Expression and localization of the renin–angiotensin system in the rat pancreas

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Abstract

The possibility of an intrinsic renin–angiotensin system (RAS) in the pancreas has been raised by previous studies in which immunohistochemical examination showed the presence of angiotensin II and its receptor subtypes, type 1 (AT_1) and type 2 (AT_2). In the present study, gene expression of several key RAS components was investigated by reverse-transcription PCR. mRNA expression for angiotensinogen, renin and angiotensin II receptor subtypes, AT_{1a}, AT_{1b} and AT_2 was shown. The presence of angiotensinogen protein, the mandatory component for an intrinsic RAS, was demonstrated by Western blotting and localized by immunohistochemistry to the epithelia and endothelia of pancreatic ducts and blood vessels respectively. Immunoblot analysis detected a predominant protein band of about 60 kDa in the pancreas. This was consistent with the predicted value for angiotensinogen as reported in other tissues. Together with previous findings, the present study shows that the rat pancreas expresses the major RAS component genes, notably angiotensinogen and renin, required for intracellular formation of angiotensin II. The data support the notion of an intrinsic RAS in the rat pancreas which may play a role in the regulation of pancreatic functions.

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Introduction

The systemic renin–angiotensin II system (RAS) plays an important role in the endocrinological regulation of blood pressure and electrolyte balance (Peach 1977). It has several key components. The physiologically active component is angiotensin II, which is synthesized from the precursor protein angiotensinogen produced in the liver. Circulating angiotensinogen is then sequentially processed by renin and angiotensin-converting enzyme, which are derived from the kidney and lung respectively. The action of angiotensin II formed in this manner is mediated through interaction with two pharmacologically defined receptor subtypes, type 1 (AT_1) and type 2 (AT_2), distributed in numerous tissues (Timmermans *et al.* 1993). In addition, angiotensin II may also be produced locally in tissues outside the humoral system. Indeed, a local RAS which may mediate tissue functions via paracrine/autocrine actions has been proposed to be present in various tissues including the brain, heart, kidney and gonads (Phillips *et al.* 1993*a*). For example, it has been suggested that an RAS in the gonads may be important in the regulation of various functions of the male and female reproductive systems, as well as affecting fertility (Ganong 1995, Vinson *et al.* 1997).

In the pancreas, binding sites for angiotensin II have been demonstrated in the rat (Ghiani & Masini 1995), indicating that it may act as a local factor in the mediation of pancreatic function. Recently, angiotensin II and its receptor subtypes, AT_1 and AT_2, have been demonstrated immunohistochemically to be predominantly present in the epithelia of the ductal system and endothelia of the blood vessels in the pancreas of rodents, suggesting a paracrine or autocrine role for RAS in regulating exocrine pancreatic anion secretion and local blood flow (Leung *et al.* 1997, 1998*a*). Interestingly, anion secretion by cystic fibrosis pancreatic duct cells has been shown to be mediated through AT_1 (Chan *et al.* 1997), further supporting the notion that locally formed angiotensins may be important in the regulation of exocrine pancreatic function. The present study is therefore aimed at elaborating the expression and localization of key RAS components in the rat pancreas, with special emphasis on angiotensinogen, which is the mandatory component for local production of angiotensin II.

Materials and Methods

Preparation of total RNA from tissues

Adult Sprague–Dawley rats weighing about 350 g were used. The animals were bred in the Laboratory Animal Services Centre, Chinese University of Hong Kong. Total RNA was isolated from the pancreas, adrenal, liver and

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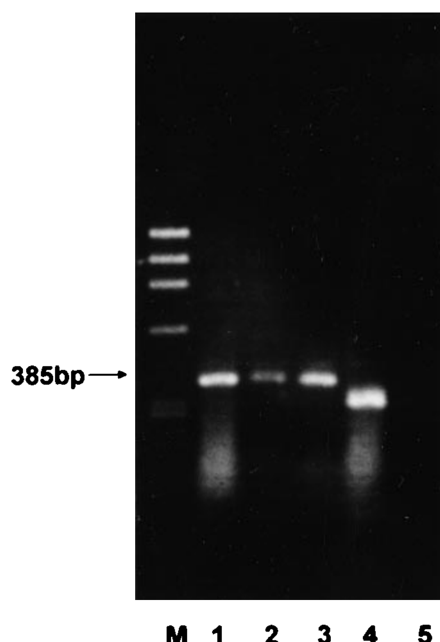

