Candesartan, an Angiotensin II Receptor Antagonist, Suppresses Pancreatic Inflammation and Fibrosis in Rats

Tamaki Yamada, Atsushi Kuno, Kazuhiko Masuda, Kumiko Ogawa, Mitsue Sogawa, Soichi Nakamuro, Tomoaki Ando, Hitoshi Sano, Takahiro Nakazawa, Hirotaka Ohara, Tomoyuki Nomura, Takashi Joh, and Makoto Itoh,

Department of Surgical Medicine, Gastroenterological Surgery (K.M.)
Department of Pathology and Pathophysiology, Experimental Pathophysiology and Tumor Biology (K.O.), Nagoya City University Graduate School of Medical Sciences, and
Department of Gastroenterology, Graduate School of Medicine (M.S.), Osaka City University
Running Title:
Angiotensin II and Its Receptors in Chronic Pancreatitis

Address for Reprints and Correspondence:
Tamaki Yamada, M.D., Ph.D.
Dept. of Comprehensive Medicine, Internal Medicine and Bioregulation
Nagoya City University Graduate School of Medical Sciences
1 Kawasumi, Mizuho-cho, Mizuho-ku,
Nagoya, Aichi, Japan, 467-8601
TEL: 81-52-853-8211
FAX: 81-52-852-0952
e-mail: yamtmaki@med.nagoya-cu.ac.jp

Number of text pages: 33
Number of tables: 2
Number of figures: 3
Number of references: 40
Number of words in the Abstract: 237
Number of words in the Introduction: 668
Number of words in the Discussion: 996

Abbreviations
renin-angiotensin system: RAS, angiotensin II: AT-II,
angiotensin-converting enzyme: ACE, hepatic stellate cells: HSC,
transforming growth factor: TGF
angiotensin II receptor 1: AT1 receptor, angiotensin II receptor 2: AT2
receptor, pancreatic stellate cells: PSC, smooth muscle actin: SMA
Wistar Bonn/Kobori: WBN/Kob, myeloperoxidase: MPO, Reverse
Transcription-Polymerase Chain Reaction: RT-PCR, tumor necrosis factor:
TNF, interleukin: IL
Abstract

Background: Angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists attenuate fibrosis in the kidney, heart, and liver by suppressing transforming growth factor-β1 mRNA and decreasing production of extracellular matrix protein. We recently demonstrated that lisinopril, an angiotensin-converting enzyme inhibitor, alleviates pancreatic inflammation and fibrosis in male Wistar Bonn/Kobori rats. The involvement of angiotensin II receptor and its receptor interaction in the pathogenesis of spontaneous chronic pancreatitis was assessed in this model.

Methods: Candesartan, an angiotensin II receptor antagonist, was administered in drinking water (10.5, 42, or 125 mg/L) to 10-week-old male WBN/Kob rats for 10 weeks and inflammatory parameters, fibrosis, and gene expression of renin-angiotensin system components and transforming growth factor-β1 was assessed in the pancreas. Immunostaining for α-smooth muscle actin was also performed.

Results: Candesartan significantly suppressed decrease in pancreatic weight and increases in pancreatic myeloperoxidase activity, hydroxyproline content, ratio of fibrous tissue, histological scores, and ratio of α-smooth muscle actin positive cells (activated pancreatic stellate cells) at 20 weeks. The high dose enhanced the expression of angiotensinogen and angiotensin II receptor type 2 mRNA and suppressed the overexpression of transforming growth factor-β1.
growth factor-ß1 mRNA.

Conclusion: Candesartan alleviates chronic pancreatitis and fibrosis by suppressing the overexpression of transforming growth factor-ß1, resulting in prevention of activation of pancreatic stellate cells in male WBN/Kob rats. We propose that angiotensin II receptor type 1 antagonists may be useful for the treatment of chronic pancreatitis involving angiotensin II interaction with its receptor.
The renin-angiotensin system (RAS) plays an important role in regulation of the systemic blood pressure, body fluid, and electrolyte balance. Angiotensin II (AT-II), an octapeptide produced by proteolytic cleavage of its precursor angiotensin by the angiotensin-converting enzyme (ACE), is a physiologically active product of the RAS (Matsusaka and Ichikawa, 1997). It stimulates the proliferation of mesangial cells, cardiac fibroblasts, and hepatic stellate cells (HSCs) and their increased synthesis of extracellular matrix proteins through induction of transforming growth factor (TGF)-β1 expression both in vivo and in vitro (Matsusaka and Ichikawa, 1997; Bataller et al., 2000).

