Presence of Aldose Reductase Inhibitors in Tea Leaves

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ABSTRACT—Water extract from commercial English tea has a potent inhibitory activity against human placenta aldose reductase (NADPH oxidoreductase, E.C.1.1.1.21). Inhibitory activity was separated into five major fractions by one-step chromatography with a C-18 reverse phase column. The most active fraction was further subjected to reverse phase column chromatography. As a result, a well-known flavone-glycoside, isoquercitrin, was isolated as the most potent chemical. The inhibitory character of isoquercitrin for aldose reductase was a mix of uncompetitive and noncompetitive inhibitions, and its IC_{50} was 1×10^{-6} M. In rat sciatic nerve tissue preparations, sorbitol accumulation in the presence of high concentrations of glucose (30 mM) was inhibited by 38% at 5×10^{-4} M of isoquercitrin. The flavone-glycoside isoquercitrin is the active inhibitor of aldose reductase inhibitor present in English tea. Given the ability of aldose reductase inhibitors to prevent diabetic complications, an epidemiological study of the effect of tea consumption on the pathogenesis and progression of diabetic complications would be interesting.

Keywords: Aldose reductase, Enzyme inhibitor, Tea leaf, Human placenta, Diabetes

Recent clinical observations have shown that strict long-term control of blood glucose in diabetic patients significantly reduces the onset or progression of diabetic complications such as retinopathy, neuropathy and nephropathy (1, 2). While the mechanism underlying the transition to diabetic complications is unclear, some complications could be due to, in part, the hyperglycemia-activated polyol metabolic pathway catalyzed by aldose reductase (alditol: NADPH oxidoreductase, E.C.1.1.1.21), resulting in excessive accumulation of the glucose metabolite sorbitol in tissues. Supporting this assumption, sorbitol accumulation has been observed in the crystalline lens of experimental diabetic rats, resulting in induction of osmotic stress followed by tissue damage (3–6).

Since 1981, a number of aldose reductase inhibitors (ARIs) have been investigated (7–9), and shown to prevent the development of experimental cataracts in animals, as well as the functional anatomic abnormalities of diabetic retinopathy and nephropathy. However, clinical trials of these ARIs do not clearly support the experimental results (10–13). Of the ARIs evaluated, only tolrestat (14, 15) and epalrestat (16) were reported to have clinical efficacy in the early stages of diabetic neuropathy.

In the course of searching for ARIs in extracts of natural

plants, we found inhibitory activity against aldose reductase in common English tea. In this report, we analyze and identify one of the active constituents of tea leaves and discuss the significance of this to diabetic complications.

MATERIALS AND METHODS

Materials

Fresh human placenta was obtained immediately after normal term delivery of an uncomplicated pregnancy. Mothers gave informed consent for use of placental tissue. The tissue was perfused with cold saline, excised of membranes and connective tissue, and then used immediately or stored at -70°C . Nicotinamide adenine dinucleotide phosphate (NADPH), D- and L-glyceraldehyde, and D-glucose were obtained from Wako Pure Chemical Industry (Tokyo). Protease inhibitors, antipain and leupeptin, were obtained from Peptide Institute, Inc. (Osaka).

Preparation of aldose reductase

A 30-mg sample of human placenta was homogenized in 5 vol of 10 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 2 mM β -mercaptoethanol, and 2 $\mu\text{g}/\text{ml}$ each of antipain and leupeptin. Following centrifugation of the homogenate at $105,000 \times g$ for 30 min, the supernatant was diluted 2 times with distilled water, subjected to 40% ammonium sulfate precipitation and centrifuged. The resulting

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supernatant was subjected to 70% ammonium sulfate precipitation followed by centrifugation. The precipitate was suspended in 10 ml of phosphate buffer, pH 7.4, containing 2 mM β -mercaptoethanol and 2 μ g/ml each of antipain and leupeptin; this was then used as an enzyme source of aldose reductase (17).

Inhibition assay

Routine assay of aldose reductase inhibition was carried out in a reaction mixture containing 50 mM sodium phosphate, 100 mM ammonium sulfate, 0.1 mM NADPH, 5 mM glyceraldehyde, enzyme and sample (inhibitor) in a total volume of 1 ml. The reaction was initiated by the addition of glyceraldehyde and monitored at 340 nm for 5 min at 25°C with a Ubest-50 Spectrophotometer (Jasco, Tokyo). As a control, absorbance at 340 nm was monitored in the absence of inhibitor.