Figure 1 RT-PCR analysis of AT_{1a} mRNA in rat pancreatic tissue as well as adrenal and kidney tissue of mature rats. Lane M, DNA marker (φX174 RF/HaeIII fragments); lane 1, AT_{1a} mRNA expression in pancreatic tissue; lane 2, AT_{1a} mRNA expression in adrenal gland; lane 3, AT_{1a} mRNA expression in kidney; lane 4, GAPDH mRNA expression in pancreatic tissue; lane 5, control in which no reverse transcriptase was added to the first-strand cDNA synthesis for RT-PCR of GAPDH in the pancreas. The arrow (385 bp) indicates the expected size of amplified products from AT_{1a}.

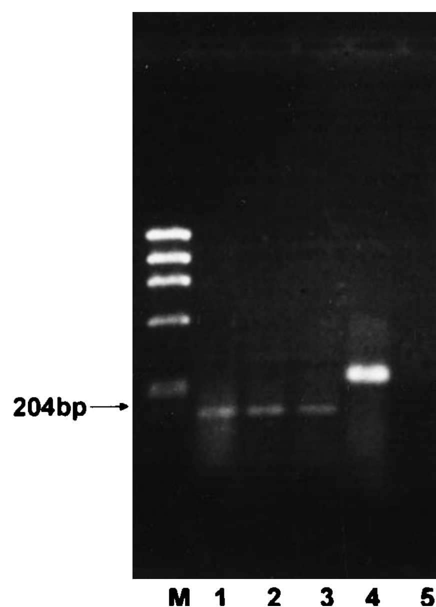

Figure 2 RT-PCR analysis of AT_{1b} mRNA in rat pancreatic tissue as well as adrenal and kidney tissue. Lane M, DNA marker; lane 1, AT_{1b} mRNA expression in pancreatic tissue; lane 2, AT_{1b} mRNA expression in adrenal gland; lane 3, AT_{1b} mRNA expression in kidney; lane 4, GAPDH mRNA expression in pancreatic tissue; lane 5, control in which no reverse transcriptase was added for RT-PCR of GAPDH in the pancreas. The arrow (204 bp) indicates the expected size of amplified products from AT_{1b}.

kidney by the acid guanidinium thiocyanate–phenol–chloroform protocol (Chomczynski & Sacchi 1987). Briefly, rat tissues were homogenized in 4 M guanidinium thiocyanate solution and repeatedly extracted with water-saturated phenol. After extraction with chloroform, RNA was precipitated with isopropanol. The resultant pellet was finally resuspended in water treated with diethyl pyrocarbonate. Total RNA was studied by gel electrophoresis and quantified by spectrophotometry.

Reverse-transcription PCR (RT-PCR)

Total RNA (10 μg) was subjected to first-strand cDNA synthesis using random hexamer primers and Superscript II transcriptase (Gibco-BRL, Grand Island, NY, USA) in a final volume of 20 μl. After incubation at 42 °C for 1 h, the reaction mixture was treated with RNase H before PCR analysis. The final mixture (2 μl) was directly used for PCR amplification.

Different sets of oligonucleotide primers based on the corresponding RAS component genes were synthesized (Gibco-BRL) for PCR analysis. mRNAs for AT_{1a}, AT_{1b}, AT_2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified using primers specific for their respective sequences as described previously (Leung *et al.* 1998*b*). Angiotensinogen mRNA was detected using primers flanking positions 245–557 of the rat angiotensinogen cDNA as described previously (Sernia *et al.* 1994). The oligonucleotide primers used for amplification of renin mRNA were based on rat renin cDNA as reported (Okura *et al.* 1991). PCRs were carried out in a volume of 50 μl containing the corresponding sense and antisense primer sequences using the PCR Reagent System (Gibco-BRL). PCR conditions were as follows: denaturation at 94 °C for 1 min; annealing at 58 °C for 1 min; elongation at 72 °C for 2 min. The reaction was repeated for 40 cycles. The amplified mixture (10 μl) was finally analysed by 2% agarose gel electrophoresis, and the amplified DNA bands were detected using ethidium bromide staining.

