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Inhibitory Effects of Tenilsetam on the Maillard Reaction

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ABSTRACT

It has been hypothesized that advanced Maillard reaction *in vivo* could explain some of the age- and diabetes-related changes. Furthermore, involvement of the Maillard reaction with Alzheimer's disease has also been suggested, as advanced glycation end products, such as pyrraline and pentosidine, were demonstrated to localize in lesions of the disease. Although aminoguanidine has been studied extensively and established as an inhibitor of the Maillard reaction, other candidates have not been investigated thoroughly. In the present study, we examined the inhibitory effect of tenilsetam [(±)-3-(2-thienyl)-2-piperazinone], an antidementia drug, on the Maillard reaction. Tenilsetam inhibited glucose- and fructose-induced polymerization of lysozyme in a concentration-dependent manner *in vitro*. Reduced enzymatic digestibility of collagen incubated with 100 mM glucose for 4 weeks was also restored to a control level by coincubation with 100 mM tenilsetam. To determine whether tenilsetam inhibits the Maillard reaction *in vivo*, streptozotocin-induced diabetic rats were treated with tenilsetam (50 mg/kg·day). Elevated levels of advanced glycation end-product-derived fluorescence and pyrraline in renal cortex and aorta of diabetic rats were suppressed by the administration of tenilsetam for 16 weeks. These inhibitory effects of this agent on advanced glycation in diabetic rats suggested its potential therapeutic role in controlling diabetic complications. (*Endocrinology* **138:** 1886–1892, 1997)

THE MAILLARD reaction is initiated by the nonenzymatic reaction of reducing sugars with free amino groups on protein to form Amadori product. The Amadori product irreversibly undergoes a variety of dehydration and rearrangement reactions, leading to the formation of advanced glycation end products (AGEs). Accumulation of AGEs has been thought to play a role in the pathogenesis of diabetic complications as well as the aging process (1–3). Recent immunochemical studies by several investigators proved the presence of AGEs *in vivo* (4–7) as well as Amadori product (8). Although AGEs are known to alter structural and functional properties of proteins (9–11), the exact pathway leading to the formation of AGEs has not yet been completely identified. However, despite their complexity, the structures of several AGEs have been described, *i.e.* those of pyrraline (12), pentosidine (13), crossline (14), and pyrropyridine (15). Furthermore, oxidation of Amadori product forms carboxymethyllysine (16). Kimura *et al.* recently suggested that carboxymethyllysine accumulated in human hippocampal neurons with age (17). Dicarbonyl compounds, including 3-deoxyglucosone (3-DG), are also known to form as reactive intermediates of an advanced stage of the Maillard reaction (18). The extent of AGE formation in tissue has been estimated by measuring the fluorescence derived from AGEs at excitation/emission = 370/440 nm (19) and 328/378 nm (20).

It has been suggested that inhibition of the Maillard reaction may prevent the progress of diabetic complications and slow the aging process. Since Brownlee *et al.* focused on a nucleophilic hydrazine compound, aminoguanidine (AG) (21), several lines of evidence now suggest that AG could inhibit the Maillard reaction both *in vitro* and *in vivo* (22–26). The action of AG is probably due to trapping of intermediates of advanced Maillard reaction, such as 3-DG, leading to the inhibition of further progress of the Maillard reaction (27). The roles of other agents as potential inhibitors of the Maillard reaction have not yet been investigated thoroughly. Tenilsetam [(±)-3-(2-thienyl)-2-piperazinone] is an antidementia drug used for the treatment of senile dementia and in clinical trials in Alzheimer's disease (28–32). Recently, the involvement of AGE modification in the lesion of Alzheimer's disease has been proposed (33, 34). Immunohistochemical analysis by Smith *et al.* (33) demonstrated that pyrraline and pentosidine were localized in lesions of Alzheimer's disease. In addition, Vitek *et al.* (34) suggested that AGEs contributed to amyloidosis in Alzheimer's disease. Considering that senile dementia is an aging process, a positive action of tenilsetam on aging could include inhibition of the Maillard reaction. We, therefore, examined in the present study the effect of tenilsetam on the Maillard reaction *in vitro*. Subsequently, we compared AGE formation in the tissues of diabetic rats with and without administration of tenilsetam. In addition, the reactivity of tenilsetam to 3-DG was investigated using a specific HPLC assay for 3-DG to compare the action of tenilsetam with that of AG.

Materials and Methods

Reagents and materials

Lysozyme (6 × crystallized, from egg white) was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Type I collagen (from bovine Achilles tendon), collagenase (type VII), AG (hemisulfate salt), and streptozotocin (STZ) were purchased from Sigma Chemical Co. (St. Louis, MO). Tenilsetam was supplied by Cassella (Frankfurt, Germany). 3-DG was supplied by Dr. H. Kato (University of Tokyo, Tokyo, Japan);

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the substance was synthesized according to a modified method of Khadem *et al.* (35, 36). All other chemicals and substances were of analytical reagent grade unless stated otherwise.

