Antioxidative Activity of Natural Isorhapontigenin

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ABSTRACT—Isorhapontigenin (ISOR), isolated from *Belamcanda chinensis*, is a derivative of stilbene. Its chemical structure is very similar to that of resveratrol, with a potent antioxidative effect. In the present study, we investigated the antioxidative activity of ISOR in vitro. Oxidative damage of rat liver microsomes, brain mitochondria and synaptosomes was induced by Fe²⁺-Cys, VitC-ADP-Fe²⁺ and H₂O₂, respectively. The formation of malondialdehyde (MDA), decrease of reduced glutathione (GSH) and increase of ultra-weak chemiluminescence during the lipid peroxidation process were determined. In addition, the characteristic ultra-weak chemiluminescence of oxidative DNA damage induced by CuSO₄-Phen-VitC-H₂O₂ system was studied. The results showed that ISOR significantly inhibited MDA formation in liver microsomes, brain mitochondria and synaptosomes induced by Fe²⁺-Cys. Also, ISOR markedly prevented the decrease of GSH in mitochondria and synaptosomes induced by H₂O₂ and the increase of ultra-weak chemiluminescence during lipid peroxidation induced by VitC-ADP-Fe²⁺ as well as oxidative DNA damage induced by CuSO₄-Phen-VitC-H₂O₂. The effects of ISOR at 10⁻⁵ and 10⁻⁶ mol/L on the MDA formation and decrease of GSH were similar to that of the classical antioxidant vitamin E (10⁻⁴ mol/L). It may be concluded that ISOR possessed potent antioxidative activity and was much more potent than vitamin E.

Keywords: Isorhapontigenin, Antioxidant, Chemiluminescence, Oxidative DNA damage

Resveratrol (3,4,5-trihydroxystilbene), one of stilbene derivatives, is a naturally occurring phytoalexin produced by some spermatophytes such as grapevines in response to injury and fungal attack. Red wine contains abundant resveratrol, even up to 15 mg/L, while white wine contains a very small amount of resveratrol because it is present in grape berry skins but not in the flesh (1). Resveratrol possesses multiple bioactivities such as anticancer activity and anti-inflammatory activity (1) and has protective effects against some diseases related to oxidative stress such as cardiovascular diseases, Parkinson's disease and Alzheimer's disease (AD) (2, 3). The antioxidant activity of resveratrol is closely related to the phenolic hydroxyl groups in its structure. Isorhapontigenin (ISOR, Fig. 1) isolated from *Belamcanda chinensis* is also a derivative of stilbene, and its chemical structure is very similar to that of resveratrol (4). It is quite possible that ISOR also has antioxidative activity. The present study was designed to investigate the antioxidant effect of ISOR.

MATERIALS AND METHODS

ISOR was prepared by M. Lin (5); its purity was over 98% and dissolved in ethanol before use. Tris, cysteine (Cys), ADP, vitamin E (Vit E), vitamin C (Vit C), *o*-phthalaldehyde (OPT) and reduced glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DNA, trichloroacetic acid (TCA), thiobarbituric acid (TBA), 1,10-phenanthroline (Phen) and other chemicals were obtained from Beijing Chemical Factory (Beijing, China) and Shanghai Biochemical Factory (Shanghai, China). Both sexes of Wistar rats (200–300 g) were purchased from the Experiment Animal Center of Chinese Academy of Medical Sciences.

Effect of ISOR on lipid peroxidation (LPO) of rat liver microsomes

Preparation of rat liver microsomes (6): Rats were sacrificed by decapitation and 20% liver homogenate was made with ice-cold TMS buffer (0.05 mol/L Tris-HCl, 3 mmol/L MgCl₂ and 0.2 mol/L sucrose, pH 7.4). The suspension was centrifuged at 10,000 × *g* for 15 min at 4°C, and then the obtained supernatant was centrifuged at

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Fig. 1. Chemical structures of isorhapontigenin and resveratrol.

105,000 $\times g$ for 1 h. The pellet was resuspended in ice-cold TMS buffer. Microsomal protein was determined by the Lowry method (7). The microsomal suspensions were stored in small portions at -70°C .

