



$\mu\text{g/ml}$  cytochalasin B (Sigma, St. Louis, MO) for 5 min at  $37^\circ\text{C}$  and further treated with  $1\ \mu\text{M}$  ionomycin (Sigma) for 20 min at  $37^\circ\text{C}$ . After centrifugation for 10 min at  $4^\circ\text{C}$  at  $750g$ , the supernatant was obtained.

**Treatment of Rats with LPS.** Male SD rats (7–9 weeks old; SLC Inc., Hamamatsu, Japan) weighing 180 to 200 g were housed in an air-conditioned room with free access to CE-2 commercial food pellets (Clea, Tokyo, Japan). The diet was changed to a synthetic basic diet, which consisted of 38% corn starch, 25% casein, 10%  $\alpha$ -starch, 8% cellulose powder, 6% minerals, 5% sugar, 2% vitamins, and 6% linoleic acid (Oriental Yeast Co., Tokyo, Japan) 1 week before the experiment. Three rats were assigned to each experimental group. Animals were maintained and handled according to the Guidelines for the Regulation of Animal Experimentation Committee of the University of Shizuoka. The rats fasted overnight, received an i.v. injection of LPS (*Escherichia coli* 0111:B4; Difco, Detroit, MI) at a dose of  $750\ \mu\text{g/kg}$ , and were anesthetized with ethyl ether 4.5 h after injection. The rat blood was withdrawn from the abdominal aorta into EDTA-treated tubes, and the plasma was prepared by centrifugation for 10 min at  $900g$  for  $\beta$ -glucuronidase assay. Luteolin ( $50\ \mu\text{mol/kg}$ ) was administered to rats by gastric intubation 3.5 h after LPS injection, and the plasma was prepared 0.5, 1, and 4.5 h after oral administration of luteolin for HPLC analysis.

**Sample Preparation of Human Serum.** Venous blood of patients on hemodialysis and healthy volunteers who gave informed consent was collected in vacuum containers early in the morning at the Social Insurance of Chukyo Hospital (Nagoya, Japan). The serum was prepared by centrifugation for 10 min at  $900g$ .

**$\beta$ -Glucuronidase Assay.**  $\beta$ -Glucuronidase activity in rat plasma and human serum was assayed using the FluorAce  $\beta$ -glucuronidase reporter assay kit (Bio-Rad, Hercules, CA). Briefly,  $50\ \mu\text{l}$  of assay buffer and  $10\ \mu\text{l}$  of sample were added to each sample well of a 96-well microplate. The microtiter plate was incubated at  $37^\circ\text{C}$  for 30 min.  $\beta$ -Glucuronidase hydrolyzes 4-methylumbelliferyl-glucuronide resulting in release of the fluorescent molecule 4-methylumbelliferone (Fig. 1). The fluorescence of this molecule was measured on a microplate fluorometer using an excitation wavelength of 360 nm and emission wavelength of 460 nm.

To know whether  $\beta$ -glucuronidase released from the stimulated human neutrophils is able to hydrolyze flavonoid glucuronides to free aglycone, supernatants, which were obtained from the culture medium after neutrophils from two human healthy volunteers had been stimulated with or without ionomycin/cytochalasin B, were reacted with methanol extracts of plasma of rats orally administered luteolin at  $37^\circ\text{C}$  for 2 h. Methanol extracts of plasma were obtained by the method described previously (Shimoi et al., 1998). In brief, rat blood was withdrawn from the abdominal aorta into heparinized tubes after administration of luteolin ( $50\ \mu\text{mol/kg}$  in propylene glycol) by gastric intubation. The plasma (0.5 ml) was acidified with the same volume of 0.01 M oxalic acid. This solution was applied to a Sep-Pak  $\text{C}_{18}$  cartridge. After washing the cartridge with 0.01 M oxalic acid, methanol/water/0.5 M oxalic acid (25:73:2, v/v/v), and distilled water, the methanol eluate was obtained. The eluate was evaporated to dryness. The reaction mixture of the supernatant and methanol extract dissolved in a small amount of ethanol was acidified with the same volume of 0.01 M oxalic acid. This solution was applied to a Sep-Pak  $\text{C}_{18}$  cartridge, and then the methanol eluate, obtained by the same method as mentioned above, was evaporated to dryness. The residue dissolved in methanol was subjected to HPLC. The ratio of luteolin to luteolin monoglucuronide was determined by the peak areas on HPLC chromatograms.