Lysozyme polymerization

We incubated 10 mg/ml lysozyme solution with 100 mM glucose, fructose, or 10 mM 3-DG in the presence and absence of tenilsetam (10 or 100 mM) for 28 days in 0.1 M sodium phosphate buffer (PB; pH 7.4) at 37 C. The samples were later dialyzed against distilled water to remove excess sugars and tenilsetam. All samples were then evaporated to dryness after determining the concentrations of proteins according to the method of Bradford (37). Samples were reconstituted in a sample buffer of 50 mM Tris-HCl, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, and 5% (vol/vol) β-mercaptoethanol (pH 6.8) at a protein concentration of 1 mg/ml, then boiled for 4 min at 95 C and subjected to SDS-PAGE, conducted on a 15% separating gel (38). An electrophoresis calibration kit for low molecular mass proteins (Pharmacia Co., Piscataway, NJ) was used as a molecular mass marker. Gels were stained with Coomassie brilliant blue R-250 (Nacalai Tesque Co., Kyoto, Japan) and destained with 40% methanol-10% acetic acid.

Measurement of collagen digestibility

We incubated type I collagen solution (10 mg/ml) with 100 mM glucose or fructose in the presence or absence of 10 or 100 mM tenilsetam in PB for 28 days at 37 C. Collagen was also incubated with buffer alone as a control. All samples were then dialyzed against distilled water and digested with an appropriate volume of type VII collagenase in 0.02 M HEPES buffer (pH 7.5) containing 0.1 M $CaCl_2$ for 48 h at 37 C. Soluble and insoluble fractions were separated by centrifugation at 8000 $\times$ *g* for 10 min. Each fraction was acid hydrolyzed in 6 N HCl for 24 h at 110 C. Finally, the amount of collagen in each fraction was estimated by the hydroxyproline assay of Stegmann and Stalder (39), and digestibility was calculated as the ratio of collagen in the soluble fraction to the total collagen content.

In addition, we examined whether the glucose concentration, comparable to blood glucose level in nontreated diabetic subjects, still had the suppressive effect on collagen digestibility. Collagen was incubated with 30 mM glucose in the presence or absence of tenilsetam for 28 days, followed by estimation of digestibility as described above. The data were expressed as the mean $\pm$ SD of three samples.

Animals

Male Sprague-Dawley rats (4–6 weeks old, 120–150 g BW) were randomly subdivided into three groups: group A, controls (n = 10); group B, STZ-induced diabetic rats (n = 8); and group C, tenilsetam-treated diabetic rats (n = 9). Diabetes was induced by a single iv injection of STZ (65 mg/kg BW) freshly dissolved in 200 μl sterile citrate buffer (pH 4.5). Only animals with plasma glucose levels over 20 mM after 1 week of injection were included in the study. Control rats were sham injected with the citrate buffer. Animals were provided with water *ad libitum* and fed standard laboratory chow. Group C rats were administered tenilsetam (50 mg/kg·day) orally using a catheter for 16 weeks. Principles of laboratory animal care were followed. Plasma glucose level was determined by glucose oxidase method (40), whereas glycated hemoglobin (Hb) was assayed using a boronate affinity chromatography with Gly-Affin-Ghb (Seikagaku Kogyo Co., Tokyo, Japan).

Tissue preparation

After 16 weeks of treatment, the animals were killed under ether anesthesia. The tissue preparation was carried out by the modified method of Monnier *et al.* (3). Briefly, the renal cortex and aorta from each animal were dissected, minced with a blade, and washed in cold PBS, followed by delipidation using 5 ml chloroform-ethanol (2:1, vol/vol) with gentle shaking overnight at room temperature. Two milliliters of methanol and 0.5 ml water were added in the middle of this period. The sample was centrifuged at 1500 $\times$ *g* for 10 min at 4 C, and the supernatant was removed by aspiration. The pellet was consecutively washed twice with 5 ml methanol, three times with distilled water, and twice with 0.02 M HEPES buffer (pH 7.5) containing 0.1 M $CaCl_2$ (buffer A). The buffer was then removed, and the pellet was resuspended in 2 ml buffer A containing 400 U type VII collagenase. Two microliters of toluene were added as a preservative. The enzymatic digestion was thoroughly performed under constant gentle shaking at 37 C for 60 h. After centrifugation at 8000 $\times$ *g* for 10 min at 4 C, the collagen content in collagenase-soluble and insoluble fractions from each sample was estimated using the hydroxyproline assay as described previously (39). Thus, it appeared that more than 95% of each tissue collagen was solubilized. The soluble fraction of each sample was subsequently used to measure collagen-linked fluorescence and the level of pyrraline. Collagenase alone was incubated under the same conditions mentioned above as a blank.

Fluorescence measurement

The collagen concentration of each digested tissue sample was adjusted to 3 mg/ml in HEPES buffer solution for fluorescence measurement. Fluorescence intensity was measured in duplicate with excitation/emission at 370/440 nm and 328/378 nm on the Hitachi F-4010 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Fluorescence values were expressed as arbitrary units (AU) per mg collagen.

