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Research article

In vitro study of hesperetin Schiff bases antioxidant activity on rat liver mitochondria

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Abstract: *Three hesperetin Schiff bases: hesperetin thiosemicarbazone (HTSC), hesperetin isoniazone (HIN) and hesperetin benzhydrazone (HHSB) have been synthesized and characterized by using analytical and spectral techniques. The influence of substituents on hesperetin antioxidant activity has been studied in vitro using mitochondrial assays. The studied compounds have been found to exhibit both antioxidant and pro-oxidant activity.*

Keywords: *hesperetin, Schiff bases, isolated mitochondria, antioxidant activity, pro-oxidant activity.*

Introduction

Mitochondria play a key role in the oxidative processes in a cell due to their function in cellular respiration that make them simultaneously a source and the final target of reactive oxygen species. Mitochondrial function is particularly susceptible to oxidative stress, leading to decreased mitochondrial ATP synthesis, induction of mitochondrial permeability transition, all of which predispose cells to necrosis or apoptosis [1, 2]. However, a risk of toxic liver damage has markedly increased in recent years due to the exposure to environmental toxins, pesticides and chemotherapeutics. Many compounds, including useful drugs, can cause liver cell damage through their metabolic conversion to highly reactive substances and the generation of free radicals. It was suggested that reactive metabolite formation, glutathione depletion, mitochondrial membrane pore formation, intramembranous protein release, and the diminished capacity to ATP synthesis, are critical events in hepatotoxicity [3]. Reduced oxygen consumption and mitochondrial enzyme activities as well as a diminished rate of ATP production were observed under intoxication [4]. Therefore, in recent years, there is an interest in determining whether antioxidants will reduce mitochondria oxidative damage and prevent the

appearance of oxidative stress. Modeling of the influence oxidative stress in vitro is a useful method to study the mechanisms of oxidative cell injury, and to evaluate the sensitivity of cells and cellular components to oxidative metabolic stress [5, 6].

Phenolic compounds are known for their antioxidant properties. They act as free radical scavengers and their antioxidant potential depends on the substituent present and the extent of structure conjugation [7, 8]. An example of the phenolic compounds is plant flavonoids which are important part of the diet because of their effects on human nutrition. Generally, flavonoids can demonstrate both antioxidant and prooxidant activity because it is related with their structure, in particular, the number of hydroxyl substitutions in their backbone structure [9, 10]. They can directly scavenge naturally occurring radicals such as $O2^{\bullet-}$, $HO^{\bullet}$ and artificial systems such as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ by single electron transfer.

Hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) is one such member of the flavonoids. It occurs in plants, fruits, flowers and foods of plant origin [11]. Hesperetin demonstrates multiple biological and pharmacological activities, including antioxidant properties [12, 13]. Flavonoids, including hesperetin, possess a carbonyl group in their chemical structure and can be condensed with amino groups to form corresponding Schiff bases [14, 15]. Hydrazines and thiosemicarbazides are frequently used to synthesize Schiff bases [16, 17]. In the present study, three hesperetin Schiff bases (HTSC, HIN and HHSB) and hesperetin alone were used (Figure 1). Their synthesis and influence on free radical scavenging activity were demonstrated in [18]. The mentioned work revealed that the conjugation of hesperetin with the hydrazides: thiosemicarbazide (HTSC), isoniazid (HIN), benzhydrazide (HHSB) was found to affect its antioxidant activity.

The goal of this study is to identify three hesperetin Schiff bases and hesperetin alone as compounds with the potential to reduce mitochondrial oxidative damage and prevent the appearance of oxidative stress.

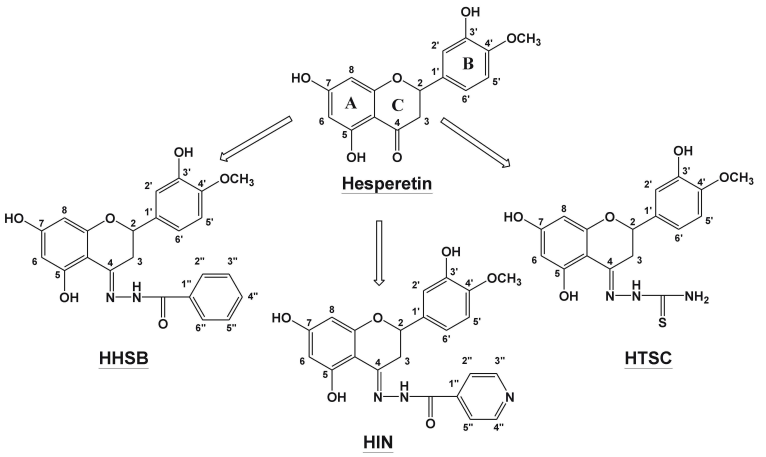

Figure 1. Studied compounds [18]

Experimental

Materials

Hesperetin, benzhydrazide, thiosemicarbazide, isoniazid, *tert*-Butyl hydroperoxide (*t*BHP), 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), calcium chloride dehydrate, thiobarbituric acid (TBA), trichloroacetic acid (TCA) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Physical measurements