**HPLC Analysis.** HPLC analysis was carried out by the method described previously (Shimoi et al., 1998). Briefly, the samples were analyzed chromatographically by a JASCO HPLC system (JASCO, Inc., Tokyo, Japan) using a Capcell Pak  $\text{C}_{18}$ -UG120 column ( $150 \times 4.6\ \text{mm}$ ; Shiseido, Tokyo, Japan) and UV detection (349 nm). Elution was performed using methanol with 0.03% trifluoroacetic acid (solvent A) and water with 0.01% trifluoroacetic acid (solvent B) in gradient conditions at the flow rate  $0.7\ \text{ml/min}$ .

**Statistical Analysis.** Statistical analysis was performed using Student's *t* test. Any difference between the two groups with a value of  $P < 0.05$  was considered significant.

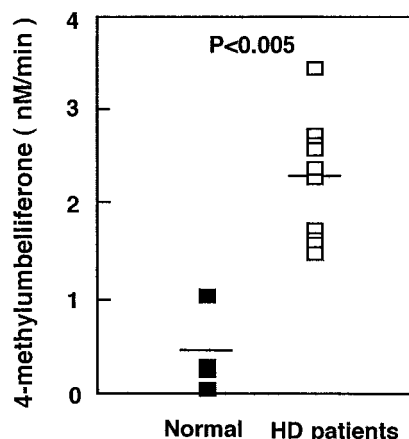


FIG. 2.  $\beta$ -Glucuronidase activity in serum of healthy volunteers ( $n = 4$ ) and patients on hemodialysis ( $n = 10$ ).

The serum was prepared by centrifugation for 10 min at  $900g$ . The  $\beta$ -glucuronidase activity in each plasma was assayed by liberation of 4-methylumbelliferone from 4-methylumbelliferyl-glucuronide.

## Results and Discussion

$\beta$ -Glucuronidase, which hydrolyzes glucuronides to liberate free aglycones, is located in the lysosomes and in endoplasmic reticulum (Wakabayashi, 1970; Paigen, 1989). This enzyme plays an important role in the hydrolysis of estrogen glucuronides (Zhu et al., 1996) and oligosaccharides (Buddecke and Hoefele, 1966). Moderate oxidative stress was found to rapidly induce partial lysosomal rupture, which was followed by apoptosis and further loss of intact lysosomes. The release of hydrolytic enzymes from the lysosomal compartment to the cytosol is an important initiating event in the apoptotic process (Brunk Ulf, 1999).  $\beta$ -Glucuronidase activity in both the liver and kidney increases with age perhaps because increasing fragility of lysosomal membrane allows these enzyme to leak out (Cristofalo and Kabakjian, 1975). In inflammation,  $\beta$ -glucuronidase is known to be released from granulocytes including neutrophils (Marshall et al., 1988). It has been reported that serum levels of proinflammatory cytokines such as interleukin-1 and C-reactive protein, circulating markers of inflammation, were increased in patients on hemodialysis (Docci et al., 1990; Pereira, 1995). Therefore, we measured  $\beta$ -glucuronidase activity in serum from patients on hemodialysis and healthy volunteers. As shown in Fig. 2,  $\beta$ -glucuronidase activity in serum from patients on hemodialysis increased significantly to  $2.26 \pm 0.66\ \text{nM/min}$  (mean  $\pm$  S.D.) compared with that from healthy volunteers,  $0.42 \pm 0.44\ \text{nM/min}$  (mean  $\pm$  S.D.) ( $P < 0.005$ ).

Supernatants, which were obtained from the culture medium after neutrophils from two human healthy volunteers who had been stimulated with or without ionomycin/cytochalasin B, were incubated with the methanol extract of rat plasma containing luteolin and luteolin monoglucuronide. Deglucuronidation activity in the supernatants was investigated by HPLC analysis. Figure 3A shows the HPLC chromatogram of the methanol extract of rat plasma used in this assay. The ratio of luteolin to luteolin monoglucuronide in the methanol extracts after treatment with the supernatants from stimulated neutrophils increased from 0.44 to 1.73 and 3.69, respectively (Fig. 3B). These results indicate that luteolin monoglucuronide can be a substrate for  $\beta$ -glucuronidase released from neutrophils.