Enzyme-linked immunosorbent assay (ELISA) for pyrraline

Pyrraline levels of collagenase-digested renal cortex and aorta were assayed as described previously (41). Briefly, the concentration of collagenase-digested tissues was adjusted to 3 mg collagen/ml in HEPES buffer solution. They were preincubated with the same volume of 3000 times diluted monoclonal antibody against pyrraline for 3 h at 37 C and then applied to a well of a microtiter plate coated with BSA conjugated with caproyl pyrraline. After incubation for 1 h under gentle shaking, the well was washed three times with 0.1% ovalbumin (grade V, Sigma) in PBS and incubated with 1000 times diluted goat antimouse IgG conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) for 1 h at room temperature. After washing as described above, 100 μl *p*-nitrophenyl phosphate (Sigma) substrate solution were added, and gentle shaking was maintained to develop coloration. Absorbance at 405 nm of each well was measured by ELISA reader (Bio-Rad, Richmond, CA). Quantitation was performed in duplicate, with lysyl pyrraline as a standard.

Reactivity of tenilsetam to 3-DG

3-DG, a highly reactive intermediate of the Maillard reaction, is suggested to also be a precursor of AGEs such as pyrraline and pentosidine. A 50-μM concentration of 3-DG was incubated in the presence or absence of 5 mM tenilsetam in PB (pH 7.4) at 37 C. AG was also incubated with 3-DG under the same conditions as those used for tenilsetam. Incubated samples were obtained periodically to measure the level of the remaining 3-DG by HPLC as described previously (42). Briefly, an aliquot of the sample was incubated overnight with 100 μl 0.1% 2,3-diaminonaphthalene (Aldrich Chemical Co., Milwaukee, WI) at 4 C in the presence of 50 μl 0.05% 3,4-hexanedione (Tokyo Kasei Organic Chemicals, Tokyo, Japan) as an internal standard. The reaction mixture was extracted using 4 ml ethyl acetate, followed by evaporation to dryness. The dried extract was reconstituted with 200 μl methanol and applied to HPLC. Conversion of 3-DG to a stable compound, 2-(2,3,4-trihydroxy butyl)-benzo-[*g*]quinoxaline, was accomplished by reaction with 2,3-diaminonaphthalene as mentioned above. The derivative had a characteristic UV spectrum, which was determined at 268 nm using HPLC. A peak height ratio of a 3-DG-derived peak to an internal standard peak was calculated. Quantitation of 3-DG was performed by comparing the peak height ratio with that of authentic 3-DG.

Statistical analysis

Data were expressed as the mean $\pm$ SD. Welch and Student's *t* tests were used for statistical analysis. $P < 0.05$ denoted statistical significance.

Results

Effect of tenilsetam on lysozyme polymerization

The rates of lysozyme polymerization induced by glucose, fructose, and 3-DG were analyzed by SDS-PAGE (Fig. 1).

