JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 1581–1590

Received March 21, 2005
Accepted August 12, 2005
DOI 10.1211/jpp.57.12.0008
ISSN 0022-3573

# Cytoprotective mechanism of baicalin against endothelial cell damage by peroxynitrite

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## Abstract

To evaluate the mechanism responsible for the cytoprotective effects of baicalin, an antioxidant flavonoid isolated from *Scutellaria baicalensis*, we investigated its effects against peroxynitrite ($ONOO^-$)-induced endothelial cell (EC) damage. Baicalin showed efficient antioxidative actions by its ability to scavenge $ONOO^-$ and inhibit $ONOO^-$-mediated nitrotyrosine formation in vitro. Using an EC (YPEN-1) culture system, baicalin exhibited cytoprotective effects against cell death by $ONOO^-$ that was induced exogenously with tert-butyl hydroperoxide (t-BHP) in the YPEN-1 model. Baicalin was also found to reduce the intracellular precursors of $ONOO^-$, $NO^{\cdot}$ and $O_2^-$ in the t-BHP-treated ECs. Evidence from Western blotting further revealed down-regulated expressions of iNOS and COX-2, endogenous sources of $NO^{\cdot}$ and $O_2^-$ by baicalin treatment. In addition, pre-incubation of baicalin with EC suppressed t-BHP-induced nuclear factor kappa-B binding activity as determined by the transfection assay and Western blot analysis, further indicating baicalin's inhibition of iNOS and COX-2 expression. Based on the present data, we propose that baicalin scavenges $ONOO^-$ and protects cells against injury. Based on these data, it was concluded that baicalin is potentially a useful antioxidant against $ONOO^-$ and $NO^{\cdot}$ and an inhibitor of iNOS and COX-2.

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**Acknowledgements and funding:** This work was supported by a grant from the KOSEF (ROI-2000-000-00120-0). We express our thanks to Dr Byung Pal Yu for his constructive comments and evaluation of the manuscript.

## Introduction

Baicalin is a flavonoid isolated from *Scutellaria baicalensis* (*S. baicalensis*) (Tang & Eisenbrand 1992) that is known to have multiple biological functions, including the inhibition of: aldose reductase (Lin & Shieh 1996), HIV infection (Li et al 2000) and nitric oxide producing activity (Kim et al 1999). In addition, baicalin has been shown to exert a multitude of other beneficial antioxidative efficacies by its ability to modulate reactive oxygen species (ROS) (Shen et al 2003), pro-matrix metalloproteinase (Li et al 2004), pro-inflammatory cytokines, nitric oxide (NO) and prostaglandin $E_2$ (Chon et al 2003) in leukocytes, gingival fibroblasts, periodontal ligament cells, and microglial and neuron cells (Lin & Shieh 1996; Chon et al 2003; Li et al 2003, 2004; Song et al 2004). However, whether or not baicalin can attenuate the potent oxidant peroxynitrite ($ONOO^-$) and its deleterious effects in endothelial cells (ECs) has not been reported.

ECs are vulnerable to oxidative insult both within and outside the cell. In ECs, superoxide ($O_2^-$) and nitric oxide ($NO^{\cdot}$) are produced spontaneously from activated cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), forming large amounts of peroxynitrite ($ONOO^-$), which is also an effective nitrating agent that reacts with lipids, proteins, carbohydrates and deoxyribonucleic acids (Rubbo et al 1994; Pryor & Squadtrito 1995; Salgo et al 1995; Beckman 1996). $ONOO^-$ has been reported to cause protein nitration, which causes a decrease in biologically functioning proteins such as mitochondrial manganese-superoxide dismutase (Ischiropoulos et al 1992), surfactant protein A (Haddad et al 1993), protein tyrosine kinases (Kong et al 1996) and chemokines (Sato et al 2000). Furthermore, $ONOO^-$ directly inhibits the activity of mitochondrial respiratory chain enzymes and changes membrane potential and permeability (Ju et al 2000). Indeed, $ONOO^-$ inflicts cellular damage and cell death by modifying cell metabolism and signalling pathways (Kooy et al 1994). Recently, toxicity by $ONOO^-$ has been reported in inflammatory-related and

neurodegenerative diseases such as atherosclerosis, Alzheimer's disease and Parkinson's disease (Darley-Usmar et al 1995).

Perhaps more relevant to the current study, at present no effective endogenous scavenger is known for $ONOO^-$ inactivation therefore the aim of our present study was to document the function of baicalin as a potential scavenger for $ONOO^-$ and to delineate the molecular mechanism underlying this protective action against $ONOO^-$ damage in ECs.

## Materials and Methods

### Materials

Bovine serum albumin (BSA) type V, DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid), tert-butyl hydroperoxide (t-BHP) and trolox were obtained from Sigma Chemical Co. (St Louis, MO). Dihydrorhodamine 123 (DHR 123), 2′,7′-dichlorodihydrofluorescein diacetate and $ONOO^-$ were from Molecular Probes (Eugene, OR). Antibody for nitrotyrosine was from Upstate Biotechnology (Lake Placid, NY). Antibodies against COX-2, iNOS, I$\kappa$Ba and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham Life Science (Buckinghamshire, UK).