Two subtypes of AT-II receptors, namely type 1 (AT1 receptor) and type 2 (AT2 receptor), have been identified (Matsusaka and Ichikawa, 1997; Matsubara, et al., 1998). AT1 receptor, which mediates most of the biological results of AT-II action, including vasoconstriction, cell proliferation, and production of extracellular matrix proteins, is found in the kidney, liver, adrenal cortex, and blood vessels. It is well documented that AT-II induces cell hypertrophy and ion transport in the kidney, heart, and liver, primarily through AT1 receptor (Matsusaka and Ichikawa, 1997; Bataller et al., 2000). In contrast, AT2 receptor is expressed at high levels in the various organs of developing fetus, while, in the adults, it is mainly present in the brain, adrenal medulla, uterus, and heart. This form, which is
increased during inflammation and ischemia, suppresses synthesis of DNA, protein, and extracellular matrix proteins, as well as cell proliferation (Matsusaka and Ichikawa, 1997; Matsubara, 1998). ACE inhibitors suppress functions of both AT1 and AT2 receptors by depleting AT-II. On the other hand, AT-II may stimulate AT2 receptor in the presence of an AT1 receptor antagonist.

It has been demonstrated that mRNAs for RAS components, including angiotensinogen, renin, and ACE are active intrinsically in the pancreas and their levels are enhanced during acute pancreatitis and chronic pancreatic hypoxia in experimental animals (Leung et al., 2000; Chan et al., 2000). Isolated pancreatic stellate cells (PSCs) have similar properties to their counterparts in the liver, HSCs, with regard to shape, presence of vitamin A, positive staining for desmin and glial fibrillary acidic protein, and expression for α-smooth muscle actin (SMA) in the active phase (Apte et al., 1998; Schneider et al., 2001). It was reported that TGF-β1 induces proliferation of PSCs and their increased production of extracellular matrix proteins (Apte et al., 1999) and PSCs are involved in the pathogenesis of pancreatic fibrosis in both experimental animals and humans (Haber et al., 1999). AT-II induces contraction and proliferation of HSCs in vitro and AT1 receptor antagonists reduce hepatic fibrosis by suppressing activation of HSCs in experimental animals (Bataller et al., 2000; Yoshiji et al., 2001).
We therefore hypothesized that RAS may be involved in the pancreatic fibrosis that occurs in chronic pancreatitis. Indeed, we recently demonstrated that lisinopril, an ACE inhibitor, in drinking water attenuates pancreatic inflammation and fibrosis by suppressing induction of TGF-β1 mRNA and activation of PSCs in a spontaneously occurring model of chronic pancreatitis, in male Wistar Bonn/Kobori (WBN/Kob) rats (Kuno et al., 2003).

ACE inhibitors increase levels of the vasodilatory prostaglandins and nitric oxide (Johnson et al., 1995; Schaefer et al., 1996), which may be involved in the protective mechanisms. Therefore, it is not clear whether AT-II and its interaction with AT1 receptor is important for the pathogenesis. Further, ACE inhibitors occasionally cause acute pancreatitis in human (Muchnick, 1999). We assumed that the effect of an AT1 receptor antagonist should be assessed to clarify the involvement of AT-II and AT1 receptor interaction and an AT1 receptor antagonist might be more suitable for treating chronic pancreatitis. However, there is no information available regarding its effect on pancreatic inflammation and fibrosis as well as pancreatic RAS. Therefore, in the present study, we assessed the effects of candesartan, an AT1 receptor antagonist, on 1) pancreatic inflammation and fibrosis, 2) the gene expression of TGF-β1 and local RAS components, including angiotensinogen, AT1 receptor, and AT2 receptor, and 3) ratio of
α-SMA positive cells (activated PSCs) in the pancreas in male WBN/Kob rats.

Materials and Animals

Male Wistar WBN/Kob and Wistar rats were purchased from SLC (Hamamatsu, JAPAN) and kept in a temperature-controlled room under dual light cycle. They were allowed free access to water and standard laboratory feed. The study protocol was approved by the Animal Care Committee of Nagoya City University. Candesartan was a generous gift from Takeda Co., Ltd. (Osaka, Japan). All other chemicals were of the highest quality available.

