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Original Article

Olmesartan and Temocapril Prevented the Development of Hyperglycemia and the Deterioration of Pancreatic Islet Morphology in Otsuka-Long-Evans-Tokushima Fatty Rats

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We investigated the impact of olmesartan and temocapril on pancreatic islet β-cells during the development of diabetes mellitus using Otsuka-Long-Evans-Tokushima Fatty (OLETF) rats. Four-week-old male OLETF rats were fed standard chow (untreated: n = 5), or chow containing either 0.005% olmesartan (n = 5) or 0.01% temocapril (n = 5) until being sacrificed at 35 weeks of age. Pancreas sections were double-stained with anti-insulin and anti-glucagon antibodies. The percent areas of β-cells, α-cells and non-α-non-β-cells were compared among groups. In untreated OLETF rats, the fasting plasma glucose (FPG) level was elevated at the 18th week and remained elevated until the 35th week. On the other hand, no significant elevation in FPG levels was observed in olmesartan- or temocapril-treated rats. Pancreatic islets from olmesartan-treated rats were significantly smaller in size as compared with those from untreated OLETF rats. Furthermore, the average area occupied by β-cells as a fraction of the total area of an individual islet was significantly higher in olmesartan- or temocapril-treated rats than that in untreated OLETF rats. Olmesartan and temocapril both prevented the development of hyperglycemia, possibly through the prevention of islet β-cell loss in spontaneously diabetic OLETF rats.

Key words: angiotensin II type-1 receptor blocker, angiotensin converting enzyme inhibitor, pancreas, insulin secretion, Type 2 diabetes mellitus

In 1999, an angiotensin-converting-enzyme inhibitor (ACEI), captopril, was for the first time reported in a large scale clinical trial to potentially decrease the incidence of new-onset Type 2 diabetes mellitus (T2DM) as compared with conventional antihypertensive therapy in patients with diastolic hypertension [1]. Other ACEIs including ramipril, enalapril and lisinopril, as well as angiotensin II type-1 receptor (AT1) blockers (ARBs) such as losartan, candesartan, and valsartan, have shown similar reductions of new-onset T2DM independent of their blood-pressure-lowering effect in non-diabetic patients with hypertension or with heart failure in randomized clinical trials [2, 3]. Hence, it is suggested that the renin-angiotensin system (RAS) blockade with whichever ACEIs or ARBs could delay or prevent the onset of T2DM [4, 5].

The mechanisms by which ACEIs and/or ARBs attenuate the onset of T2DM remain to be elucidated, but they may involve both insulin secretion from pancreatic islet β-cells and insulin sensitivity at the peripheral tissues including skeletal muscles and adi-

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pocytes [2, 6, 7]. Initially, much attention was paid to the improvement of insulin sensitivity by RAS blockade, which is often seen in diabetic patients; however, conflicting reports have mounted, thus raising controversy over whether it counts as the prevention of diabetes onset [4]. Recently, local RAS in the pancreas has been discovered [8, 9], and has been postulated to play crucial roles in the pathogenesis of diabetes in animal models [10–13].

OLETF rats are an established model of T2DM, characterized by obesity and insulin resistance [14, 15]. The morphological changes in the OLETF rats' pancreatic tissues during the course of the development of diabetes have been documented in detail [14–17]. In brief, the islets show a typical appearance of "peripheral α-cells and central β-cells" at the 10th week [16], and some islets start to enlarge and develop new blood vessels and ducts in the periphery of the islet at the 20th week, followed by the destruction of the "peripheral α-cells and central β-cells" appearance by fibrous tissues at the 40th week. Such changes in pancreatic histology were not observed in non-diabetic LETO rats until 40 weeks, and hence are considered to be alterations specific to diabetic animals [17]. Recently, Ko *et al.* reported that ramipril suppressed islet fibrosis in OLETF rats [10].

Olmesartan and temocapril, similarly to other ARBs and ACEIs, respectively, have been shown to possess actions beneficial for the improvement of glucose intolerance [18–21]. Therefore, it is expected that these agents could also reduce the incidence of the new onset of T2DM in future clinical trials. In the present study, we examined the impact of olmesartan and temocapril on the pathogenesis of diabetes in OLETF rats, focusing on the diabetogenic alterations of pancreas morphology.