### Extraction and isolation of baicalin from *S. baicalensis*

Dried and pulverized roots (1.2 kg) of *S. baicalensis* were refluxed with MeOH (3 × 18 L) for 3 h. The extract (400 g) was suspended in distilled water and partitioned with $CH_2Cl_2$ (65 g), EtOAc (35 g), n-BuOH (130 g) and $H_2O$ (140 g) in sequence. The EtOAc fraction (35 g) was first chromatographed over a silica gel (Merck, 70–230 mesh, 1 kg) column (12 cm × 1.5 m). The column was eluted using mixtures of $CH_2Cl_2$/MeOH under gradient conditions (25:1 ~ 5:1) to yield 33 subfractions (F1–F33). Wogonin (2 g) was obtained from F1 by recrystallization. The F8 to F11 fractions (4.5 g) were combined and subjected to column chromatography over a silica gel column with $CH_2Cl_2$/MeOH (25:1) to give baicalein (2.1 g) and 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone (40 mg). Baicalin (800 mg) was obtained from silica gel column chromatography with $CH_2Cl_2$/MeOH (10:1) of F30–F33 (3.8 g). The isolated compounds were identified by comparison of their $^1H$ and $^{13}C$ NMR spectral data in the reported literature (Ishmaru et al 1995).

### Measurement of $ONOO^-$, $O_2^-$, and $NO^\cdot$ scavenging activity

$ONOO^-$ scavenging activity was measured by monitoring the oxidation of fluorescent dye DHR 123 by a modified method of Kooy et al (1994). Briefly, a working solution of 5 $\mu$M DHR 123 was loaded into a 96-well-plate containing 10 $\mu$L of sample (compounds of various concentrations) or 10 $\mu$L of vehicle as control in 50 mM sodium phosphate buffer (pH 7.4) containing 90 mM NaCl and 5 mM KCl. Just before use, 100 $\mu$M diethylenetriaminepentaacetic acid and 200 $\mu$M $ONOO^-$ were added to the reaction mixture. After 5 min of incubation at room temperature, the oxidation status of DHR 123 by $ONOO^-$ was measured using a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, Winooski,VT) at excitation and emission wavelengths of 485 and 530 nm, respectively. The $ONOO^-$ scavenging activity was calculated by comparing the fluorescence intensities generated from the control and from samples.

$O_2^-$ and $NO^\cdot$ scavenging activities were determined by measuring the changes in the fluorescence intensity of 2,7-dichlorodihydrofluorescin diacetate ($H_2$DCFDA), oxidized to fluorescent 2,7-dichlorofluorescin (DCF), and NO indicator, 4,5-diaminofluorescein diacetate (DAF-2DA) (Chung et al 2001), respectively. Briefly, 20 mM menadione (as $O_2^-$ inducer) or 2 mM sodium nitroprusside (as $NO^\cdot$ donor) was loaded into a 96-well-plate containing 10 $\mu$L of sample (compounds of various concentrations) and vehicle as control in 50 mM sodium phosphate buffer (pH 7.4); 5 min later 25 $\mu$M $H_2$DCFDA or 0.5 $\mu$g DAF-2DA was added to this mixture. The fluorescence intensity of DCF or DAF-2DA was measured by FL 500 (excitation 485 nm and emission 530 nm) every 5 min for 30 min. The $O_2^-$ and $NO^\cdot$ scavenging activities were calculated by comparing the fluorescence intensity changes per minute that were generated from the control and from samples.

IC50 was calculated by the following method. First, the scavenging activity curve of each compound was obtained through three independent experiments, then IC50 values were derived from the average of three doses in which 50% of oxidants was scavenged.

### Spectrophotometric analysis of the reactions of baicalin with $ONOO^-$

The interaction of baicalin, tyrosine and $ONOO^-$ was investigated as follows. The reaction of $ONOO^-$ with tyrosine in the presence or absence of baicalin was carried out in 50 mM phosphate buffer (pH 7.0) at room temperature for 10 min. After the reaction, a spectrophotometric scan was performed (Ultrospec 2000, Pharmacia Biotech) with wavelengths from 190 to 600 nm.

### Cell culture system measurements of intracellular $NO^\cdot$, $O_2^-$ and $ONOO^-$

The endothelial cell line YPEN-1 was purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured with DMEM medium (Nissui Co., Tokyo, Japan) supplemented with 5% fetal bovine serum, 233.6 mg $mL^{-1}$ glutamine, 0.25 $\mu$g $mL^{-1}$ amphotericin B (Gibco, Grand Island, NY) and 72 $\mu$g $mL^{-1}$ gentamicin (Sigma). The mixture was adjusted to pH 7.4–7.6 by $NaHCO_3$ (Gibco) and maintained in a 5% $CO_2$ incubator. The fresh medium was replaced before

treatment of baicalin. Cell viability after treatment was detected by the MTT assay (Tada et al 1986).

To measure intracellular $NO^{\bullet}$, $O_2^-$ and $ONOO^-$, cells seeded at a density of $5 \times 10^4$ cells per well in a Costar 48-well plate were allowed to adhere overnight and then incubated in serum-free DMEM with various concentrations of baicalin and 2 $\mu$M t-BHP. The amounts of intracellular $ONOO^-$, $NO^{\bullet}$, and $O_2^-$ were measured by the changes in fluorescence intensity of the fluorescent dyes DHR 123, DCF and DAF-2DA that were previously incubated with cells for 15 min. The levels of $ONOO^-$, $NO^{\bullet}$ and $O_2^-$ were measured by monitoring the oxidation of the respective fluorescent dyes.

### Preparation of cytosolic/nuclear extracts and Western blot analysis

The cells were washed by ice-cold PBS and harvested. The cytosolic fractions were extracted by buffer containing 10 mM Tris (pH 8.0), with 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40 and protease inhibitors. Nuclei were separated from cytosol by centrifugation at 12 000 rpm at 4°C for 15 min, and nuclear fractions were extracted from the pellets by buffer containing 10 mM Tris (pH 8.0), with 50 mM KCl, 100 mM NaCl and protease inhibitors.