Groups of Animals and Treatment

Ten-week-old WBN/Kob rats were randomly divided into untreated (n=9), low (n=5), medium (n=5), and high (n=10) dose candesartan groups. Candesartan (100 mg) was dissolved in 1:1 mix of ethanol and polyethylene glycol (1.2 mL), warmed to 60 centigrade, and then 1 N sodium bicarbonate solution (3.3 mL) was added before being dissolved in drinking water. The high dose concentration of candesartan in drinking water, 125 mg/L, was selected on the basis of a previous report (Kim et al., 1995) and intake was estimated to be 10 mg/kg/day (Kuno et al., 2003). Lower concentrations of candesartan (42 or 10.5 mg/L) were also tested to assess dose-dependence. Untreated group was given water containing equal volume of ethanol,
polyethylene glycol, and sodium bicarbonate solution without candesartan in the same manner. The amount consumed was recorded and the solutions were refreshed every other day over 10 weeks. Body weights were recorded weekly. The doses of the drugs were calculated from consumption of solutions and body weights. The treatment period was as reported previously (Hashimoto et al., 2000; Yamada et al., 2001; Kuno et al., 2003) and is considered to be suitable to determine prophylactic effects of drugs on chronic inflammation and fibrosis since pancreatic lesions does not exist at 10 weeks of age and chronic inflammation and fibrosis almost completely involve the pancreas at 20 weeks in the present model (Mori et al., 1988).

Twenty-week-old male Wistar rats (n=5) were used for determination of body weight, pancreas weight, pancreatic myeloperoxidase (MPO) activity, pancreatic hydroxyproline content, and mRNAs for TGF-β1, angiotensinogen, AT1 receptor, and AT2 receptor in the pancreas.

**Tissue Sampling**

Twenty-week-old WBN/Kob rats treated for 10 weeks were killed with an overdose of pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA) after taking blood from the abdominal aorta and 20-week-old Wistar rats were also killed to obtain pancreas. The pancreases were resected and stored at −80 centigrade for subsequent determination of MPO activity (WBN/Kob rats: untreated, n=9; low dose,
n=5; medium dose, n=5; and high dose, n=5 and Wistar rats: n=5) and hydroxyproline content (WBN/Kob rats: untreated, n=9; low dose, n=5; medium dose, n=5; and high dose, n=10 and Wistar rats: n=5). Those from WBN/Kob rats in the untreated and high dose candesartan groups and 20-week-old Wistar rats frozen in liquid nitrogen and stored at -80 centigrade were assessed for expression of TGF-β1, angiotensinogen, AT1 receptor, and AT2 receptor mRNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Samples were also fixed in 10% buffered formalin for histological assessment and immunohistochemistry (WBN/Kob rats: untreated, n=6; low dose, n=5; medium dose, n=5; and high dose, n=5).

**Histological Analysis**

Pancreas tissue was fixed with 10% buffered formalin, processed for paraffin embedding sectioned, and stained with hematoxylin-eosin and Azan. Microscopic examination was carried out by a pathologist who was unaware of the groups of rats. The status of inflammation was evaluated in terms of the grades of inflammatory cell infiltration, interstitial edema, fibrosis, acinar cell necrosis, and hemorrhage on a scale of negligible to maximal (0-3), as reported previously (Hashimoto et al., 2000). The scores were compared among the untreated and low, medium, and high dose candesartan groups. Quantity of fibrosis was analyzed with the aid of an image processor (Image Processor for Analytical Pathology; Sumika Technoservice,
Osaka, Japan) as the ratio of anilin-blue positive fibrous tissue to total area in Azan-stained pancreas sections, excluding lymph nodes and major vessels, if present.

**Immunohistochemistry for α-Smooth Muscle Actin**

Pancreas sections fixed in 10% buffered formalin were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After washing with phosphate buffered saline, they were treated for 1 hour at room temperature with 5% fetal bovine serum to block nonspecific reactions. They were then incubated overnight at room temperature with mouse anti-human α-SMA mAb (1:750 dilution; Dako, Carpiteria, CA) which can detect rat α-SMA. After washing, the sections were incubated for 1 hour at room temperature with biotinylated rabbit anti-mouse immunoglobulin G antibody (1:500 dilution; Dako) and then for 1 hour at room temperature with horseradish peroxidase-labeled streptavidin (LSAB kit, Dako). Reaction products were visualized by treating sections for 3-5 minutes with 0.2 mg/mL 3,3’-diaminobenzine tetrahydrochloride in 0.05 M Tris-buffered saline, pH 7.4, containing 0.003% hydrogen peroxide. Nuclei were counterstained with methyl green. Positively stained areas were quantitatively determined using an image processor as described in the “Histological Analysis” and expressed as the ratio of α-SMA positive cells to the total area.
Measurement of Pancreatic Myeloperoxidase Activity

