Protective role of angiotensin II type 2 receptor signaling in a mouse model of pancreatic fibrosis

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Ulmavos B, Xu Z, Tetri LH, Inagami T, Neuschwander-Tetri BA. Protective role of angiotensin II type 2 receptor signaling in a mouse model of pancreatic fibrosis. Am J Physiol Gastrointest Liver Physiol 296: G284–G294, 2009. First published November 25, 2008; doi:10.1152/ajpgi.90409.2008.—The renin-angiotensin system contributes to pathological processes in a variety of organs. In the pancreas, blocking the angiotensin II (AII) type 1 receptor (AT1) attenuates pancreatic fibrogenesis in animal models of pancreatitis. Because the role of the AII type 2 receptor (AT2) in modulating pancreatic injury is unknown we investigated the role of AT2 in pancreatic injury and fibrosis. Pancreatic fibrosis was induced by repetitive cerulein administration in C57BL/6 wild-type (WT) or AT2-deficient (AT2−/−) mice and assessed by morphology and gene expression at 10 days. There was no difference between WT and AT2−/− mice in the degree of acute pancreatic injury as assessed by amylase release at 9 and 12 h and by histological examination of the pancreas at 12 h. In contrast, parenchymal atrophy and fibrosis were more pronounced in AT2−/− mice compared with WT mice at 10 days. Fibrosis was accompanied by activation of pancreatic stellate cells (PSC) evaluated by Western blot analysis for α-smooth muscle actin and by immunocytochemistry; PSC activation was further increased in AT2−/− mice compared with WT mice. The level of pancreatic transforming growth factor-β1 mRNA and protein after repetitive cerulein treatment was higher in AT2−/− mice than in WT mice. Our results demonstrate that, in contrast to AT1 receptor signaling, AT2 receptor signaling modulates protective antifibrogenic effects in a mouse model of cerulein-induced pancreatic fibrogenesis. We propose that the effects of AII on injury-induced pancreatic fibrosis may be determined by the balance between AT1 and AT2 receptor signaling.

chronic pancreatitis; transforming growth factor-β1; pancreatitis

CHRONIC PANCREATITIS IS CHARACTERIZED by chronic inflammation, progressive parenchymal atrophy, and extensive fibrosis of the exocrine pancreas (45). The pathophysiology of chronic pancreatitis is not well understood, and thus the treatment strategies are mostly symptomatic. The most accepted theory is that repeated pancreatic injury by alcohol abuse or other causes of repetitive injury combined with a dysregulated ability to repair the organ damage leads to activation of a fibrotic cascade and eventually to irreversible exocrine and to a lesser extent endocrine insufficiency (9). Fibrosis in the pancreas is initiated by activation and proliferation of pancreatic stellate cells (PSC), which are the primary source of the fibrotic collagen extracellular matrix (2). Although several inflammatory mediators can activate PSC, transforming growth factor-β1 (TGF-β1) is considered to play a key role in this process (24).

Recent studies have suggested that the renin-angiotensin system (RAS) in addition to its classical role as endocrine regulator of blood pressure and body-fluid homeostasis (35) also acts as an intragranular local mediator of pathophysiological processes (34). Angiotensin II (AII), the main bioactive peptide of the RAS system, is produced by proteolytic cleavage of its precursor angiotensin I by angiotensin-converting enzyme (ACE), a step that has been exploited pharmacologically to block the effects of AII. AII exerts its biological actions via two distinct types of G protein-coupled receptors, designated as angiotensin receptor type 1 (AT1) and angiotensin receptor type 2 (AT2). The majority of well-known cardiovascular and renal effects of AII are mediated through the AT1 receptor. Anti-inflammatory and antifibrotic effects of AT1 receptor blockade in animal models of fibrotic disease have been reported in the heart, kidney, liver, and pancreas (26, 37, 39, 48). These findings suggest that the AT1 receptor mediates not only hemodynamic but also inflammatory and fibrogenic functions of AII. The mechanisms involved in the antifibrotic effects of AT1 inhibitors have been widely investigated but are still not fully understood. The effect of AT1 receptor blockers may not be attributable solely to their inhibition of the AT1 receptor because increased feedback activation of RAS in the setting of AT1 inhibition may increase stimulation of AT2 receptors (32). In contrast to the AT1 receptor, the pathophysiological role of the AT2 receptor remains poorly understood and controversial. Recent studies, however, demonstrated that the AT2 receptor mediates cellular differentiation and growth, opposing the actions of AII mediated by the AT1 receptor (4, 40). Studies of AT2 receptor knockout mice have revealed that AT2 receptor signaling is protective in a mouse model of vascular injury (46), glomerulonephritis (31), and hepatic fibrosis (25).