The aliquots containing cytosol/nuclear extracts in gel-loading buffer were boiled for 5 min. Total protein equivalents for each sample were separated on 6–12% SDS-polyacrylamide mini gel using a Laemmli buffer system at 100 V and then transferred to a PVDF membrane at 100 V for 1.5 h in a wet transfer system (Bio-Rad, Hercules, CA). The membrane was immediately placed in a blocking solution (5% non-fat dry milk in TBS-T buffer containing 10 mM Tris, 100 mM NaCl and 0.1% Tween 20, pH 7.5) at 4°C overnight. The membrane was washed in TBS-T buffer for 30 min and then incubated with a first antibody at room temperature for 1–2 h. After triplicate washings in TBS-T buffer, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. After four washings in TBS-T buffer, antibody labelling was detected using ECL following the manufacturer's instructions and exposed to radiographic film. Pre-stained blue protein markers were used for molecular weight determination.

### Transfection and luciferase reporter assay for nuclear factor kappa-B activity

The activity of nuclear factor kappa-B (NF-$\kappa$B) was examined using the luciferase plasmid DNA, pTAL-NF$\kappa$B, which contains a specific binding sequence for NF-$\kappa$B (BD Bioscoences Clontech, CA). Transfection was carried out using FuGENE 6 Reagent (Roche, Indianapolis, IN). Briefly, $1.5 \times 10^4$ cells per well were seeded in 48-well plates. When cultured cells reached about 40% confluence, cells were treated with 0.1 $\mu$g DNA/0.5 $\mu$L FuGENE, six complexes in a total volume of normal media (5% serum contained) with 500 $\mu$L for 42 h. Subsequently, 0.2 $\mu$M of t-BHP was treated after exchange to serum-free media and treatments with baicalin were performed 1 h previously. After an additional incubation for 6 h, cells were washed with PBS and subjected to the Steady-Glo Luciferase Assay System (Promega, Medison, WI). Luciferase activity was measured by a luminometer (GENious, TECAN, Salzburg, Austria).

### Statistical analyses

Data are expressed as mean ± s.e.m. Results were analysed statistically using the Kruskal–Wallis test and Nemenyi's test. Values of $P < 0.05$ were considered statistically significant.

## Results

### $ONOO^-$ scavenging compounds isolated from *S. baicalensis*

The major compounds from *S. baicalensis* showing $ONOO^-$ scavenging activity are listed in Table 1. The effectiveness of these compounds to inhibit $ONOO^-$-induced DHR 123 oxidation was as follows: baicalin ($IC50 = 0.710 \pm 0.007$ $\mu$M); 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone ($IC50 = 0.872 \pm 0.026$ $\mu$M); baicalein ($IC50 = 5.555 \pm 0.284$ $\mu$M); and wogonin ($IC50 = 6.697 \pm 0.304$ $\mu$M). The scavenging activity of baicalin (structure shown in Figure 1) was even stronger than that of penicillamine, a well-known $ONOO^-$ scavenger ($IC50 = 3.753 \pm 0.086$ $\mu$M). We also analysed the raw data using the Kruskal–Wallis test. The result indicated that baicalin shows the highest activity, as indicated by its IC50 $0.710 \pm 0.007$ ($P < 0.01$ vs wogonin; $P < 0.01$ vs baicalein; $P < 0.01$ vs penicillamine; Table 1).

Because $ONOO^-$ is endogenously formed from a reaction of $O_2^-$ with $NO^{\bullet}$, it is interesting to compare the ability of the three components tested for $O_2^-$ and $NO^{\bullet}$ scavenging ability. The data shown in Table 2 indicate that compared with an $NO^{\bullet}$ standard scavenger, carboxy-PTIO ($IC50 = 2.618 \pm 0.967$ $\mu$M), baicalin had much higher activity ($IC50 = 1.893 \pm 0.249$ $\mu$M). In addition, baicalin inhibitory action ($IC50 = 2.715 \pm 0.116$ $\mu$M) was higher in $O_2^-$ scavenging ability compared with a $O_2^-$ scavenging standard, Trolox ($IC50 = 5.156 \pm 1.475$ $\mu$M).

**Table 1** $ONOO^-$ scavenging activity of compounds isolated from *Scutellaria baicalensis*

| Compounds | IC50 ($\mu$M)[a] |
|---|---|
| Baicalin | 0.71 ± 0.01 |
| Wogonin | 6.70 ± 0.30 |
| Baicalein | 5.56 ± 0.28 |
| 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone | 0.87 ± 0.03 |
| Penicillamine[b] | 3.75 ± 0.09 |

[a]Each value is the mean ± s.e.m. from three measurements. [b]Used as a reference compound for $ONOO^-$ scavenging activity.

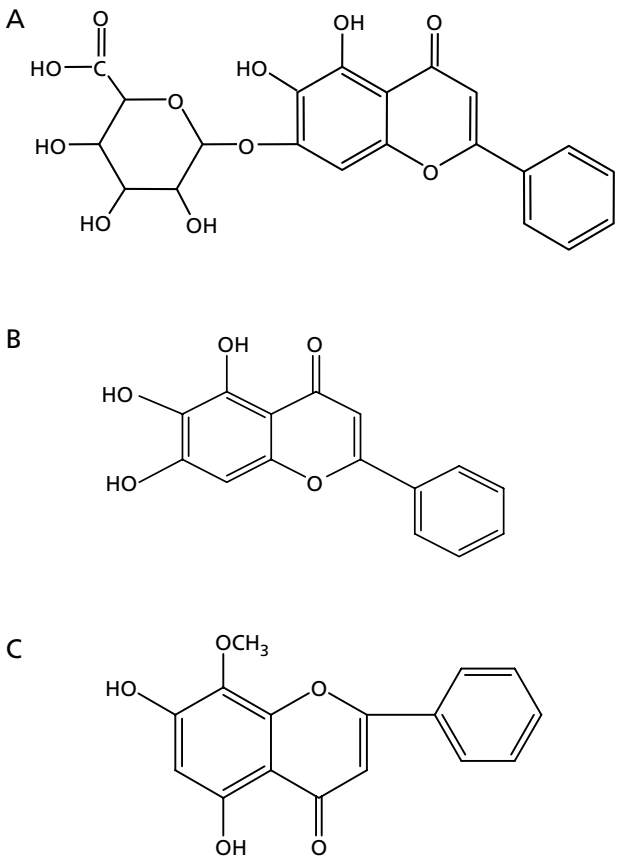


**Figure 1** Structures of baicalin (A), baicalein (B) and wogonin (C).

**Table 2** The $NO^{\cdot}$ and $O_2^-$ scavenging activity of baicalin

| Components | $NO^{\cdot}$ | $O_2^-$ |
|---|---|---|
| Baicalin | $1.89 \pm 0.25$ | $2.72 \pm 0.12$ |
| Carboxyl-PTIO[a] | $2.62 \pm 0.97$ | |
| Trolox[b] | | $5.16 \pm 1.48$ |