Extraction of angiotensinogen protein and immunoblotting

Pancreatic tissue from adult Sprague–Dawley rats (350 g) was homogenized at 4 °C in water (1:9, w/v) containing 10 mM EDTA and 1 mM phenylmethylsulphonyl fluoride. The homogenate was sedimented at 10 000 ***g*** for 30 min at 4 °C and the pellet discarded. The supernatant was adjusted to pH 4·5 with acetic acid and left for 1 h at 4 °C. The acidic supernatant was re-centrifuged at 1000 ***g***

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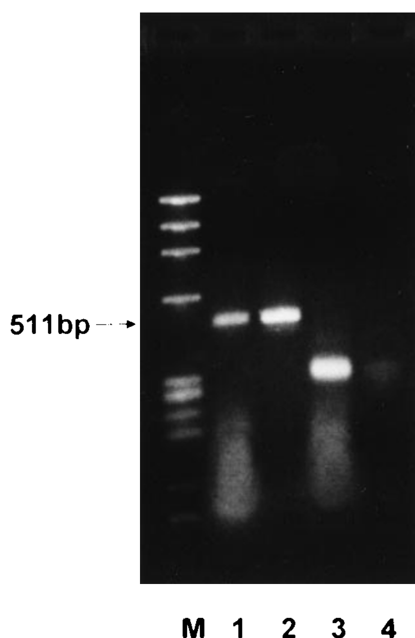

Figure 3 RT-PCR analysis of AT_2 mRNA in rat pancreatic and adrenal tissues. Lane M, DNA marker; lane 1, AT_2 mRNA expression in pancreatic tissue; lane 2, AT_2 mRNA expression in adrenal gland; lane 3, GAPDH mRNA expression in pancreatic tissue; lane 4, control in which no reverse transcriptase was added for RT-PCR of GAPDH in the pancreas. The arrow (511 bp) indicates the expected size of PCR products from AT_2.

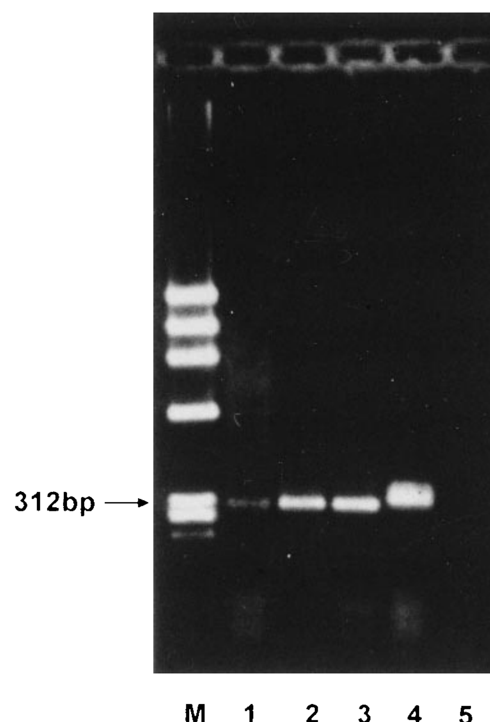

Figure 4 Expression of angiotensinogen mRNA in rat pancreas, liver and kidney. Lane M, DNA marker; lane 1, RT-PCR amplification of angiotensinogen mRNA in pancreas; lane 2, RT-PCR amplification of angiotensinogen mRNA in liver; lane 3, RT-PCR amplification of angiotensinogen mRNA in kidney; lane 4, RT-PCR amplification of GAPDH in pancreas; lane 5, control in which no reverse transcriptase was added for RT-PCR of GAPDH in the pancreas. The arrow (312 bp) indicates the expected size of PCR products from angiotensinogen.

for 20 min. The supernatant was then neutralized to pH 7·4 and assayed for protein (Bio-Rad Protein Assay) before being subjected to SDS–PAGE.