In the pancreas, all components of RAS are intrinsically present, and the level of their expression is enhanced in animal models of pancreatic diseases (for a recent review see Ref. 20). There are several reports describing the inhibition of RAS in animal models of pancreatitis. With the use of either pharmacological AT1 receptor blockers (41, 47) or AT1a knockout mice (26), blockade of the AT1 receptor ameliorates pancreatic fibrosis. Data concerning the blockade of ACE, which impairs activation of both the AT1 and AT2 receptors, with specific
ACE inhibitors are somewhat controversial. Tsang et al. (42) reported that the ACE inhibitor ramiprilat augmented pancreatic injury in a rat model of acute pancreatitis, yet Kuno et al. (19) found that lisinopril, another ACE inhibitor, alleviated chronic pancreatitis and fibrosis in rat models. The use of ACE inhibitors in humans is associated with a modest increased risk of acute pancreatitis (8). More studies are needed to understand the pathophysiological role of RAS in the pancreas before RAS inhibitors are used therapeutically in clinical practice for this purpose. The aim of present study was to clarify the role of AT2 receptor signaling in pancreatitis and pancreatic fibrogenesis.

To elucidate the role of the AT2 receptor in the pathophysiology of pancreatic injury and fibrosis, we compared the response to repetitive pancreatic injury in AT2 receptor wild-type mice (WT) and AT2 receptor deficient mice (AT2−/−) (15). Pancreatitis and fibrosis were induced using a repetitive cerulein model that we described previously (29).

**MATERIALS AND METHODS**

**Animals.** We used equal numbers of AT2−/− (female) and AT2−/−(male) mice on a C57BL/6 background. For simplicity, the term AT2−/− was used to designate both male and female AT2-deficient mice. The generation of AT2−/− mice has been described previously (15), and WT control C57BL/6 mice were obtained from Harlan (Indianapolis, IN). All mice were housed in standard facilities under controlled conditions of temperature, humidity, and a 12-h:12-h light/dark cycle and were maintained on standard rodent chow with free access to water. Animal care and all procedures were approved by the institutional animal care committee of Saint Louis University.

**Experimental pancreatitis and tissue processing.** Mice 7–8 wk old were divided into four groups of eight animals each and subjected to repeated episodes of acute pancreatitis. Pancreatitis was induced by repeated intraperitoneal injections of 50 μg/kg cerulein (Sigma, St. Louis, MO) as described previously (29). Control mice from all genotypes received comparable injections of 0.9% sodium chloride (saline). Six hourly injections given in one day constituted one treatment. To induce pancreatic fibrosis, treatments were repeated Monday, Wednesday, and Friday for 1 wk. To allow resolution of acute changes, mice were euthanized by CO2 asphyxiation 3 days after their final cerulein treatment. Pancreatic tissues were harvested, weighed, and divided into sections. Sections were immediately frozen in liquid nitrogen and stored at −80°C for subsequent protein extraction and Western blot analysis, fixed in 10% neutral buffered formalin (Sigma, St. Louis, MO) overnight for histological analysis, or placed in an RNA stabilization solution (RNAlater; Ambion, Austin, TX) and stored overnight at 4°C for RNA isolation and subsequent RT-PCR assays. Plasma amylase activity. For measurement of amylase activity, mice were subjected to a single treatment of 50 μg/kg cerulein (6 hourly injections). Mice were euthanized by CO2 asphyxiation 9 and 12 h after the first cerulein injection. Blood was collected, and plasma amylase activity was determined by a chromogenic method using Infinity Amylase Liquid Stable Reagent (ThermoTrace, Victoria, Australia) according to manufacturer’s protocol and expressed as units of amylase activity per liter.

**Histological analysis.** Formalin-fixed pancreatic sections were embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin according to standard methods. Three histopathological changes in the pancreas of acute injury, vacuolization, necrosis of pancreatic acinar cells, and inflammatory cell infiltration were blindly graded as previously described (30); the magnitude of parenchymal involvement by each feature was scored as absent or <5% = 0, 15–35% = 2, 35–50% = 3, and >50% = 4.