[a]Used as a reference compound for $NO^{\cdot}$ scavenging activity. [b]Used as a reference compound for $O_2^-$ scavenging activity.

### Effect of baicalin in reducing $ONOO^-$-induced nitrotyrosine

To determine whether or not $ONOO^-$-induced nitrotyrosine formation is reduced as $ONOO^-$ is scavenged by baicalin, we monitored the formation of nitrotyrosine spectrophotometrically in the interaction of $ONOO^-$ with tyrosine. The reaction of $ONOO^-$ (500 $\mu$M) with tyrosine (400 $\mu$M) generated a nitrotyrosine peak at 430 nm (as shown in Figure 2A-2). The reduced 3-nitrotyrosine peak at 430 nm on the addition of baicalin was taken as an indication of the inhibition of tyrosine nitration by baicalin (Figure 2A-3). The new peak at 325nm (Figure 2B-1) implies the interaction of baicalin with $ONOO^-$ (Figure 2B-2). Furthermore, we found that reaction between baicalin and $ONOO^-$ does not generate nitration products because no peak at 430nm was observed (Figure 2B-2).

To confirm the reduction of tyrosine nitration in the presence of baicalin, monoclonal antibody specific to nitrotyrosine was used to detect nitrotyrosine in BSA (0.5 mg protein $mL^{-1}$) after treatment with $ONOO^-$ for 1 h. When BSA was pre-incubated with baicalin at various concentrations, ranging from 20 to 80 $\mu$M, we observed that baicalin attenuated the $ONOO^-$-induced nitration of BSA in a dose-dependent manner (Figure 3A). In addition, we also found protective effects of 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone against $ONOO^-$-induced nitrotyrosine formation (Figure 3B), verifying our previous data on the $ONOO^-$ scavenging activity of 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone (Table 1). More importantly, the data also imply a more efficient high free radical scavenging capacity of bailcalin compared to 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone since at 40 $\mu$M 5,7,2′,5′-tetrahydroxy-8,6′-dimethoxyflavone cannot completely prevent the nitrotyrosine formation, although bailcalin did (Figure 3).

### The cytoprotective effects of baicalin against cell damage from $ONOO^-$

To assess the protective ability of baicalin against $ONOO^-$-induced cell death, we investigated the effect of baicalin on cell viability using the MTT assay. Cells were pre-incubated with baicalin at concentrations of 2, 5, 10, 20 $\mu$M or penicillamine (20 $\mu$M) used as positive control, followed by treatment with $ONOO^-$ for 6 h. Pre-incubated ECs with baicalin 10 or 20 $\mu$M before $ONOO^-$ treatment were found to have cell viabilities of about 71.8 and 93.3%, respectively (Figure 4A). In addition, baicalin did not seem to cause toxicity to the cells at the high concentration of 20 $\mu$M (Figure 4B). To further study the cytoprotective effects of bailcalin we induced cell death by t-BHP treatment. As shown in Figure 4B, baicalin reduced the cytotoxity of t-BHP, dose-dependently increasing cell viability (Figure 4B).

### Effect of baicalin on intracellular $O_2^-$ and $NO^{\cdot}$

To further understand the molecular events underlying the cytoprotective effect of baicalin, t-BHP-induced intracellular $ONOO^-$, $O_2^-$ and $NO^{\cdot}$ levels were investigated in YPEN-1 cells. The data showed that t-BHP significantly increased intracellular $ONOO^-$, $O_2^-$ and $NO^{\cdot}$. This high oxidative stress reaction due to t-BHP may be responsible for YPEN-1 cell death. It was evident that the cells pre-incubated with baicalin had decreased levels of $ONOO^-$ (A), $NO^{\cdot}$ (B) and $O_2^-$ (C), as shown in Table 3. As indicated in Table 3, the level of $ONOO^-$ was reduced nearly to the baseline with baicalin, but not completely.

COX-2 and iNOS are known as major endogenous resources of $O_2^-$ and $NO^{\cdot}$, respectively. For detection of COX-2 and iNOS expression in YPEN-1, cells were incubated with t-BHP for 6 h. As shown in Figure 5, the