Materials and Methods

Antibodies and chemicals. Guinea pig polyclonal antibody against porcine insulin antibody was purchased from Thermo Shandon (Pittsburgh, PA, USA), and rabbit polyclonal antibody against porcine glucagon was purchased from Zymed Laboratory (South San Francisco, CA, USA). Rabbit polyclonal anti-AT1 receptor antibody was kindly provided by Prof. Rakugi (Osaka University) [22]. Olmesartan and temocapril were provided by Sankyo Pharmaceutical Company.

Animals and experimental design. Animals were cared for and treated in accordance with the guidelines of the Animal Use and Care Committee at Okayama University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1985). OLETF rats were kindly provided by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). Fifteen male, 4 week-old OLETF rats were housed singly with food and water ad libitum; specifically, they were fed standard rat chow containing 5% fat (Oriental Yeast, Tokyo, Japan). When the rats were 8 weeks of age, they were randomly assigned to one of 3 groups that were fed one of the following: standard chow (untreated, $n = 5$), or chow containing either 0.005% olmesartan (olmesartan-treated, $n = 5$) or 0.01% temocapril (temocapril-treated, $n = 5$). The doses of these agents were the same as those used by Kim *et al.* [23] in order to generate compatible suppressor effects.

The following clinical parameters were determined at weeks 5, 9, 13, 18, 22, 26, 30, and 35 in all animals: body weight, blood pressure, fasting plasma glucose (FPG), fasting plasma insulin, serum fructosamine, 24-h collected urine volume, and 24-h urinary excretion of C-peptide. The pancreases were dissected out at the 35th week. Each pancreas was fixed in 10% formalin and embedded in paraffin. For histopathological examination, 4 μm sections were stained with Masson-Trichrome and subjected to the immunostaining procedures described below.

Insulin-glucagon immunostaining. Sections were first incubated with polyclonal anti-insulin antibody followed by anti-glucagon antibody at room temperature for 2h each. Secondary antibodies, namely biotin-conjugated goat anti-guinea-pig IgG and biotin-conjugated goat anti-rabbit IgG (Vector Labs, Burlingame, CA, USA), were used as the anti-insulin and anti-glucagon antibodies, respectively. The sections were labeled with avidin-biotin-complex (Vectastain Elite ABC kit, Vector Labs) for glucagon, or with ABC-alkaline phosphatase (ABC-AP) procedures (Vectastain Elite ABC-AP kit, Vector Labs) for insulin. Sections were visualized with Vector Red (Vectastain) and diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin.

AT1 receptor immunostaining. The section serial to that used for insulin-glucagon double staining was subjected to AT1 receptor immunostaining. Polyclonal anti-AT1 antibodies were labeled with the ABC procedure and visualized with DAB. Sections were microwaved at 500W for 10min to activate the epitope immunogenicity.

Morphological measurement. Using the image analysis software OPTIMAS 6.5 (Mediacybernetics, Silver Spring, MD, USA), the islet's total area, anti-glucagon antibody-stained (α-cell) area and anti-insulin antibody-stained (β-cell) area were outlined. In the present study, we defined the subtraction of the α-cell area and the β-cell area from the total islet area as the non-α-non-β-cell area. The area occupied by the β-cells, α-cells and non-α-non-β-cells in an individual islet was calculated.

Statistical analysis. Values are expressed as the means ± SE. Statistical analyses were performed using Stat View 5.0 (ABACUS Concepts, Berkeley, CA, USA). Differences between untreated OLETF rats and olmesartan-treated or temocapril-treated animals were analyzed using an unpaired Student's *t*-test. A $p < 0.05$ was considered to be significant.

Results

Clinical features. The time courses of body weight, systolic blood pressure, and FPG values are shown in Fig. 1. There were no significant differences in body weight among the groups, though temocapril-treated rats tended to gain less weight than animals in the other groups (Fig. 1A, at 35th week, temocapril; 556±25g, untreated; 651±9g, $p=0.0518$). Systolic blood pressures in olmesartan-treated and in temocapril-treated rats were significantly lower than in untreated OLETF rats (Fig. 1B, at 13th week, olmesartan; 122±2mmHg, temocapril; 123±2mmHg, untreated; 139±2mmHg, $p < 0.05$ for both vs. untreated). FPG levels in untreated OLETF rats gradually increased to over 140mg/dl at the 18th week and stayed above that level until the animals were sacrificed. Contrarily, the FPG levels in either treatment group never exceeded 140mg/dl (Fig. 1C). FPG values in temocapril-treated rats stayed at significantly lower levels than those in untreated OLETF rats throughout the study period, while the decrease in levels in olmesartan-treated rats reached statistical significance solely at the 26th week. There were no significant differences between olmesartan- and temocapril-treated rats in either systolic blood pressures or FPG values throughout the experiment (Figs. 1B, 1C).

