Chemico-Biological Interactions 197 (2012) 103–109

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Inhibitory effects of dietary flavonoids on purified hepatic NADH-cytochrome b5 reductase: Structure–activity relationships

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ARTICLE INFO

Article history:
Received 6 March 2012
Received in revised form 4 April 2012
Accepted 7 April 2012
Available online 19 April 2012

Keywords:
Flavonoids
Cytochrome b5 reductase
Enzyme inhibition
Structure–activity
IC_{50}

ABSTRACT

The structure–activity relationships of flavonoids with regard to their inhibitory effects on NADH-cytochrome b5 reductase (E.C. 1.6.2.2), a clinically and toxicologically important enzyme, are not known. In the present study, the inhibitory effects of fourteen selected flavonoids of variable structure on the activity of purified bovine liver cytochrome b5 reductase, which shares a high degree of homology with the human counterpart, were investigated and the relationship between structure and inhibition was examined. Of all the compounds tested, the flavone luteolin was the most potent in inhibiting b5 reductase with an IC_{50} value of 0.11 μM, whereas naringenin, naringin and chrysin were inactive within the concentration range tested. Most of the remaining flavonoids (morin, quercetin, quercitrin, myricetin, luteolin-7-*O*-glucoside, (−)-epicatechin, and (+)-catechin) produced a considerable inhibition of enzyme activity with IC_{50} values ranging from 0.81 to 4.5 μM except apigenin (36 μM), rutin (57 μM) and (+)-taxifolin (IC_{50} not determined). The magnitude of inhibition was found to be closely related to the chemical structures of flavonoids. Analysis of structure–activity data revealed that flavonoids containing two hydroxyl groups in ring B and a carbonyl group at C-4 in combination with a double bond between C-2 and C-3 produced a much stronger inhibition, whereas substitution of a hydroxyl group at C-3 was associated with a less inhibitory effect. The physiologically relevant IC_{50} values for most of the flavonoids tested regarding b5 reductase inhibition indicate a potential for significant flavonoid–drug and/or flavonoid–xenobiotic interactions which may have important therapeutic and toxicological outcomes for certain drugs and/or xenobiotics.

1. Introduction

NADH-cytochrome b5 reductase (EC 1.6.2.2), a flavoprotein, is an amphipathic membrane-bound protein which exists in relatively high concentration in endoplasmic reticulum of liver tissues. This enzyme transfers the electrons from NADH to cytochrome b5 which plays a central role in many diverse metabolic reactions in liver such as in fatty acid desaturation, in elongation of fatty acids, in biosynthesis of cholesterol, in plasmologen synthesis, in prostaglandin synthesis and in drug metabolism involving cytochrome P450 mixed function oxidations [1,2]. A second soluble form of cytochrome b5 reductase exists in erythrocytes where it catalyzes the reduction of methemoglobin via transferring electrons to cytochrome b5 [3].

Many reports have provided evidences that microsomal cytochrome b5 reductase and cytochrome b5 can stimulate and augment cytochrome P450 catalyzed monooxygenase reactions in vivo [4]. Apart from this, cytochrome b5 reductase alone can directly catalyze the metabolism of a wide range of xenobiotics, thus contributing to their toxicological or therapeutic effects. NADH-cytochrome b5 reductase functions as a one-electron reducing enzyme and has been shown to be responsible for the generation of free radicals from heterocyclic amines [5]. Cytochrome b5 reductase using NADH reduces heterocyclic amines to free radicals which in turn catalyze the transfer of one electron to molecular oxygen, thereby producing superoxide radical. Thus, in addition to the highly reactive DNA-adduct forming metabolites formed by cytochrome P450 isozymes through oxidative pathways, reactive oxygen species generated in the presence of cytochrome b5 reductase through redox cycling may contribute to the carcinogenic effects of heterocyclic amines.

One electron reduction pathway, on the other hand, is desirable and thought to be a major mechanism for the antitumor activities of some kinds of chemotherapy drugs. NADH-cytochrome b5 reductase has been shown to be capable of reducing anticancer drugs mitomycin C [6,7], adriamycin (an anthracycline antibiotic) [8], RB90740 (a mono-*N*-oxide bioreductive drug) [9] and bleomycin [10] to reactive and/or redox active metabolites which may account for their cytotoxic actions to tumor cells.

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0009-2797/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cbi.2012.04.003

NADH-cytochrome b5 reductase together with cytochrome b5 catalyzes the microsomal reduction of arylhydroxylamine metabolites generated from drugs such as sulfamethoxazole, dapsone, procainamide and other arylamine drugs to their parent amines. The amidoximes, used as prodrugs, are also metabolically reduced to their parent amidines by the same microsomal NADH-dependent system [11]. This microsomal enzyme system has been shown to be essential and sufficient for direct reductive detoxification of hydroxylamine metabolites (proximate mutagens) of pro-carcinogenic 4-aminobiphenyl and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP, found in grilled meats) compounds [12]. The oxidatively formed hydroxylamine metabolites are thought to be responsible for dose-dependent adverse effects of arylamine drugs and DNA-adduct forming potential of some heterocyclic and aromatic amine carcinogens. Therefore, reduction of these metabolites in human liver by cytochrome b5 reductase is necessary for their elimination. Amidoximes and other hydroxylamines can also serve as prodrugs to enhance the absorption of some antihypertensive, antimicrobial and antithrombotic drugs [13]. In this case, enzymatic reduction of these drugs by cytochrome b5 reductase is essential for their bioactivation.