Pancreatic MPO activity, an indirect quantitative index of granulocyte infiltration, was determined using the method of Grisham et al. (1990) with a minor modification. Briefly, pancreatic tissue was homogenized in 20 mM phosphate buffer (pH 7.4) and the homogenate was centrifuged at 6,000 x g for 20 min at 4 centigrade. The pellet was homogenized and sonicated with an equivalent volume of 50 mM acetic acid (pH 6.0) containing 0.5% (weight/volume) hexadecyltrimethylammonium hydroxide. MPO activity was determined by measuring the H₂O₂-dependent oxidation of 3,3′,5,5′ tetramethylbenzidine and expressed as units per gram wet weight of tissue.

Measurement of Pancreatic Hydroxyproline Content

Pancreatic hydroxyproline content, an indicator of collagen deposition, was determined by a modification of the method of Blumenkrantz and Asboe-Hansen (1973). Briefly, approximately 100 mg specimens were homogenized in water and hydrolyzed at 110 centigrade for 20 hours in 10 N HCl. HCl was evaporated under nitrogen gas and the hydrolysate was dissolved in distilled water and filtered. Then 0.5 mL aliquots were mixed with 3 mL of citrate-phosphate buffer (0.15 M citric acid and 0.6 M dibasic sodium phosphate) and 0.5 mL of 1 M periodic acid in 9 M H₃PO₄. The samples were then mixed with 1.75 mL of extract buffer...
consisting of toluene (5 parts), 2-methyl-1-propanol (5 parts), and 1-propanol (2 parts), shaken for 30 min and centrifuged. The organic phase (0.6 mL) was mixed with Ehrlich’s reagent (0.15 mL) and left for 15 min. The absorbance was measured at 565 nm and hydroxyproline levels were calculated using a standard curve made with 4-hydroxy-1-proline and expressed as micrograms per gram tissue.

**Measurement of Serum Candesartan Levels**

Serum candesartan levels were determined in the low (n=5), medium (n=5), and high (n=5) dose groups. Briefly, after serum was mixed with 0.2 M HCl and diethyl ether, the organic layer was obtained by centrifugation and freezing of the aqueous layer using a dry-ice acetone bath, then evaporated under nitrogen gas at 40 centigrade, and the residue was mixed with 0.02M KH₂PO₄/CH₃CN (8:2; vol/vol), vortexed, sonicated, and centrifuged, and the concentration of candesartan in the supernatant was determined using hyper liquid chromatography.

**Reverse Transcription-Polymerase Chain Reaction**

Total RNA was extracted from frozen pancreatic tissues with Trizol reagent (Invitrogen Co., Carlsbad, CA. USA) and 2 mg was reverse transcribed into cDNA using an Oligo (dT) 12-18 Primer (Invitrogen Co.), Superscript II RNase H-Reverse Transcriptase (Invitrogen Co.), and an RNase Inhibitor (TOYOBO Co., LTD., Osaka, Japan). The polymerase
chain reaction (PCR) was performed with reaction mixtures containing 2.5 mM dNTP, 10 mM sense and anti-sense primers, and 5 units/mL Taq DNA polymerase (TAKARA SHUZO Co. Otsu, Japan) in a thermal cycler for 1 min at 94 centigrade, 1 min at 55 (β-actin), 57 (angiotensinogen, AT1 receptor, AT2 receptor), and 62 (TGF-β1) centigrade, and 2 min at 72 centigrade, for 20 (β-actin), 28 (angiotensinogen, AT1 receptor), 32 (TGF-β1) and 35 (AT2 receptor) cycles, and then an extension reaction was carried out at 72 centigrade for 5 min.

The PCR primers, designed in line with previous reports (Bouhnik et al., 1981; Qian et al., 1990; Iwai et al., 1991; Iwai and Inagami, 1992; Koike et al., 1995), were: TGF-β1 sense 5’-GCGGACTACTACGCCAAAGA-3’, anti-sense 5’-TGGTTGTAGAGGGCAAGGAC-3’, β-actin, sense 5’-TGGCCTCACTGTCCACCTTC-3’, anti-sense 5’-TGGCCTCACTGTCCACCTTC-3’, angiotensinogen sense 5’-CGAATGGCTGACCATCCAGA-3’, anti-sense 5’-TGAGTTCTGGGTGGACAACA-3’, AT1 receptor sense 5’-ACACCACATTGGGGGTAA-3’, anti-sense 5’-TGGAACAGCCTTGGTGTT-3’, AT2 receptor sense 5’-GAAGAAAAAGCAACATCGCCA-3’, anti-sense 5’-CCCTGGCAAGCATTCTTATGT-3’, anti-sense 5’-TGGAGCAGGTAATGGGAAC-3’. PCR products were electrophoresed on 1% agarose gels, visualized by ethidium bromide staining and
photographed under ultraviolet light.

