin; 509.5 ± 26.8 μM, $p<0.001$, catechin; 420.7 ± 23 μM, $p<0.01$, quercetin; 114.1 ± 10.5 μM, $p<0.01$ as compared to the absence of added bicarbonate) were required to achieve 30% inhibition of cell cytoxicity, determined by the loss of MTT reduction.

Measurement of intracellular GSH

All phenolics tested exhibited cytoprotective effects against peroxynitrite-mediated intracellular GSH depletion in a concentration-dependent manner in the presence and absence of added bicarbonate (Fig. 5). The effectiveness of all phenolics tested in inhibiting the loss of intracellular GSH by peroxynitrite was markedly decreased in the presence of bicarbonate as higher concentrations of the phenolics tested (epicatechin and quercetin; >1000 μM, $p<0.001$, catechin; 229.2 ± 6.7 μM, $p<0.05$ and caffeic acid; 122.6 ± 0.9 μM, $p<0.05$ as compared to the absence of added bicarbonate) were needed to achieve 30% inhibition of GSH depletion. These values are significantly higher than that in the absence of added bicarbonate.

Measurement of cellular oxidant formation

All phenolics tested exerted a concentration-dependent decrease in intracellular fluorescence, which was induced by the addition of peroxynitrite to

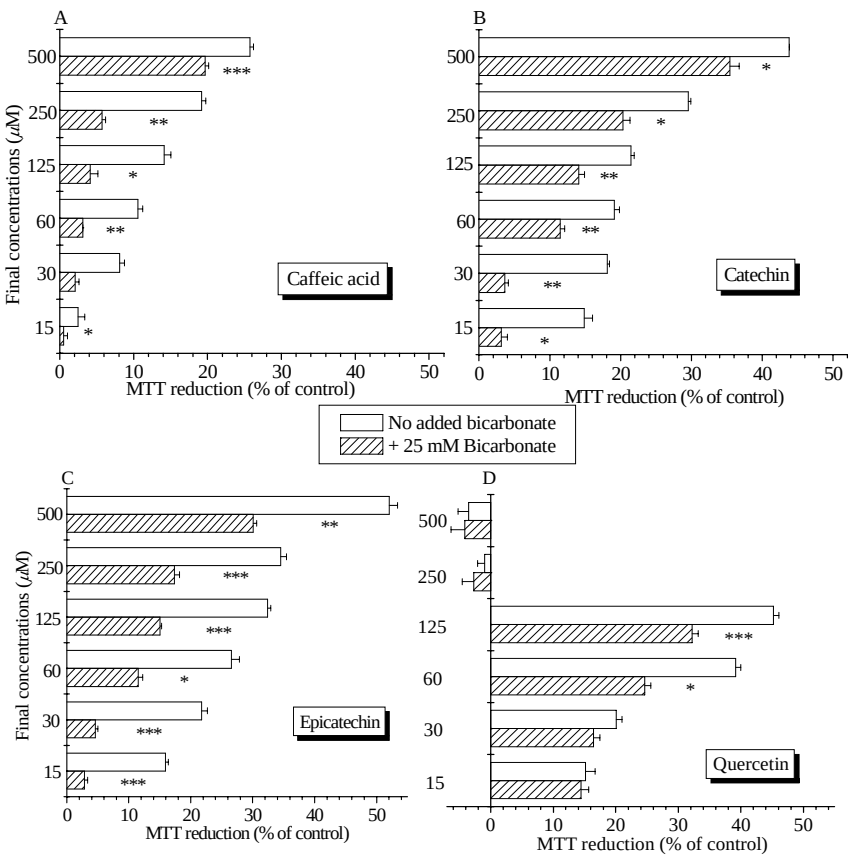

Fig 4. Cytoprotective effects of phenolics [caffeic acid (A), catechin (B), epicatechin (C) and quercetin (D)] on peroxynitrite-mediated SW1353 cell cytotoxicity determined by MTT reduction: modulation by added bicarbonate. Cell viability indicated by MTT reduction was expressed as a percentage of cells treated with peroxynitrite in the in the presence or absence of bicarbonate (100%). Values given are means ± sem for 6 determinations Statistical significance was evaluated using analysis of variance. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, comparing no added bicarbonate with 25 mM bicarbonate.

cells in the presence and absence of added bicarbonate (Fig. 6). In comparing the effects of phenolics in the presence and absence of 25 mM bicarbonate, 100% on each plot in Fig. 6 indicates an increase in fluorescence in cells treated with peroxynitrite without test compounds in the presence and absence of added bicarbonate. The abilities of all phenolics tested to inhibit cellular oxidant formation mediated by peroxynitrite was reduced to the limited extent in the presence of bicarbonate since higher concentrations of the phenolics tested (caffeic acid; 434.1 ± 3.4 μM, $p<0.05$, epicatechin; 313.5 ± 23.8 μM, $p<0.01$, catechin; 113.4 ± 4.5 μM, $p<0.05$ and quercetin; 77.2 ± 3.9 μM, $p>0.05$ as compared to no bicarbonate addition) were required to achieve 50% inhibition of intracellular oxidant formation.