Isolation and determination of ARI of tea leaves

Ten tea-bags (from Lipton, Brooke Bond or Nitto) were soaked in 1 l of hot water (90–95°C) for 30 min. The extract was left on ice overnight, centrifuged, and the clear supernatant was concentrated by lyophilization. The lyophilate was dissolved in 100 ml of distilled water and applied to a C-18 reverse phase column (5.5 $\times$ 30 cm, ODS-80TS; Tosoh, Tokyo) equilibrated with 20% acetonitrile containing 0.1% trifluoroacetic acid. The column was washed with 3 bed volumes of 20% acetonitrile and eluted with 5 bed volumes of a linear gradient of 20–40% acetonitrile containing 0.1% trifluoroacetic acid. Four-milliliter fractions were collected. Aliquots of 200 μ l were withdrawn from every other fraction, lyophilized, and examined in the inhibition assay.

For further purification of inhibitor(s), one of the active fractions from the C-18 column chromatography, fraction D (Fig. 1), was lyophilized and subjected to analytical reverse phase column chromatography (0.46 $\times$ 25 cm, TSKgel ODS-80TM; Tosoh). The bound materials were eluted with 5 bed volumes of a linear gradient of 30% to 40% acetonitrile containing 0.1% trifluoroacetic acid. The major fraction was lyophilized and subjected to structural analysis by infrared spectrometry (IR), nuclear magnetic resonance spectrometry (NMR) and mass spectrometry (MS).

Determination of sorbitol in nerve tissue

Sciatic nerve tissues were excised from rats and soaked in 3 ml of Krebs-Ringer bicarbonate buffer, pH 7.2, containing 30 mM glucose, in the presence or absence of ARI. At the indicated time, tissues were removed, placed on a filter paper to remove adherent fluid, weighed and then homogenized in 0.5 ml of cold 8% perchloric acid. Following centrifugation of the homogenate, the clear supernatant was recovered, neutralized with 2 N KOH and assayed for

sorbitol. Sorbitol content was determined enzymatically by the sorbitol dehydrogenase reaction (18, 19). The amount of sorbitol is proportional to the formation of NADPH as measured by the increase in extinction at 340 nm. Results were statistically analyzed by one way ANOVA with the Bonferroni/Dunn test as the post hoc test, and a difference is significant when $P \leq 0.05$ vs control.

RESULTS

Isolation of inhibitor from tea-leaf extract

A typical elution profile of ARIs on the C-18 reverse phase column is shown in Fig. 1. The hot water extract of tea leaves, which exhibited a potent inhibitory activity, was separated into 5 fractions (Fig. 1, designated A, B, C, D and E). Initially, partial extraction of the inhibitors from tea extract was attempted using organic solvents including ethylacetate, acetone or butanol; unfortunately, the inhibitory activity remained in the aqueous phase and not the organic phase. In addition, since pretreatment with these organic solvents showed no advantage over subsequent C-18 reverse phase column chromatography, pretreatment procedures were deleted. Among the five active fractions, fraction D (Fig. 1) exhibited the highest specific inhibitory activity; this fraction was pooled, concentrated, and applied to an analytical reverse phase column. The activity was eluted in one peak with a negligible peak on the shoulder (data not shown). The active fractions were pooled and subjected to structural analyses by IR, NMR and MS. ARI in this fraction was identified as the well-known flavone-glycoside isoquercitrin (Fig. 2).

Characteristics of isoquercitrin

The potency of isoquercitrin to human aldose reductase appeared equal to that of the known ARI, ONO-2235 (Epalrestat) (Fig. 3). The IC_{50} was 1×10^{-5} M. When an inhibitory mechanism is elucidated, the binding site(s) of the inhibitor on the enzyme should be considered: binding sites of either the substrate or cofactor (NADPH) or elsewhere on the enzyme. First, the possibility of interference with the substrate binding site was examined (Fig. 4). Isoquercitrin at 1×10^{-6} M exhibited a parallel line with the control, characteristic of uncompetitive inhibition. Increasing the inhibitor to 3×10^{-6} M, however, resulted in convergence of the K_{ms} in the reciprocal plot to show noncompetitive inhibition. Our results indicate that the enzyme is inhibited in a mixed manner and not by binding to the site of the substrate.