Elemental analysis (C, H, N) was carried out on an EuroVector 3018 analyzer. The melting point of the ligands was determined on a Electrothermal 9200 microscopic melting point apparatus. The IR spectra were obtained on Nicolet 6700 (Thermo-Scientific) FT-IR spectrometer, in the 4500-500 cm-1 region. 1H NMR spectra were recorded on a Bruker AV200 200MHz spectrometer in DMSO (dimethyl sulfoxide)-d6 with TMS (tetramethylsilane) as internal standard. Mass spectra were performed on a Finnigan MAT 9 instrument.
Electronic absorption (UV-VIS) spectra were recorded with a Perkin-Elmer Lambda 11 spectrophotometer. The absorbance measurements in all biochemical assays were performed using a spectrophotometer V-530 Jasco.

Methods

Preparation of hesperetin Schiff bases

Three hesperetin Schiff bases: hesperetin thiosemicarbazone (HTSC), hesperetin isoniazone (HIN) and hesperetin benzhydrazone (HHSB) have been prepared according to reference [18]. Briefly, hesperetin Schiff bases were synthesized by the reaction of hesperetin with thiosemicarbazide/isoniazide/benzhydrazide in molar ratio 1:1 in methanol (targeted compounds are shown in Figure 1). As catalyst of the reaction, acetic acid was added. pH of the mixture was about 4. The solution was refluxed on an oil-bath for one day with stirring. After that, the reaction solution was concentrated and cooled, and a solid was separated out. The precipitate was filtered, washed with excess of water, and dried in a desiccator to give the one of the above mentioned Schiff base. The products have been characterized on the basis of elemental analysis, melting point, ^{1}H NMR, mass spectra, UV–Vis spectra and IR spectra. All data are placed in the reference [18].

Isolation of rat liver mitochondria

All experiments have been performed according to the international rules "Guide for the Care and Use of Laboratory Animals". Male Wistar rats weighing approximately 200-300 g were killed by decapitation after an overnight fast, and their livers were removed for immediate isolation of mitochondria. Mitochondria were isolated as described [19] with slight modification. Briefly, tissues were rinsed with saline, dried with filter paper, weighed and homogenized in a glass-teflon homogenizer with icecold isolation medium containing 250 mM sucrose, 20 mM Tris-HCl and 1 mM EDTA, pH 7.2 at 4°C. The homogenate was

centrifuged at 600 g for 10 min, and the supernatant was centrifuged at 8.500 g for 10 min at 4°C. The obtained pellet was washed in buffer containing 250 mM sucrose, 20 mM Tris-HCl, pH 7.2 (at 4°C). The protein concentration was about 35-40 mg/ml and determined by the Lowry method [20].

Biochemical measurements

In order to test the effect of hesperetin and synthesized HHSB, HIN and HTSC on the antioxidant activity, the initial pre-incubations of isolated rat liver mitochondria in the presence of 40 μM compounds were carried out for 20 min at 25ºC. After the preincubation to induce oxidative stress in isolated rat liver mitochondria, *tert*-Butyl hydroperoxide (*t*BHP) (1.5 mM) was added. The reaction mixture was incubated for 30 min at 25°C. The protein concentration in the mitochondria samples was 15 mg/ml. The concentration of reduced glutathione (GSH), total (TSH) and protein (PSH) thiols in mitochondria was determined spectrophotometrically by the Ellman method [21] using the molar absorption coefficient $\varepsilon_{412} = 1.36 \times 10^4\ M^{-1}cm^{-1}$. Mixed disulfides (GSSP) formed by glutathione and accessible sulfhydryl groups of mitochondrial proteins were determined by the method described by Rossi et al. [22]. The level of accumulated lipid peroxidation products (thiobarbituric acid-reactive substances, TBARS) was determined according to Stocks and Dormandy method [23]. The activity of glutathione peroxidase (mtGPx) in the mitochondrial pellet was measured by the method of Martinez et al. [24]. The activity of mitochondrial glutathione-S-transferase (mtGST) was determined employing the method of Habig et al. [25].

Results and discussion

The present biochemical measurement was designed to evaluate antioxidant activity of hesperetin Schiff bases and hesperetin alone on the oxidative effects of *tert*-Butyl hydroperoxide (*t*BHP) in rat liver mitochondria. In these studies, fisetin and its metal chelates were also used and discussion of the effect of these compounds on mitochondrial cells has been presented by Łodyga-Chruścińska *et al.* [26]. The concentrations of hesperetin and Schiff bases used in present study were within the range of flavonoid concentration used in the assessment of antioxidant activity in cell cultures by [27, 28]. In an *in vitro* study of Bawazeer et al. [27], citrus flavonoid - hesperetin regulates the LDL receptor (LDLr) gene in the human liver, HepG2 cells, in various concentrations such as 25, 50, 100, 150 and 200 μM. The concentrations used in our study did not exceed 50 μM. Flavonoid aglycones have greater bioavailability than their glycosides and can accumulate in cells under prolonged contact [29, 30]. Various modifications of the flavanoid structures can have impact on their bioavailability and activity [31].

