Introduction

The pathogenesis of a wide range of lifestyle diseases such as hypertension and cardiovascular disease is not fully understood. However, the role of oxidative stress on the genesis and maintenance of these diseases has been revealed in experiments involving administration of several types of antioxidants, with a consequent lowering of the risk of these diseases (Rodríguez et al., 2002; Vasdev et al., 2001; Schnackenberg and Wilcox, 1999). Oxidative stress initiated by reactive oxygen species (ROS) might be a factor in these pathological conditions via oxidation of biological macromolecules such as lipids, protein, and DNA. Thus, there is growing interest in the many antioxidative components in foods for the prevention of ROS-induced oxidative damage.

Flavonoids are widely distributed in many kinds of plant food sources. The beneficial effects of flavonoids on human health have been widely studied, and antioxidative activity was proposed as one of the most important causes of these effects (Duarte et al., 1993; Duarte et al., 2001; Yamamoto and Oue, 2006; Gryglewski et al., 1987; Yu et al., 2006; Villar et al., 2002). Quercetin is the most abundant flavonol type of flavonoid, and a wide range of biological actions including vasodilation, antihypertensive, and antithrombotic effects have been reported (Duarte et al., 1993; Duarte et al., 2001; Yamamoto and Oue, 2006). Chrysin is a flavone-type flavonoid, and is present in foods like honey and propolis, and in many kinds of fruits and vegetables at low concentrations. Additionally, chrysin is commercially available as an androgen-boosting supplement (Kao et al., 1998). Previous experiments have suggested that chrysin possesses in vitro antioxidative and free radical scavenging activities (Rackova et al., ...
in assessing the in vivo physiological effects of chrysin, Villar et al. observed an antihypertensive effect in spontaneously hypertensive rats (SHR) (Villar et al., 2002), which has not been found in any study on normotensive rats.

The consumption of a high-fat diet or a high-fat high-sucrose (HFS) diet induces a significant rise in oxidative stress, accompanied by hyperlipidemia, hypercholesterolemia, and hypertensive effects (Wilde et al., 2000; Roberts et al., 2000; Roberts et al., 2005). These diet-induced adverse effects can be used as a model of lifestyle-related diseases. In the present study, the effects of dietary chrysin on blood pressure and oxidative status of normotensive rats fed a HFS diet was examined in comparison with quercetin. Also, the effect of chrysin on in vitro antioxidative activity was also investigated.

Materials and Methods

**Animals and experimental diet** Twenty-eight 6-week-old male Sprague-Dawley rats were obtained from SLC Japan, Inc (Hamamatsu, Japan), and were housed individually in cages with wire mesh bottoms in a room kept at 22 ± 1°C and under a 12:12-h light-dark cycle (light period from 8:00 to 20:00 h). The animals were given free access to water and a basal diet for the first 5 days, and were subsequently randomly divided into four groups of seven animals each to average the initial body weight and systolic blood pressure (SBP). One group received a control diet (CON group), and the other four groups were fed a high-fat high-sucrose (HFS) diet with or without chrysin or quercetin (HFS, HFS, and HFSQ groups). The concentration of added flavonoid was 0.25%, as 0.2% or 0.5% quercetin was shown to be antioxidative and antihypertensive in our previous experiment (Yamamoto and Oue, 2006). The composition of the control diet was equal to that of the basal diet and was as follows (wt%): soybean oil, 7.0; mineral mixture, 3.5; vitamin mixture, 1.0; choline bitartrate, 0.25; casein, 20.0; cellulose, 5.0, and α-cornstarch to make 100. The composition of the HFS diet was as follows (wt%): soybean oil, 7.0; lard, 15.0; cholesterol, 0.5, mineral mixture, 3.5; vitamin mixture, 1.0; choline bitartrate, 0.25; casein, 20.0; sucrose, 40.0, cellulose, 5.0, and α-cornstarch to make 100. The composition of the flavonoid-added diets was the same as the HFS diet except that 0.25% of the cellulose was replaced with chrysin or quercetin. Chrysin and quercetin were purchased from Wako Pure Chemicals (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. The mineral mixture was AIN-93G-MX and the vitamin mixture was AIN-93-VX (Reeves, 1997). Freshly prepared experimental diets were served, and food and water were given ad libitum for the 4-week duration of the experiment. All experiments were conducted according to the Ethics Guidelines for Animal Experiments of Osaka City University.

