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Nitrite–glucose–glucose oxidase system directly induces rat heart homogenate oxidation and tyrosine nitration: Effects of some flavonoids

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ABSTRACT

Protein tyrosine nitration is a common post-translational modification occurring under conditions of nitrative/oxidative stress in a number of diseases. It has been found that in the presence of nitrite and hydrogen peroxide, hemoprotein catalyzes protein tyrosine nitration. In this paper, it was found that in heart homogenate, protein nitration and oxidation could be induced by a nitrite–glucose–glucose oxidase system without addition of exogenous heme or hemoprotein. Several structural diversity flavonoids (quercetin, rutin, baicalein, baicalin, apigenin, puerarin, and (+)-catechin) could, more or less, protect rat heart homogenate from oxidative and nitrative injury induced by nitrite–glucose–glucose oxidase *in vitro*. The inhibitory effects of flavonoids on protein nitration and lipid peroxidation were consistent with their antioxidant activities, whereas the inhibitory effects on protein oxidation were almost contrary to their antioxidant activities. These results mean that nitrite–glucose–glucose oxidase system can cause heart homogenate protein nitration and protein oxidation in different pathways, and those flavonoids with strong antioxidant activities may contribute their protective effect partly through inhibiting protein nitration.

1. Introduction

Oxidative injury has been implicated in pathogenesis of numerous diseases including cardiovascular diseases, neurodegenerative diseases and inflammation which involve reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Turko and Murad, 2002). Excessive generation of ROS/RNS can damage numerous molecules such as protein, DNA and polyunsaturated fatty acids, and may lead to oxidative stress and a variety of diseases (Djuric et al., 2001; Peluffo and Radi, 2007).

3-Nitrotyrosine (3-NT) has been revealed as a relevant biomarker of pathological process and oxidative stress (Turko and Murad, 2002). Over the past several years, substantial evidence has been accumulated that the major pathways of protein tyrosine nitration *in vivo* include peroxynitrite ($ONOO^-$) and heme peroxidase (hemoprotein)-dependent reaction in which ROS/RNS radicals are involved (Peluffo and Radi, 2007). As a hemoprotein-rich organ, heart tissue may be a fragile site for nitrative attack catalyzed by hemoprotein (Bian et al., 2003; Kanski et al., 2005). Cardiovascular pathologies, such as heart failure and ischemia-reperfusion injury, are typically characterized by sudden formation of oxygen radicals and NO overproduction, which can lead to extensive nitration of protein tyrosine residues (Turko and Murad, 2002; Kanski et al., 2005; Zhang et al., 2007). Hence, besides lipid peroxidation and protein oxidation, the formation of 3-NT, which influences catalytic activity, cell signaling and cytoskeletal organization, may be involved in the pathogenesis of many heart diseases (Schopfer et al., 2003; Turko and Murad, 2002).

Flavonoids are polyphenolic compounds which present in plants, fruits and vegetables (Rice-Evans et al., 1995; Havsteen, 2002). They may have beneficial health effects because of their antioxidant activities, including metal-chelating, free radical-scavenging and lipid peroxidation-inhibiting (Havsteen, 2002; Heim et al., 2002). Evidence for the potential antioxidant activities suggests that the dietary intake and the therapeutic use of flavonoids can be associated with significant heart benefits (Katan, 1997; Hertog et al., 1997; Erdman et al., 2007). In France, the low incidence of heart disease and arteriosclerosis has been ascribed to a higher intake of flavonoids from red wine (Katan, 1997). Many flavonoids were found to be better antioxidants than the antioxidant nutrients such as vitamin C, vitamin E and β-carotene on a mole for mole basis (Rice-Evans et al., 1995). Many researches indicated that flavonoids could effectively inhibit $ONOO^-$-mediated nitration (Pannala et al., 1997; Ketsawatsakul et al., 2000; Schroeder et al., 2001; Sadeghipour et al., 2005). However, few studies deal with the effect of flavonoids on NO_2^-–H_2O_2-triggered nitration. Considering there is more possibility of hemoprotein–NO_2^-–H_2O_2 caused

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oxidation and nitration present in heart, we introduced a NO_2^-–glucose (G)–glucose oxidase (GO) system, which is more likely to happen in heart pathological process, as an *in vitro* model, to assess the effects of flavonoids on protein nitration and oxidation. We chose seven flavonoids, i.e. quercetin, rutin, baicalein, baicalin, apigenin, puerarin, and (+)-catechin, which belong to flavonol, flavone, isoflavone and flavane sub-family, respectively, as the representatives of flavonoids. The structures of tested flavonoids are shown in Fig. 1. The study will not only give the relationship between antioxidant activity and protein nitration, but also provide a new method for primary evaluation of flavonoids as heart protector.

