JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 1729–1732

Received May 12, 2007
Accepted August 13, 2007
DOI 10.1211/jpp.59.12.0016
ISSN 0022-3573

Protective effect of *icariin* on DNA against radical-induced oxidative damage

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Abstract

Icariin (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone) is a flavonoid with a rhamnose as ligand. It is the major component in *Herba epimedii*, widely used for the treatment of atherosclerosis and neuropathy in Chinese traditional medicine, and its antioxidative property has attracted much scientific interest. The major objective of this work is to determine the antioxidative effect of *icariin* against oxidative DNA damage induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The oxidative damage of DNA was followed by measuring the formation of carbonyl compounds that can react with thiobarbituric acid (TBA) to form thiobarbituric acid reactive substance (TBARS). We found that *icariin* protects DNA against AAPH-induced oxidative damage in a concentration-dependent manner, although it does not affect the rate of AAPH-induced DNA damage. This result indicates that *icariin* is a concentration-dependent chemopreventor in protecting DNA against radical-induced damage.

Introduction

The relationship between health and reactive oxygen and nitrogen species (Kirkwood & Austad 2000), and the supplementation of antioxidants to maintain health (Finkel & Holbrook 2000; Peake & Suzuki 2004) has aroused much scientific interest. The discovery and validation of antioxidants from natural (Galvez et al 2005; Maldonado et al 2005) or synthesized sources (Gaboriau et al 2005) are of major interest in pharmacology research. Explanation of the antioxidative mechanism (Giannakopoulos et al 2005; Valavanidis et al 2005) and the development of new methods to evaluate antioxidant capacity (Antolovich et al 2002) are attractive topics in chemistry and other related fields. Antioxidants extracted from medicinal herbs are superior to synthesized ones, because the complex structures of natural antioxidants are difficult to synthesize (Liao et al 2005).

Herba Epimedii has been extensively used in traditional Chinese medicine for the treatment of atherosclerosis and neuropathy. *Icariin* (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone) (Figure 1) is the major component of *Herba Epimedii*, and has antioxidative properties. The antioxidant properties of *icariin* have been evaluated in 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH, R-N=N-R)-induced haemolysis of human erythrocytes (Liu et al 2004), and in peroxidation of linoleic acid dissolved in micelles (Liu 2006). The major objective of this work was to determine the protective effect of *icariin* against AAPH-induced oxidative DNA damage. The process of DNA damage was followed by measuring carbonyl compounds that can react with thiobarbituric acid (TBA) to form thiobarbituric acid reactive substance (TBARS).

Materials and Methods

Materials

AAPH and DNA sodium salt were purchased from AROS (via J & K Co. Ltd, Beijing, China), and *icariin* was obtained from the Institute of Pharmaceutical and Biological Reagents, Beijing, China. AAPH and DNA sodium salt were dissolved in phosphate-buffered solution (PBS; composition 8.1 mM Na_2HPO_4; 1.9 mM NaH_2PO_4; 0.2 mM EDTA).

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Acknowledgement: We would like to thank the National Natural Science Foundation, China, for financial support (20572033).

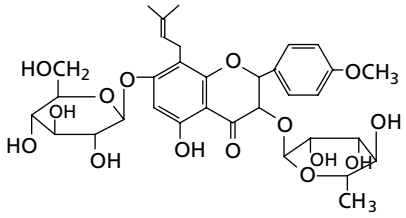

Figure 1 Structure of *icariin* (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone).

The AAPH-induced oxidative damage of DNA is measured in aqueous solution, but *icariin* cannot be dissolved in PBS; thus, it was dissolved in 0.1 M SDS/PBS for addition to the aqueous phase in the experiment (Liu 2006).

Measurement of oxidative DNA damage

AAPH-induced oxidation of DNA was measured using the method described by Aeschbach et al (1994) with minor modifications. In brief, *icariin* and AAPH were added to DNA to form a homogenous solution, in which the final concentrations of DNA and AAPH were 2.0 mg mL^{-1} and 40 mM, respectively. Although different concentrations of *icariin* were used in this experiment, each experiment contained the same concentration of SDS in order to eliminate any influence of SDS. Aliquots (2 mL) of the above solution were transferred into test tubes, which were incubated in a water bath at 37°C to initiate the reaction. Three tubes were taken out at appropriate intervals and cooled immediately, to which was then added 1.0 mL 1.0% TBA (dissolved in 100 mM NaOH as the stock solution) and 1.0 mL 3.0% aqueous trichloroacetic acid. The tubes were heated in a boiling water bath for 15 min. After cooling, 2.0 mL *n*-butanol was added and the tubes were shaken vigorously to extract pink oxidative products, for determination at 535 nm.

