Effect of Enzymatically Modified Isoquercitrin in Spontaneously Hypertensive Rats

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Summary Enzymatically modified isoquercitrin (EMIQ) is a water-soluble glycoside of quercetin produced from rutin by enzymatic treatment. We investigated the anti-hypertensive effect of orally administered EMIQ in spontaneously hypertensive rats (SHR). The systolic blood pressure (SBP) in SHR administered EMIQ at a dose of 3 and 26 mg/kg/d was significantly lower than that in the control group on d 22, 36 and 50 of administration. The effect of EMIQ (26 mg/kg/d) was higher than equimolar administration of quercetin. Diltiazem administered as a positive control also suppressed the increase in SBP, and the effect was stronger than that of EMIQ. In the control group, the mean values of mean blood pressure (MBP) and diastolic blood pressure (DBP) were increased after the start of administration. Although diltiazem suppressed the increase in MBP, no significant changes were observed in the EMIQ groups. Compared with the control group, EMIQ groups showed the incidental changes of MBP and heart rate on day 22 of administration only. These results indicate that EMIQ suppressed the increase in SBP in SHR dose-dependently, and was more effective than the aglycone quercetin. It was also speculated that EMIQ showed higher anti-hypertensive effect than quercetin due to the high bioavailability, and the mechanism of SBP suppression is possibly through the improvement of endothelial NO production. In conclusion, our results suggest that EMIQ shows possibility as a naturally-derived safe food material which has an antihypertensive effect.

Key Words enzymatically modified isoquercitrin, flavonoid, hypertension, blood pressure, spontaneously hypertensive rats

Hypertension, which is closely related to the life-threatening diseases arteriosclerosis and cardiovascular disease, is gathering increasing concern worldwide. There are many evidence-based antihypertensive medicines, such as calcium antagonist, angiotensin-converting enzyme (ACE) inhibitors and sympathetic blocking agents. However, with the recent increase of lifestyle-related diseases, there is growing concern about their prevention by dietary modification. There are many factors involved in hypertension, such as insufficient protein intake, excess intake of NaCl, and shortage of calcium and zinc, which are attributed to a disturbed nutritional status.

Recently, oxidative stress due to reactive oxygen species (ROS), such as the superoxide radical and hydroxyl radical, has been reported to be involved in the development of hypertension (1, 2). Negishi et al. reported an increase in the oxidative stress marker, urine 8-OHdG in a hypertensive patient compared with normotensive subjects suggesting that hypertensive patients have a high level of oxidative stress (3). Nitric oxide (NO) has a vasodepressor effect, and is closely related with oxidative stress (4). The increased ROS can decrease the half-life of NO. Certain reports suggested that the increase of ROS and simultaneous decrease of NO and antioxidants such as SOD and vitamin E occurs in essential hypertension (2, 5). Tsukahara et al. reported that rats lead to hypertension by N-nitro-L-arginine methyl ester (L-NAME) showed a decrease of excretion of NO2− and NO3− and increase of 8-OHdG in urine (6). These results indicated that the oxidative stress marker is inversely related to NO content, and that prevention of oxidative stress by antioxidants is effective against hypertension due to the inhibition of NO decrease. Recent studies have also demonstrated that antioxidative polyphenols and flavonoids suppressed hypertension (7–9).

Enzymatically modified isoquercitrin (EMIQ) is a mixture of quercetin glycoside, which consists of isoquercitrin (IQC) and its α-glucosylated derivatives, with 1–7 additional linear glucose moieties (10). EMIQ is manu-
factured from rutin, which exists widely in natural products such as citrus fruits, red beans, and buckwheat, according to the manufacturing procedure shown in Fig. 1. EMIQ has strong antioxidant activity and high water solubility, and is commonly used as an antioxidant in various foods and beverages in Japan (11). EMIQ has been found to be safe by many evaluation tests, acute toxicity test, 4-wk toxicity test, 13-wk toxicity test, chronic/carcinogenic toxicity test and mutagenicity test (Ames assay), and is approved as a food additive under the Japan Food Sanitation Law (12). Recently, certain flavonoids, quercetin, catechin, chrysin and hesperidin which have antioxidative activity, have been found to significantly lower blood pressure in SHR (13–16). Therefore, EMIQ, which has a quercetin framework, is expected to be useful as an antihypertensive agent.

In the present study, we assessed the effect of long-term administration of EMIQ on the blood pressure in SHR in comparison with that of its aglycone quercetin and established antihypertensive medicine, diltiazem.