**Sampling procedures** After the 4-week experimental period, the rats were fasted overnight, anesthetized, and their blood was collected in a heparinized syringe from the abdominal aorta. The plasma was separated by centrifugation at 10,000 × g at 4°C for 15 min and was used to measure lipid peroxide levels. Immediately after blood sampling, the thoracic aorta was excised, rinsed gently with chilled saline, blotted, and then stored at −80°C while awaiting analysis of the NADPH oxidase and xanthine oxidase activities. The liver was harvested, cleaned, blotted, and stored at −80°C until analysis. The epididymal fat tissue was harvested and weighed.

**Blood pressure** The SBP was measured at the start of the experiment and at 2-week intervals using the tail-cuff method with a blood pressure monitor (BP98; Softron, Tokyo, Japan).

**Lipid peroxide levels in the plasma** Plasma lipid peroxide levels were measured as thiobarbituric acid reactive substances (TBARS) by the spectrometric method (Ohkawa et al., 1979) and were expressed as the amount of malondialdehyde in the plasma.

**NADPH oxidase and xanthine oxidase in the aortic homogenate** Activities of NADPH oxidase and xanthine oxidase were measured by the chemiluminescent superoxide anion probe method (Rajagopalan et al., 1996). Aortic segments (3 to 4 cm) were homogenized with a glass-to-glass homogenizer on ice in 50 mmol/L phosphate-buffered saline (pH 7.0) with 0.01 mmol/L EDTA. The homogenate was centrifuged for 10 min at 1,000 × g, and the supernatant was used for measurement of oxidase activities. The reaction mixture contained 100 μmol/L NADPH or xanthine and 50 μmol/L lucigenin (Bis-N-methylacridinium), and the reaction was started by the addition of the aortic supernatant. The activity was measured by lucigenin-enhanced chemiluminescence using a Luminescensor AB-2200 (Atto Corp. Tokyo, Japan). Activities were expressed as the amount of generated superoxide anion per protein. Protein was measured by the method of Lowry et al. (Lowry et al., 1951).

**Determination of total glutathione** The liver was homogenized with 5 volumes of 10% trichloroacetic acid and then centrifuged at 3,000 × g for 10 min. A portion of the supernatant was subjected to total glutathione measurement using 5,5′-dithio-bis-2-nitrobenzoic acid according to Anderson (Anderson, 1985).

**In vitro antioxidative activity** For the in vitro measurement of antioxidative activity on lipid peroxidation, an aqueous emulsion was prepared with 25 mg of linoleic acid and 10 mL of 0.1 mol/L sodium phosphate buffer (pH 7.0) containing 100 mg of Tween 20 by sonicating in ice-cold water for 5 min. The emulsion with or without added flavonoids (final concentration: 50 μmol/L) was incubated at 40°C for 4 days. Oxidation was spectrophotometrically measured as the amount of produced TBARS (Ohkawa et al., 1979), and the results were expressed as increased optical density at 550 nm. Chrysin and quercetin were employed as the flavonoids, and were first dissolved in dimethyl sulfoxide and diluted with water.

**Statistical analysis** Each data value for the animal experiments and the in vitro experiment were expressed as the mean ± SEM. Differences between the four groups in the animal experiment and the in vitro antioxidative activity on lipid peroxidation were assessed by a post-hoc test of Bonferroni's method when ANOVA was significant. Differences were considered significant at P < 0.05.
the three groups in the in vitro experiment were assessed by a one-way analysis of variance (ANOVA) and multiple-range comparison by Tukey’s honestly significant difference (HSD) test. A p value of < 0.05 was considered significantly different.

Results

Growth and dietary intake  Body weight gain and epididymal fat weight of the four experimental groups did not differ (Table 1). Food intake in the HFS group was lower, while food efficiency and liver weight were higher, than the CON group. Food efficiency and liver weight in the HFSC and HFSQ groups did not differ from those of the HFS group.

Systolic blood pressure  The SBP of the HFS group was significantly higher than that of the CON group at 4 weeks, and the SBP of the HFSC and HFSQ groups was significantly lower than that of the HFS group (Fig. 1).

In vivo antioxidative activity  In vivo antioxidative activity of chrysin was estimated by the change in NADPH oxidase and xanthine oxidase activities in the aortic homogenate, the plasma TBARS value, and liver total glutathione.