To assess pancreatic collagen content, sections were stained with Sirius red. Sections were pretreated to remove paraffin and stained in 0.1% of Sirius red (F3B) solution in saturated picric acid for 1 h. Slides were then washed in two changes of 0.09 N acetic acid, dehydrated in three changes of 100% ethanol, cleared in xylene, and finally mounted in Permount. The extent of collagen accumulation was evaluated by morphometric analysis (10). Images were captured with all exposures manually set at equal times using a Zeiss research light microscope equipped with an Olympus (Tokyo, Japan) digital camera by an investigator blinded to treatment group. Eight nonoverlapping images from each pancreas were acquired using the ×25 objective. Image analysis was performed using ImageJ software (ImageJ 1.37v; National Institutes of Health, Bethesda, MD). Images were converted from red, green, and blue (RGB) color (as captured to the grayscale RGB stack, then differentiated into three grayscale images representing red, green, and blue. For analysis, the green-gray scale images were used (14). Several pilot images were used to set the lower grayscale threshold for discrimination of Sirius red-positive material. To measure Sirius red-positive staining, the examination area was chosen and kept constant for all images, and the same threshold was applied to all images, and the integrated density for each image (the sum of the pixels in the selection) was calculated by the program. An image of a blank area of the slide was used for background correction. The amount of collagen was expressed relative to the amount of collagen in WT saline control group.

**Hydroxyproline assay.** Tissue samples (30–60 mg) were hydrolyzed in 2 ml 6 N HCl under argon for 16–24 h. The hydrolysates were filtered (0.2 μm) and derivatized with 9-fluorenemethylchloroformate (FMOC) for HPLC analysis. Briefly, 10 μl of hydrolysate was neutralized and buffered with 60 μl 1 M Na2CO3 and 35 μl 0.5 M potassium tetraborate (Sigma). To this, 10 μl of FMOC, 2.5 mg/ml in acetonitrile (Agilent Technologies, Santa Clara, CA) was added, and, after 10 min at room temperature, the samples were diluted by adding 200 μl water. The FMOC derivative of hydroxyproline was measured by HPLC using an Agilent 1100 series pump and fluorescence detector. A 10-μl aliquot was eluted isocratically with 30% acetonitrile, 70% 0.5 M sodium acetate, pH 4.4, through a Zorbax Eclipse AAA reverse phase column (3.0 × 150 mm, 35 μl, Agilent) equilibrated with a Zorbax Eclipse AAA guard column (4.6 × 12.5 mm, 5 μm) at a flow rate of 0.7 ml/min. The FMOC derivative of hydroxyproline was detected fluorometrically (excitation 265 nm, emission 315 nm); the peak area was calculated using Agilent Chemstation software and compared with a standard curve using derivatized trans-4-hydroxy-1-proline (Sigma).

**Immunohistochemistry.** Formalin-fixed pancreatic tissue sections were pretreated to remove paraffin and blocked with 3% bovine serum albumin (Sigma Chemical). Next, sections were incubated with primary antibody to α-smooth muscle actin (α-SMA, Sigma) overnight at 4°C. After being washed, slides were incubated with secondary antibodies (Alexa Fluor labeled; Invitrogen, Carlsbad, CA) at room temperature for 1 h, washed again, and mounted in the mounting solution (80% glycerol, 1% N-propyl gallate in PBS; Fisher Scientific, Pittsburgh, PA). Fluorescently labeled tissues were imaged on a Bio-Rad laser-scanning confocal microscope (Hercules, CA).

**Western blotting.** Pancreatic tissue samples were homogenized in ice-cold RIPA buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, and a freshly added mixture of a protease inhibitor cocktail (Sigma). Protein extracts (20 μg total protein) were resolved by SDS-PAGE and blotted to polyvinylidene fluoride membranes. Blots were blocked in 5% nonfat dried milk in Tris-buffered saline-Tween buffer (10 mM Tris·HCl, pH 7.4, 0.9% NaCl, 0.05% Tween-20) and probed with a monoclonal antibody to α-SMA (Sigma) in 1% nonfat dried milk. For the loading, control blots were probed with an antibody to histone deacetylase protein (HDAC2) (Santa Cruz Bio-technology, Santa Cruz, CA). Signals were developed using horseradish peroxidase-conjugated anti-mouse IgG (Sigma) for α-SMA and
anti-rabbit IgG (Santa Cruz Biotechnology) for HDAC2 and ECL Plus Western Blotting Detection Reagent (Amersham, Buckinghamshire, UK) followed by detection with X-ray film. Protein band intensities were quantified using Personal Densitometer SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Determination of TGF-β1 in pancreatic homogenates.** Pancreases were frozen in liquid nitrogen and kept at −80°C until they were homogenized in 1× RIPA buffer. Debris was removed by centrifugation. TGF-β1 activity was measured using a TGF-β1 ELISA kit (Quantikine; R and D Systems, Minneapolis, MN) following the manufacturer’s protocol and expressed as amount of TGF-β1 (picograms) per the amount of total protein (milligrams).