Discussion

Apparently, CO_2/HCO_3^- is possibly a crucial source of biological oxidants responsible for cellular peroxynitrite reactivity. Hence, the reaction of $ONOO^-$ with CO_2/HCO_3^- is of particular relevance *in vivo* because it does not detoxify peroxynitrite due to its fast reaction at physiological conditions to form $ONOOCO_2^-$-derived reactive species, $^{\bullet}NO_2$ and $CO_3^{\bullet-}$. Considerable evidence has shown the effects of the presence of physiological concentrations of bicarbonate on peroxynitrite-mediated oxidation and nitration as discussed previously. Therefore, there have been revisions on the biological activity-dependent pathophysiological significance of peroxynitrite and its derived radicals involving CO_2/HCO_3^- [17].

Although many defense mechanisms within the organism limit the levels of reactive oxidants including peroxynitrite and the damage they cause, endogenous antioxidant defenses may not be sufficient during pathological conditions. Thus, administration of exogenous antioxidants has received much attention and there is a need to develop potentially therapeutic antioxidants. A number of *in vitro* and *in vivo* evidence suggests that diet-derived compounds including phenolics with catechol group (e.g., caffeic acid, catechin, epicatechin and quercetin) can potentially

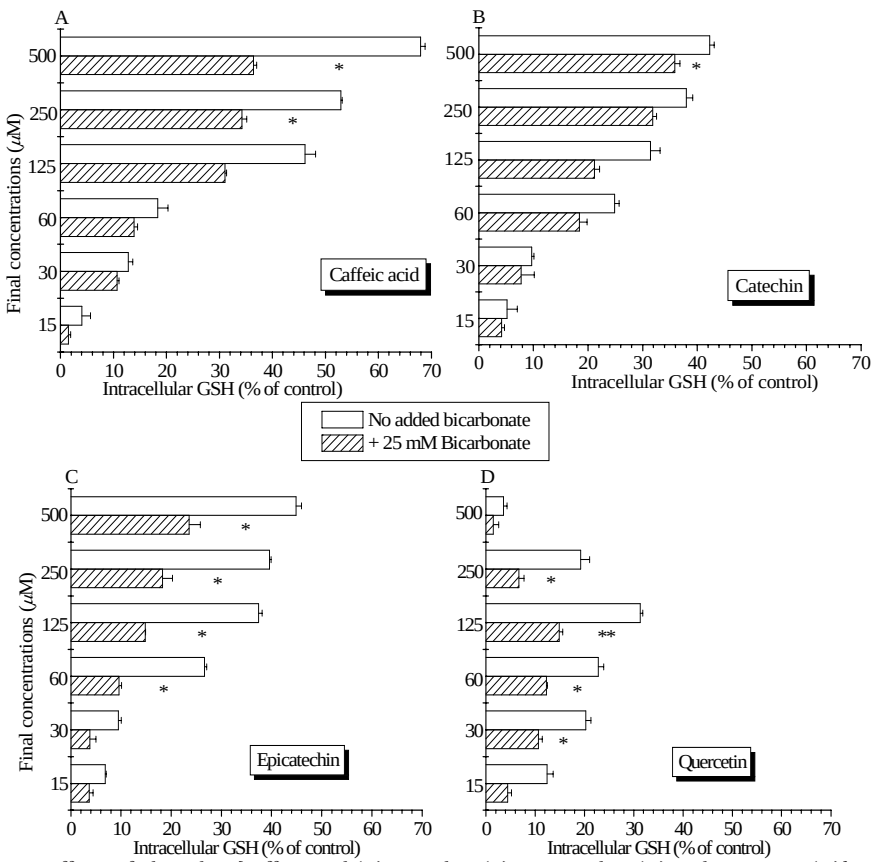

Fig 5. Cytoprotective effects of phenolics [caffeic acid (A), catechin (B), epicatechin (C) and quercetin (D)] on peroxynitrite-mediated GSH depletion in SW1353 cells: modulation by added bicarbonate. Cellular GSH levels were related to the protein concentration and expressed as a percentage of cells treated with peroxynitrite in the in the presence or absence of bicarbonate (100%). Values given are means ± sem for 6 determinations. Statistical significance was evaluated using analysis of variance. *$p\leq0.05$, **$p\leq$£0.01, ***$p\leq0.001$, comparing no added bicarbonate with 25 mM bicarbonate.

