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Myricitrin protects against peroxynitrite-mediated DNA damage and cytotoxicity in astrocytes

Wei Chen [a,b,*], Jingjing Zhuang [b], Ya Li [b], Yang Shen [b], Xiaodong Zheng [a,*]

[a] Department of Food Science and Nutrition, College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310058, China
[b] College of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310035, China

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ABSTRACT

Peroxynitrite, a potent oxidising and nitrating species, has been implicated in the pathogenesis of neurodegenerative diseases. This study was undertaken to investigate the protective effect of myricitrin on peroxynitrite-mediated toxicity and the underlying mechanism. Our results showed that the presence of myricitrin was found to significantly inhibit peroxynitrite-mediated DNA damage. EPR spectroscopy demonstrated that myricitrin potently diminished the DMPO-hydroxyl radical adduct signal from peroxynitrite. Further study showed that glutathione (GSH) depletion caused by peroxynitrite can be effectively prevented by pre-incubation of astrocytes with myricitrin. Moreover, co-incubation of astrocytes with myricitrin and buthionine sulfoximine (BSO) eliminated the myricitrin-induced GSH increase. In contrast, co-incubation of myricitrin with BSO slightly protected astrocytes against cytotoxicity and DNA damage mediated by peroxynitrite. These results revealed that myricitrin can protect against peroxynitrite-induced DNA damage and cytotoxicity, which might have implications for myricitrin-mediated neuroprotection.

1. Introduction

In recent decades, accumulating evidence has demonstrated that oxidative stress plays an important role in the progression of many chronic diseases, including neurodegenerative diseases, cancer, diabetes and cardiovascular diseases (Corsinovi, Biasi, Poli, Leonarduzzi, & Isaia, 2011; Fearon & Faux, 2009; Houstis, Rosen, & Lander, 2006; Lin & Beal, 2006; Visconti & Grieco, 2009). One such mediator of oxidative stress is peroxynitrite ($ONOO^-$), which can be generated from the bi-radical reaction of nitric monoxide (NO) and superoxide ($O_2^{\cdot-}$) (Beckman & Koppenol, 1996). Peroxynitrite is highly toxic to cultured neurons, and has been implicated in the pathogenesis of neurodegenerative disorders (Chen, Feng, Huang, & Su, 2012a; Mancuso et al., 2007; Vauzour et al., 2008). Moreover, peroxynitrite can induce the oxidation of DNA, lipids, and protein sulfhydryls (Bartesaghi et al., 2006; Chen et al., 2010; Pacher & Szabot, 2008). The mechanisms underlying peroxynitrite-mediated cytotoxicity are multiple, among them, induction of DNA strand breaks and the subsequent activation of poly (ADP-ribose) polymerase-1 (PARP-1) have been demonstrated to be critical events (Szabo, 2003). In this context, DNA strand breakage triggered by peroxynitrite may lead to cell death (Komjati, Besson, & Szabo, 2005). Moreover, studies have also indicated that some natural compounds are able to protect against peroxynitrite-mediated DNA damage due to their peroxynitrite-scavenging activity (Klotz & Sies, 2003; Matsunaga et al., 2009).

Chinese bayberry (*Myrica rubra Sieb. et Zucc.)* has been cultivated mainly in southern China for more than 2000 years. In Chinese markets, bayberry is one of the most popular and valuable fruits, due to its special sweet/sour taste. It also has been widely used as a functional food to treat gastric intestinal problems, such as diarrhoea and gastroenteritis. In addition to the fruit, the leaf and bark of bayberry are also used in traditional Chinese medicine for skin diseases, wounds, and ulcers. Myricitrin (3,3′,4′,5,5′,7-hexahydroxyflavone 3-*O*-rhamnoside), a naturally occurring flavonoid, is a bioactive component of the bark, leaf and fruit of bayberry (Bao, Cai, Sun, Wang, & Corke, 2005; Matsuda et al., 1995; Matsuda, Yamazaki, Matsuo, Asanuma, & Kubo, 2001). Recent studies showed that myricitrin possesses anti-inflammatory, anti-allodynic, antinociceptive and anxiolytic properties (Meotti et al., 2006, 2007, 2008; Pereira et al., 2011). However, there is little information available concerning the neuroprotective efficacy of