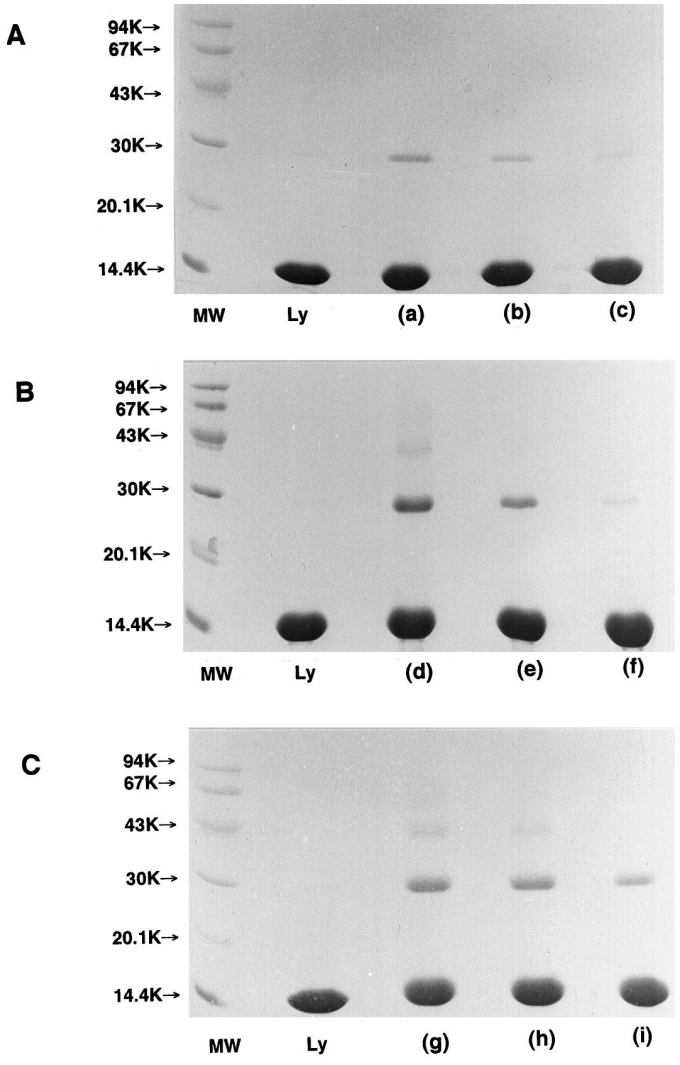

FIG. 1. SDS-PAGE of lysozyme incubated with glucose, fructose, or 3-DG in the presence and absence of tenilsetam. Effect of tenilsetam on glucose (A)-, fructose (B)-, or 3-DG (C)-induced polymerization of lysozyme was investigated. Lysozyme solution (10 mg/ml) was incubated with 100 mM glucose or fructose or with 10 mM 3-DG in the presence and absence of tenilsetam for 28 days in 0.1 M PB (pH 7.4) at 37 C as described below. Each sample was applied to SDS-PAGE, conducted on a 15% separating gel. The rate of polymerization was suppressed by tenilsetam. MW, Standard proteins (molecular mass in kilodaltons, listed on the *left*). Ly, Lysozyme alone. A: a, Lysozyme plus glucose; b, lysozyme, glucose, and 10 mM tenilsetam; c, lysozyme, glucose, and 100 mM tenilsetam. B: d, lysozyme plus fructose; e, lysozyme, fructose, and 10 mM tenilsetam; f, lysozyme, fructose, and 100 mM tenilsetam. C: g, lysozyme plus 3-DG; h, lysozyme, 3-DG, and 10 mM tenilsetam; i, lysozyme, 3-DG, and 100 mM tenilsetam.

Lysozyme incubated with 100 mM glucose showed dimer formation. Tenilsetam suppressed dimer formation in a dose-dependent manner (Fig. 1A). The same amount of fructose polymerized lysozyme more efficiently than glucose, yielding a dimer and a trimer. Formation of the latter was inhibited by 10 mM tenilsetam, whereas dimer formation was significantly suppressed by 100 mM tenilsetam (Fig. 1B). 3-DG caused dimer and trimer formation of the lysozyme when used at an even lower concentration. The polymer formation was also inhibited by coincubation with tenilsetam in a dose-dependent manner (Fig. 1C).

Effect of tenilsetam on collagen digestibility

The amount of digested collagen reached 77.9 ± 3.2% of the original total content when the collagen was incubated under the control conditions (buffer alone). The mean value was used as 100% to compare digestibility under other conditions. Incubation with 100 mM glucose significantly ($P < 0.005$) reduced collagen digestibility to 74.1 ± 4.9% compared with the control value. The digestibility of collagen was significantly recovered to 93.1 ± 8.6% with 10 mM tenilsetam and to 99.0 ± 2.1% with 100 mM ($P < 0.05$ and $P < 0.005$ *vs.* glucose alone, respectively). The rate of collagen digestibility was further decreased by fructose (39.1 ± 2.6% of control; $P < 0.001$). The reduction in digestibility was recovered to 61.4 ± 4.6% with 10 mM tenilsetam ($P < 0.005$ *vs.* fructose alone) and 76.2 ± 6.6% with 100 mM tenilsetam ($P < 0.001$ *vs.* fructose alone; Fig. 2).

When collagen was incubated with glucose at a diabetic blood sugar level such as 30 mM, the decrease in digestibility was still significant ($P < 0.05$) compared with the control value, as shown in Table 1. Even 10 mM tenilsetam efficiently recovered the decreased digestibility, whereas 100 mM tenilsetam almost completely restored it to the control level.

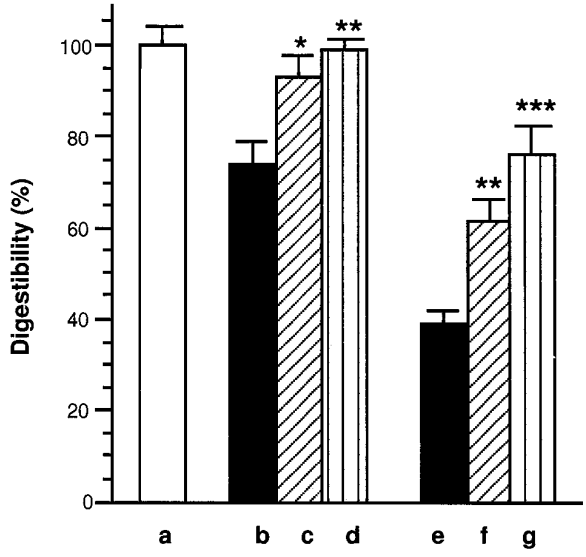

FIG. 2. Enzymatic digestibility of collagen incubated with glucose or fructose in the presence and absence of tenilsetam. The collagen concentration was 10 mg/ml in 0.1 M PB. Incubation was performed with 100 mM glucose or fructose in the presence and absence of tenilsetam at 37 C for 4 weeks. Each sample was digested by type VII collagenase, and the digestibility was expressed as a percentage of the digestibility of collagen incubated with PB alone. The digestibility of collagen was significantly reduced by the incubation with glucose and fructose ($P < 0.005$ and $P < 0.001$, respectively). The reduced digestibility of collagen was normalized by the coincubation with tenilsetam in a dose-dependent manner. Data are the mean ± SD (n = 3). a, Collagen alone; b, collagen and glucose; c, collagen, glucose, and 10 mM tenilsetam; d, collagen, glucose, and 100 mM tenilsetam; e, collagen plus fructose; f, collagen, fructose, and 10 mM tenilsetam; g, collagen, fructose, and 100 mM tenilsetam. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$ (*vs.* collagen incubated with the corresponding sugar alone).