Statistics

Data are expressed as arithmetic means ± standard deviation. Statistical differences between two groups or among groups were identified using the Student $t$ test or one-way analysis of variance, followed by multiple comparisons using the least significant difference method, respectively. The histological scores and quantitative ratio of fibrous tissue underwent Kruskal-Wallis rank analysis and Scheffe's correction of raw score data.
Results

Doses and Serum Levels of Candesartan, Body Weight, and Pancreas Weight

The average doses of candesartan calculated from water consumption and body weights of low, medium, and high dose groups were $0.80 \pm 0.08$, $4.34 \pm 0.32$, and $13.92 \pm 0.98$ mg/kg/day, respectively, during the experimental period. Serum candesartan levels were $55.0 \pm 24.6$, $442.0 \pm 112.6$, and $914.6 \pm 382.0$ ng/mL, respectively, in the low, medium, and high groups at the end of the experiment (Table 1). The pancreas weights were significantly lower in the untreated group than those in the age matched male Wistar rats (Table 1). The body weights were significantly lower in the medium and high dose groups than the untreated and low dose groups, while the pancreas weights increased significantly in all treatment groups compared with the untreated group (Table 1).

Macroscopic Findings

The pancreases in the untreated and low dose groups were severely atrophic and had widely spread brown and red foci. In contrast, the pancreases in the high dose group had an almost intact appearance, and the medium dose group had only a few brown and red foci.

Histopathological Analysis

In the untreated rats, severe inflammation was focally observed
around pancreatic ducts (Fig. 1A). It was characterized by neutrophil and lymphocyte infiltration, interstitial edema, hemorrhage and occasional acinar cell necrosis (Fig. 1A; left-half side). Fibrosis was noted as replacement of vanished acini and was also seen between acini and fine to thick collagen fibers were stained in blue in the untreated group (Fig. 1B; left-half side). The administration of candesartan dramatically reduced the inflammatory changes in a dose-dependent manner. Table 2 summarizes the average scores for each factor and the quantitative data for fibrous tissue. The inflammatory cell infiltration and interstitial edema were significantly reduced in the medium and high dose groups (Table 2). Focal inflammatory changes were only slight around the ducts and fine collagen fiber is present in the high dose group (Fig. 1C and 1D; arrows).

**Immunohistochemistry for α-Smooth Muscle Actin**

α-SMA positive cells with the morphology of activated PSCs were localized in the peri-acinar fibrotic areas and vascular walls in the untreated group (Fig. 2A; right-half side). In contrast, α-SMA positive cells were observed only in the vascular walls in the high dose candesartan group (Fig. 2B), demonstrating significant decrease (Table 2).

**Pancreatic Myeloperoxidase Activity**

Pancreatic MPO activity, an index of granulocyte infiltration, was significantly increased in untreated group compared with the Wistar rats.
(6.22 ± 0.48 vs. 1.93 ± 0.39 units/g, p<0.005). It was significantly decreased in the medium and high dose groups, compared with the untreated and low dose groups (2.92 ± 0.62 and 2.22 ± 0.48 vs. 6.00 ± 4.07 and 6.22 ± 0.48 units/g, p<0.05).

**Pancreatic Hydroxyproline Content**

Pancreatic hydroxyproline content, an indicator of collagen deposition, was significantly increased in untreated group compared with the Wistar rats (537.6 ± 292.9 vs. 139.6 ± 38.4 µg/g, p<0.005). The high dose of candesartan significantly suppressed the increase in pancreatic hydroxyproline content compared with the untreated, low, and medium dose groups (146.6 ± 56.4 vs 537.6 ± 292.9, 390.9 ± 198.5 and 385.9 ± 90.7 µg/g, p<0.01, respectively).