Statistical analysis

Since each experiment was repeated three times, non-parametric statistical methods (Kruskal–Wallis test and Mann–Whitney U test) were used to analyse the data obtained, using SPSS software (version 10.0, Chicago, IL, USA). A P value below 0.05 was taken as significant.

Results and Discussion

AAPH-induced oxidative damage of DNA is usually tested in-vitro because the radicals derived from the decomposition of AAPH are able to convert supercoiled DNA strands into open circular and then linear forms. This process can be observed qualitatively by electrophoresis (Zheng et al 2006). The linear form of DNA can be further oxidized by radicals to generate more than 20 carbonyl species, which are small molecules and cannot be observed by electrophoresis. However, the carbonyl species can be detected by spectrometry after reaction with thiobarbituric acid (TBA) to form thiobarbituric acid reactive substance (TBARS) (Aeschbach et al 1994).

Figure 2 shows that the absorbance at 535 nm increased with the reaction period. In particular (as shown as the inset in Figure 2), the absorbance correlates quantitatively with the reaction period, which can be expressed as $A_{535} = (\text{slope} \times t) + \text{intercept}$, where A_{535} is the absorbance at 535 nm and t is the reaction period. Thus, this method can be used to detect whether *icariin* can protect DNA against AAPH-induced oxidative damage.

Figure 3 shows that TBARS still increases with the reaction period in the presence of various concentrations of *icariin*. Linear regression was used to analyse the points, and the trend lines are shown in Figure 3. Statistical analysis showed that the correlation coefficients between A_{535} and t are 1.000.

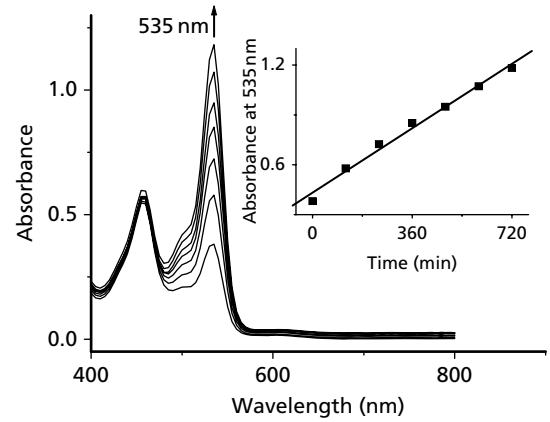

Figure 2 Ultraviolet spectra of TBARS related to the oxidative products from the AAPH-induced oxidative damage of DNA. The inset shows the linear increase in absorbance at 535 nm with the reaction period. Each experiment was repeated three times.

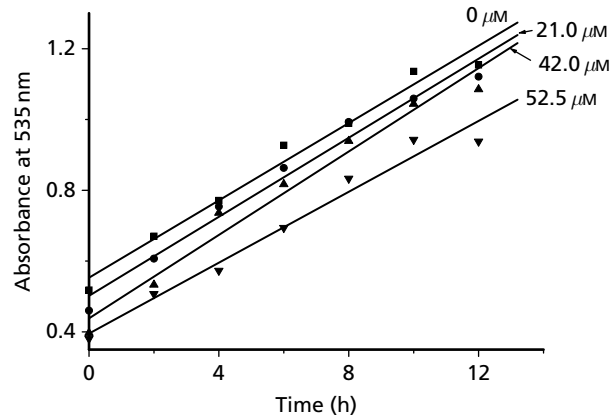

Figure 3 The linear relationship between the absorbance at 535 nm (A_{535}) and the reaction period (t) when the concentration of *icariin* is, from top to bottom, 0, 21.0, 42.0 and 52.5 μM. Each experiment was repeated 3 times; error bars have been omitted for clarity. Statistical analysis showed the correlation coefficients of these trend lines to be 1.000.

The equations for the linear relationships in the presence of various concentrations of *icariin* are listed in Table 1. The slopes have similar values to that in the absence of *icariin*, but the intercept values change remarkably. The slope indicates the increase in TBARS with reaction period and thus gives the rate of the formation of TBARS, that is, the rate of AAPH-induced oxidative damage of DNA. Similar values in the presence or absence of *icariin* show that the rates of AAPH-induced oxidative damage of DNA do not vary in the presence of *icariin*.

The intercept value is the extent of oxidation of DNA. The intercept values decrease with increasing concentrations of *icariin*. The low intercept value implies that the amount of TBARS decreases at high concentrations of *icariin*. The following equation can be used to express the quantitative relationship between the concentration of *icariin* (C_{icariin}) and the intercept value ($A_{535(\text{intercept})}$): $A_{535(\text{intercept})} = -0.00295\ C_{\text{icariin}} + 0.558$ (correlation coefficient = 0.9939).