Serum fructosamine, fasting plasma insulin and 24-h urinary C-peptide. Levels of serum

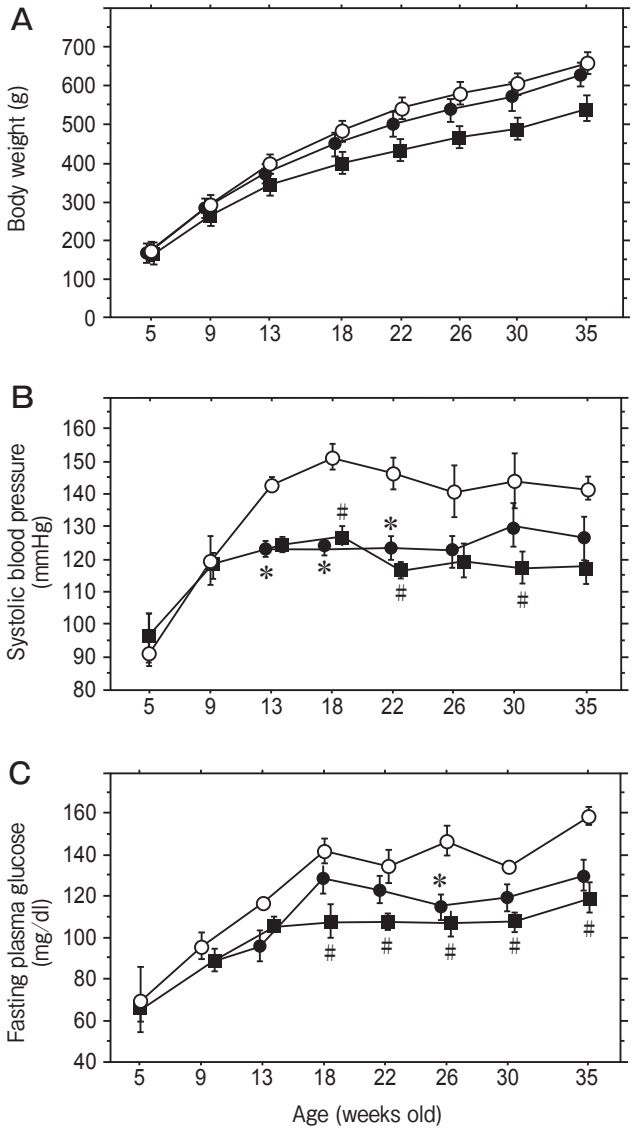

Fig. 1 Changes of body weight (**A**), systolic blood pressure (**B**), and fasting plasma glucose levels (**C**) in untreated (open circle), olmesartan-treated (closed circle), and temocapril-treated (closed box) OLETF rats.

#$p < 0.05$ notes olmesartan-treated versus untreated. *$p < 0.05$ notes temocapril-treated versus untreated.

fructosamine, fasting plasma insulin, and 24-h collected urinary C-peptide at the time of sacrifice are shown in Table 1. Both insulin and C-peptide levels in olmesartan- or temocapril-treated rats tended to be higher than those in untreated OLETF rats; however, statistically significant differences were not observed among the groups.

Table 1 Levels of fructosamine, fasting plasma insulin and 24-h urine C-peptide of untreated, olmesartan-treated, or temocapril-treated OLETF rats at 35 weeks

	untreated	olmesartan	temocapril
Fructosamine (μmol/l)	217±88	223±9	192±4
Plasma insulin (ng/ml)	1.2±0.2	2.0±0.1	1.8±0.1
24-h urine C-peptide (ng/mgCr)	2.3±0.05	2.8±0.1	3.8±0.23

Values are means ± SE. No significant differences were observed among groups.

Pathohistological alterations in pancreatic islets at 35th week. In Fig. 2A, the pancreas of a non-diabetic LETO rat at 35 weeks of age is shown as a reference for the typical appearance of "peripheral α-cells and central β-cells". In the present study, we observed various alterations in the appearance of islets (representatives are shown in Figs. 2B–2D), as well as in their size and number (summarized in Fig. 2F). The islet size was distributed widely, ranging from small (2,500–10,000 μm^2) to huge (> 70,000 μm^2). Huge islets were occasionally found only in untreated OLETF rats, and there was a significant difference in average islet size between untreated and olmesartan-treated rats. Small islets were seen in all groups. There were many tiny islets (< 2,500 μm^2) in all groups, although we have ignored them in the current study for technical reasons. It is notable that small islets and tiny islets were found most frequently in untreated islets, suggesting islet generation (data not shown).