**Real-time RT-PCR.** To prepare total RNA, pancreatic tissue in RNA stabilization solution (RNAlater, Ambion) was extracted with TRizol reagent (Invitrogen) according to the manufacturer’s instructions. The quantity and purity of RNA was verified by measuring absorbance at 260 and 280 nm. Furthermore, the integrity of RNA was confirmed by electrophoresis on a formaldehyde-denaturing agarose gel. For real-time RT-PCR assays, 5 μg of total RNA was treated with DNase 1 (Ambion) and reverse transcribed to complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was performed with MiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories) using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions. PCR primers were either designed using Primer Express software (Applied Biosystems, Foster City, CA) or were chosen from Primer Bank (44). AT1a primers were designed according Kudoh et al. (18). Primer sequences of transcripts evaluated in real-time quantitative PCR are shown in Table 1. Threshold cycle numbers were determined using iCycler software version 1.0 (Bio-Rad). Amplification products were verified by melting curves. Reactions were performed in duplicate, and threshold cycle numbers were averaged. Control reactions in the absence of template were used as negative controls. Results were calculated with normalization to acidic ribosomal phosphoprotein P0 (ARP) mRNA. We used ARP in this study because it was shown to be a reliable housekeeping gene for detecting fibrotic changes in the pancreas (12, 23). Relative changes in mRNA abundance were calculated using the comparative threshold cycle (Ct) method (21).

**PSC isolation.** Mouse PSC were prepared similar to the method described previously for rats (1, 38). Briefly, the dissected pancreases from 10 mice were finely chopped and placed in digestion solution containing 0.03% collagenase type 2 (Worthington Biomedical, Lake-wood, NJ) and 0.1% DNase I (Sigma). After digestion for 30 min at 37°C with shaking, cells were mechanically dispersed by pipetting, and undigested tissue was removed by filtration through a nylon mesh. The cell suspension was centrifuged to obtain a pellet that was then resuspended in 12% OptiPrep density gradient medium (Sigma). A layer of HBSS (Sigma) was placed on the top of the gradient medium, and cells were centrifuged for 20 min at 1,400 g at 4°C. The stellate cells were collected from the band between the OptiPrep/HBSS interface. After being washed with HBSS, the isolated cells were plated onto plastic culture plates and maintained in 10% fetal bovine serum in DMEM (Sigma) and antibiotics at 37°C in a humidifying incubator with 5% CO₂.

**Statistical analysis.** The results were expressed as means ± SE. Statistical analysis was performed using one-way ANOVA with P values < 0.05 being considered statistically significant. Post hoc pairwise comparisons were performed using the t-test, and multiple pairwise comparisons were performed using the Holm-Sidak method (SigmaPlot 9.0; Systat Software, San Jose, CA).

**RESULTS**

Agt, AT1, and AT2 receptor expression in the pancreas. Real-time RT-PCR was used to assess the expression of angiotensinogen and angiotensin receptors in pancreas of WT and AT2−/− mice in normal and cerulein-treated mice. Steady-state pancreatic expression of angiotensinogen (Agt) mRNA increased approximately fourfold following repetitive cerulein administration (Fig. 1A). The level of Agt expression in AT2−/− mice showed a trend toward being slightly higher than in AT2+/+ mice, but the difference was not statistically significant. To determine whether repetitive injury also changed the expression of AT1a receptors, the mRNA abundance for AT1a, AT1b, and AT were examined. Whereas in humans AT1 is encoded by one gene, in rodents it is encoded by two highly similar genes identified as AT1a and AT1b. Whether the two are functionally dissimilar is unknown, but repetitive cerulein treatment differentially regulated the pancreatic mRNA of AT1a and AT1b receptors, downregulating AT1a and upregulating AT1b mRNA (Fig. 1B and C). The specificity of the PCR primers used to identify AT1a and AT1b expression was confirmed by using these primers to amplify mRNA isolated from pancreas of AT1a and AT1b knockout mice that were kindly provided by Dr. Thomas Coffman (Duke Univ.) (Fig. 1E). Expression of AT2 receptor mRNA could not be detected in cerulein-treated AT2−/− mice or in saline-treated WT mice. After repetitive cerulein treatment, AT2 mRNA expression could be detected in the pancreas of WT mice although at significantly lower levels than AT1 mRNA (Fig. 1D).

**Effects of AT2 receptor deletion on acute pancreatic injury induced by cerulein.** To evaluate the possibility that the absence of AT2 receptor in AT2−/− mice might influence the degree of acute pancreatic injury induced by cerulein treatment, we evaluated pancreatic weight, histological changes, and plasma levels of amylase activity after a single episode of cerulein-induced acute pancreatitis. One treatment of six hourly intraperitoneal injections of cerulein significantly increased the pancreatic weight at 12 h after the first cerulein injection.