Supernatant (5–10 μg protein/lane) was electrophoresed on a 12% polyacrylamide gel in SDS (Laemmli 1970), and the gel subsequently processed for electroblotting to polyvinylidene difluoride (PVDF) membrane. The blotted PVDF membrane was first saturated with 5% (w/v) skimmed milk in PBS, pH 7·4, and 0·1% (v/v) Tween 20 for 1 h at room temperature. The membrane was incubated overnight at 4 °C in rabbit anti-rat angiotensinogen serum (1:5000 dilution). This angiotensinogen antibody did not cross-react with angiotensin I and angiotensin II as reported previously (Thomas & Sernia 1988). The membrane was then incubated in peroxidase-labelled anti-rabbit IgG (1:500 dilution) for 1 h at room temperature. After thorough washing, the positive band was revealed using ECL Western blotting detection reagents and autoradiography film (Amersham International, Amersham, Bucks, UK).

Immunohistochemical localization of angiotensinogen

The pancreas was dissected from the rat (*n*=10), and fats and other connective tissues were removed under a stereomicroscope. The pancreas was then divided into its head and splenic parts and prepared for frozen sectioning as described previously (Leung *et al.* 1997). Consecutive cryosections (8 μm) were cut on a cryotome (Shandon AS 620, Cryotome, Cheshire, UK). After the sections had been fixed with cold freshly prepared paraformaldehye (4%) for 30 min, they were processed for indirect immunoperoxidase staining. They were first rinsed in methanol containing 0·3% H_2O_2 for 30 min and then incubated overnight at 4 °C in anti-rat angiotensinogen serum (Thomas & Sernia 1988) diluted 1:500. The primary antibody was detected using an avidin–biotin conjugate kit (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Positive immunoreactivity for angiotensinogen was visualized by reacting sections with Vector VIP substrate kit (Vector Laboratories). Sections were slightly counterstained with haematoxylin, dehydrated and mounted. The following controls were used: (1) replacement of primary antibodies with buffer; (2) incubation with rabbit non-immune serum; (3) preadsorption of angiotensinogen antibody with excess angiotensinogen purified from plasma.

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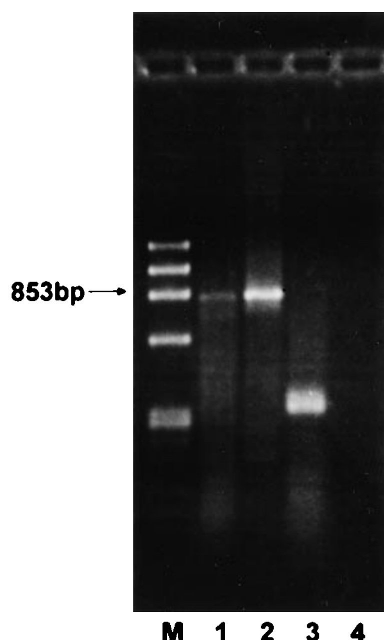

Figure 5 Expression of renin mRNA in rat pancreas and kidney. Lane M, DNA marker; lane 1, RT-PCR amplification of renin mRNA in pancreas; lane 2, RT-PCR amplification of renin mRNA in kidney; lane 3, RT-PCR amplification of GAPDH in the pancreas; lane 4, control in which no reverse transcriptase was added for RT-PCR of GAPDH in the pancreas. The arrow (853 bp) indicates the expected size of PCR products from renin.

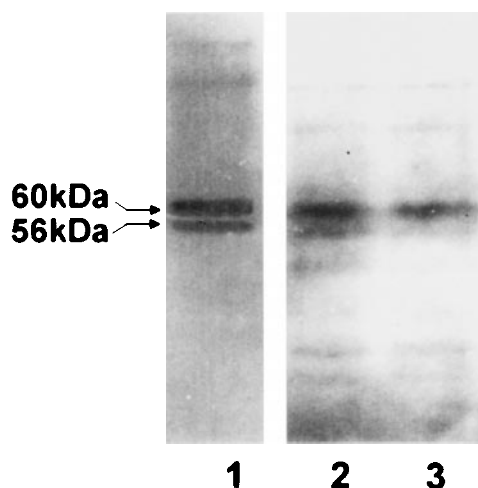

Figure 6 Western blot analysis for angiotensinogen of SDS–PAGE separated proteins from homogenates of rat pancreas. Lane 1 shows the detection of two isoforms of angiotensinogen with molecular sizes of about 60 and 56 kDa respectively from plasma of nephrectomized rats. A major protein band of about 60 kDa was detected in homogenates (10 µg protein (lane 2); 5 µg protein (lane 3)) of the rat pancreas.