The  $\beta$ -glucuronidase activity in rat plasma after LPS treatment increased significantly from  $1.38 \pm 0.86\ \text{nM/min}$  (mean  $\pm$  S.D.) to  $10.5 \pm 3.0\ \text{nM/min}$  (mean  $\pm$  S.D.) ( $P < 0.005$ ; Fig. 4A). The ratio of luteolin to luteolin monoglucuronide in plasma of LPS-treated rats also increased after luteolin administration (Fig. 4B). A significant

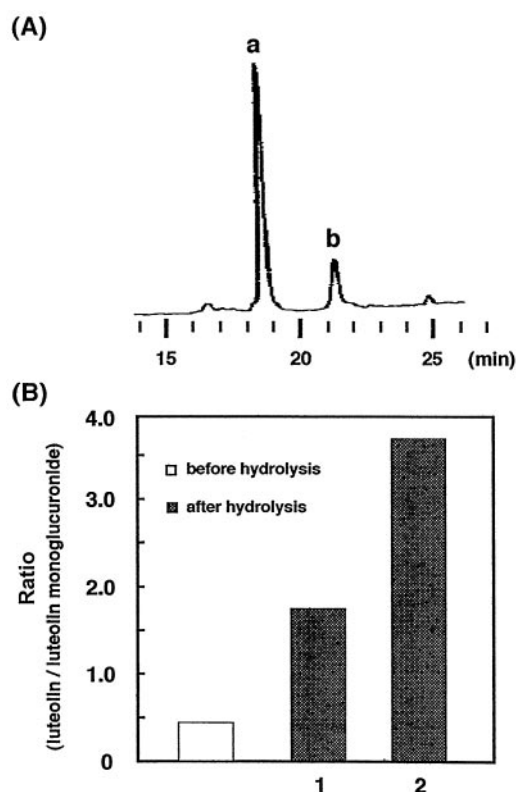


Fig. 3. A, HPLC chromatogram of the methanol extract of rat plasma 30 min after oral administration of luteolin (50  $\mu\text{mol/kg}$ ).

Peak a, luteolin monoglucuronide; peak b, luteolin. B, the ratio of luteolin to luteolin monoglucuronide of the methanol extract of plasma of rat orally administered luteolin after treatment with or without the supernatant that contains  $\beta$ -glucuronidase released from the stimulated human neutrophils. Neutrophils were isolated from venous blood of two healthy volunteers (1,  $5.4 \times 10^6$  cells; 2,  $7.6 \times 10^6$  cells). The reaction mixtures were analyzed by HPLC. The ratio of luteolin to luteolin monoglucuronide was determined by the peak areas on HPLC chromatograms.

increase was observed 0.5 and 1 h after luteolin administration [ $0.23 \pm 0.02$  to  $0.78 \pm 0.26$  (mean  $\pm$  S.D.),  $P < 0.005$ ;  $0.25 \pm 0.03$  to  $0.74 \pm 0.41$  (mean  $\pm$  S.D.),  $P < 0.01$ , respectively]. No difference in the plasma concentration of luteolin was observed between LPS-treated rats and nontreated rats. However, the concentration of luteolin monoglucuronide decreased by about half in the LPS-treated rats (data not shown). Although there is a possibility that  $\beta$ -glucuronidase for deglucuronidation is more active than UDP-glucuronyltransferase for glucuronidation in the liver when inflammation occurs, the results suggest that  $\beta$ -glucuronidase released from human granulocytes, including neutrophils or certain injured cells, appears in the blood or body fluids and hydrolyzes glucuronide conjugates of flavonoids to free aglycones at the inflammation site. The tissue fluid at the inflammation site has a low pH, which is favorable for  $\beta$ -glucuronidase, because  $\beta$ -glucuronidase exhibits activity at a very low pH (pH 4–5).