Table 1 The equations for absorbance at 535 nm (A_{535}) as a function of reaction period (t) in the presence of various concentrations of *icariin*

Icariin concn (μM)	Equation of A_{535} reaction period (t)
0	$A_{535} = 0.0546\ (\pm 0.0037)\ t + 0.553\ (\pm 0.027)$
21.0	$A_{535} = 0.0558\ (\pm 0.0038)\ t + 0.502\ (\pm 0.027)$
42.0	$A_{535} = 0.0587\ (\pm 0.0045)\ t + 0.441\ (\pm 0.033)$
52.5	$A_{535} = 0.0501\ (\pm 0.0037)\ t + 0.395\ (\pm 0.027)$

This high correlation coefficient implies that *icariin* is a concentration-dependent antioxidant, protecting against AAPH-induced oxidative damage of DNA.

We have reported the antioxidative role of *icariin* in protecting human erythrocytes against AAPH-induced haemolysis (Liu et al 2004), and the mechanism by which *icariin* scavenges radicals by means of chemical kinetics (Liu 2006). The antioxidative activity of *icariin* is due to the stabilization function derived from intramolecule hydrogen bonds when the hydrogen atom in OH of *icariin* is abstracted by a radical (Liu 2006). This mechanism may also be available for the experimental system of AAPH-induced oxidative damage of DNA and is shown in Figure 4. The radicals derived from the decomposition of AAPH attack DNA directly in the absence of *icariin*. However, OH in *icariin* can scavenge the initiation radical derived from AAPH, to form an *icariin* radical (**I**). The radical (**I**) transfers its single electron to the allyl position to generate the radical (**II**). The same transfer takes place in radical (**II**) to form radical (**III**). Finally, the single electron is dispersed to form a stable resonance structure (**IV**). The fact that *icariin* decreases the extent of oxidative damage rather than influencing the oxidative rate implicates that it protects DNA against AAPH-induced oxidative damage by exhausting the initiation radical.

Conclusion

The present work together with our previous reports demonstrate that *icariin* is a concentration-dependent antioxidant

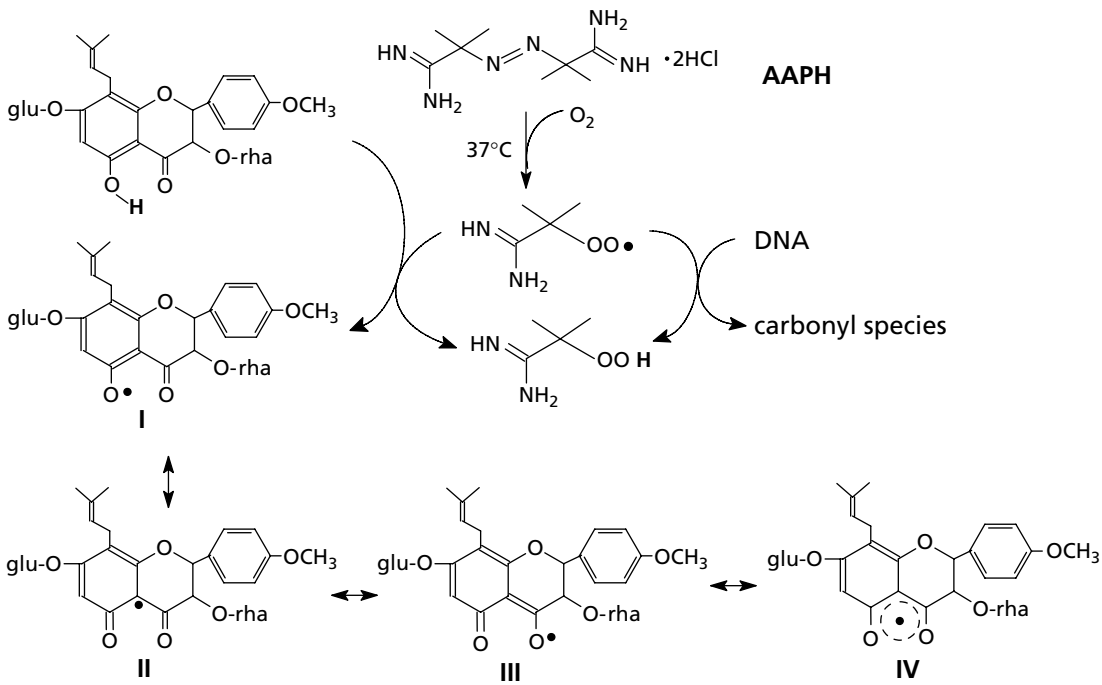

Figure 4 A possible mechanism of the antioxidative effect of *icariin* on 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative damage of DNA. glu = glucose; rha = rhamnose.

that is able to protect DNA and human erythrocytes against AAPH-induced oxidation. When *icariin* protects DNA against AAPH-induced oxidative damage of DNA, it scavenges the initiation radical derived from AAPH, and generates an intramolecule hydrogen bond to stabilize the *icariin* radical. This information may be helpful in the clinical usage of *icariin*.

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