**MATERIALS AND METHODS**

**Animals and diet.** Male SHR/Izm rats aged 4 wk were purchased from Japan SLC. Prior to the study, the animals were quarantined for 5 d and then acclimatized for 2 or 4 d. The animals were kept in an animal room on a 12-h light and 12-h dark cycle (lights on at 6:00) with a temperature range of 20–26˚C, a relative humidity range of 40–70%, and air change of 12 times per hour. The animals were housed individually in stainless steel 5-compartment cages and were given free access to solid feed (CRF-1, Oriental Yeast Co., Ltd., Japan) and to tap water. Then, the animals were grouped on the first dosing day by a random sampling method so that the mean body weight, mean value of systolic blood pressure (SBP), and variance stratified by computer were made as equal as possible among the groups.

EMIQ and quercetin (San-EI Gen F.F.I., Inc., Japan), diltiazem hydrochloride (Wako Pure Chemicals Industries, Ltd., Japan), and water for injection (Otsuka Pharmaceutical Co., Ltd., Japan) served as the test substance, positive control substance, and vehicle respectively (10). The required amount of EMIQ and diltiazem were dissolved in water and were orally administered. The dosage of diltiazem was expressed as weight excluding the hydrochloride base. The required amount of quercetin was suspended in water and was orally administered. Each dosing preparation was made immediately before use. The dosage of EMIQ was set at 26 mg/kg/d, which was found to significantly lower blood pressure in SHR, and another dosage was set at 3 mg/kg/d which was about a tenth of this amount (13 and 1.5 mg/kg twice a day) (17). The dosage of quercetin was set at 10.4 and 1.2 mg/kg/d (5.2 and 0.6 mg/kg twice a day) which was the concentration equimolar to each EMIQ dosage as converted using the absorbance ratio of the flavonoid framework. The dosage of diltiazem, the positive control article, was set at 120 mg/kg/d (60 mg/kg twice a day), at which its inhibitory effect on increase in blood pressure was confirmed by Yoshida et al. (18) and Narita et al. (19). A group treated with the water alone was employed as the control and given the same dosing volume as the group treated with EMIQ.

**Blood pressure and heart rate measurement.** Blood pressure was measured before the first dosing and on days 22, 36, and 50 of administration. During the dosing period, blood pressure was measured during the period from about 1 h after morning treatment to the time of afternoon treatment. Using an automated noninvasive blood-pressure meter (BP-98A, Softron Co., Japan), SBP, diastolic blood pressure (DBP) and heart rate were measured for unanesthetized animals by the tail-cuff method, and mean blood pressure (MBP) was measured for additional reference data. Each of these parameters was measured 3 times in each animal, and the mean of the 3 readings was regarded as the value of each parameter in each animal. To raise the body temperature of the animals, the animals were placed in a heated box (38˚C) for about 5 min before measurement. Blood pressure was measured in a soundproof booth.

**Statistics.** Each data point represents the mean of replicated samples and the result is expressed as the mean±SD. Two-way ANOVA was performed to determine the main effects of administered chemicals and the time course changes, and their interactions. Significant differences between means were compared by using Dunnett test as a post-hoc analysis. Differences were considered significant at p<0.05. All data were analyzed using the SAS software (SAS institute Inc., USA).
RESULTS

General signs and body weight

Figure 2 shows the body weight change in each group. During the dosing period, there were no notable changes in general signs in any group, and normal body weight changes were noted in all groups.

Systolic blood pressure

Figure 3 shows the antihypertensive effect of EMIQ on SBP measurement. In the control group, the mean value of SBP was 137 mmHg before the first dosing (day 0), but it increased to 191 mmHg on day 50 of administration. EMIQ administration significantly suppressed increase of SBP on days 22, 36 and 50; even the dosage of 3 mg/kg/d was effective compared with the control group. EMIQ suppressed the increase in SBP dose-

Fig. 2. Effects of EMIQ and quercetin on body weight in SHR. Each data point shows the mean of 10 rats.

Fig. 3. Effects of EMIQ and quercetin on systolic blood pressure in SHR. Blood pressure was measured on days 0, 22, 36 and 50 of administration. Each data point shows the mean of 10 rats. Significantly different from control group (*p<0.05, **p<0.01). Significantly different from EMIQ 3 mg/kg group (§p<0.01). Significantly different from EMIQ 26 mg/kg group (†p<0.01).

Fig. 4. Effects of EMIQ and quercetin on mean blood pressure in SHR. Blood pressure was measured on days 0, 22, 36 and 50 of administration. Each data point shows the mean of 10 rats. Significantly different from control group (*p<0.05, **p<0.01).
dependently. In the group treated with diltiazem, SBP was significantly lower than for the control group on days 22, 36, and 50 of administration. The suppressive effect of diltiazem was significantly stronger than that of 3 mg/kg/d EMIQ and 26 mg/kg/EMIQ on day 50 of administration, indicating that diltiazem suppressed the increase in SBP more than EMIQ. In the group treated with quercetin, SBP was significantly lower than the control group on days 22, 36, and 50 of administration, indicating that quercetin suppressed the increase in SBP. However, compared with equimolar administration of 26 mg/kg/d quercetin, the suppressive effect of 10.4 mg/kg/d quercetin was significantly weak on days 36 and 50 of administration. This result indicates the suppressive effect of EMIQ was stronger than that of aglycone quercetin. On analyzing the increase of SBP, a significant main effect of test substance \( (p<0.01) \) and dosing period \( (p<0.01) \) and interaction between test substance and dosing period \( (p<0.01) \) were observed.