Manach et al. (1998) have reported that the conjugated derivatives of quercetin significantly delayed the  $\text{Cu}^{2+}$ -induced oxidation of lipoproteins. However, the magnitude of this inhibition was about one-half that measured with the aglycone. It has been demonstrated that quercetin-conjugated metabolites, such as quercetin-3' (or 4')-glucuronide, inhibit xanthine oxidase and lipoxygenase at 0.25  $\mu\text{M}$  ( $K_i$ ). This concentration is within the expected range after a meal rich in flavonols (Manach et al., 1998; Day et al., 2000). However, it is not clear at the present time whether or not the cells take up the flavonoid glucuronides.

In inflammatory responses such as those which are involved in the

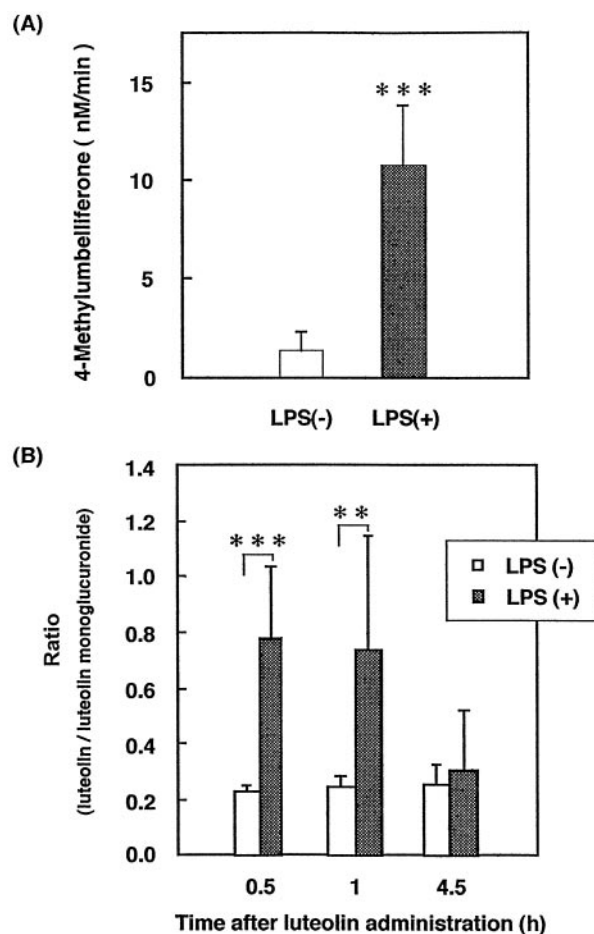


Fig. 4. A,  $\beta$ -glucuronidase activity in plasma of rats treated with or without LPS.

The blood was collected from the abdominal aorta into EDTA-treated tubes 4.5 h after i.v. injection of LPS (750  $\mu\text{g/kg}$ ).  $\beta$ -Glucuronidase activity was assayed with 4-methylumbelliferone, as described in the legend to Fig. 2. The values are mean  $\pm$  S.D. ( $n = 3$ ; \*\*\*,  $P < 0.005$ ). B, the ratio of luteolin to luteolin monoglucuronide in plasma of LPS-treated rats at different times after luteolin administration. Luteolin (50  $\mu\text{mol/kg}$ ) was administered to rats by gastric intubation 3.5 h after LPS injection. The ratio of luteolin to luteolin monoglucuronide was determined by the peak areas on HPLC chromatograms. The values are mean  $\pm$  S.D. ( $n = 3$ ; \*\*\*,  $P < 0.005$ ; \*\*,  $P < 0.01$ ).

pathogenesis of atherosclerosis, cancer, rheumatoid arthritis, diabetic nephropathy, etc., neutrophils generate  $\text{O}_2^-$  and release lysosomal enzymes such as  $\beta$ -glucuronidase. Because the released  $\beta$ -glucuronidase can hydrolyze glucuronide conjugates of flavonoids to free aglycones, flavonoids in the free form become more antioxidant. They come into the cells and exert biological effects at inflammation sites. The flavonoids might play a role in the prevention of diseases mentioned above.

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## References

- Brunk Ulf T (1999) Lysosomal leakage causes apoptosis following oxidative stress, growth-factor starvation and fas-activation, in *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease* (Kumpulainen JT and Saloonen JT eds) pp 57–66, The Royal Society of Chemistry, Cambridge.