In vivo studies

Table 2 shows the profile of animals in the present study. Although the increase in body weight was suppressed in diabetic rats (groups B and C), the administration of tenilsetam did not influence body weight. In the 16th week, the mean levels of plasma glucose and glycated Hb were significantly higher in groups B and C compared with the control values ($P < 0.01$). However, no differences in plasma glucose and glycated Hb levels between nontreated and tenilsetam-treated diabetic rats were observed.

After 16 weeks of diabetes, control rats did not exhibit abnormalities in the lens, whereas five of eight diabetic rats developed signs of lens opacity. In contrast, only one of nine tenilsetam-treated diabetic rats developed lens opacity.

Fluorescence intensity of renal cortex and aorta

After 16 weeks of diabetes, the fluorescence intensity (excitation, 370 nm; emission, 440 nm) of the renal cortex in diabetic rats was significantly higher than that in the control group (25.3 ± 2.9 *vs.* 18.8 ± 5.6 AU/mg collagen; $P < 0.01$). Fluorescence intensity returned to the control level with tenilsetam treatment (19.5 ± 2.3 AU/mg collagen), as shown in Fig. 3. The other fluorescence intensity (excitation, 328 nm; emission, 378 nm) characteristic for pentosidine increased significantly in the renal cortex of diabetic rats compared with the control value (60.5 ± 13.5 *vs.* 44.5 ± 11.6 AU/mg collagen; $P < 0.01$; Fig. 3). Concerning this type of fluorescence, the preventive action of tenilsetam against the increase in fluorescence intensity was similar to that observed in conventional fluorescence described above, so that the fluorescence intensity was not different from the control value (40.7 ± 9.9 AU/mg collagen).

These inhibitory effects of tenilsetam were confirmed by investigating the fluorescence intensity of aortas in the same animals (Fig. 4). The fluorescence intensity (excitation, 370 nm; emission, 440 nm) of the aorta in diabetic rat was significantly higher than that in the control group (21.4 ± 9.9 *vs.* 10.2 ± 2.7 AU/mg collagen; $P < 0.01$). The fluorescence intensity returned to the control level with tenilsetam treatment (8.1 ± 1.4 AU/mg collagen). The other fluorescence intensity (excitation, 328 nm; emission, 378 nm) also increased significantly in aortas of diabetic rats compared with that in the controls (64.3 ± 22.0 *vs.* 25.3 ± 4.4 AU/mg collagen; $P < 0.01$; Fig. 4). Fluorescence intensity was restored to the control level with tenilsetam treatment (27.5 ± 3.1 AU/mg collagen).

Pyrraline level in the rat renal cortex and aorta

As shown in Fig. 5, the level of pyrraline in the collagenase digest of renal cortex increased significantly in diabetic rats 16 weeks after the induction of diabetes compared with that in control rats (24.7 ± 2.4 *vs.* 19.7 ± 2.1 nmol/mg collagen; $P < 0.01$). Tenilsetam significantly prevented the increase in pyrraline (20.2 ± 3.3 nmol/mg collagen; $P < 0.01$ *vs.* untreated diabetic rats). The pyrraline level in the collagenase

TABLE 1. Effect of tenilsetam on the digestibility of collagen incubated with 30 mM glucose

Incubation of collagen	Enzymatic digestibility (%)
Control (buffer alone)	100 ± 4.1
30 mM glucose	89.0 ± 1.8[a]
30 mM glucose with 10 mM tenilsetam	97.4 ± 0.5[b]
30 mM glucose with 100 mM tenilsetam	99.8 ± 0.9[c]

Collagen was incubated with 30 mM glucose in the presence and absence of tenilsetam for 28 days, followed by the estimation of the digestibility. The mean digestibility under control conditions was considered to be 100% to compare the digestibility under other conditions. The data were expressed as the mean ± SD of three samples.

[a] $P < 0.05$ compared with control.

[b] $P < 0.005$ compared with 30 mM glucose.

[c] $P < 0.001$ compared with 30 mM glucose; $P < 0.05$ compared with 30 mM glucose plus 10 mM tenilsetam.