Determination of malondialdehyde (MDA) formation in liver microsomes induced by Fe^{2+} -Cys (8): The microsomal protein (1 mg) was incubated with ISOR (10^{-4} , 10^{-5} and 10^{-6} mol/L), Vit E (10^{-4} mol/L) or vehicle in 1 ml 0.1 mol/L PBS (pH 7.4) at 37°C for 15 min, and then 50 $\mu\text{mol/L}$ FeSO_4 and 200 $\mu\text{mol/L}$ Cys were added; 20 min later, 1 ml of 20% TCA was added and then the suspension was centrifuged at 200 $\times g$ for 10 min. The supernatant (1 ml) was mixed with 1 ml 0.67% TBA and reacted in a boiling water bath for 10 min. The absorbance was determined at 532 nm on a semi-automatic biochemical reader (Citizen, Tokyo).

Measurement of the ultra-weak chemiluminescence during LPO in liver microsomes induced by VitC-ADP- Fe^{2+} (9): The microsomal protein (2 mg) was incubated with ISOR (10^{-4} , 10^{-5} and 10^{-6} mol/L), Vit E (10^{-4} mol/L) or vehicle in 1 ml 0.1 mol/L PBS (pH 7.4) at 37°C for 15 min and then mixed with Vit C (1 mmol/L) - ADP (4 mmol/L) - FeSO_4 (0.2 mmol/L); the intensity of ultra-weak chemiluminescence was determined immediately by a Ultra-Weak Chemiluminescence Analyzer (BPCL, Beijing, China).

Effect of ISOR on oxidative damage of rat cerebral cortex mitochondria and synaptosomes

Preparation of mitochondria and synaptosomes (10): Rats were sacrificed by decapitation and 10% cerebral cortex homogenate was made in ice-cold 0.32 mol/L sucrose solution. The suspension was centrifuged at 4°C , 120 $\times g$ for 10 min, and then the supernatant was centrifuged at 20,000 $\times g$ for 10 min. The precipitate was resuspended in 0.32 mol/L sucrose solution. The suspension was centrifuged by sucrose gradient centrifugation (0.8 and 1.2 mol/L sucrose solution) at 150,000 $\times g$ for 20 min. The mitochondria and synaptosomes were collected from the bottom and the interface between two layers, respectively. The mitochondria and synaptosomes were resuspended in

0.32 mol/L sucrose solution and washed twice at 4°C , 20,000 $\times g$. The mitochondrial and synaptosomal protein was determined by the Lowry method (7). The prepared mitochondria and synaptosomes suspensions in small portions were stored at -70°C .

Determination of MDA formation in mitochondria and synaptosomes induced by Fe^{2+} -Cys (11): The mitochondrial and synaptosomal protein (0.1 mg) were incubated with ISOR (10^{-5} , 10^{-6} and 10^{-7} mol/L), Vit E (10^{-4} mol/L) or vehicle in 1 ml 0.1 mol/L PBS (pH 7.4) at 37°C for 15 min, respectively. The following procedure for MDA determination was the same as in LPO of liver microsomes induced by Fe^{2+} -Cys.

Determination of the decrease of GSH in mitochondria and synaptosomes induced by H_2O_2 (12): The mitochondrial and synaptosomal protein (0.2 mg) were incubated with ISOR (10^{-5} , 10^{-6} and 10^{-7} mol/L), Vit E (10^{-4} mol/L) or vehicle in 0.5 ml 0.1 mol/L PBS - 0.5 mmol/L EDTA buffer (pH 7.4) at 37°C for 10 min; then 0.2 mmol/L H_2O_2 was added into the reaction system; and 5 min later, 50 μl 0.5% Triton X-100 and 50 μl 20% metaphosphoric acid were added. After oscillation, the incubation mixture was centrifuged at 240 $\times g$ for 10 min. The supernatant (0.2 ml) was mixed with 1.7 ml PBS-EDTA buffer and 0.1 ml OPT (1 mg/ml) for 15 min. The fluorescence intensity of GSH was determined at emission wavelength 420 nm and excitation wavelength 350 nm on a MPF-4 Fluorescence Spectrophotometer (Hitachi, Tokyo). The GSH standard curve was made by the above-described method.

Effect of ISOR on the ultra-weak chemiluminescence of oxidative DNA damage induced by CuSO_4 -Phen-VitC- H_2O_2 system (13)

DNA (1 μg) was mixed with the CuSO_4 -Phen-VitC- H_2O_2 system (5×10^{-5} mol/L CuSO_4 , 3.5×10^{-4} mol/L Phen, 3.5×10^{-4} mol/L Vit C, H_2O_2 0.5%) and ISOR (10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} mol/L) or vehicle in 1 ml 0.1 mol/L sodium acetate-acetic acid (pH 5.5) buffer. The ultra-weak chemiluminescence was determined immediately at room temperature.