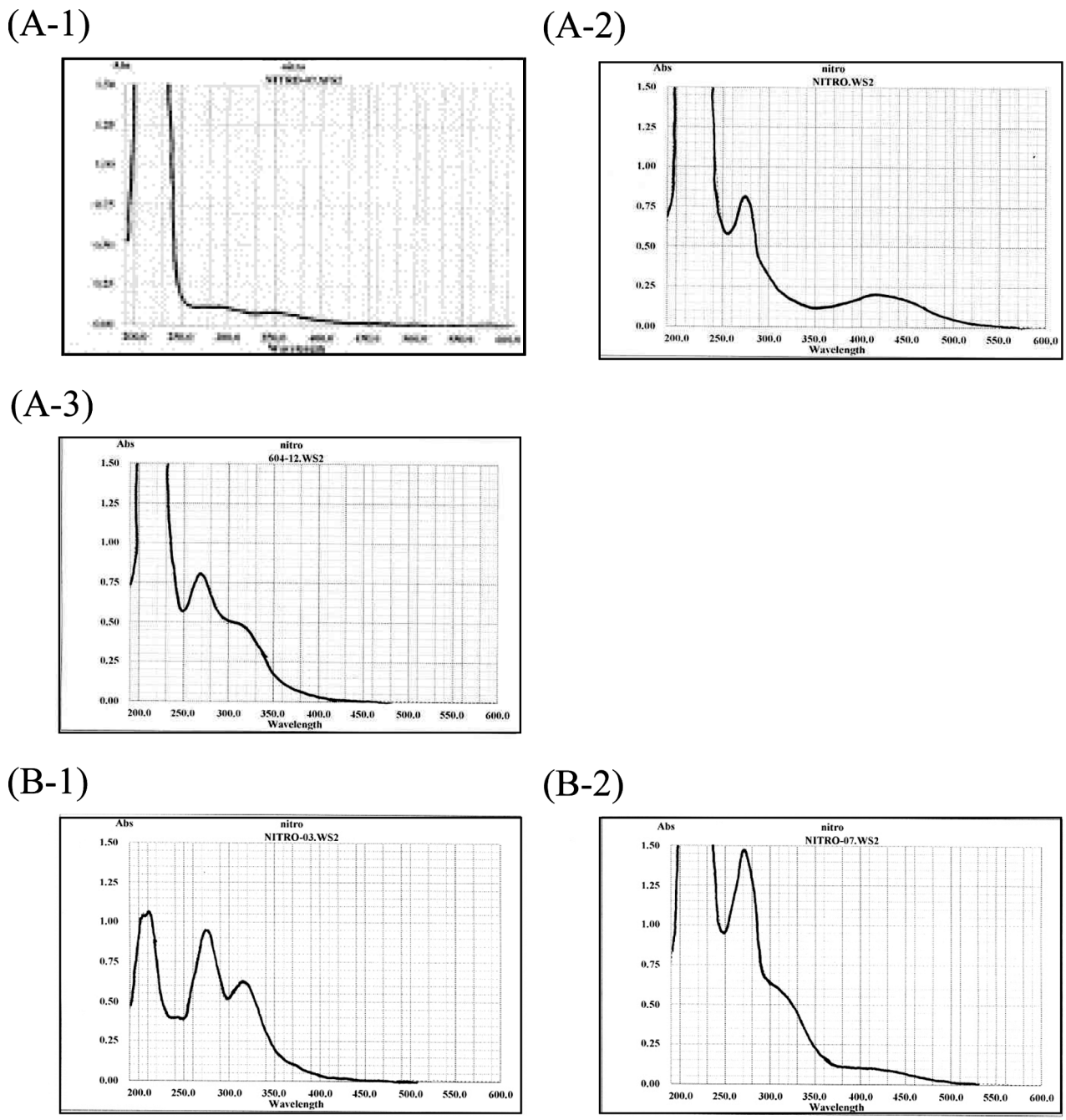


**Figure 2** Spectrophotometric analysis of baicalin reacting with $ONOO^-$. (A) $ONOO^-$ (A-1), reaction of $ONOO^-$ (500 $\mu$M) and tyrosine without (A-2) and with (A-3) baicalin. (B) Baicalin (50 $\mu$M) incubated without (B-1) and with (B-2) $ONOO^-$ (500 $\mu$M). Each mixed solution was incubated at 37°C with shaking for 1 h and scanned between 190 and 600 nm by spectrophotometric analysis. The spectra of the peak displayed at 430 nm reflect the formation of 3-nitrotyrosine.

expression of these two enzymes was up-regulated by t-BHP and, importantly, was suppressed by baicalin.

### Inhibition of NF-κB activation by baicalin

The expression of COX-2 and iNOS is well known to be strongly influenced by NF-$\kappa$B activation; therefore we investigated the effect of baicalin on NF-$\kappa$B activity related to the expression of iNOS and COX-2. The protein levels of major components of the NF-$\kappa$B complex, p65 and I$\kappa$B$\alpha$, were studied by Western blotting of nuclear extracts. For detection of changed p65 and I$\kappa$B$\alpha$ levels, cells were cultured for 2 h in the presence of 10 $\mu$M t-BHP. As shown in Figure 6, the pre-incubation of the cells with

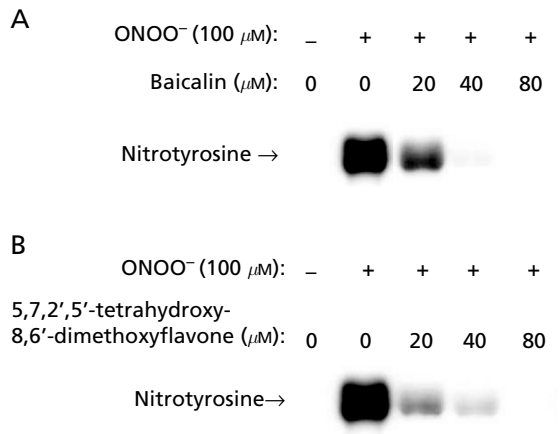


**Figure 3** Active components reduce nitrotyrosine triggered by $ONOO^-$. Nitrotyrosine was induced by reaction of BSA (0.5 mg protein $mL^{-1}$) and $ONOO^-$ (100 $\mu$M) with/without active components. The nitration level was analysed by the Western blot method with antibody specific for nitrotyrosine.