Huge islets contained noticeable areas of non-α-non-β-cells with disrupted arrangements of "peripheral α-cells" (Figs. 2B, 2F). Mid-range-sized islets (10,000–70,000 μm^2) were found in the pancreas in all groups with a similar incidence (Fig. 2F); however, their appearances differed markedly between the untreated and treated groups. Namely, the islets from untreated OLETF rats appeared to be down-sized copies of huge islets, while those from olmesartan- or temocapril-treated rats showed the "peripheral α-cells and central β-cells" appearance (Figs. 2C, 2D). The average areas occupied by β-cells, α-cells and non-α-non-β-cells in individual islets are shown in Fig. 2F. The occupancy rates of β-cells and α-cells in either olmesartan- or temocapril-treated rats were significantly higher than those in untreated OLETF rats (Fig. 2F). The area occupied by non-α-non-β-cells consisted mainly of connective tissues and microvessels under Masson-Trichrome stain (data not shown).

AT1 receptor localization in islets. In the serial section analysis, AT1 receptors were recognized in the rat pancreas, predominantly in acinar cells and to a lesser extent in islets (Fig. 3B). There were no differences in the cellular distribution and intensity of AT1 receptor immunostaining among experimental groups (data not shown). Figs. 3C and 3D demonstrated relatively strong AT1 receptor staining in areas occupied by β-cells and non-α-non-β-cells in islets but not in areas occupied by α-cells.

Discussion

Male OLETF rats are a useful model of T2DM, characterized by obesity and insulin resistance starting around 16 weeks of age [17]. Simultaneously, hyperinsulinemia develops in order to cope with peripheral insulin resistance, accompanied by morphological changes in the pancreas including islet enlargement and an increase in the number of islets and β-cells. FPG starts to rise at around 18 to 24 weeks, reflecting the limitation of β-cells' compensation. Meanwhile, connective tissues emerge and proliferate in islets, separating them in clusters to develop a "nodular" appearance. By the age of 40 weeks, severe hyperglycemia is seen as a consequence of deteriorated insulin secretion and the fibrotic destruction of islets [14–17].

Pancreases from untreated animals in the present study showed the above-mentioned diabetogenic morphology at the 35th week (Figs. 2B, 2F). Olmesartan or temocapril treatment clearly attenuated the morphological alterations both in islet size and islet number (Figs. 2C, 2D, 2E). Since islet enlargement requires an increase in the β-cell number, there may be β-cell generation, duplication or differentiation. Furthermore, the decreasing trend in the number of