**Table 1. GenBank accession numbers and primer sequences of genes evaluated by real-time quantitative PCR**

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<td>5'-CGAGGACGAGGACGACTG-3'</td>
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<tr>
<td>TGF-β1</td>
<td>NM_011577</td>
<td>5'-CTCTGACGGAGGACGACTG-3'</td>
<td>5'-CTCTGACGGAGGACGACTG-3'</td>
</tr>
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ARP, acidic ribosomal phosphoprotein; Agt, angiotensinogen; AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor, TGF-β1, transforming growth factor-β1.
injection (Fig. 2A) and plasma levels of amylase at 9 and 12 h after the first cerulein injection (Fig. 2B). There was no significant difference in the pancreatic weight or level of plasma amylase activity between WT and AT2/H/H/H mice with saline treatment, and there was no difference between the two groups after cerulein treatment. Histological manifestations of acute pancreatitis (Fig. 2C) were assessed by blinded scoring at 12 h after the first cerulein injection and were not significantly different between WT and AT2/H/H/H mice (acinar necrosis 2.5 ± 0.5 vs. 2.9 ± 0.5, vacuolization 2.5 ± 0.5 vs. 3.0 ± 0.5, and inflammatory cell infiltration 2.8 ± 0.5 vs. 3.0 ± 0.5 in WT and AT2/H/H/H mice, respectively).

Effect of AT2 receptor deletion on the severity of pancreatic changes following repeated episodes of acute pancreatitis. To explore the role of the AT2 receptor in pancreatic remodeling and fibrosis associated with repeated injury, 7-wk-old mice (8 mice per group) were subjected to three episodes of acute cerulein injury per week and euthanized 3 days after the last cerulein injection to allow for the resolution of the acute changes. The ratios of pancreatic weight to body weight as well as histological changes in the pancreas were evaluated for the cerulein-treated mice and saline-treated control groups. Pancreatic weights were significantly lower in cerulein-treated groups compared with control mice, suggesting significant organ atrophy with repetitive injury. The AT2/H/H/H mice were found to have more markedly decreased pancreatic weight compared with WT mice after cerulein treatment (Fig. 3A). There was no significant difference in pancreatic weight between WT and AT2/H/H/H mice without cerulein treatment.

Morphological changes in the pancreas were assessed by hematoxylin and eosin staining (Fig. 3B). In the normal pancreas, the acinar units were tightly packed (Fig. 3Ba), and there was no difference in appearance between AT2/H/H/H and WT mice without cerulein treatment (Fig. 3Bb). Repetitive cerulein treatment induced striking changes in the pancreas. Intra-acinar lumina became dilated, and some acinar units dedifferentiated into tubular complexes; inflammatory cell infiltration and increased cellularity of periacinar stroma also developed (Fig. 3Bc). AT2/H/H/H mice had more severe pancreatic morphological alterations; in fact, in some areas, acinar cell architecture was completely disrupted and replaced by fibrous stroma around residual tubular structures without evidence of residual acinar cells (Fig. 3Bd).
Effect of AT2 receptor deletion on collagen deposition following repeated episodes of acute pancreatitis. Sirius red staining (22) and hydroxyproline content were used as measures of collagen deposition in the pancreas. In saline-treated mice, Sirius red staining was detected only in interlobular areas and around vessels and pancreatic ducts (Fig. 4Aa). AT2 receptor deletion did not change the pattern of Sirius red staining under these conditions (Fig. 4Ab). Repetitive cerulein treatment induced interlobular staining in WT mice (Fig. 4A); this was further accentuated in mice with AT2 receptor deletion with the presence of marked intralobular and enhanced interlobular staining compared with WT cerulein-treated mice (Fig. 4Ad). To quantify these fibrogenic changes, the extent of Sirius red staining was evaluated with morphometric image analysis (Fig. 4B). This analysis confirmed that the amount of pancreatic Sirius red-positive staining increased ~170% after repetitive cerulein treatment in WT mice and ~270% in AT2−/− knockout mice.

Pancreatic collagen accumulation was also assessed by measuring hydroxyproline, a posttranslationally modified amino acid found in collagen. Pancreatic hydroxyproline content increased 2.5-fold in WT mice treated with cerulein compared with saline-treated control mice. Cerulein-treated AT2−/− mice had further increased hydroxyproline content compared with cerulein-treated WT mice (Fig. 4C).