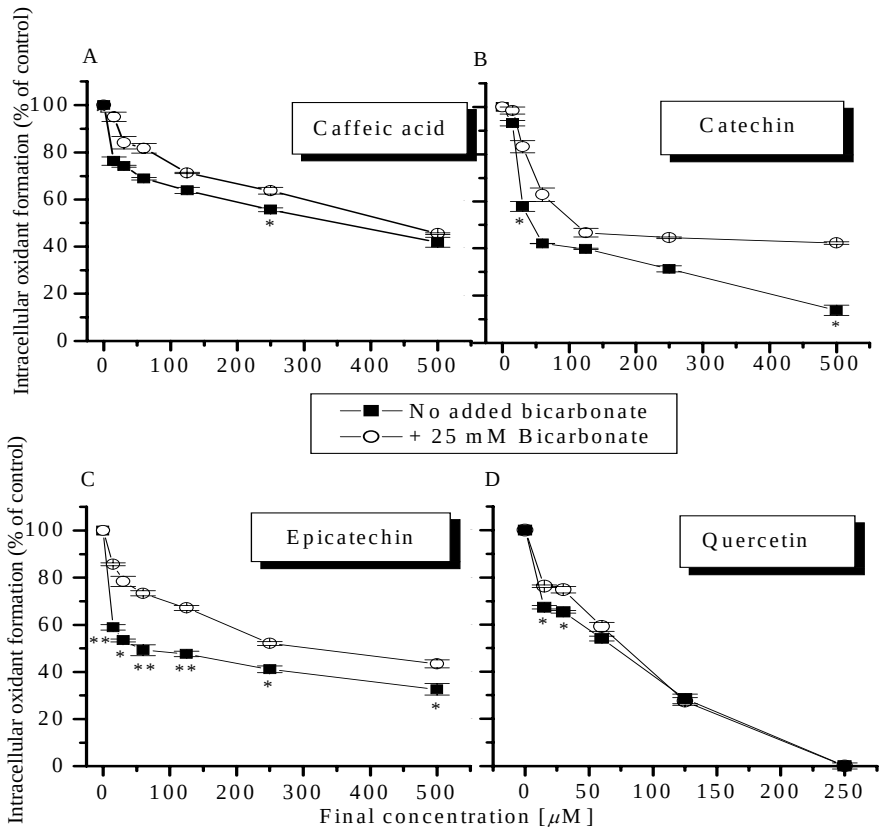

Fig 6. Cytoprotective effects of phenolics [caffeic acid (A), catechin (B), epicatechin (C) and quercetin (D)] on peroxynitrite (PN)-mediated intracellular oxidant formation in SW1353 cells: modulation by added bicarbonate. Intracellular peroxides indicated by RFU were expressed as a percentage of cells treated with peroxynitrite in the in the presence or absence of bicarbonate (100%). Values given are means ± sem for 6 determinations. Statistical significance was evaluated using analysis of variance. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, comparing no added bicarbonate with 25 mM bicarbonate.

protect against RNS (including $ONOO^-$ and $^{\bullet}NO$)-induced damage of various biomolecules and cell types[18]. However, *in vitro* studies showed that physiological concentrations of bicarbonate substantially modify the abilities of phenolics to prevent peroxynitrite-mediated reactions[19]. Therefore, the cytoprotective effects of diet-derived phenolics (catechin, epicatechin, quercetin and caffeic acid) on the inhibition of peroxynitrite-induced cytotoxicity of human SW1353 chondrosarcoma cells were investigated in the presence and absence of added bicarbonate.

Whereas CVS assay can determine cell death induced by peroxynitrite, the MTT and GSH assays can identify metabolic dysfunction and DCFHDA assay measures oxidative stress induced by peroxynitrite in cells. Therefore, these four cytotoxicity assays can be used to study cell injury and death.

Peroxynitrite-induced cytotoxicity of SW1353 cells: the modulation by bicarbonate

Peroxynitrite is found in both intra- and extracellular compartments since cells such as neutrophils, neurons, endothelial cells and fibroblasts produce both $^{\bullet}NO$ (10-100 nM) and $O_2^{\bullet-}$ (0.1-1 nM) under physiological conditions. However, much higher concentrations of both $^{\bullet}NO$ (up to 10 μM) and $O_2^{\bullet-}$ (> 2.5 μM) can be produced by activated human neutrophils to produce a 100-fold increase in the rate of peroxynitrite formation[20]. It is suggested that peroxynitrite at rates of 0.25–1.0 mM.10^6 cells^{-1}.min^{-1} could be formed under certain pathological conditions. Such concentrations correlate with amount of peroxynitrite (0.25–1 mM) used to mediate cell cytotoxicity in this study. Peroxynitrite is expected to have free access to hydrophobic compartments in cells and its half-life is long enough to travel some distances (<10-20 μm) across extra- and/or intracellular compartments. Once at the target cell surface, peroxynitrite can penetrate both via anion channels (e.g., as $ONOO^-$) and by passive diffusion of the protonated form (ONOOH), and then undergo intracellular reactions, thereby resulting in cell damage and death[21]. Indeed, peroxynitrite caused SW1353 cell injury identified by CVS assay, loss of MTT