The NADPH oxidase activity in the HFS group was significantly higher than the CON group and lower in the HFSQ group than the HFS group, whereas the activity of the HFSC group did not differ from that of the CON group (Fig. 2A). Xanthine oxidase activity in the HFS group with or without flavonoids did not differ from that of the CON group (Fig. 2B).

The plasma TBARS value in the HFS group was higher than the CON group (Fig. 3). The value of the HFSQ group was lower than the HFS group, but the value of the HFSC group did not differ from that of the HFS group.

Liver total glutathione concentration of the HFS group was significantly lower than the CON group. The concentration of the HFSQ group was higher than that of the HFS group, but the value of the HFSC group did not differ from that of the HFS group (Fig. 4).

In vitro antioxidative activity  Accumulation of lipid peroxidation products in the linoleic acid emulsion was not altered by the addition of chrysin but was significantly suppressed by the addition of quercetin (Fig. 5).

Discussion

Flavonoids have been reported to exert a wide range of biological actions, including vasodilation, antithrombotic, and antihypertensive effects (Duarte et al., 1993; Duarte et al., 2001; Yamamoto and Oue, 2006; Gryglewski et al., 1987; Yu et al., 2006; Villar et al., 2002). Most of these reports dealt with quercetin, and only one report focused on the in vivo biological effects of chrysin. Villar et al. showed the antihypertensive effect of orally administered chrysin on SHR (Villar et al., 2002); however, the effects of chrysin on normotensive rats remained unknown. In the present study, dietary chrysin suppressed the increase in blood pressure of normotensive rats fed a HFS diet. The effect of chrysin was similar to that observed for quercetin. From these results, dietary chrysin is expected to alleviate diet-induced hypertension.

One of the proposed mechanisms to suppress the elevation of

![Fig. 1. Effect of chrysin or quercetin dietary supplementation on the systolic blood pressure (SBP) of rats fed a HFS diet. Rats were fed a control diet or a HFS diet with or without 0.25% chrysin or quercetin for 4 weeks, and SBP was measured at the start of the experiment and at 2-week intervals using the tail-cuff method. Values with different superscript letters in each assay are significantly different from each other by Tukey’s HSD test (p < 0.05). ○: control, □: HFS, ●: HFSC, ■: HFSQ](image)
Fig. 2. Effects of chrysin or quercetin dietary supplementation on NADPH oxidase (A) and xanthine oxidase (B) activities in the aorta of rats fed a HFS diet.

Rats were fed a control diet or a HFS diet with or without 0.25% chrysin or quercetin for 4 weeks, and activities of NADPH oxidase and xanthine oxidase in an aortic suspension were measured by the chemiluminescent superoxide anion probe method. Activities are expressed as the amount of generated superoxide anion per protein. Values with different superscript letters in each assay are significantly different from each other by Tukey’s HSD test ($p < 0.05$).

Fig. 3. Effects of chrysin or quercetin dietary supplementation on plasma TBARS of rats fed a HFS diet.

Rats were fed a control diet or a HFS diet with or without 0.25% chrysin or quercetin for 4 weeks, and lipid peroxides in the blood plasma were measured as thiobarbituric acid reactive substances (TBARS) and are expressed as the amount of malondialdehyde in the plasma. Values with different superscript letters in each assay are significantly different from each other by Tukey’s HSD test ($p < 0.05$).

Fig. 4. Effects of chrysin or quercetin dietary supplementation on liver glutathione concentration in rats fed a HFS diet.

Rats were fed a control diet or a HFS diet with or without 0.25% chrysin or quercetin for 4 weeks, and total liver glutathione level was measured using 5,5′-dithio-bis-2-nitrobenzoic acid. Values with different superscript letters are significantly different from each other by Tukey’s HSD test ($p < 0.05$).

Fig. 5. Effect of chrysin or quercetin on in vitro lipid peroxidation of a linoleic acid emulsion. Values with different letters are significantly different from each other ($p < 0.05$) by Tukey’s HSD test.

○: not added, ●: chrysin, ■: quercetin
blood pressure involves antioxidant activity (Vasdev et al., 2001; Chen et al., 2001; Schnackenberg and Wilcox, 1999). Superoxide
is known to react quickly with NO (Thomson et al., 1995; Huie and Padmaja, 1993), and its consequent deactivation of NO is
responsible for the retardation of acetylcholine-mediated vascular relaxation. Thus, the possibility that this antioxidative activity
might be a mechanism of the antihypertensive effects of chrysin and quercetin was investigated in this experiment.