As the above-mentioned studies demonstrate, it appears that the microsomal cytochrome b5 reductase is a clinically and toxicologically important enzyme and differences in the activity of this enzyme may have implications in drug toxicity, prodrug bioactivation and possibly carcinogenesis. However, despite its important role in the metabolism of xenobiotics, to our knowledge, the interaction of cytochrome b5 reductase with dietary flavonoids has not been investigated in detail. Flavonoids are polyphenolic compounds found in significant quantities in foods of plant origin and exhibit a broad spectrum of biological and pharmacological activities including antitumor, antioxidant, antimicrobial, antimutagenic effects and inhibition of several enzymes [14]. Therefore, these considerations prompted us to study modulation of cytochrome b5 reductase activity by dietary flavonoids of variable structures.

In our experiments, we used NADH-cytochrome b5 reductase purified from bovine liver microsomes. Aminoacid sequence data demonstrate that a high degree of identity greater than 90% exists between human cytosolic and bovine liver microsomal cytochrome b5 reductases [15,16]. These sequencing studies have also confirmed that the cytosolic soluble form of b5 reductase lacks a hydrophobic segment at the NH_2 terminus which normally present in the microsomal reductase. Thus, the present study was conducted to investigate the structural requirements necessary for inhibition of cytochrome b5 reductase activity by structurally related flavonoids using purified enzyme from bovine liver microsomes. Such compounds, by modulating the activity of cytochrome b5 reductase, could have potential effects in the therapeutic effectiveness of a wide range of drugs or in the toxicities associated with certain drugs or carcinogens.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in this study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany) at the highest grade of purity available. The distilled water used in this study was Milli-Q® filtered.

2.2. Preparation of bovine liver microsomes

The livers from well-bled healthy bovine (about 1–2 years old) were obtained from a local slaughterhouse immediately after killing. Bovine liver microsomes were prepared by a standard differential ultracentrifugation as previously described [17]. Homogenization of tissues was performed in 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ε-ACA and 0.1 mM PMSF. The washed microsomal pellets were finally suspended in 25% glycerol containing 1 mM EDTA at a volume of 0.5 ml for each gram of liver. Microsomal suspensions were gassed with nitrogen in small plastic bottles and stored at −86 °C in a deep freezer until used for purification studies.

2.3. Purification of bovine liver NADH-cytochrome b5 reductase

Purification of NADH-cytochrome b5 reductase was accomplished according to the methods described by Güray and Arinç [18] and Arinç and Çakir [19] for the purification of sheep lung and sheep liver b5 reductases, respectively, with slight modifications [20].

Briefly, the purification procedure involved anion-exchange chromatography of the detergent solubilized microsomes on two successive DEAE-cellulose columns followed by affinity chromatography of the partially purified enzyme on adenosine 5′-diphosphate-agarose. The key step in the purification of enzyme was the affinity chromatography on adenosine 5'-diphosphate-agarose.

The purity of the final enzyme preparation was evaluated by polyacrylamide gel electrophoresis under denaturing conditions. The final enzyme preparation was highly pure with respect to microsomes and produced a single protein band on SDS–PAGE (Supplemental Fig. 1).

The protein concentrations were determined according to the method of Lowry et al. [21] and crystalline bovine serum albumin was used as a standard.

2.4. Measurement of NADH-ferricyanide reductase activity of purified bovine liver NADH-cytochrome b5 reductase

The NADH-ferricyanide reductase activity of the purified enzyme was assayed by measuring the rate of potassium ferricyanide reduction at 420 nm, spectrophotometrically, according to the method of Strittmatter and Velick [22]. The assay mixture contained 0.1 M potassium phosphate buffer, pH 7.5 containing 200 nmoles potassium ferricyanide, 120 nmoles NADH and appropriate amounts of purified enzyme preparation in a final volume of 1 mL. The reaction was started by addition of cofactor NADH and reduction of ferricyanide was followed for 2 min by recording the absorbance decrease at 420 nm using Shimadzu UV-1800 double beam spectrophotometer with cuvettes of 1.0 cm light path. Since ferricyanide was also reduced chemically with NADH, the reaction rate was corrected by subtracting the reaction rate of ferricyanide in the absence of enzyme. The enzyme activity was calculated using the extinction coefficient of 1.02 mM^{-1} cm^{-1} for the difference in absorbance between reduced and oxidized form of ferricyanide. One unit of reductase was defined as the amount of enzyme catalyzing the reduction of 1 μmole of potassium ferricyanide per minute under the described conditions.

2.5. Inhibition studies of flavonoids

To assess the inhibitory effects of flavonoids on bovine liver cytochrome b5 reductase, test compounds dissolved in dimethyl sulfoxide (DMSO) were added at various final concentrations between 0.05 and 100 μM to the assay medium containing 0.1 M potassium phosphate buffer, 0.2 mM potassium ferricyanide and appropriate amounts of diluted enzyme. After 5 min pre-incubation, the reaction was started by addition of cofactor NADH at a final concentration of 0.12 mM. Enzyme assays were carried out at room temperature (24–25 °C). The final concentration of DMSO was 1% (v/v) to avoid the interference with enzyme activity. The rate of enzyme activity