**Mean blood pressure**

Figure 4 shows the results of measurement of MBP. In the control group, the mean value of MBP was 106 mmHg on day 0, but it increased to 147 mmHg on day 50 of administration. Compared with the control group, a significant change of MBP was observed in the 26 mg/kg/d EMIQ group on day 22 of administration only. In the quercetin group, no significant difference was observed for the treatment. On the other hand, MBP was lower in the group treated with diltiazem, the positive control substance, than in the control group on days 22 and 50 of administration; diltiazem suppressed the increase in MBP. On analyzing the increase of MBP, a significant main effect of test substance \( (p<0.01) \) and dosing period \( (p<0.01) \) was observed; however no significant interaction was observed between test substance and dosing period \( (p=0.34) \).

**Diastolic blood pressure**

Figure 5 shows the results of measurement of DBP. In the control group, the mean value of DBP was 91
mmHg before dosing, but it increased to 125 mmHg on day 50 of administration. Compared with the control group, DBP was higher in the EMIQ group on days 36 and 50 of administration, but not significantly. On analyzing the increase of DBP, only a significant main effect of dosing period (p<0.01) was observed. No significant main effect of test substance (p=0.33) or interaction between test substance and dosing period (p=0.72) was observed.

Heart rate

Figure 6 shows the results of measurement of heart rate. In the control group, the mean value of heart rate was 426 beats/min before the start of administration, but it decreased to 366 beats/min on day 50 of administration. Compared with the control group, heart rate was significantly lower in the 26 mg/kg/d EMIQ groups on day 22. Since no significant changes were observed thereafter, the significantly lower heart rate in the EMIQ groups was not a continuous change. On analyzing the heart rate, only a significant main effect of dosing period (p<0.01) was observed. No significant main effect of test substance (p=0.11) or interaction between test substance and dosing period (p=0.21) was observed.

DISCUSSION

SHR is a good experimental model of hypertension: the blood pressure rises sharply after 3–4 wk age, and simultaneous development of oxidative stress is observed (3, 20). Using the SHR model rats, we can study the entire period of hypertension development which is difficult to study in humans. The hypertensive effect of various compounds has been investigated using SHR. We investigated the antihypertensive effect of EMIQ on SHR in order to consider the possibility of utilizing it as a functional food for preventing hypertension.

Flavonoids are phytochemicals reported to have various pharmacologic effects. Flavanoids and flavonoid-rich food show cardioprotective and antihypertensive effects (21–23). Oxidized low-density lipoprotein (LDL) is one of the main factors involved in the development of cardiovascular disease, especially atherosclerosis. Atherosclerosis is characterized by an accumulation of arterial foam cells mainly derived from oxidized LDL-loaded macrophages. Macrophage-scavenging receptors recognize oxidized LDL, but not native LDL. LDL containing cholesterol, fatty acid and phospholipids is easily oxidized by ROS. Hence, inhibiting LDL oxidation may help prevent the development of cardiovascular disease. Numerous studies have revealed that flavonoids such as quercetin and myricetin showed a strong inhibitory effect on LDL oxidation in vitro and ex vivo (24–26). Although we can not directly apply these results to the in vivo LDL oxidation, some epidemiologic studies have revealed the cardioprotective effects of flavonoids and quercetin-containing foods (21, 22). Many flavonoids showed antihypertensive effect in hypertensive animal models. Duarte et al. reported that quercetin administration significantly decreased SBP, DBP and MBP in SHR but not in WKY, and enhanced the endothelium-dependent relaxation to acetylcholine (13). Park et al. reported that isoflavone decreased SBP in SHR, and elevated the serum NO and radical-trapping antioxidant potential (27). Hara and Tonooka reported that tea catechin significantly suppresses the increase of blood pressure without a change in body weight in SHR (14). Ohtsuki et al. reported that long-term administration of hesperidin and its glycoside decreased the blood pressure of SHR (16). Thus, cardioprotective and antihypertensive effect of flavonoids has already been demonstrated.

In the present study, administration of EMIQ to SHR at a dosage of 3 and 26 mg/kg/d significantly suppressed the increase in SBP dose-dependently, and the effect was stronger than that of aglycone quercetin. However, compared with the antihypertensive medicine diltiazem, the effect of EMIQ was significantly weak. In the control group, the MBP and DBP values increased after the start of administration, and diltiazem significantly suppressed the increase of MBP, but only an incidental change was observed in the EMIQ group. EMIQ did not significantly affect the MBP, DBP or heart rate. EMIQ decreased the SBP significantly and showed a tendency to increase the DBP. The decrease of SBP and the increase of DBP mean the decrease of pulse pressure. It is suggested that EMIQ has a possibility of protection against deterioration of the arterial vessel elasticity. Thus, these results indicated that EMIQ at a low dose is an effective antihypertensive with a mild effect of decrease in SBP.