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; DMPO, 5,5-dimethylpyrroline-N-oxide; DMPO-OH, DMPO-hydroxyl adduct; DMPO-OOH, DMPO-superoxide spin adduct; EPR, electron paramagnetic resonance; SOD, superoxide dismutase; GSH, reduced glutathione; BSO, buthionine sulfoximine.

* Corresponding authors. Address: Department of Food Science and Nutrition, College of Biosystems Engineering and Food Science, Hangzhou 310058, China. Tel.: +86 571 88982398; fax: +86 571 88982861 (W. Chen). Tel.: +86 571 86098861; fax: +86 571 86971139 (X. Zheng).

E-mail addresses: zjuchenwei@zju.edu.cn (W. Chen), xdzheng@zju.edu.cn (X. Zheng).

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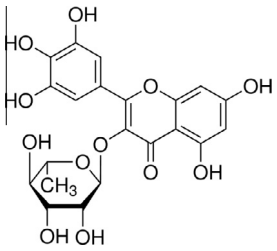

Fig. 1. Chemical structure of myricitrin.

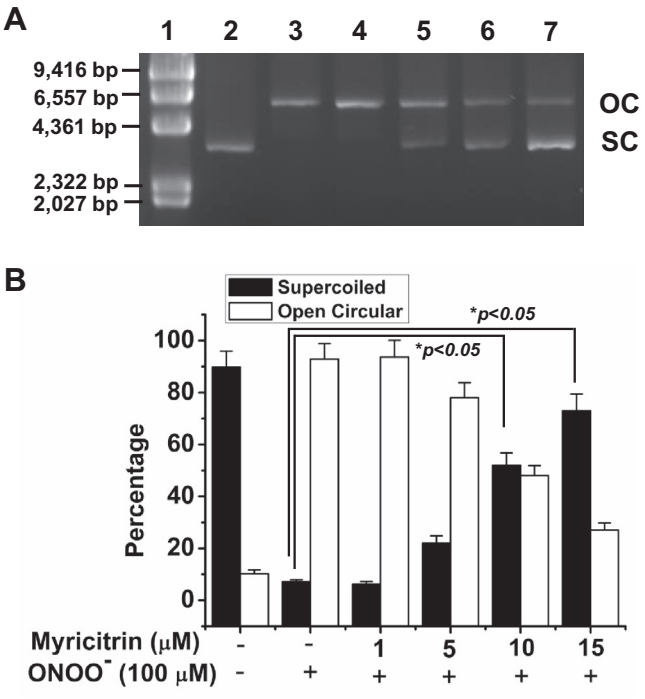

Fig. 2. DNA cleavage caused by peroxynitrite in the presence or absence of myricitrin. Plasmid DNA was incubated with peroxynitrite ($ONOO^-$) in the presence or absence of myricitrin for 60 min. (A) A representative gel picture of plasmid DNA after incubation with 100 μM peroxynitrite in the presence or absence of various concentrations of myricitrin for 60 min. Lane 1: marker; lane 2: control; lane 3: $ONOO^-$ 100 μM; lane 4: $ONOO^-$ 100 μM + myricitrin 1 μM; lane 5: $ONOO^-$ 100 μM + myricitrin 5 μM; lane 6: $ONOO^-$ 100 μM + myricitrin 10 μM; lane 7: $ONOO^-$ 100 μM + myricitrin 15 μM; (B) the quantitative data of panel A (*$p < 0.05$ versus $ONOO^-$ treatment group).

myricitrin. Since peroxynitrite is involved in the pathogenesis of neurodegenerative diseases, and induction of DNA strand breakage is a critical initial lesion leading to cell death caused by peroxynitrite (Szabo, 2003), in the present study, using φX-174 plasmid DNA and rat primary astrocytes as model systems, we investigated the efficacy of myricitrin on peroxynitrite-mediated DNA damage and cytotoxicity.