Data analyses and statistics

Results were expressed as the mean $\pm$ S.D. Statistical significance was assessed by Dunnett's test. *P* values less than 0.05 were considered to be significant statistically.

RESULTS

Effect of ISOR on MDA formation in LPO of rat liver microsomes induced by Fe^{2+} -Cys

As shown in Fig. 2, LPO of microsomes was successfully induced by the Fe^{2+} -Cys system as the formation of MDA increased significantly. In comparison with the control, ISOR at 10^{-4} and 10^{-5} mol/L significantly inhibited the formation of MDA ($P < 0.01$) by 94.7% and 97.6%, respectively. The potency of ISOR was much more potent than that of Vit E at the same concentration ($P < 0.01$).

Effect of ISOR on the intensity of chemiluminescence of LPO of rat liver microsomes induced by VitC-ADP- Fe^{2+}

Lipid proxyl radicals ($\text{ROO}^{\cdot}$) are produced during the LPO of microsomal membrane induced by the VitC-ADP- Fe^{2+} system, which can be detected by the ultra-weak chemiluminescence technique. As shown in Fig. 3, the intensity of ultra-weak chemiluminescence was notably increased during LPO of microsomes induced by the VitC-ADP- Fe^{2+} system. ISOR (10^{-4} , 10^{-5} and 10^{-6} mol/L) significantly decreased the chemiluminescence intensity as compared with the control, indicating that ISOR also inhibited the generation of $\text{ROO}^{\cdot}$ during microsomal LPO induced by the VitC-ADP- Fe^{2+} system.

Fig. 2. Effect of isorhapontigenin on the MDA formation during liver microsomal lipid peroxidation induced by Fe^{2+} -Cys ($n = 3$). A, Untreated microsomes; B, microsomes treated with Fe^{2+} -Cys only; C, D, E, microsomes treated with Fe^{2+} -Cys and isorhapontigenin at 10^{-6} , 10^{-5} and 10^{-4} mol/L, respectively; F, microsomes treated with Fe^{2+} -Cys and Vit E at 10^{-4} mol/L. * $P < 0.05$, ** $P < 0.01$ vs Fe^{2+} -Cys control (B). Each bar is the mean $\pm$ S.D. of 4 determinations.

Fig. 3. Effect of isorhapontigenin on the increase of ultra-weak chemiluminescence during liver microsomal lipid peroxidation induced by VitC-ADP- Fe^{2+} system. A, Microsomes treated with VitC-ADP- Fe^{2+} system only; B, microsomes treated with VitC-ADP- Fe^{2+} system and Vit E at 10^{-4} mol/L; C, D, E, microsomes treated with VitC-ADP- Fe^{2+} system and isorhapontigenin at 10^{-6} , 10^{-5} and 10^{-4} mol/L, respectively; F, untreated microsomes.

Effect of ISOR on MDA formation during LPO of rat brain mitochondria and synaptosomes induced by Fe^{2+} -Cys

As indicated in Table 1, ISOR at 10^{-5} and 10^{-6} mol/L significantly inhibited MDA formation during LPO of mitochondria and synaptosomes induced by the Fe^{2+} -Cys system. ISOR at a concentration of 10^{-7} mol/L still displayed significant inhibitory effect on MDA formation of synaptosomes ($P < 0.05$), but had no effect on that of mitochondria. The inhibitory effect of Vit E at 10^{-4} mol/L on MDA formation in mitochondria and synaptosomes was less potent than that of ISOR at 10^{-6} mol/L. In other words, ISOR was almost 100 times more potent than Vit E in the inhibition of MDA formation.