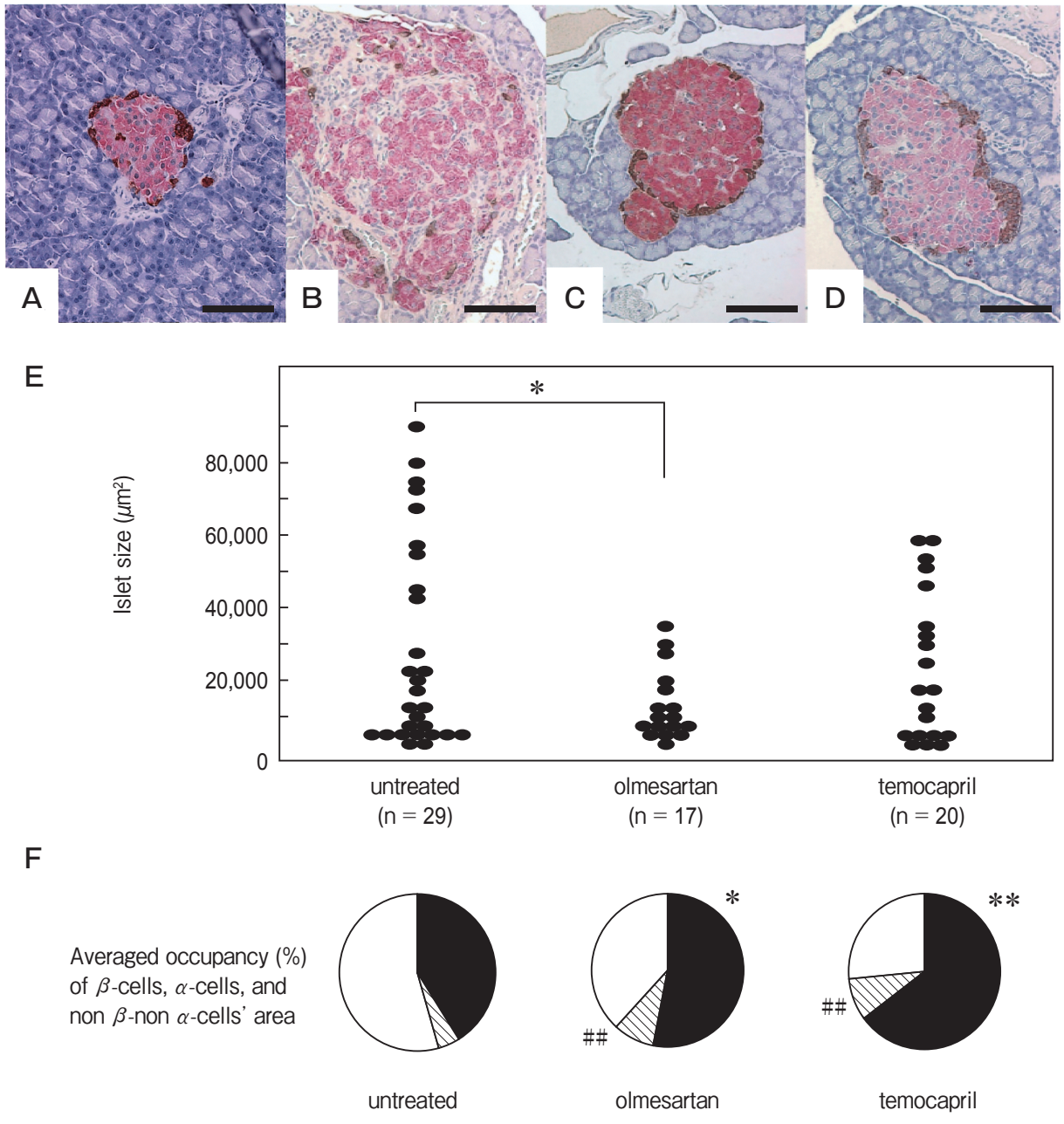

Fig. 2 Immunohistological visualization of β-cells (red) and α-cells (brown) in pancreatic islets and morphological analysis in untreated, olmesartan-treated and temocapril-treated OLETF rats at 35 weeks.

A, Typical appearance of pancreas at 35 weeks of age from a non-diabetic LETO rat displaying peripheral α-cells (brown) regularly surrounding the central cluster of β-cells (red) (served as reference). **B-D**, Representative appearances of huge islets from an untreated OLETF rat (**B**), a moderately enlarged islet from an olmesartan-treated OLETF rat (**C**), and a moderately enlarged islet from a temocapril-treated OLETF rat (**D**). **E**, Islet size in untreated OLETF rats was distributed widely from tiny (less than 2,500 μm^2) to huge (larger than 70,000 μm^2), whereas that in olmesartan-treated OLETF rats never exceeded 40,000 μm^2. * denotes significant ($p<0.05$) difference in average islet size between untreated and olmesartan-treated rats. **F**, Averaged area occupied by β-cells (closed), alpha-cells (stripe), and non-α-non-β-cells' area (open), * $p<0.05$ vs. untreated, ** $p<0.01$ vs. untreated, ## $p<0.01$ vs. untreated OLETF rats. scale bar: 100 μm