- Buddecke E and Hoefele O (1966) Reinigung und eigenschaften der  $\beta$ -glucuronidase aus der aorta des rindes. *Hoppe-Seyler's Z Physiol Chem* **347**:173–191.
- Cristofalo CA and Kabakijiam J (1975) Lysosomal enzymes and aging in vitro: subcellular enzyme distribution and effect of hydrocortisone on cell life-span. *Mech Aging Dev* **4**:19–28.
- Day AJ, Bao Y, Plumb GW, Kroon PA, O'leary KA, Morgan MRA, and Williamson G (2000) Quercetin glucuronidation and effect of conjugation position on biological activity, in *Polyphenols Communications 2000* (Martens S, Treutter D, and Forkmann G eds) Technische Universitat Munchen, Freising-Weiheinstephan, Germany.
- Docci D, Bilancioni R, Buscaroli A, Baldralti L, Capponcini C, Mengozzi S, Turci F, and Feletti C (1990) Elevated serum levels of C-reactive protein in hemodialysis patients. *Nephron* **56**:364–367.
- Ferriola PC, Cody V, and Middleton E Jr (1989) Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *Biochem Pharmacol* **38**:1617–1624.
- Heneghan JB (1988) Alimentary tract physiology: interaction between the host and its microbial flora, in *Role of the Gut Flora in Toxicity and Cancer* (Rowland IR ed) pp 39–78, Academic Press, San Diego, CA.
- Manach C, Morand C, Crespy V, Demigne C, Texier O, Regerat F, and Remesy C (1998) Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett* **426**:331–336.
- Marshall T, Shult P, and Busse WW (1988) Release of lysosomal enzyme beta-glucuronidase from isolated human eosinophils. *J Allergy Clin Immunol* **82**:550–555.
- Paigen K (1989) Mammalian beta-glucuronidase: genetics, molecular biology, and cell biology. *Prog Nucleic Acid Res Mol Biol* **37**:155–205.
- Pereira BJ (1995) Cytokine production in patients on dialysis. *Blood Purification* **13**:135–146.
- Sadzuka Y, Sugiyama T, Shimoi K, Kinae N, and Hirota S (1997) Protective effect of flavonoids on doxorubicin-induced cardiotoxicity. *Toxicol Lett* **92**:1–7.
- Scalbert A and Williamson G (2000) Dietary intake and bioavailability. *J Nutr* **130**:2073S–2085S.
- Shimoi K, Masuda S, Furugori M, Esaki S, and Kinae N (1994) Radioprotective effect of antioxidative flavonoids in  $\gamma$ -ray irradiated mice. *Carcinogenesis* **15**:2669–2672.
- Shimoi K, Okada H, Furugori M, Goda T, Takase S, Suzuki M, Hara Y, Yamamoto H, and Kinae N (1998) Intestinal absorption of luteolin and luteolin 7-O- $\beta$ -glucoside in rats and humans. *FEBS Lett* **438**:220–224.
- Wakabayashi M (1970)  $\beta$ -Glucuronidases in metabolic hydrolysis, in *Metabolic Conjugation and Metabolic Hydrolysis* (Fishman WH ed) vol 2, pp 520, Academic Press, New York.
- Yamamoto H, Sakakibara J, Nagatsu A, and Sekiya K (1998) Inhibitors of arachidonate lipoygenase from defatted perilla seed. *J Agric Food Chem* **46**:862–865.
- Yasukawa K, Takido M, Takeuchi M, and Nakagawa S (1989) Effect of chemical constituents from plants on 12-O-tetradecanoylphorbol-13-acetate-induced inflammation in mice. *Chem Pharm Bull* **37**:1071–1073.
- Zhu BT, Evaristus EN, Antoniak SK, Sarabia SF, Ricci MJ, and Liehr JG (1996) Metabolic deglucuronidation and demethylation of estrogen conjugates as a source of parent estrogens and catecholestrogen metabolites in Syrian hamster kidney, a target organ of estrogen-induced tumorigenesis. *Toxicol Appl Pharmacol* **136**:186–193.