NADH/NADPH oxidase is well known as a major source of
vascular superoxide in addition to two other enzyme systems,
namely xanthine oxidase and NO synthase uncoupling (Cai and Harrison, 2000). Among these three systems, the importance of
NADH/NADPH oxidase for the production of superoxide in the
vessel wall has been suggested (Mohazzab et al., 1994; Guzik et al., 2000). In this study, accelerated oxidative stress in rats fed a
HFS diet was observed using indicators such as elevated aortic
NADPH oxidase activity, increased plasma lipid peroxides, and
decreased liver total glutathione. The increase in NADPH oxidase
activity was inhibited by the dietary addition of quercetin. However, chrysin supplementation did not significantly affect
NADPH oxidase activity, suggesting that the suppression of
NADH/NADPH oxidase-dependent superoxide production was not
the mechanism responsible for the antihypertensive effect of
chrysin.

An in vitro experiment revealed that chrysin and quercetin
strongly inhibited xanthine oxidase activity, and suggested that a
hydroxyl group at C-5 and/or C-7 of the A-ring of the flavonoid
was essential for the inhibitory activity (Lin et al., 2002). In
contrast, aortic xanthine oxidase activity was not inhibited by the
addition of chrysin or quercetin in this experiment. These
differences in the in vivo and in vitro effects of flavonoids on
xanthine oxidase activity might be attributable to the low
physiological bioavailability of chrysin (Walle et al., 2001) and
quercetin (Hollman and Katan, 1999).

As chrysin did not significantly decrease NADPH oxidase and
xanthine oxidase activities, the antihypertensive effect of chrysin
was not explained by the inhibition of superoxide production.
Another possible mechanism is a decrease in NO availability due
to its radical scavenging activity. There are few reports dealing
with the effects of chrysin on the in vivo oxidative status of rats.
Free radical scavenging activity of flavonoids were relatively weak,
and their antioxidative activities against lipid peroxidation were not
detected in previous in vitro experiments (Burda and Oleszek,
2001; Vinson et al., 1995). Also, the results of this experiment
suggested that chrysin was not antioxidative against the increase in
lipid peroxidation products in the linoleic acid emulsion.

Many previous studies have investigated and postulated a
number of hypotheses on the relationship between structure and
antioxidative activity of flavonoids. The ortho-hydroxylation on the
B-ring, the number of free hydroxyl groups, and a C2-C3 double
bond in the C-ring are all proposed as conditions of radical
scavenging activity (Rackova et al., 2005; Cao et al., 1997; Foti et al.,
1996). Also, a free hydroxyl group at the C-3 position in
flavonol has been proposed as a structural contributor to the high
antioxidative activity against the progress of lipid peroxidation and
the increase in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical
(Burda and Oleszek, 2001). The flavonol quercetin has five
hydroxyl groups, the 3', 4'-hydroxylation on the B-ring and free
hydroxyl groups at C-3, C-5, and C-7, with all of these structural
characteristics, and is the most potent antioxidant flavonoid. On the
other hand, the flavone chrysin has only two hydroxyl groups, at
C-5 and C-7, and does not possess a hydroxyl group at the C-3 position
and in the B-ring. From these structural characteristics,
chrysin was not expected to express efficient antioxidative activity.

A probable role of other mechanism(s) was suggested for
chrysin's suppressive effect on the genesis and maintenance of
hypertension in rats fed a high-fat high-sucrose diet regime. The
vasoconstrictor angiotensin II is important in blood pressure
control, and the effect of chrysin on angiotensin II production may
be another possible mechanism for the suppression of blood
pressure elevation. To our knowledge, there are no reports showing
the suppression of angiotensin II production by chrysin. Further
studies are needed to elucidate whether chrysin would be effective
for suppressing angiotensin II production.

In summary, it was demonstrated that the dietary addition of
the chrysin suppressed the elevation of blood pressure in
normotensive rats fed a high-fat high-sucrose diets. Further studies
are needed to clarify the mechanisms, other than antioxidative
activity, involved in the antihypertensive effect of chrysin.
Furthermore, studies employing other flavonoids should be
conducted to elucidate the relationship between structure and
antihypertensive activity of flavonoids.

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