In the present study, the application of *t*BHP led to a significant decrease (75%) in the reduced form of glutathione (GSH) and three-fold increases in the content of the mixed glutathione-protein disulfide (GSSP) and lipid peroxidation products (TBARS), which are known to be markers of the impairment of the antioxidant defense system and membranes of mitochondria (Table 1).

HIN and HTSC prevented the development of oxidative stress and protected mitochondrial membranes from oxidative damage more effectively than HHSB and hesperetin, significantly reducing levels of TBARS (Table 1).

Total (TSH) and protein (PSH) thiols were not significantly changed under *tert*-Butyl hydroperoxide action but in the presence of hesperetin Schiff bases the statistically significant TSH and PSH increase can be observed. There can be many reasons for this fact, such as the Schiff bases in contact with protein disulfides within the mitochondrial matrix could regenerate the protein thiols [32, 33]. Moreover, the growth of TSH value could be caused by recovery, for example, of cysteine from its disulfide form or GSH from GSSG [34]. Both glutathionylation and the formation of intraprotein disulfides can dramatically affect the activity of enzymes and transcription factors, enabling them to respond reversibly to the ambient GSH/GSSG ratio, just as proteins are regulated by reversible phosphorylation [32].

Tert-Butyl hydroperoxide at a concentration of 1.5 mM had no effect on the enzyme activities of glutathione transferase (mtGT) or glutathione peroxidase (mtGPx), which play a crucial role in the antioxidant defense of mitochondria. However, it should be noted that hesperetin and the Schiff bases had the effect of activating glutathione transferase (mtGT), leading to increased mtGT activity both with respect to the control group and to the *t*BHP group (Table 1). Hesperetin activity was also observed for mitochondrial glutathione peroxidase at 668.0±35.5 nmol/mg protein (Table 1). Modifications made to hesperetin had various effects on the activity of the enzyme. HHSB and HIN had no effect on glutathione peroxidase activity, while HTSC had a pronounced inhibitory effect (enzyme activity decreased by 45% compared to control). These differences in the action of hesperetin and modified hesperetin on glutathione peroxidase activity are probably due to differences in the structure of their molecules. In particular, modified HTSC with a thiosemicarbazide molecule in its structure is capable of breaking the hydrogen bonds and hydrophobic regions in glutathione peroxidase (mtGPx), leading to changes in functional activity of the enzyme.

Summarizing, the tested compounds exhibit antioxidant properties by increasing levels of TSH, PSH, mtGT, partially restoring level of GSH (in the case of HIN), and by decreasing level of TBARS (in the case of HIN and HTSC). In addition, they can modify the activity of mitochondrial glutathione peroxidase. Probably, the lowest level of mtGPx may point out the pro-oxidant activity of HTSC. However, this is one of the mechanisms. The pro-oxidant and antioxidant properties could be a very important function because such activity is responsible for the induction of apoptosis in cancer cells [34].

Table 1. Protein thiol (PSH) groups, reduced gluthatione (GSH), mixed glutathione-protein disulfides (GSSP), lipid peroxidation products (TBARS), glutathione transferase (mtGT) and glutathione peroxidase (mtGPx) levels in rat liver cell mitochondria

Parameters	Compounds [average ± SD]						
	Control		Hesp	HHSB	HIN	HTSC	Control+HIN
TSH [nmol/mg protein]	91.40±2.46	83.94±1.21	82.90±1.93	90.64±1.94*	106.00±7.12*	100.20±6.80*	102.2±4.70*
GSH [nmol/mg protein]	14.04±0.68	3.57±0.214*	3.15±0.24*	3.46±0.21*	4.20±0.12*#	3.83±0.18*	13.19±0.19
PSH [nmol/mg protein]	77.36±1.52	80.37±2.35	79.80±1.10	87.17±2.15*	101.80±5.72*	96.37±3.25*	89.01±4.71*
TBARS [nmol/mg protein]	0.028±0.003	0.084±0.004*	0.074±0.007*	0.062±0.010*	0.043±0.007*#	0.050±0.005*#	0.022±0.002
GSSP [nmol/mg protein]	0.487±0.035	1.562±0.099*	1.554±0.017*	1.478±0.083*	1.452±0.025*	1.288±0.096*	0.575±0.089
mtGT [nmol/mg protein	26.72±0.5	25.00±2.5	28.93±1.0*	31.49±2.8	31.91±0.2***#	32.29±2.0	31.74±1.8*
mtGPx [nmol/mg protein	522.3±74.7	460.5±102.4	668.0±35.5*	490.4±65.5	479.7±31.0	288.2±26.2*	748.5±176.7

*statistically significant in comparison with control, $p < 0.05$

#statistically significant in comparison with *t*BHP, $p < 0.05$.

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