Effects of AT2 receptor deletion on PSC activation following repeated episodes of acute pancreatitis. Pancreatic fibroblast-like cells that are immunoreactive for α-SMA likely represent activated PSC. Mice were treated with repeated episodes of cerulein injections or saline injections as described in MATERIALS AND METHODS, and pancreatic tissues were stained for α-SMA. Pancreatic tissues from saline-treated control mice contained no interstitial α-SMA-positive staining cells, whereas normal vascular smooth muscle cells around blood vessels stained prominently (Fig. 5Aa). AT2-deficient saline-treated mice had the same pattern of α-SMA staining as WT mice (Fig. 5Ab). In contrast, pancreatic tissues from cerulein-treated mice contained α-SMA-positive cells in the periacinar spaces (Fig. 5Ac). Pancreas from cerulein-treated AT2−/− mice had increased α-SMA staining in periacinar space. Western blot analysis demonstrated that α-SMA expression was significantly increased in cerulein-treated mice and that AT2−/− mice expressed more α-SMA than WT mice after cerulein treatment (Fig. 5, B and C).

Fig. 2. Parameters of pancreatic injury after a single course of acute pancreatitis. Acute pancreatitis was induced by cerulein administration (50 μg/kg per h for 6 hourly intraperitoneal injections). Saline-injected mice were used as controls. Mice were euthanized at 9 and 12 h after the first cerulein injection. Pancreatic weight relative to total body weight was measured at 12 h (8 mice per group) and shows similar increases in both AT2 WT and AT2−/− mice (A). Plasma amylase levels measured at 9 and 12 h (6 and 8 mice per group, respectively) were also similarly elevated in both groups (B). Results represent means ± SE; *P < 0.001. Histological changes in the pancreas were evaluated at 12 h by hematoxylin and eosin stain (original magnification ×200) using the scoring system of vacuolization, necrosis, and inflammation as described in MATERIALS AND METHODS (8 mice per group) (C). Pancreas from saline-treated WT (a) and AT2−/− mice (b) appeared normal, whereas the pancreas from cerulein-treated WT (c) and cerulein-treated AT2−/− (d) mice showed a similar extent of acute injury. Blinded scoring did not identify differences between the treated groups (8 mice per group).
Effects of AT2 deletion on TGF-β1 mRNA and protein expression in the pancreas. TGF-β1 is a central mediator of extracellular matrix deposition in the development of pancreatic fibrosis (24). The transcriptional level of TGF-β1 mRNA expression in the pancreas was analyzed using real-time RT-PCR in WT as well as AT2+/−/− mice following control saline or repetitive cerulein treatment. TGF-β1 mRNA was found to be significantly increased with cerulein treatment and further increased in the AT2+/−/− group compared with the WT group (Fig. 6A). The changes in mRNA were reflected in corresponding changes in active TGF-β1 protein in the pancreas (Fig. 6B).

AT1 and AT2 receptor mRNA expression in activated PSC. Because PSC play a critical role in the pathogenesis of pancreatic fibrosis, we isolated PSC from WT and AT2+/−/− mice and analyzed the expression of AT1 and AT2 receptors in activated PSC. All three AII receptor subtype transcripts were detected in PSC activated by culture on plastic plates (Fig. 7). The relative amount of AT2 transcript was about 20-fold higher than AT1a or AT1b transcripts in PSC cultured without serum for 3 days. Because the mRNA expression of AT2 receptor in mouse R3T3 fibroblasts has been shown to be strongly downregulated by serum (3), we evaluated the effect of 10% FBS on the mRNA expression of angiotensin receptors in PSC and found that serum dramatically (∼60-fold) downregulated mRNA levels of the AT2 receptor, whereas the mRNA expression of AT1 receptors was not significantly changed. AII did not affect the mRNA expression of AT1 or AT2 receptors in vitro. The expression of AT1a and AT1b receptors in PSC isolated from AT2+/−/− mice did not vary significantly from their expression in PSC isolated from WT mice (data are not shown).