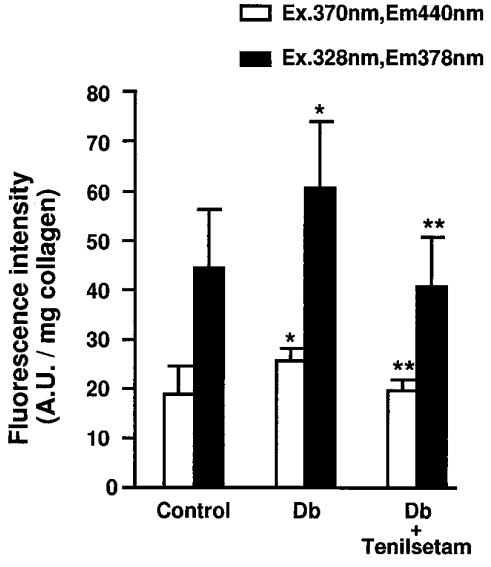

FIG. 3. Fluorescence intensity in the collagenase-soluble fraction of renal cortex measured at 370/440 and 328/378 nm excitation/emission. After 16 weeks of STZ treatment, the renal cortex of each animal was extracted and solubilized with collagenase. Fluorescence intensities in the soluble fraction of renal cortex were measured and corrected by collagen content. The fluorescence intensity was elevated in diabetic rats compared with that in controls and was suppressed in diabetics administered tenilsetam. *, $P < 0.01$ *vs.* control rats; **, $P < 0.01$ *vs.* untreated diabetic rats.

TABLE 2. Characteristics of rats used in the present experiment

Exp group	No.	BW (g)	Plasma glucose (mM)	Glycated Hb (%)
Control rats	10	516 ± 42	7.3 ± 0.9	7.2 ± 1.9
Untreated diabetic rats	8	226 ± 40[a]	31.2 ± 5.7[a]	20.5 ± 3.4[a]
Tenilsetam-treated rats	9	235 ± 30[a]	32.0 ± 2.8[a]	20.9 ± 3.2[a]

Male Sprague-Dawley rats were randomly subdivided into three groups: A) controls, B) diabetic rats, and C) tenilsetam-treated diabetic rats. Diabetes was induced by a single iv injection of STZ (65 mg/kg BW) freshly dissolved in 200 μl sterile citrate buffer (pH 4.5). Group C rats were given tenilsetam (50 mg/kg · day, orally). Body weight, plasma glucose level, and glycated Hb of each animal were measured after 16 weeks.

[a] $P < 0.01$ compared with control rats.

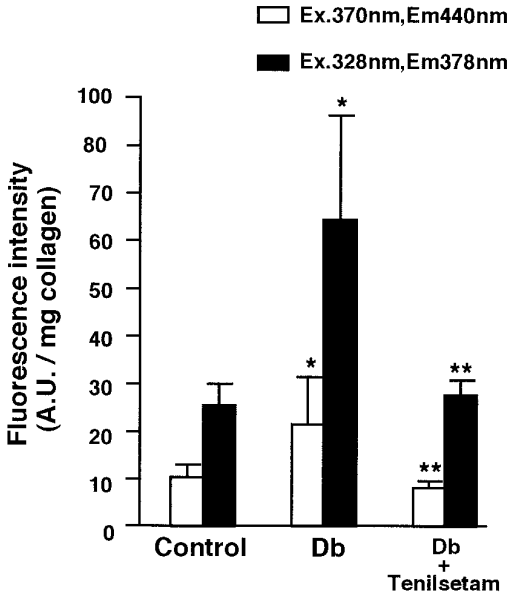

FIG. 4. Fluorescence intensity in the collagenase-soluble fraction of aorta measured at 370/440 and 328/378 nm excitation/emission. Fluorescence intensities of solubilized aorta were corrected by collagen content measured by hydroxyproline assay. The fluorescence intensity was elevated in diabetic rats compared with that in controls and was suppressed in diabetics administered tenilsetam. *, $P < 0.01$ *vs.* control rats; **, $P < 0.01$ *vs.* untreated diabetic rats.

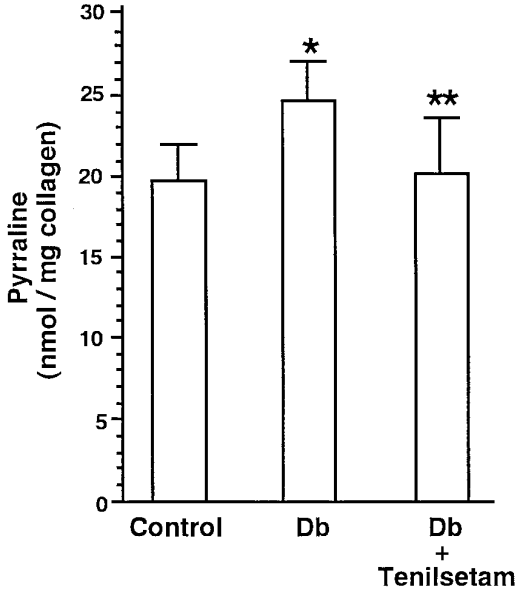

FIG. 5. Pyrraline level in the collagenase-soluble fraction of renal cortex. The pyrraline level in the renal cortex of diabetic rats, determined by ELISA using monoclonal antibody to pyrraline, was significantly elevated compared with that in control rats. The elevated level was suppressed to the control level by the oral administration of tenilsetam. *, $P < 0.01$ *vs.* control rats; **, $P < 0.01$ *vs.* untreated diabetic rats.