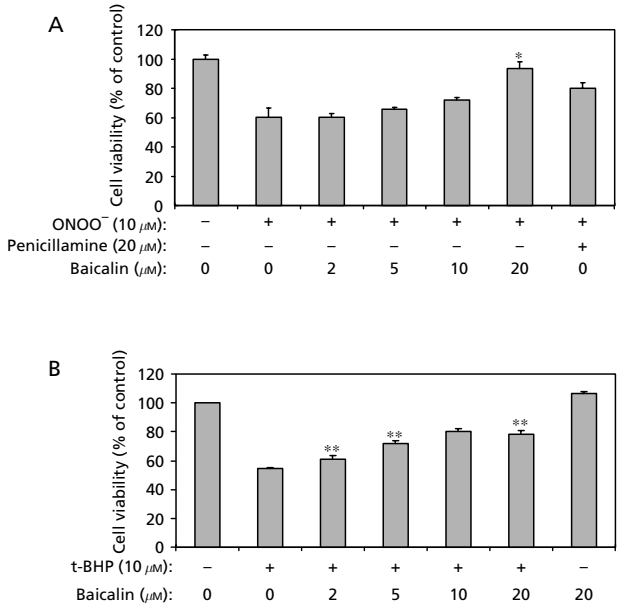


**Figure 4** Effect of baicalin on endothelial cell viability. The cells were pretreated with various concentrations of baicalin for 1 h and followed by treatment with or without 10 $\mu$M $ONOO^-$ (A) for 24 h or 10 $\mu$M t-BHP (B) for 6 h. The MTT assay was done as described in the Materials and Methods section. Data were mean ± s.d. of triplicate measurements. $^*P < 0.05$, $^{**}P < 0.01$ vs t-BHP (10 $\mu$M).

baicalin resulted in a decrease of p65 in the nuclear extracts, while I$\kappa$B$\alpha$ in cytosol increased (Figure 6A). To validate our finding that baicalin suppressed NF-$\kappa$B activity, we performed transient transfection of pTAL-NF-$\kappa$B to YPEN. The luciferase activity was increased after t-BHP treatment in the transfected cells and baicalin suppressed this luciferase activity in a dose-dependent manner (Figure 6B). The decrease was especially evident at a dose of 40 $\mu$M of baicalin, which resulted in a significant decrease in NF-$\kappa$B activity.

## Discussion

Our study was able to document that baicalin is not only a strong $ONOO^-$ scavenger but also an effective inhibitor of intracellular free radical generation. This conclusion was reached based on our experimentation in which baicalin's scavenging activity was compared (as shown in Tables 1 and 2) with other well-known $ONOO^-$, $NO^\bullet$ and $O_2^-$ scavenging agents, namely penicillamine (Pannala et al 1997), carboxy-PTIO (Yoshida et al 1998) and Trolox (Satoh et al 1997). Another salient finding of the present study is the ability of baicalin to scavenge $NO^\bullet$ and $O_2^-$, which makes baicalin more versatile against a broad spectrum of reactive species.

To evaluate the safety and possible untoward cytotoxic effects of baicalin, we carried out EC viability tests. Under experimental conditions of less than 40 $\mu$M, baicalin was able to maintain cell viability, even under the challenged condition of endogenously added t-BHP. The latter finding is significant because t-BHP, as a strong oxidant on its own, can elicit a redox disruption and enhance the cellular oxidative stress responsible for lipid peroxidation, DNA adducts formation and the induction of apoptosis (Haidara et al 2002).

Another salient finding coming from our experiments with baicalin is that it was clearly shown to suppress NF-$\kappa$B activation. Although NF-$\kappa$B rapidly modulates the gene expression needed for host defences, uncontrolled, chronic activation of NF-$\kappa$B can lead to various pathogenic conditions, such as the chronic pro-inflammatory conditions related to atherosclerosis, cancer and dementia (Baldwin 2001). Although the involvement of NF-$\kappa$B in $ONOO^-$ induced cell death has not yet been investigated in detail, it is conceivable that NF-$\kappa$B could promote $ONOO^-$ generation by up-regulating COX-2 and iNOS activation. The present data seem to indicate such a possibility by baicalin's ability to suppress the activation of NF-$\kappa$B by inhibiting its nuclear translocation by maintaining a high level of inhibitory I$\kappa$B$\alpha$ in the cytosol. In line with this evidence, baicalin reduced the translocation of P65, the NF-$\kappa$B component.

The effects of baicalin in modulating NF-$\kappa$B activation were further confirmed by the luciferase gene transfection assay using endothelial cells. The relative luciferase activity increased after t-BHP treatment and baicalin suppressed this activity in a dose-dependent manner. Thus, baicalin may down-regulate COX-2 and iNOS expression by modulating NF-$\kappa$B activity. Inhibition of NF-$\kappa$B was measured 2 h after treating t-BHP: p65 was not completely blocked by baicalin, which may be related to the lower concentration of baicalin used in the experiments or the fact that bailcalin only partially blocked the oxidative stress-induced NF-$\kappa$B activation.

Thus, data from our experiments show the effectiveness of the antioxidative action of baicalin on $ONOO^-$ scavenging activity. The protective ability of baicalin against

**Table 3** The intracellular $ONOO^-$, $NO^{\cdot}$ and $O_2^-$ scavenging activity of baicalin in YPEN-1 challenged by 10 $\mu$M t-BHP

| Treatment | Intracellular free radicals | | |
|---|---|---|---|
| | (A) $ONOO^-$ | (B) $NO^{\cdot}$ | (C) $O_2^-$ |
| Control | $100 \pm 1.49$ | $100 \pm 2.26$ | $100 \pm 8.19$ |
| t-BHP | $248.92 \pm 1.01$ | $390.67 \pm 3.81$ | $393.78 \pm 45.09$ |
| t-BHP + baicalin (2 $\mu$M) | $127.43 \pm 0.50$* | $277.53 \pm 4.37$* | $277.72 \pm 37.38$ |
| t-BHP + baicalin (5 $\mu$M) | $111.75 \pm 0.98$* | $306.52 \pm 12.12$* | $299.87 \pm 39.47$ |
| t-BHP + baicalin (10 $\mu$M) | $109.34 \pm 0.96$* | $175.32 \pm 5.29$* | $175.11 \pm 18.77$* |
| t-BHP + baicalin (20 $\mu$M) | $100.71 \pm 1.40$* | $184.86 \pm 3.17$* | $177.35 \pm 17.45$* |
| t-BHP + baicalin (40 $\mu$M) | $95.73 \pm 1.93$* | $180.11 \pm 4.00$* | $220.41 \pm 16.38$* |