Fig. 3. Severity of cerulein-induced pancreatitis after repetitive episodes of acute pancreatitis. Mice were subjected to 3 episodes of acute pancreatitis (6 cerulein treatments per day every other day for 3 days) and euthanized 3 days after the last treatment. A: pancreatic weight relative to total body weight. Note the decrease in pancreatic weight of cerulein-treated AT2+/−/− mice compared with cerulein-treated WT mice (8 mice per group), *P < 0.001. B: histological changes in the pancreas of WT and AT2+/−/− mice after repetitive episodes of acute pancreatitis (6 mice per group, hematoxylin and eosin stain, original magnification ×200). Control pancreas from WT (a) and AT2+/−/− mice (b) after control saline treatment. Pancreas from cerulein-treated WT (c) and cerulein-treated AT2+/−/− (d) mice. Note more severe morphological alterations such as disruption of acinar cell architecture and inflammatory cell infiltration in cerulein-treated AT2+/−/− mice (d) compared with cerulein-treated WT mice (c).
Fig. 4. Collagen content in the pancreas after repetitive episodes of acute pancreatitis. Mice were subjected to 3 episodes of acute pancreatitis (6 cerulein treatments per day every other day for 3 days) and euthanized 3 days after the last treatment. A: representative Sirius red staining of pancreatic sections of control pancreas from WT (a) and AT2−/− (b) mice and from cerulein-treated WT (c) and AT2−/− (d) mice. Collagen staining appears red (original magnification ×250). B: graph represents the relative amount of pancreatic collagen quantified by morphometric analysis as described in MATERIALS AND METHODS. Note the increased collagen content of cerulein-treated AT2−/− mice vs. WT mice. Results are expressed as means ± SE, n = 6, *P < 0.001. C: hydroxyproline content of the pancreas. Similar to Sirius red staining, biochemical measurement of hydroxyproline demonstrated increased collagen after cerulein treatment in WT mice with further increases in AT2−/− mice. There was a trend to increased hydroxyproline in untreated AT2−/− mice compared with control mice that did not reach statistical significance. Cerulein treatment induced significantly increased hydroxyproline in WT and AT2−/− compared with the corresponding untreated mice. Pancreatic hydroxyproline in AT2−/− mice was significantly higher than WT mice after cerulein treatment. Data are expressed as means ± SE, n = 3, *P < 0.05.
Fig. 5. Activation of pancreatic stellate cells (PSC) during the course of cerulein-induced pancreatitis. Mice were subjected to 3 episodes of acute pancreatitis (6 cerulein treatments per day every other day for 3 days) for 3 treatments and euthanized 3 days after the last treatment. α-Smooth muscle actin (α-SMA) was used as a marker of PSC activation. A: representative immunofluorescent staining for α-SMA in pancreatic sections of saline-treated WT (a) and AT2−/− (b) mice and from cerulein-treated WT (c) and AT2−/− (d) mice (original magnification ×600). In control mice without cerulein treatment, α-SMA staining was located only around the blood vessels; in cerulein-treated mice α-SMA staining was observed also around the acini and occasionally in the interstitial space (c and d) with more intense staining in AT2−/− mice. B: Western blotting of pancreatic extracts confirmed the presence of increased α-SMA in AT2−/− mice. Histone deacetylase protein (HDAC2) was used as a loading control. C: densitometric analysis of α-SMA protein expression after repetitive cerulein treatment. Note the increase in α-SMA protein expression in cerulein-treated AT2−/− mice compared with cerulein-treated WT mice. Data are means of 6 mice per group ± SE. *P < 0.05.
DISCUSSION

The tissue RAS plays an important role in organ remodeling and fibrogenesis in different organs including the pancreas (20, 34). Recent studies in AT1a knockout mice have demonstrated that the AII-AT1a receptor pathway is essential in the development of pancreatic fibrosis and that the use of AT1 receptor antagonists may be beneficial in the treatment of chronic pancreatitis (26). Interestingly, repetitive cerulein administration caused differential regulation of AT1 receptor isoform mRNA, increasing AT1b transcript levels while decreasing AT1a transcript levels. The significance of this differential regulation is unknown. The function of the AT1b gene in rodents has been largely ignored, and it is unclear which isoform functions most like the human AT1 gene product. Although genetic deletion of AT1b is not associated with significant phenotypic changes (6), specifically blocking the AT1 receptor could be antifibrotic, whereas nonspecifically impairing signaling through both receptors with the use of ACE inhibitors might not have the desired antifibrotic effect (16). The goal of this study was to understand the role of AT2 receptor signaling in a mouse model of pancreatic fibrosis induced by repetitive acute injury. We used genetically modified AT2−/− C57BL/6 mice (15) given repetitive cerulein treatments, which we described earlier for Swiss-Webster mice (28, 29). We found that the C57BL/6 mice were strikingly more sensitive to repetitive cerulein treatment than Swiss-Webster mice and after 1 wk of treatment developed pancreatic changes similar to those seen after 6 wk of treatment of the Swiss-Webster strain.