reduction, intracellular GSH depletion and the rise in intracellular oxidant formation. Apparently, the roles of peroxynitrite-mediated cellular apoptosis and necrosis have been widely studied. However, modulation of its cytotoxic effect by bicarbonate remains unclear.

The presence of 25 mM bicarbonate had an inhibitory effect on peroxynitrite-mediated cytotoxicity of SW1353 cells indicated by the measurement of CV-stained cells, MTT reduction and cellular oxidant formation. The possible explanation is that the protection could be attributed in part to diffusion limitation due to competitive CO_2-catalyzed isomerization of peroxynitrite to nitrate before it could reach its biological target sites. The half-life of peroxynitrite is therefore reduced from about 1 s to less than 20 ms in the presence of bicarbonate due to the rapid reaction of peroxynitrite and CO_2/HCO_3^- and consequently the diffusion distance of peroxynitrite is much shortened[22]. O=N-$OOCO_2^-$-derived $^{\bullet}NO_2/CO_3^{\bullet-}$ might thus be responsible for the reaction with biomolecules and subsequent cytotoxicity of SW1353 cells. In spite of the fact that $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ are strong oxidants, they have a half-life even shorter to that of $ONOO^-$ and $CO_3^{\bullet-}$ has poor diffusivity across cell membranes. Hence, the extracellular reactions of $ONOO^-$ with CO_2/HCO_3^- can attenuate the extent of toxicity due to a significant decrease in the amount of peroxynitrite approaching the target cell. Previous studies showed that 25 mM bicarbonate was found to inhibit peroxynitrite-induced oxidation of hemoglobin in erythrocytes and membrane lipids[23]. Therefore, extracellular CO_2/HCO_3^- would be possible to protect cells from exogenous peroxynitrite.

The inhibitory effects of phenolics on peroxynitrite-induced cytotoxicity of SW1353 cells: the modulation by bicarbonate

Whereas the presence of added 25 mM bicarbonate diminished SW1353 cell cytotoxicity induced by peroxynitrite, its presence aggravated GSH depletion, probably, because O=N-$OOCO_2^-$-derived $^{\bullet}NO_2$ which readily permeates cell membranes could be more active than peroxynitrite in oxidizing intracellular GSH[7]. In addition, all phenolics tested were less effective to inhibit cytotoxic effects including mitochondrial dysfunction, GSH depletion and overall oxidative stress in the presence of added 25 mM bicarbonate. The explanation is that flavonoids were possibly unable to outcompete the reaction of O=N-$OOCO_2^-$-derived $CO_3^{\bullet-}/^{\bullet}NO_2$ with biomolecules and therefore $^{\bullet}NO_2$ could lead to oxidation and nitration of intracellular biomolecules (such as thiol and tyrosine). Furthermore, $CO_3^{\bullet-}/^{\bullet}NO_2$ could rapidly oxidize catechol-type compounds to their semiquinone/quinone derivatives, which have only a weak ability to scavenge oxidants and may even themselves provide some cytotoxic effects[24].

In conclusion, the presence of CO_2/HCO_3^- appears to modulate the reactivity of peroxynitrite, possibly, because various radicals derived from peroxynitrite generate different reactions in the absence and presence of bicarbonate such as $^{\bullet}OH$ and $^{\bullet}NO_2$ or $CO_3^{\bullet-}$ and $^{\bullet}NO_2$, respectively. Our study demonstrated that peroxynitrite yields weaker reactivity in inducing cytotoxicity of SW1353 cells in CVS, MTT and DCFDA, but not GSH assay, in the presence of 25 mM bicarbonate. Furthermore, phenolics tested here are inhibitors of peroxynitrite-dependent cell death, metabolic impairment, loss of intracellular GSH and cellular oxidant formation, although they were less effective in the presence of added bicarbonate. Since peroxynitrite- and O=N-$OOCO_2^-$-derived species may selectively react with different biomolecules in various mechanisms, phenolics could exhibit differential reactivities and efficiencies with biological oxidants (e.g. ONOOH, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$). Thus, the effects of scavengers in four assays would not give identical results and should be assessed for their peroxynitrite scavenging activities using several types of assays. In addition, in developing potential peroxynitrite scavengers, there is a need to conduct experiments in the presence of physiological concentrations of bicarbonate.

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