Results

Expression of RAS component mRNAs

As a first step to identifying RAS component mRNAs, RT-PCR coupled with specific primers for the corresponding angiotensinogen, renin and angiotensin receptor subtype genes was performed to elucidate the expression levels of these key RAS components in the rat pancreas. Results from RT-PCR showed that mRNAs of angiotensin II receptor subtype genes, namely AT_{1a} (Fig. 1), AT_{1b} (Fig. 2) and AT_2 (Fig. 3), were consistently expressed in the rat pancreatic tissue. Angiotensinogen mRNA, which is the indispensable component of a local RAS, was found to be expressed in the rat pancreas, although its level of expression appeared to be low when compared with that in the liver and kidney (Fig. 4). In addition, renin mRNA which is the determining enzyme in the conversion of angiotensinogen into the physiologically active component of the RAS, i.e. angiotensin II, was also demonstrated to be present in the rat pancreas (Fig. 5). Therefore the pancreas appears to express the major RAS component genes, especially that for the precursor protein angiotensinogen.

Expression of angiotensinogen protein

In order to study the expression of the precursor angiotensinogen at the protein level, SDS–PAGE and Western blot analysis were performed on the homogenate of rat pancreas. A major protein band of about 60 kDa was apparently expressed in the rat pancreas. This molecular mass is in close agreement with that of one of the angiotensinogen isoforms purified from plasma, which was used as a positive control in the experiment (Fig. 6).

Localization of angiotensinogen

The precise localization of angiotensinogen was studied by immunohistochemistry using a specific antibody against the purified rat protein. Immunohistochemical examination of the rat pancreas showed that immunoreactivity for angiotensinogen appeared to be localized exclusively in the pancreatic ducts and blood vessels (Fig. 7*a*). Under closer examination of the pancreatic sections, intense immunostaining for angiotensinogen was specifically localized to the endothelial lining of the blood vessels (Fig. 7*b*). Immunostaining for angiotensinogen was also predominantly localized in the cytoplasm of the epithelial cells from pancreatic ducts (Fig. 7*c*). Intense immunostaining for angiotensinogen was also localized in the endothelial lining of the blood vessels (Fig. 7*c*). The specificity of the immunostaining was validated by negative control experiments in which specific antibody was preabsorbed in excess with its purified angiotensinogen (Fig. 7*d*) or in which the primary antibody was replaced with rabbit non-immune serum or buffer (data not shown).

Discussion

In the present study, the expression and localization of key components of the RAS from the rat pancreas have been