There are several antihypertensive mechanisms such as inhibition of ACE, improving the bioavailability of endothelium NO, and modulation of the sensitivity of the vascular smooth muscles to NO, but the antihypertensive mechanism of flavonoids remains unknown (28, 29). The antihypertensive effect of flavonoids has been attributed to the induction of increase of NO production. Several animal studies have demonstrated that administration of foods containing quercetin and flavonoids induced an increase of NO production and relaxation of the arterial vessel (30–32). Machha and Mustafa reported that long-term administration of quercetin to SHR decreased SBP and increased the endothelium-dependent relaxation response to acetylcholine, and suggested these are effects attributable to an increase of NO production (33). Taubert et al. also demonstrated that flavonoids enhanced the endothelial NO release of isolated porcine coronary arteries (34). Quercetin and IQC, a component of EMIQ, induced NO release, and the NO-stimulating activity of the flavonoids was uniformly positively correlated with their vasorelaxing activity. These results suggest that the antihypertensive effect of EMIQ, which has a quercetin framework, may be due to the increase of NO.

Our results showed that EMIQ suppressed the increase of SBP more effectively than quercetin, while Taubert et al. reported that administration of quercetin increased NO release (Δ[NO]>8.5 nM) more effectively than IQC (5 nM<Δ[NO]<8.5 nM), which is a compo-
The intestinal sugar carriers and solubilization in water may play a role in the mechanisms of the high bioavailability of EMIQ. Recent evidence suggests that quercetin glucoside is absorbed from the small intestine by a mechanism involving the glucose transport pathway. Wolffram et al. demonstrated that quercetin-3-glucoside is transported by the glucose carrier sodium-dependent glucose transporter (SGLT1) across the brush border membrane of the rat small intestine using several inhibitors of SGLT1 (38). EMIQ has higher water solubility than quercetin because of enzymatic glycosylation. Piskula and Terao demonstrated that solubilization of quercetin against vehicles enhanced its concentration in rat plasma after oral administration (39). Thus, at an equimolar concentration, EMIQ treatment was more effective against hypertension in SHR than quercetin because of the high bioavailability which may be due to the sugar carriers and solubilization.

In conclusion, this study demonstrated that EMIQ suppressed the increase of SBP during the stage of transition to hypertension in SHR dose-dependently. This effect was stronger than quercetin, which is an aglycone of EMIQ, but milder than the antihypertensive medicine, diltiazem. The antihypertensive effect of EMIQ was suggested to be due to the improved endothelial NO production because of the high bioavailability. Previous in vitro studies revealed that EMIQ is decomposed by α-amylase treatment to IQC and a mono- or diglucoside. IQC and the mono- or diglucoside form are absorbed more readily than quercetin (35). Hollman et al. reported that absorption is enhanced by conjugation with glucose: the absorption of quercetin glucosides from onions was 52%, and that of quercetin aglycone was 24% in ileostomy volunteers (36). Morand et al. reported that after administration of the 20 mg equivalent of quercetin-3-glucoside (IQC) and quercetin, the concentration of quercetin in rat plasma was 33.2 μM and 11.2 μM respectively (37). Their results also showed that the quercetin-3-glucoside was hydrolysed before and during its intestinal absorption, and 59 to 65% of the quercetin in the plasma was methylated, and intact quercetin-3-glucoside was not detected. These results demonstrated glycosylation of quercetin improves the absorption of quercetin. Thus, EMIQ, which is absorbed in the form of quercetin mono- or diglucoside, has higher bioavailability than quercetin.

We speculate that the intestinal sugar carriers and solubilization in water may play a role in the mechanisms of the high bioavailability of EMIQ. Recent evidence suggests that quercetin glucoside is absorbed from the small intestine by a mechanism involving the glucose transport pathway. Wolffram et al. demonstrated that quercetin-3-glucoside is transported by the glucose carrier sodium dependent glucose transporter (SGLT1) across the brush border membrane of the rat small intestine using several inhibitors of SGLT1 (38). EMIQ has higher water solubility than quercetin because of enzymatic glycosylation. Piskula and Terao demonstrated that solubilization of quercetin against vehicles enhanced its concentration in rat plasma after oral administration (39). Thus, at an equimolar concentration, EMIQ treatment was more effective against hypertension in SHR than quercetin because of the high bioavailability which may be due to the sugar carriers and solubilization.

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REFERENCES


EMURA K et al.