**Expression of TGF-β1, Angiotensinogen, AT1 receptor and AT2 receptor mRNA**

RT-PCR revealed TGF-β1 mRNA to be overexpressed in the pancreas in the untreated group, while it was only detected at low levels in male Wistar rats (Fig. 3). The high dose of candesartan suppressed the overexpression of TGF-β1 mRNA in WBN/Kob rats (Fig. 3). The expressions of angiotensinogen and AT2 receptor were enhanced in the high dose group compared with untreated group and Wistar rats, whereas that of AT1 receptor was only slightly increased (Fig. 3).
Discussion

The present study demonstrated that candesartan, an AT1 receptor antagonist, attenuates chronic pancreatic inflammation and fibrosis in male WBN/Kob rats. This protective effect was quantitatively confirmed by significant increase in pancreatic weight (an index of atrophy) and decreases in pancreatic MPO activity (an index of granulocyte infiltration), pancreatic hydroxyproline content (an index of collagen deposition), histological scores, and the ratio of fibrous tissue. The high dose of candesartan also suppressed overexpression of TGF-β1 mRNA in the pancreas and decreased the ratio of α-SMA positive cells (activated PSCs), while enhancing expression of angiotensinogen and AT2 receptor mRNA in the pancreas. We can conclude that candesartan, an AT1 receptor antagonist, alleviated pancreatic inflammation and fibrosis by suppressing gene expression of TGF-β1 and preventing activation of PSCs, suggesting that AT-II and its interaction with AT1 receptor may be intimately involved in the pathogenesis in the present model.

Male WBN/Kob rats spontaneously develop chronic pancreatitis (Mori et al., 1988). We previously demonstrated induction of acinar cell apoptosis, with infiltration of CD8 and CD4 T cells and tacrolimus, an immunosuppressant, attenuates this apoptosis and chronic inflammation in this model (Hashimoto et al., 2000; Yamada et al., 2001). Clinical
investigations have demonstrated that infiltration of CD4 and CD8 T cells and high expression of major histocompatibility complex class I and class II antigens by pancreatic epithelial cells is observed in human chronic pancreatitis, suggesting that cell-mediated autoimmune mechanisms are at work (Anderson et al., 1988; Bedossa et al., 1990, Jalleh et al., 1993; Yoshida et al., 1995). Further, autoimmune chronic pancreatitis has been recognized as a new entity in humans defined in terms of lymphocyte infiltration, autoantibody production, and effectiveness of steroid administration (Okazaki et al., 2000). We can speculate that the present model, at least in part, may reflect chronic pancreatitis in man and may thus be useful for exploring clinical therapeutic strategies.

AT-II, a potent constrictor of vascular smooth muscle cells, and its receptor interaction play important roles in endocrine regulation of pancreatic blood flow in rodents (Leung et al., 1997) and humans (Tahmasebi et al., 1999). Many investigations have demonstrated that AT-II and AT1 receptor interaction is also involved in the pathogenesis of fibrosis in the kidney, heart, and liver during chronic inflammation (Matsusaka and Ichikawa, 1997; Bataller et al., 2000; Yoshiji et al., 2001). Furthermore, it was documented that RAS mRNAs and proteins are present in the pancreas and enhanced in acute experimental pancreatitis and chronic pancreatic hypoxia (Leung et al., 2000; Chan et al., 2000). We recently demonstrated
that lisinopril, an ACE inhibitor, attenuated pancreatic inflammation and fibrosis in male WBN/Kob rats by suppressing TGF-β1 gene expression, resulting in prevention of PSC activation (Kuno et al., 2003). However, ACE inhibitors activate the kallikrein-kinin system and preserve bradykinin (White, 1998). The latter is a vasodilator and increases levels of the vasodilatory prostaglandins and nitric oxide (Johnson et al., 1995; Shaefer et al., 1996). Thus, the protection mediated by an ACE inhibitor may not mean direct involvement of AT-II and AT1 receptor in the pathogenesis of pancreatic inflammation and fibrosis. We confirmed an importance of AT-II and its interaction with an AT1 receptor in the present model. Our findings further suggested that the protective effect of ACE inhibitor observed in the present model may be mediated by inhibiting conversion of AT-I to AT-II although involvement of vasodilatory factors may not completely be denied.