All components of RAS are present in the pancreas although the data are sometimes controversial and not consistent among species (for the recent review see Ref. 20). In this study, we evaluated Agt and AII receptor subtype (AT1a, AT1b, and AT2) mRNA expression in normal mouse pancreas and in the pancreas after the repetitive cerulein treatment. The Agt transcript was detected in mouse pancreas, and cerulein treatment caused a significant increase in Agt mRNA expression, indicating that repetitive injury may provide a stimulus for the local intraorgan activation of RAS. We also confirmed the presence of AT1a and AT1b receptor mRNA in mouse pancreas (26). Interestingly, repetitive cerulein administration caused differential regulation of AT1 receptor isoform mRNA, increasing AT1b transcript levels while decreasing AT1a transcript levels. The significance of this differential regulation is unknown. The function of the AT1b gene in rodents has been largely ignored, and it is unclear which isoform functions most like the human AT1 gene product. Although genetic deletion of AT1b is not associated with significant phenotypic changes (6),
it does partially replace the function of AT1a when AT1a is deleted (7, 43). The level of AT2 mRNA expression in normal mouse pancreas was extremely low. In fact, we were not able to detect the transcript using the same real-time PCR conditions that we used for the detection of AT1a and AT1b receptor mRNA, consistent with previous observations that AT2 expression occurs primarily during development and in response to injury (11). Indeed, repetitive cerulein treatment raised the level of pancreatic WT mouse AT2 mRNA expression well into a measurable range. Similar differential regulation of AII receptors as we demonstrated here with selective increases in AT1b and AT2 but no change in AT1a mRNA was also reported in rat pancreas in response to chronic hypoxia (5).

Because of the possibility that increased severity of acute pancreatic injury could be responsible for the increased severity of pancreatic fibrosis in AT2−/− mice, we evaluated the role of AT2 receptor deletion in acute pancreatic injury. We found that there was no significant difference in acute injury between AT2−/− and WT mice as assessed by analyzing changes in pancreatic weight, level of plasma amylase activity, and histological evaluation after one cerulein treatment (consisting of six hourly injections).

In the present study, we provide evidence for enhanced pancreatic remodeling and fibrosis in AT2−/− mice compared with WT C57BL/6 mice by measuring changes in pancreatic weight and by histological evaluation. The pancreatic damage was associated with pancreatic fibrogenesis in WT mice as determined by Sirius red staining (a marker of collagen deposition) and pancreatic hydroxyproline content, and these fibrogenic changes were increased in AT2−/− mice compared with WT mice.

In the pancreas, PSC play a key role in fibrogenesis (33). In response to the injury, they develop an activated myofibroblast-like phenotype-expressing α-SMA, proliferate, and secrete extracellular matrix proteins. In the present study, PSCs were activated to a greater degree in AT2−/− mice than in WT mice as indicated by α-SMA immunostaining of pancreatic sections as well as by Western blot for α-SMA protein. This raises the possibility that the increased fibrosis in the pancreas of AT2−/− mice is mediated by increased activation and proliferation of PSCs. The profibrogenic cytokine TGF-β1 plays an essential role in fibrogenesis in different organs, including the pancreas (27). We found that AT2−/− mice expressed higher amounts of pancreatic TGF-β1 mRNA and protein than WT mice in response to repetitive cerulein administration, suggesting that absence of AT2 receptors increases the production of TGF-β1 in pancreas in response to repetitive injury.

To begin to understand the mechanism of AT2 receptor signaling in pancreatic fibrosis, we analyzed the expression of AT1 and AT2 receptors in PSC isolated from WT and AT2−/− mice. Although previous studies have identified AT1 receptors in rat PSC, the data regarding AT2 expression in rat PSC are somewhat controversial; one group has reported the absence of AT2 receptor expression in rat PSC as detected by RT-PCR and by immunostaining (36), yet other groups have demonstrated the expression of the AT2 receptor in rat PSC by Western blot (13) or by real-time RT-PCR (17). We demonstrated the expression of both AT1 and AT2 receptor mRNA in mouse PSC by real-time RT-PCR and found that, similar to other cell types (3), AT2 receptor expression can be modulated by changing extracellular conditions. Technical issues with available antibodies prevent the reliable detection of AT2 protein in tissue or cell lysates.

In summary, the finding of enhanced pancreatic remodeling and fibrosis in AT2 knockout mice suggests that AT2 receptor signaling has protective antifibrotic effects in the repetitive cerulein model of chronic pancreatitis. Taken together with the data showing that AT1a receptor signaling enhances pancreatic fibrosis, our data imply that AT1a and AT2 receptors have opposing roles in the development of pancreatic fibrosis and that a balance between AT1 and AT2 receptor signaling plays an important role in the development of the disease. This may have significant therapeutic implications in how the RAS might be best manipulated to prevent pancreatic fibrogenesis.

GRANTS
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REFERENCES