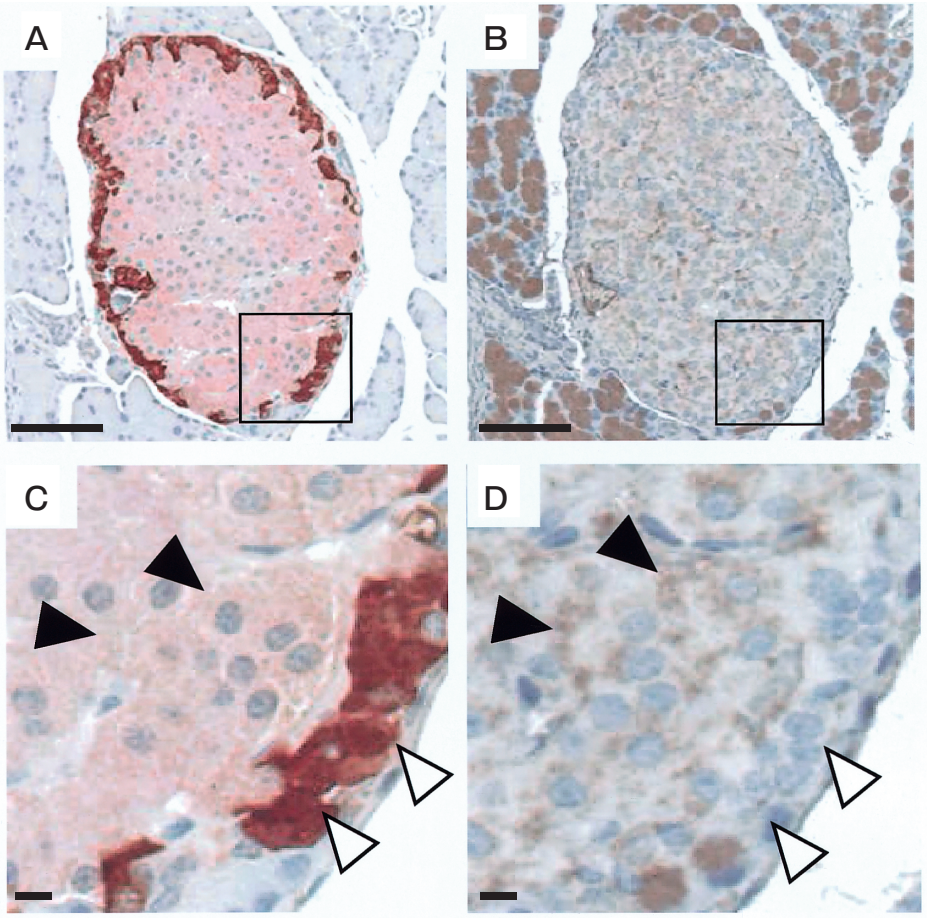

Fig. 3 AT1 receptor localization in rat pancreatic islets.
A and **C**, Double staining with anti-insulin (red) and anti-glucagon (brown) antibodies. **B** and **D**, AT1 receptors (brown) were immunohistologically recognized in the pancreas, predominantly in the acinar cells, to a less extent in islet β-cells (black arrow head), and not at all in α-cells (white arrow head). **A** and **B** are serial sections, and **C** and **D** are magnified images of **A** and **B**. Scale bar: 100μm (**A**, **B**); 10μm (**C**, **D**)

islets (Fig. 2E), in particular the number of tiny islets (data not shown), in treated rats, prompted us to speculate that islet generation occurring in untreated OLETF rats might have been prevented by RAS inhibitors.

FPG levels in animals treated with RAS inhibitors were significantly lower than those in untreated animals from the 18th week to the 35th week (Fig. 1C). Since no significant differences were observed in levels of serum fructosamine, fasting plasma insulin, or urinary C-peptide among groups, post-fed plasma glucose levels might not have differed greatly between treated rats and untreated rats. Indeed, urinary C-peptide levels were within normal range in all groups (Table 1). Thus, we have demonstrated the prevention of the very early steps of diabetogenesis in OLETF rats.

It is apparent that olmesartan and/or temocapril inhibited connective tissue growth in pancreatic islets (Fig. 2F). Both agents have been shown to mediate antifibrotic actions in animal models of cardiovascular and kidney diseases where local RAS plays important roles [23–25]. Recently, Ko *et al.* suggested that ramipril potentially prevented the islet destruction by fibrosis in OLETF rats. Here, we have demonstrated that both olmesartan and temocapril also prevented islet destruction, supporting the emerging concept that pancreas RAS might play a crucial role in the diabetogenic process in T2DM [4, 6]. The precise mechanisms remain to be elucidated; however, they may include the blockade of angiotensin II action on cellular proliferation, apoptosis, TGF-β activation

[10], and reactive oxygen generation [26]. In addition, a direct action of zofenoprilat and enalaprilat, ACEIs, on pancreatic β-cell protection has been suggested [27]. We have demonstrated the predominant distribution of AT1 receptors on β-cells in islets. Further studies are needed to elucidate whether these AT1 receptors play a role in β-cell duplication, which is possibly involved in islet enlargement.

In conclusion, both olmesartan and temocapril protected pancreatic islets, and β-cells in particular, from the morphological deterioration of diabetogenesis. AT1 receptors expressed on β-cells may be involved in the mechanism underlying such favorable actions of RAS inhibitors.

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