It was reported that ischemia may be involved in the pathogenesis of chronic pancreatitis in the WBN/Kob rats (Mori et al., 1988). We speculate that blockade of AT1 receptor by an AT1 receptor antagonist may act by normalizing pancreatic blood flow, resulting in reduced pancreatic inflammation in the present model. It was also demonstrated that an AT1 receptor antagonist inhibits the binding of N-formylmethyonyl-leucyl-phenylalanine to neutrophil receptors in vitro (Raiden, et al., 1997) and decreases plasma levels of tumor necrosis factor
(TNF)-α and interleukin (IL)-6 and adhesion molecules in patients with chronic heart failure (Tsukamoto et al., 2000). Further, an AT1 receptor antagonist ameliorates increased expressions of Mac-1 and intercellular adhesion molecule-1 in stroke-prone spontaneously hypertensive rats (Takemori et al., 2000) and reduces hepatic ischemia-reperfusion injury by decreasing production of cytokines, including TNF-α and IL-6 in rats (Araya et al., 2002). Pancreatic inflammation and fibrosis is associated with up-regulation of tissue expressions of TNF-α and IL-6 in the present model (Xie et al. 2001). Taken together, we assumed that candesartan may reduce inflammation by suppressing cytokines and adhesion molecules. However, additional studies are needed to clarify these points.

It has been demonstrated that AT2 receptor is up-regulated by insulin, insulin-like growth factor, or cytokines (Matsubara, 1998). We presented that treatment with high dose of candesartan enhanced AT2 receptor expression in the pancreas. Although the mechanisms by which candesartan enhances AT2 receptor expression remains to be elucidated, AT-II would be expected to preferentially interact with enhanced AT2 receptor in the presence of an AT1 receptor antagonist, resulting in decreased cell proliferation and extracellular matrix protein synthesis.

Autocrine and paracrine stimulation of HSCs by TGF-β results in production of extracellular matrix protein and suppression of matrix
degradation by metalloproteinases, resulting in the deposition of extracellular matrix protein in the liver (Okuno et al., 2001; Friedman et al., 2000). TGF-ß1 also induces proliferation of PSCs and stimulates their synthesis of extracellular matrix proteins (Apte et al., 1999). These findings support the idea that down-regulation of TGF-ß1 production may be effective for attenuating pancreatic fibrosis. Indeed, candesartan clearly suppressed the expression of TGF-ß1 mRNA and decreased pancreatic collagen deposition in the present model.

In conclusion, the present study demonstrated efficacy for prophylactic administration of candesartan, an AT1 receptor antagonist, in suppressing chronic inflammation and fibrosis in the pancreas in male WBN/Kob rats. This is associated with suppressed TGF-ß1 expression, enhanced AT2 receptor expression, and reduced activation of PSCs. We speculate that AT-II and its interaction with AT2 receptor may be involved in attenuation of pancreatic inflammation and fibrosis. Our results suggest a new strategy for the treatment of human chronic pancreatitis.
References


186:729-741.


Leung PS, Chan HC, Fu LXM and Wong PYD (1997) Localization of


White CM (1998) Pharmacologic, pharmacokinetic, and therapeutic
differences among ACE inhibitors. Pharmacology 18:588-599.


Figure Legends

Figure 1

Representative histological appearance of pancreas tissue in untreated and high dose candesartan groups (A, C: hematoxyline-eosin staining and B, D: Azan staining). (x 400).

Untreated group

A: Infiltration of neutrophils, lymphocytes and plasma cells, disappearance of acinar cells and replacement with fibrous tissue are evident. This view illustrates scores 3 for inflammatory cell infiltration, 2 for fibrosis, acinar cell necrosis, and hemorrhage, and score 1 for edema (left–half side of the photograph).

B: Fine to thick collagen fibers are stained blue. The ratio of fibrosis is 52.3% in this specimen (left–half side).

High dose candesartan group

C: Focal inflammatory changes are slight around a duct (arrows). This view illustrates scores 1 for inflammatory cell infiltration, acinar cell necrosis, hemorrhage, and fibrosis, and 0 for other categories.

D: Fine collagen fibers are seen around the ducts (arrows). The ratio of fibrosis is 14.4% in this specimen.

Figure 2

Immunohistochemical staining for α-SMA in pancreas tissue of the untreated
(A) and high dose candesartan (B) groups. (400x).

**A: Untreated group**

α-SMA positive cells with the stellate morphology (activated pancreatic stellate cells) are abundant in the peri-acinar fibrotic areas and vascular walls (right–half side).