digest of aorta also significantly increased in diabetic rats compared with that in control rats (4.3 ± 1.8 *vs.* 0.3 ± 0.4 nmol/mg collagen; $P < 0.01$; Fig. 6). The administration of tenilsetam in diabetic rats significantly suppressed the in-

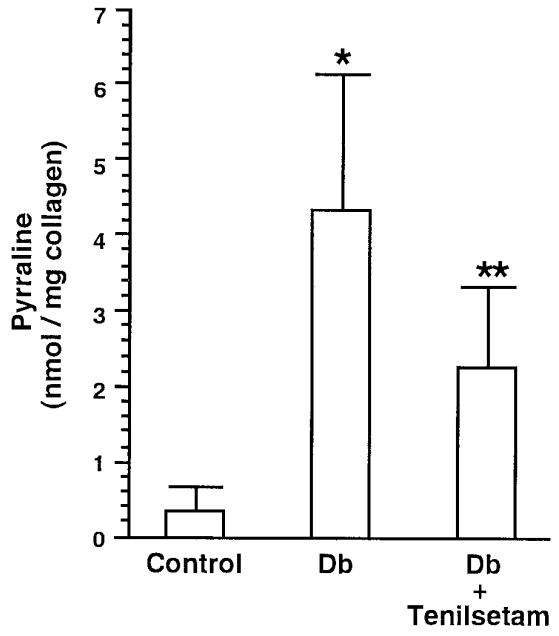

FIG. 6. Pyrraline level in the collagenase-soluble fraction of aorta. The pyrraline level in the soluble fraction of aorta from diabetic rats was significantly elevated compared with that in control rats, as determined by ELISA. It was suppressed to the control level by oral administration of tenilsetam. *, $P < 0.01$ *vs.* control rats; **, $P < 0.05$ *vs.* untreated diabetic rats.

crease in the pyrraline level of aorta (2.3 ± 1.1 nmol/mg collagen; $P < 0.05$).

In vitro direct reactivity of tenilsetam to 3-DG

A 50-μM concentration of 3-DG solution incubated with PB alone showed a negligible change in 3-DG concentration throughout the incubation period. The level of 3-DG was 42.4 ± 2.0 μM 72 h after incubation with the buffer (Fig. 7). Incubation with 5 mM tenilsetam also did not produce a significant change in the 3-DG concentration. Even after 72 h of incubation with tenilsetam, the concentration of free 3-DG was still 40.9 ± 1.0 μM. In contrast, 50 μM 3-DG solution incubated with 5 mM AG decreased the concentration of 3-DG to 24.1 ± 8.8 μM after 6 h of incubation and to an undetectable level after 24 h of incubation.

Discussion

Since the first description of the inhibitory effect of AG on the Maillard reaction, several other suppressive effects of the agent on the progression of diabetic complications have been reported (22–26). The present study focused on another agent, tenilsetam, previously used in human volunteers and patients with dementia of the Alzheimer type (29–32). Our results indicated that tenilsetam suppressed protein polymerization due to an *in vitro* glycation. Enzymatic collagen digestion is known to decrease after advanced glycation (43, 44). Tenilsetam prevented the reduction of collagen digestibility in a dose-dependent manner. Our results also demonstrated an inhibitory effect of tenilsetam on fructosylation of proteins. Fructose is known to react with proteins more efficiently than glucose (45). Our *in vitro* observations

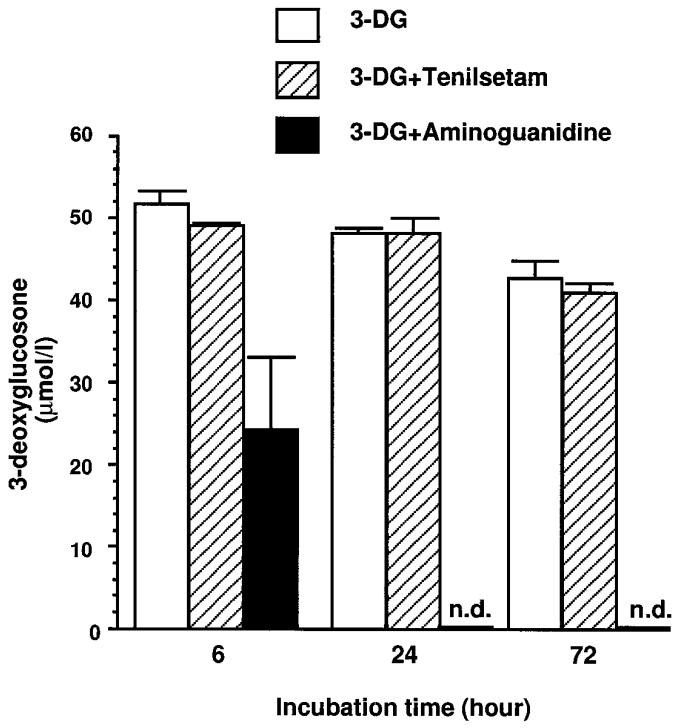

FIG. 7. Reactivity of tenilsetam to 3-DG. 3-DG solution (50 μM in PB, pH 7.4) was incubated in the presence and absence of 5 mM tenilsetam at 37 C. AG (5 mM) was also incubated with 3-DG under the same conditions as those used for tenilsetam. The remaining free 3-DG levels in the reaction mixtures were assayed periodically by HPLC. Tenilsetam did not significantly react with 3-DG, whereas AG directly trapped 3-DG.