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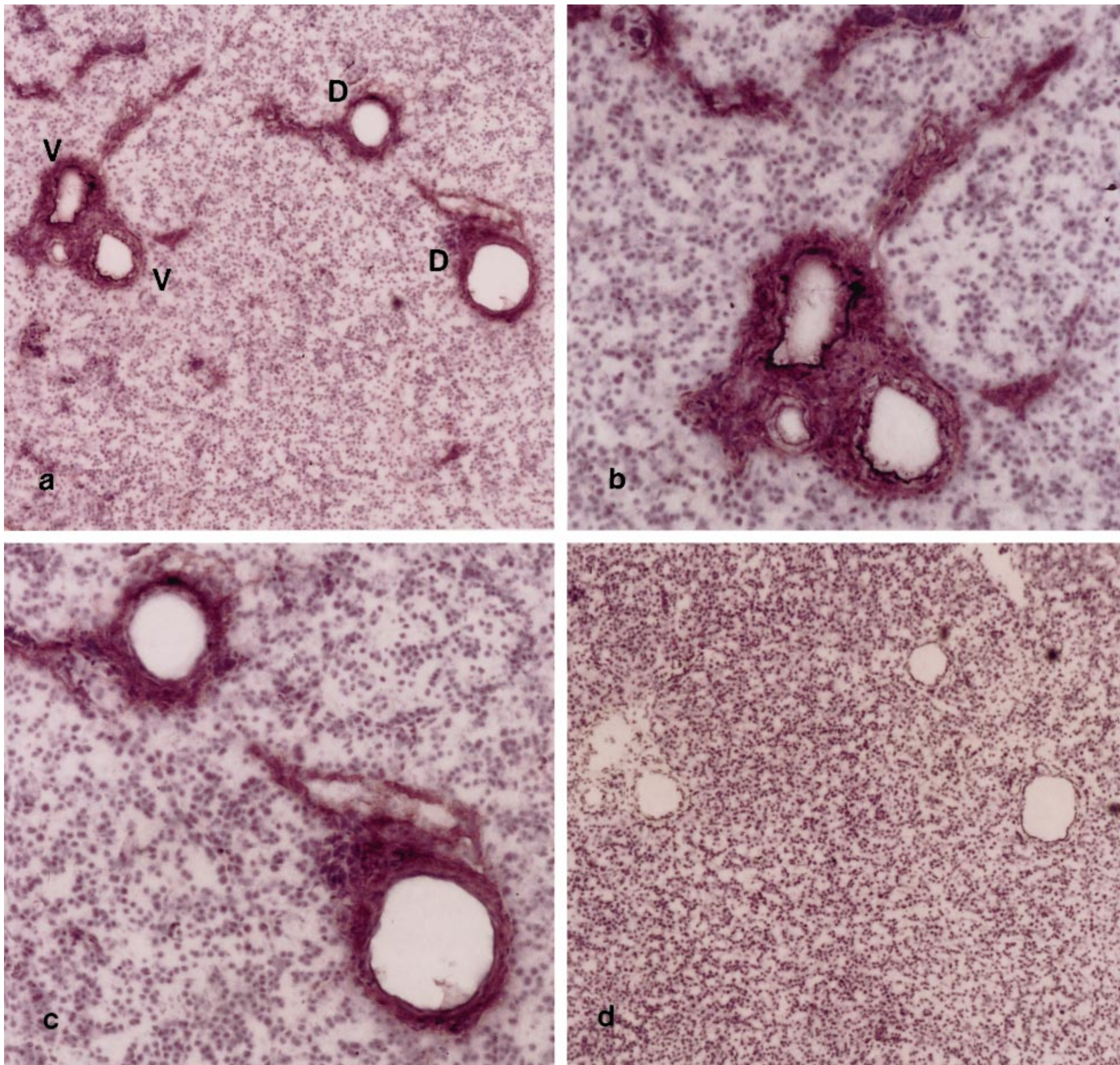

Figure 7 Immunohistochemical localization of angiotensinogen protein in rat pancreas. (a) Angiotensinogen immunoreactivity was localized predominantly in the epithelial cells of pancreatic ducts (D) as well as in the endothelial lining of pancreatic blood vessels (V). Magnification × 100. (b) Higher magnification of pancreatic sections showing intense immunostaining in vascular endothelium. Magnification × 200. (c) Higher magnification of pancreatic sections showing intense immunostaining in pancreatic ductal epithelium. Magnification × 200. (d) Consecutive pancreatic section showing the specificity of immunostaining in which the primary antibody was preabsorbed in excess with its purified angiotensinogen. Magnification × 100.

elucidated at the gene and protein levels using molecular biological and immunohistochemical approaches. RT-PCR showed that the rat pancreas expresses mRNAs of angiotensinogen, renin and angiotensin II receptor subtypes AT_{1a}, AT_{1b} and AT_2. Western blot analysis and immunohistochemical examination of the rat pancreas using specific antibody raised to rat angiotensinogen provided evidence for expression and localization of angiotensinogen protein, which is the obligatory component for the formation of angiotensin II. Angiotensinogen immunoreactivity was specifically localized in the pancreatic ductal epithelia and vascular endothelia of the pancreas. The present study is the first to present evidence that the rat pancreas possesses the major components of an intrinsic RAS.