**High dose candesartan group**

**B: α-SMA positive cells are present only in the vascular walls.**

**Figure 3**

Expression of angiotensinogen, AT1 receptor, AT2 receptor, and TGF-ß1 mRNAs analyzed by RT-PCR. Findings are for untreated and high dose candesartan groups and 20 week-old male Wistar rats. The β-actin mRNA is included as an internal control.
Table 1. Body Weight and Pancreas Weight in 20-Week-Old Male Wistar Rats and All Groups of WBN/Kob Rats and Calculated Doses and Serum Levels of Candesartan in the Low, Medium, and High Dose Groups of 20-Week-Old Male WBN/Kob Rats

<table>
<thead>
<tr>
<th></th>
<th>Concentration in Drinking Water (mg/L)</th>
<th>Candesartan Calculated Dose (mg/kg/day)</th>
<th>Serum Level@ (pg/mL)</th>
<th>Body Weight (g)</th>
<th>Pancreas Weight (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wistar</strong> (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Dose (n=5)</td>
<td>10.5</td>
<td>0.80 ± 0.08</td>
<td>55.0 ± 24.6</td>
<td>367.0 ± 2.9</td>
<td>2.42 ± 0.52#</td>
</tr>
<tr>
<td>Medium Dose (n=5)</td>
<td>42</td>
<td>4.34 ± 0.32</td>
<td>442.0 ± 112.6</td>
<td>326.8 ± 21.8++</td>
<td>3.01 ± 0.39##,**</td>
</tr>
<tr>
<td>High Dose (n=10)</td>
<td>125</td>
<td>13.92 ± 0.98</td>
<td>914.6 ± 382.0</td>
<td>316.5 ± 17.3++</td>
<td>2.81 ± 0.38##</td>
</tr>
</tbody>
</table>

Candesartan in drinking water, refreshed every other day, was given for 10 weeks. The amount consumed was measured and body weights were recorded weekly. The dose of candesartan was calculated from the amount consumed and body weight.

@ Serum levels of candesartan were determined in the low (n=5), medium (n=5), and high (n=5) dose group.
++: p<0.01 vs untreated and low dose groups.
*: p<0.05 vs low dose group.
#, ##,: p<0.05 vs untreated group, respectively.
Table 2. Effects of Low, Medium, and High Doses of Candesartan in Drinking Water on Histological Scores, Ratio of Fibrous Tissue, and Ratio of α-Smooth Muscle Actin Positive Cells of the Pancreas in 20-Week-Old Male WBN/Kob Rats

<table>
<thead>
<tr>
<th></th>
<th>Untreated (n=6)</th>
<th>Low Dose (n=5)</th>
<th>Medium Dose (n=5)</th>
<th>High Dose (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratios of</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrous Tissue (%)</td>
<td>45.2 ± 11.6</td>
<td>39.0 ± 9.0</td>
<td>35.8 ± 17.2</td>
<td>12.2 ± 3.8***</td>
</tr>
<tr>
<td>α-Smooth Muscle Actin Positive Cells (%)</td>
<td>9.21 ± 6.85</td>
<td>10.24 ± 6.13</td>
<td>3.30 ± 1.93</td>
<td>2.86 ± 0.79 **</td>
</tr>
</tbody>
</table>

Histological Score &

<table>
<thead>
<tr>
<th></th>
<th>Inflammatory Cell Infiltration</th>
<th>Interstitial Edema</th>
<th>Acinar Cell Necrosis</th>
<th>Hemorrhage</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (n=6)</td>
<td>2.2 ± 1.0</td>
<td>1.2 ± 0.8</td>
<td>1.0 ± 0.6</td>
<td>1.5 ± 0.5</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Low Dose (n=5)</td>
<td>1.6 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>0.8 ± 0.8</td>
<td>1.2 ± 0.4</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Medium Dose (n=5)</td>
<td>1.0 ± 0.0**</td>
<td>0.4 ± 0.5*,#</td>
<td>0.2 ± 0.4</td>
<td>1.0 ± 0.0</td>
<td>0.6 ± 0.5**,#</td>
</tr>
<tr>
<td>High Dose (n=5)</td>
<td>1.0 ± 0.0**</td>
<td>0.2 ± 0.4*,#</td>
<td>0.2 ± 0.4</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

*: Scores for each factor are averages and standard deviations for grade (none, mild, moderate and severe, 0-3).
***, **, *: p<0.001, 0.01 and 0.05 vs the untreated group, respectively.
#, ##, ###: p<0.001, 0.01 and 0.05 vs the low dose group, respectively.
+: p<0.05 vs the low dose group.

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 1A
Figure 1B
Figure 1C
Figure 1D
Figure 2B
Figure 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>WBN/Kob Untreated</th>
<th>WBN/Kob Candesartan</th>
<th>Wistar Untreated</th>
<th>Wistar Candesartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>723bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>566bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>616bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>554bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>658bp</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>