The cells were incubated in serum-free media at indicated concentrations of baicalin. (A) Inhibition of t-BHP-induced $ONOO^-$ by baicalin. $ONOO^-$ levels were measured by monitoring equal amounts of DHR 123 in cell homogenates. (B) Inhibition of t-BHP-induced $NO^{\cdot}$ by baicalin. $NO^{\cdot}$ levels were measured by monitoring equal amounts of DAF-2 in cell homogenates. (C) Inhibition of t-BHP-induced $O_2^-$ by baicalin. $O_2^-$ levels were measured by monitoring equal amounts of DCF in cell homogenates. *$P < 0.01$.

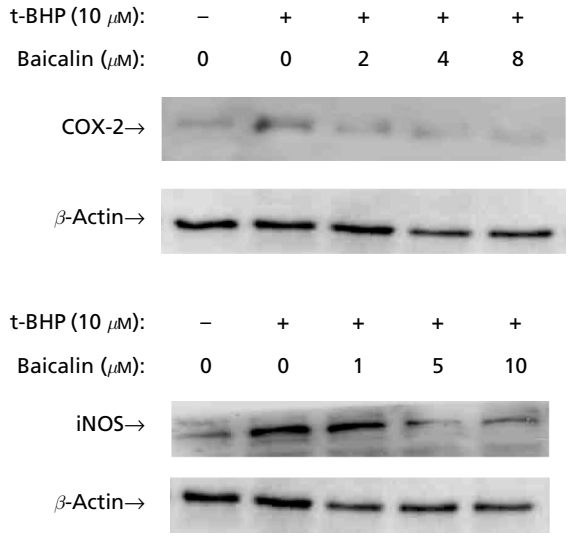


**Figure 5** Effect of baicalin on t-BHP-induced COX-2 and iNOS expression in endothelial cells. The cells were pretreated with various concentrations of baicalin, followed by incubation with t-BHP for 6 h. Cytosol protein was separated on SDS-polyacrylamide gels and blotted with antibody specific for COX-2 and iNOS as described in the Materials and Methods section.

$ONOO^-$ damage may be attributed to two of its properties: its ability to directly scavenge $ONOO^-$ and its ability to downregulate iNOS and COX-2 through the suppression of NF-$\kappa$B activation. In the scavenging capacity test (Table 3), pretreatment of YPEN-1 with baicalin for 1 h caused the reduction of intracellular $ONOO^-$, NO and $O_2^-$, which was followed by the suppression of COX-2 and iNOS. These data were taken as an indication that baicalin may first interact with $ONOO^-$ in the cell and then lead to the inhibition of COX-2 and iNOS. Thus, the net result of baicalin $ONOO^-$ scavenging ability is a combination of free radical reduction and inhibition of precursor-generating proteins.

It is interesting and worth discussing to consider some structural aspects of baicalin with respect to its action. Baicalin is a member of a family of flavonoids that are naturally occurring plant polyphenols. Recent reports (Choi et al 2002) point out that the $ONOO^-$-scavenging activities of flavonoids are dependent on a hydroxyl group and its location in the phenol ring. An increase in the number of hydroxyl groups is generally thought to enhance the antioxidant activity of flavonoids (Heijnen et al 2001) and there is some correlation with the number of hydroxyl groups, as previously reported (Cao et al 1997). In this study, baicalin, baicalein and wogonin, all of which contain hydroxyl groups, showed different efficiencies in their ability to scavenge $ONOO^-$, as shown in Tables 1 and 2. Baicalin showed a far better scavenging effect than either baicalein or wogonin, and its greater reactivity might be explained by differences in its chemical structure. Although baicalin has one less hydroxyl group than baicalein due to the attachment of a glucuronide to one hydroxyl group, it is expected that the ortho-dihydroxyl group in the baicalin structure would facilitate the release of a reduced hydrogen ion due to delocalization of the aromatic nucleus (Figure 1).

The exact reaction mode by which antioxidants scavenge $ONOO^-$ has not been fully defined. However, in the case of phenolic compounds having hydroxyl groups such as those of baicalin, modes of either nitration or electron donation with $ONOO^-$ have been proposed (Pannala et al 1998). Nitration formation by $ONOO^-$ gives a peak at 430 nm due to the breakdown of $ONOO^-$ to the nitrogen dioxide radical ($NO_2$) or the nitronium ion. In our study, the absence of a typical nitration peak at 430 nm following baicalin's reaction with $ONOO^-$ (Figure 2A and B) indicates that no nitration occurs. In addition, a reduced nitrotyrosine peak around 430 nm by baicalin, as shown in Figure 2A-3, implies that electron donation by baicalin is a likely reaction mechanism to $ONOO^-$ (Pannala et al 1998).