2. Materials and methods

2.1. Reagents

Baicalein (Ba), (+)-catechin (Ca), sodium nitrite ($NaNO_2$), glucose (G), glucose oxidase (GO), butylated hydroxytoluene (BHT), 2,4-dinitrophenylhydrazine (DNPH), rabbit polyclonal antibody against nitrotyrosine and dinitrophenyl (DNP) were purchased from Sigma. 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was purchased from Amresco. Quercetin (Qu), rutin (Ru), baicalin (BaG), apigenin (Ap), and puerarin (Pu) were purchased from Shanxi Huike Botanical Development Co. Ltd., and recrystallized in methanol; the purities of the flavonoids were more than 98% based on HPLC analysis. All solvents and other reagents were the highest purity commercially available. Male Wistar rats (body weight, 180–220 g) were obtained from Tongji Medical School, Huazhong University of Science and Technology (China).

2.2. Tissue treatment

Rats were fasted overnight and anesthetized with ethyl ether, then decapitated, and the hearts were carefully removed and perfused with saline at 4 °C to remove blood cells. The perfused hearts were then blotted on filter paper, weighed and homogenized with a glass homogenizer in ice-cold 100 mM phosphate-buffered saline (PBS, pH 7.4) containing D-glucose (10 mM) and protease inhibitors (final concentration: 5 mTIU/ml aprotinin; 10 μg/ml leupeptin; 200 μM phenylmethane sulfonate fluoride). After centrifugation at 10,000*g* for 15 min, the protein concentration in the supernatant was measured by Bradford protein assay. The heart homogenate supernatant (8 mg protein/ml) was obtained and stored at −20 °C for further use. The samples were preincubated with or without tested flavonoids at 37 °C for 5 min, and then $NaNO_2$ and glucose oxidase were added (for lipid peroxidation detection, only glucose oxidase were added), the mixture was further incubated at 37 °C for 60 min. The reacted heart homogenate supernatant was used in later assays.

2.3. Protein nitration and oxidation detection by SDS–PAGE and Western blotting

The heart homogenate supernatant was incubated with different concentration of nitrite–glucose–glucose oxidase (NO_2^-–G–GO) system at 37 °C for 60 min, and then 80 μl aliquots were mixed with 20 μl 5× sample loading buffer, heated at 100 °C for 3 min, and loaded on 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) for electrophoresis. For detection of protein oxidation, the carbonyl groups in proteins were first derivatized with DNPH in the presence of SDS (3%). After 1 h incubation at room temperature, the reaction was stopped with the neutralization solution, and then samples were mixed with loading buffer and subjected to SDS–PAGE.

For Western blotting, after electrophoresis, proteins were transferred to nitrocellulose membrane and immunoblotted with a rabbit polyclonal antibody against 3-nitrotyrosine or DNP. The antibody was detected using an anti-rabbit secondary antibody conjugated with horseradish peroxidase. Chemiluminescence was used to identify specific proteins according to the ECL system (Pierce).

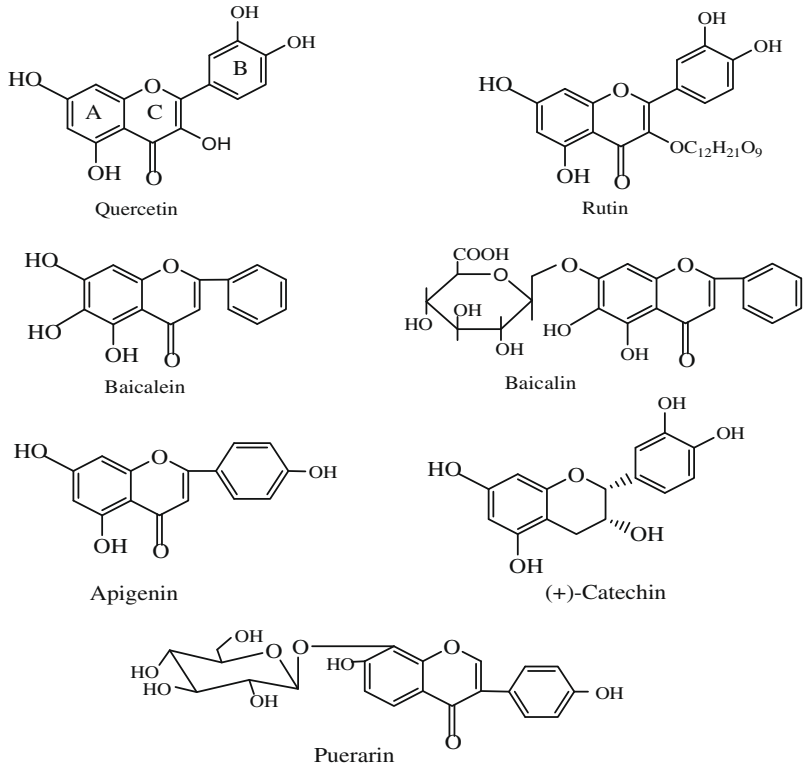

Fig. 1. The chemical structures of tested flavonoids.