2. Materials and methods

2.1. Reagents

The Lambda DNA-*Hind*III digest and φX-174 RF I DNA were obtained from New England Biolabs (Beverley, MA). Peroxynitrite was purchased from Calbiochem (San Diego, CA). Myricitrin (purity >98%) was obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Other chemicals were purchased from Sigma Chemical (St. Louis, MO).

2.2. Examination of DNA cleavage

DNA cleavage was determined by the conversion of supercoiled φX-174 RF I double-stranded DNA to open circular forms (Chen et al., 2009). Briefly, 0.2 μg DNA were incubated with peroxynitrite in the presence or absence of myricitrin at room temperature. Following incubation, the samples were loaded into 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer. After electrophoresis, the gels were stained with 0.5 μg/ml ethidium bromide for 30 min, followed by 30 min destaining in water. The gels were then visualised under ultra-violet illumination and digitally photographed using a gel documentation system and Quantity-one™ image analysis software (Bio-Rad, Hercules, CA).

2.3. Electron paramagnetic resonance (EPR) spin-trapping assay

EPR spectra were recorded at room temperature using a Bruker A300 EPR spectrometer, operating at X-band with a TM cavity and capillary tube. For peroxynitrite reaction, reactants containing 80 mM DMPO, 0–15 μM myricitrin, 0–500 μM $ONOO^-$ were mixed in test tubes to a final volume of 100 μl and the reaction mixture was then transferred to a capillary tube for EPR detection at room temperature. For the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$), reactants containing 80 mM DMPO, 0–15 μM myricitrin, 250 μM ferrous sulfate and 250 μM H_2O_2 were mixed in test tubes to a final volume of 100 μl and the reaction mixture was then transferred to a capillary tube for EPR detection at room temperature. The EPR spectrometer settings were: modulation frequency, 100 kHz; X band microwave frequency, 9.5 GHz; microwave power, 15 mW; modulation amplitude, 6.0 G; time constant, 160 s; scan time, 200 s; and receiver gain, 4×10^5. Spectral simulations were performed on the EPR data by matching directly with the spectra as described previously (Pieper, Felix, Kalyanaraman, Turk, & Roza, 1995).

2.4. Cell culture

Primary cultures of cortical astrocytes, derived from 5-day-old Sprague–Dawley rats, were prepared as previously described (Chen, Su, Huang, Feng, & Nie, 2012; Choi & Kim, 1998). Primary astrocytes were cultured in Eagle's minimal essential medium supplemented with 10% foetal bovine serum with an atmosphere of 95% air/5% CO_2.

2.5. Comet (single-cell gel electrophoresis) assay

Astrocytes were incubated with various concentrations of myricitrin (0–15 μM) in combination with peroxynitrite (500 μM) for 12 h. Cells were harvested by trypsinisation and centrifuged at 1000 rpm. The supernatants fractions were removed and the pelleted cells were used for single-cell gel electrophoresis as previously described, with some modifications (Singh, McCoy, Tice, & Schneider, 1988). Briefly, Cells (1×10^5/ml) were combined with molten low melting agarose at 37 °C at a ratio of 1:10 (v/v), and 80 μl of the mixture were added immediately onto Comet-Slides. After treatment with lysis buffer (1% Triton X-100, 1% sodium lauryl sarcosinate, 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, pH 10), the slides were transferred to a horizontal electrophoresis unit containing buffer (1 mM EDTA and 0.3 M NaOH, pH 12.4), and electrophoresis was performed at 300 mA for 30 min. After electrophoresis, the slides were stained with ethidium bromide and determined using a fluorescence microscope (Nikon EFD-3, excitation filter BP 543/10 nm, emission barrier filter 590 nm). Comet analysis system (Komet 3.1, Kinetic Imaging Ltd, Nottingham,