showed that the rate of polymerization of lysozyme incubated with fructose was greater than that with glucose, and digestibility of collagen declined after incubation with fructose more than after incubation with glucose. Tenilsetam also inhibited these fructosylations of proteins in a dose-dependent manner. Furthermore, even 30 mM glucose, comparable to diabetic blood sugar level, showed a suppressive effect on collagen digestibility *in vitro*, although it was not as much as 100 mM glucose. This effect of 30 mM glucose was very efficiently inhibited by tenilsetam. It would be conceivable that longer exposure to high blood glucose *in vivo* would lead to more remarkable AGE formation on tissue collagen due to the time-dependent reaction, so that the effect of tenilsetam might become more noticeable.

Our following *in vivo* studies actually indicated that AGE formation in tissues occurred predominantly in diabetic rats and was inhibited by the administration of tenilsetam. The results demonstrated that tenilsetam suppressed to control levels the formation of two different types of fluorescence characteristic of AGEs in the renal cortex and aorta of diabetic rats. One type of fluorescence (excitation/emission = 370/440 nm) has been conventionally used as a marker of AGEs (19). The other fluorescence (excitation/emission = 328/378 nm) was recently demonstrated by analysis of three-dimensional fluorescence spectra to represent fluorescence in glycated proteins (20). Furthermore, the latter fluorescence is similar to the maximum fluorescence characteristic of pentosidine, one of the fluorophores formed in advanced Maillard reaction (13, 46). Our results were consistent with those of Dyer *et al.* (47), confirming that the fluorescence intensity in tissues at an excitation/emission = 328/378 nm was twice as much as that at an excitation/emission = 370/440 nm.

Furthermore, our results revealed an inhibitory effect of tenilsetam on the formation of pyrraline in the collagenase-soluble fraction of diabetic renal cortex and aorta. Using immunohistochemical techniques with monoclonal antibody to pyrraline, Miyata and Monnier (41) recently described the presence of pyrraline, a nonfluorescent product in advanced Maillard reaction, in a glomerular lesion and an arteriosclerotic lesion of a diabetic patient. In this regard, tenilsetam may be able to inhibit the formation of nonfluorescent advanced glycation products as well as fluorescent products, thus having a potential role in arresting or preventing the pathological process of diabetic angiopathy. Another observation on the cataract suggests that tenilsetam not only reduces the Maillard reaction in renal cortex and aorta of diabetic rats, but also influences diabetic-induced abnormalities in the lens, probably by inhibiting the Maillard reaction in the eye.

Several investigators reported that tenilsetam improved impaired cognitive functions in animal models (28) and in geriatric and Alzheimer's patients (29–32). As the Maillard reaction may play a role in normal aging and in Alzheimer's disease, it is conceivable that tenilsetam may also inhibit the Maillard reaction in this process. Although the precise mechanism of tenilsetam to inhibit AGE formation is not fully elucidated, the finding by Munch *et al.* (48) that radiolabeled tenilsetam was incorporated into glycated proteins may provide a potential mechanism. They suggested that the beneficial effect of tenilsetam was due to a covalent attachment to sugar-derived moieties of glycated proteins, thereby blocking the reactive sites for further polymerization reactions. With regard to the action site of AG, dicarbonyl compounds such as 3-DG have been suggested to be one of the major targets (27). Our *in vitro* investigation using a specific assay for 3-DG also showed that AG trapped 3-DG with time. On the other hand, there was little direct reaction between tenilsetam and 3-DG. However, tenilsetam proved to inhibit *in vitro* polymerization of lysozyme with 3-DG. Furthermore, tenilsetam inhibited AGE formation in the renal cortex and aorta, including pyrraline that had been reported to form via 3-DG in part (41, 49). The latter is also known to be a potential precursor of pentosidine (46). We observed inhibition of the fluorescence characteristic of pentosidine in diabetic renal cortex and aorta by tenilsetam administration. Thus, our data suggest that tenilsetam acts on a post-3-DG stage in advanced Maillard reaction to inhibit AGE formation. Considering our previous finding (42) that the plasma 3-DG level was elevated in diabetic rats, it would be effective to inhibit the post-3-DG stage in terms of preventing tissues from accumulating AGEs. Vasan *et al.* (50) recently showed that a compound, *N*-phenacylthiazolium bromide, cleaved advanced glycated product at the step after the formation of dicarbonyl intermediate. The mechanism of tenilsetam action might be compatible with their potential therapeutic approach, although it remains to be further elucidated. As the mechanism of action of tenilsetam seems different from

that of AG, the combined use of tenilsetam and AG or of tenilsetam alone may prove to be beneficial in diabetic complications in the future.

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