Table 1. Effect of ISOR on MDA formation of rat brain cortex mitochondria and synaptosomes induced by Fe^{2+} -Cys system

Group	MDA content (nmol $\cdot$ L ⁻¹ /mg protein)	
	mitochondria	synaptosomes
Normal	8.38 $\pm$ 7.74**	9.34 $\pm$ 7.74**
Fe^{2+} -Cys	24.63 $\pm$ 11.88	33.23 $\pm$ 11.57
ISOR 10^{-5} mol/L	9.97 $\pm$ 7.10**	12.20 $\pm$ 7.42**
10^{-6} mol/L	15.39 $\pm$ 7.42*	18.58 $\pm$ 9.02*
10^{-7} mol/L	24.63 $\pm$ 10.93	21.44 $\pm$ 7.11*
Vit E 10^{-4} mol/L	12.52 $\pm$ 6.79*	16.03 $\pm$ 9.65**

Data are expressed as the mean $\pm$ S.D. of 4 determinations. Compared with the control group using Dunnett's test, * $P < 0.05$, ** $P < 0.01$.

Effect of ISOR on the decrease of GSH in rat cerebral cortex mitochondria and synaptosomes induced by H₂O₂

As shown in Table 2, ISOR at 10⁻⁵ and 10⁻⁶ mol/L significantly prevented the decrease of GSH in mitochondria induced by 0.2 mmol/L H₂O₂ ($P < 0.01$ and $P < 0.05$, respectively), while ISOR at 10⁻⁵, 10⁻⁶ and 10⁻⁷ mol/L all markedly prevented GSH decrease in synaptosomes ($P < 0.01$). The preventive effect of Vit E at 10⁻⁴ mol/L on the decrease of mitochondrial and synaptosomal GSH corresponded to that of ISOR at 10⁻⁵ mol/L.

Protective effect of ISOR on the oxidative DNA damage induced by CuSO₄-Phen-VitC-H₂O₂

Free guanines produced during breakage of the DNA doublestrand induced by oxidative damage possess characteristic ultra-weak chemiluminescence that reflects the extent of DNA damage indirectly. As shown in Fig. 4, the characteristic ultra-weak chemiluminescence of guanines markedly increased during oxidative DNA damage induced by CuSO₄-Phen-VitC-H₂O₂ system. ISOR (10⁻⁷, 10⁻⁶, 10⁻⁵ and 10⁻⁴ mol/L) markedly reduced the chemiluminescence in a dose-dependent manner, particularly ISOR at 10⁻⁵ and 10⁻⁴ mol/L almost completely decreased the production of chemiluminescence.

DISCUSSION

Various reactive oxygen species (ROS) are generated from enzymatic or nonenzymatic processes during the normal metabolism in cells. If excessive ROS are not eliminated promptly, the accumulation of ROS can produce cytotoxic effects (14). Lipid peroxidation of polyunsaturated fatty acids (PUFA) in the biomembranes are prone to be initiated by ROS, which leads to impairment of biomembrane structure and functions. In the present study, Fe²⁺-Cys and VitC-ADP-Fe²⁺ systems were used to generate ROS for initiating LPO of liver microsomes, brain mitochondria and synaptosomes. In the early stage of the LPO process, the

Fig. 4. Effect of isorhapontigenin on the increase of ultra-weak chemiluminescence during oxidative DNA damage induced by CuSO₄-Phen-VitC-H₂O₂ system. A, Control, DNA treated with CuSO₄-Phen-VitC-H₂O₂ system; C, DNA treated with CuSO₄-Phen-VitC-H₂O₂ system and vit E at 10⁻⁴ mol/L; B, D, E and F, DNA treated with CuSO₄-Phen-VitC-H₂O₂ system and isorhapontigenin at 10⁻⁷, 10⁻⁶, 10⁻⁵ and 10⁻⁴ mol/L, respectively.

secondary ROO[•] of PUFA is highly susceptible to peroxidative breakdown (9) and may internally cyclize to form a five-member endoperoxide, which is assumed to decompose to low molecular weight ketones and aldehydes, predominantly MDA. Mitochondria and synaptosomes are important organelles; particularly, mitochondria are the energy plant of cells and are an abundant source of ROS, allowing the occurrence of plentiful biochemical reactions. Consequently, the double-membrane layers of mitochondria are the susceptible target of ROS attack. Our results showed that ISOR even at 10⁻⁶ mol/L could significantly inhibit MDA formation during biomembrane LPO induced by Fe²⁺-Cys, and the anti-MDA formation of ISOR is about 100 times more potent than that of the classical antioxidant Vit E at the same concentration. It appears that ISOR could protect cell organelles against oxidative damage at the early stage of the LPO process.