Secondly, the possibility of competitive binding of the inhibitor to the site of NADPH binding was examined with fixed concentrations of substrate and inhibitor (Fig. 5). At lower concentrations of inhibitor (1×10^{-5} M), the inhibitory manner was uncompetitive, whereas at higher con-

Fig. 1. Typical elution profile of aldose reductase inhibitors of tea extract. Water extract of tea leaves was applied on a C-18 reverse phase column. After washing with 40 ml of 20% acetonitrile (∇), inhibitors were eluted with linear gradients of 20% to 40% acetonitrile (----). Elution profile was monitored by absorbance at 260 nm (—), and inhibitory activities of fractions were monitored by the human placenta aldose reductase assay (o---o).

Fig. 2. Chemical structure of isoquercitrin.

centrations, it was noncompetitive. These results indicate that inhibition occurred at a site independent from that of the cofactor. ONO-2235 (20) and sorbinil (18) are ARIs with a similar mixed type of inhibition. So far, all ARIs which have been subjected to enzyme kinetic studies have demonstrated activity by binding at a site independent of the substrate or cofactor (NADPH) binding sites (20–22). However, structural analysis of crystallized human aldose reductase by electron microscopy has not revealed the inhibitor binding site (23). Nevertheless, these inhibitors have been shown to be effective in preventing some of the complications of diabetes in animal models.

Fig. 3. Inhibition of human placenta aldose reductase. Aldose reductase activities in the presence of various concentrations of isoquercitrin ($\square$) or ONO-2235 (----) were measured as described in Materials and Methods. Reductase activity in the absence of inhibitor was defined as 100%.

Effect of isoquercitrin on sorbitol accumulation in sciatic nerve tissue

When sciatic nerve tissue is incubated in 30 mM glucose, sorbitol accumulated linearly over time. Sorbitol levels increased 11-fold in 4 h and 15-fold in 6 h (data not shown).

Fig. 4. Effect of isoquercitrin on the Lineweaver-Burke plot of aldose reductase activity. The ordinate and abscissa represent the reciprocals of initial velocity and glyceraldehyde concentration, respectively. Activity was measured in the absence (■) or presence of isoquercitrin at concentrations of 1×10^{-6} M (□) or 3×10^{-6} M (▲).

Fig. 5. Effect of isoquercitrin on the Lineweaver-Burke plot of aldose reductase activity. The ordinate and abscissa represent the reciprocals of initial velocity and NADPH concentration, respectively. Activity was measured in the absence (■) or presence of isoquercitrin at concentrations of 1×10^{-6} M (□) or 3×10^{-6} M (▲).

In the presence of isoquercitrin, sorbitol accumulation was inhibited in a dose-dependent manner (Fig. 6). At 5×10^{-4} M, sorbitol accumulation was inhibited by 38%. In comparison with ONO-2235, which exhibits an IC_{50} of $0.39 \mu\text{g/ml}$, isoquercitrin was less potent.

DISCUSSION

In this study, we demonstrated ARIs in common English tea. One of these inhibitors was the flavone-glycoside isoquercitrin. Since the potency of isoquercitrin is equivalent to that of ONO-2235 in enzyme assays of aldose reductase, we expected the same inhibitory activity as ONO-2235 on

Fig. 6. Effect of isoquercitrin on sorbitol accumulation in nerve tissue. Isolated rat sciatic nerve tissue was incubated in the presence of 30 mM glucose with or without 5×10^{-4} M of isoquercitrin for 4.5 h, and sorbitol content in tissue was measured as described in Materials and Methods. Sorbitol accumulation (mean $\pm$ S.D., $n = 4$) in the tissue was significantly inhibited at the higher concentrations of isoquercitrin than 1×10^{-5} M ($P \leq 0.05$ vs control).

sorbitol accumulation in nerve tissue. Supposing its minimal effective dose is 1 mg/day, we can expect its pharmacological benefit from daily consumption of more than 10 tea bags. However, isoquercitrin had much less activity than ONO-2235, presumably due to its low lipophilicity and poor permeability, as noted by its poor solubility in organic solvents (see our purification procedure). Despite its relatively weak activity as an ARI in tissue, in combination with other bioactivities of isoquercitrin (24, 25), tea consumption might have a beneficial chronic effect. There are no epidemiological data on the relationship between tea consumption and the pathogenesis or progression of diabetic complications. It would be interesting to determine whether drinking tea daily affects sorbitol accumulation and the pathogenesis of diabetic complications.

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