The existence of a canine pancreatic RAS was previously suggested on the basis of the presence of

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angiotensin, angiotensinogen protein and angiotensinogen mRNA as revealed by chromatographic and Northern blot analysis (Chappell *et al.* 1991). Later, angiotensin II receptor subtypes were also characterized pharmacologically in the dog pancreas (Chappell *et al.* 1992). In studies from this laboratory, angiotensin II (Leung *et al.* 1998*a*) and its receptor subtypes AT_1 and AT_2 (Leung *et al.* 1997) were predominantly localized in epithelial cells of the ductal system and endothelial lining of the blood vessels in the rodent pancreas, although weak immunoreactivity for AT_1 and AT_2 was also found in the pancreatic acini. The localization of angiotensin II and its receptor subtypes in specific regions of rodent pancreas suggested the local production of a major RAS with possible roles in the regulation of pancreatic anion secretion and pancreatic blood flow (Leung *et al.* 1997, 1998*a*). Interestingly, an intrinsic RAS has also been reported in the rat pancreas, and it may play a role in regulation of insulin secretion and pancreatic blood flow (Carlsson *et al.* 1998). Nevertheless, in a previous study by Campbell & Habener (1986), angiotensinogen mRNA could not be detected in the rat pancreas by Northern blot. This could have been due to a combination of low expression of angiotensinogen mRNA because of its degradation during extraction from the RNase-rich pancreas and the limited sensitivity of the Northern blot method. In the present study, these potential problems were avoided by using RT-PCR with primers known to be specific for RAS components. mRNAs for the angiotensin II receptor subtypes AT_{1a}, AT_{1b} and AT_2 were clearly observed using the RT-PCR conditions described previously (Leung *et al.* 1998*b*); the results are consistent with previous immunohistochemical data demonstrating the presence of angiotensin II receptor subtypes in the rat pancreas (Leung *et al.* 1997). More importantly, the present RT-PCR analysis also showed the unequivocal expression of angiotensinogen mRNA in the rat pancreas, although its level of expression appeared to be low compared with that in liver and kidney, which are known to express angiotensinogen mRNA (Phillips *et al.* 1993*b*). The identity of the angiotensinogen component in the rat pancreas was further confirmed at the protein level. Using Western blot analysis, a major protein band of about 60 kDa was apparently detected in the rat pancreas. This was consistent with the predicted value for angiotensinogen as purified from plasma of nephrectomized rats (Hilgenfeldt & Hackenthal 1982). Furthermore, the localization of the angiotensinogen protein was investigated by immunohistochemistry with a specific antibody to rat angiotensinogen and shown to be present in the endothelial cells of pancreatic blood vessels and the epithelial cells of pancreatic ducts, which agrees with the immunohistochemical localization of angiotensin II, AT_1 and AT_2. The cellular coincidence of these RAS components and the presence of renin mRNA strongly suggest the existence of a local RAS which generates intracellular angiotensin II for autocrine or paracrine functions. However, it remains possible that intracellular angiotensin II is biosynthesized through a renin-independent pathway. For example, a previous study reported that canine pancreas contains kallikrein (Hojima *et al.* 1977), an enzyme capable of converting angiotensin II directly from its precursor angiotensinogen (Arakawa & Maruta 1980). It is also possible that locally formed angiotensinogen is secreted and then processed extracellularly to angiotensin II by circulating plasma renal renin. These three alternative models of angiotensin II-generating systems have previously been proposed for tissues in which angiotensinogen mRNA is present but renin mRNA may or may not be expressed (Dzau *et al.* 1986, 1988). The RT-PCR results from the present study show the presence of renin mRNA in the rat pancreas, and therefore a renin-dependent RAS appears to be the more likely mechanism operating in this tissue. However, further investigations are needed to confirm the renin-dependence and the precise site of angiotensin II formation.

In summary, the present study has provided evidence that all the major RAS components are present in the rat pancreas, especially angiotensinogen, which is indispensable for the production of angiotensin II. The data support the existence of an intrinsic RAS in the rat pancreas, with possible paracrine or autocrine roles in the regulation of local pancreatic blood flow and ductal anion secretion.

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