All secondary ROO[•] of LPO can act as potential precursors of photoemissive species (9). At a later stage of LPO, collision of two ROO[•] is assumed to generate two different electronically excited species, singlet molecular oxygen (¹O₂) and a compound in the triplet state (³R = O). Photons are generated during conversion of ³R = O into R = O, termed as ultra-weak chemiluminescence, which can be regarded as one branch of LPO occurring later than MDA formation. In the present study, the intensity of ultra-weak chemiluminescence significantly increased during microsomal LPO induced by the VitC-ADP-Fe²⁺ system. ISOR at 10⁻⁵ and 10⁻⁴ mol/L almost inhibited this reaction completely, and even ISOR at the concentration of 10⁻⁶ mol

Table 2. Effect of ISOR on decrease of glutathione (GSH) in rat brain mitochondria and synaptosomes induced by H₂O₂

Group	GSH content (μg/mg protein)	
	mitochondria	synaptosomes
Normal	2.51 ± 0.05**	4.14 ± 0.14**
Control	1.86 ± 0.03	2.54 ± 0.08
ISOR 10 ⁻⁵ mol/L	2.21 ± 0.07**	3.34 ± 0.21**
10 ⁻⁶ mol/L	1.94 ± 0.04*	2.97 ± 0.26**
10 ⁻⁷ mol/L	1.99 ± 0.17	2.79 ± 0.08**
Vit E 10 ⁻⁴ mol/L	2.21 ± 0.04**	3.65 ± 0.47**

Data are expressed as the mean ± S.D. of 4 determinations. Compared with the control group using Dunnett's test, * $P < 0.05$, ** $P < 0.01$.

/L was still effective, suggesting that ISOR could prevent microsomes from oxidative damage at the later stage of LPO.

GSH plays an important role in eliminating ROS in cells. Besides oxidative damage during the LPO chain reaction, ROS can cause lesions in biomacromolecules such as proteins through other pathways (15):

Although removal of H_2O_2 by GSH and GSH peroxidase extinguishes a direct oxidative threat to cells, formation of GSSG can bring deleterious consequences. GSSG reacts spontaneously with thiol groups in proteins (Protein-SH) to form protein mixed disulfides (Protein-SSG). When the affected protein thiols are essential for biologic activity, their functions will be suppressed, such as in the cases of succinate dehydrogenase, NADH dehydrogenase, ATPase, isocitrate dehydrogenase and succinate-supported mitochondrial electron transport, enzymes that are all essential for energy metabolism (16, 17). Through the LPO and GSSG pathways, ROS induces essential enzymes suppression and leakage, and eventually, energy metabolism is arrested and cellular viability is decreased. Our results showed that ISOR not only significantly protected rat brain mitochondria and synaptosomes against LPO damage, but also reduced the consumption of endogenous antioxidant GSH, possibly as a result, the damage to thiol proteins induced by GSSG accumulation may be alleviated.

Oxidative damage to DNA induced by ROS can occur through many routes including the oxidative modification of the nucleotidebases and sugars or by forming cross-links. Such modifications may lead to mutation, carcinogenesis, cellular aging and death (18). A recent study (19) suggested that the brain in AD and aged people may be subject to the insult of oxidative stress, and an increase in levels of brain DNA oxidation exists in both aging and AD. The level of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) has been regarded as the predominant marker of oxidative DNA damage, and significant elevations of free 8-OH-dG were observed in cerebrospinal fluid of AD subjects as compared with age-matched control subjects. Various antioxidants could markedly reduce the level of 8-OH-dG during oxidative DNA damage induced by ROS (20). As shown in the present study, hydroxyl free radicals generated in the CuSO_4 -Phen-VitC- H_2O_2 system caused oxidative DNA damage expressed in the remarkable increase of characteristic ultra-weak chemiluminescence of free guanines. ISOR significantly decreased the intensity of luminescence during oxidative DNA damage induced by the system, suggesting that ISOR could prevent oxidative DNA

damage induced by ROS. By using the electron spin resonance (ESR) technique, Fang found that ISOR has the activity to scavenge O_2^- and $\cdot\text{OH}$ in activated neutrophils (studies performed as part of the dissertation of YN Fang: Effect of isorhapontigenin and several related compounds on neutrophil's function and the oxidation of low density lipoprotein, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing (1997)). All the above results suggest that ISOR has protective action against ROS oxidative damage at different levels.

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