Another hypothesis for a possible reaction for the $ONOO^-$ scavenging action is the interaction of $ONOO^-$

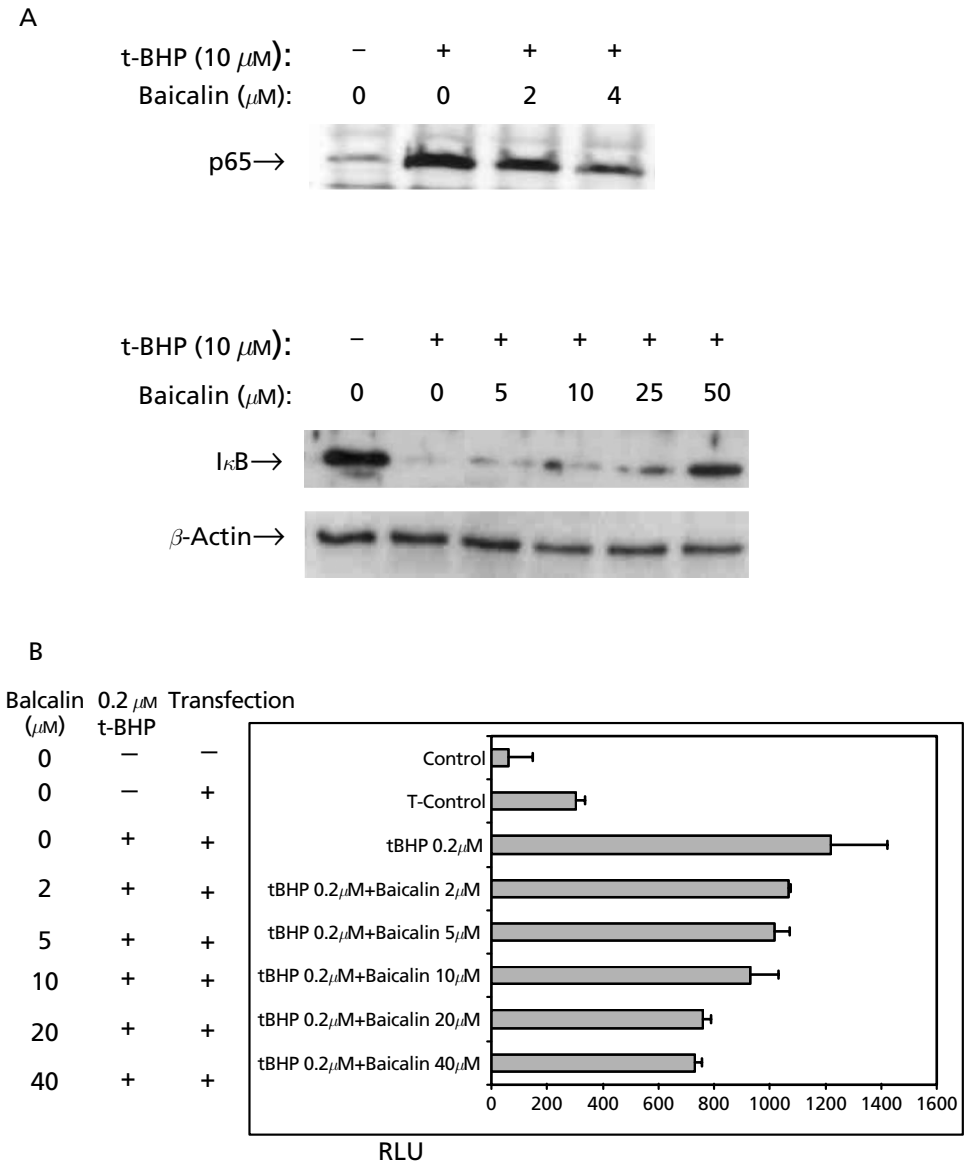


**Figure 6** Effect of baicalin on t-BHP-induced NF-κB activity. (A) Baicalin suppressed p65 translocation and maintained IκB levels. The cells were pretreated with various concentrations of baicalin and t-BHP treated for 6 h. Nuclear and cytosol protein was separated on SDS-polyacrylamide gels and blotted with antibody specific for p65 and IκBa, respectively, as described in the Materials and Methods sections. (B) Baicalin reduced t-BHP-induced NF-κB activity. The cells were co-treated with 0.2 μM t-BHP and 2, 5, 10, 20 and 40 μM of baicalin for 6 h. Luciferase activity is reported as percentage activity compared with control cells. Statistical significance of differences in luciferase activity of NF-κB between the untreated control and treated groups was determined using one-way analysis of various (ANOVA), followed by Duncan's multiple range test.

with the tyrosine moiety of proteins (Schroeder et al 2001), which proposes that flavonoids inhibit nitrotyrosine formation by the interaction of flavonoids with intermediate tyrosyl radicals and not with $ONOO^-$ itself. Thus, further study is required to clarify the exact mechanism responsible for baicalin's ability to reduce nitrotyrosine formation, which was confirmed by the Western blot analysis of nitrated albumin in the present study. It should also be mentioned that baicalin contains the glucuronide form that could act as a free radical scavenger, as shown with rheumatoid arthritis (Sato et al 1988).

Based on our evidence, we conclude that baicalin can protect cells from the oxidative damage elicited by $ONOO^-$. The significant $ONOO^-$ scavenging property of baicalin was further highlighted in its effectiveness in modulating pro-inflammatory NF-κB activity and possibly COX-2 and iNOS expression.

## Conclusions

$ONOO^-$ is produced by the reaction of $O_2^-$ with $NO^{\cdot}$. $ONOO^-$, a powerful oxidant, causes damage to proteins,

lipids and DNA through nitration or oxidation. However, due to the lacking of endogenous defence components against $ONOO^-$ in the body, a supplement of scavengers is needed.

The present study suggests that the active bioflavonoid component baicalin can scavenge reactive species efficiently. Baicalin treatment led to an inhibition of $ONOO^-$-mediated nitration of tyrosine through electron donation, and it also showed significant dose-dependent inhibition of BSA nitration from $ONOO^-$. Baicalin was shown to reduce t-BHP-induced reactive species equally well and it suppressed NF-$\kappa$B luciferase activity and its related gene expression. The significant $ONOO^-$ scavenging properties of baicalin make it a possible antioxidant against $ONOO^-$.

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