The recovery of some components of the renin angiotensin system in the rat pancreas after chronic exposure to hypoxic condition

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Abstract

Previous studies have shown that the expression of the major components from a local pancreatic renin–angiotensin system (RAS) was upregulated after chronic exposure to oxygen deprivation (10% oxygen). In the present study, the reversibility of expression for the pancreatic RAS affected by chronic hypoxia was investigated in the pancreas. Rats were first subject to hypoxia for one month and they were then returned to normoxic conditions for a varying period of time (1, 2, 3 and 4 weeks). The degree of recovery in the expression of RAS components was analyzed with standard curve-quantitative competitive-reverse transcription-polymerase chain reaction (SC-QC-RT-PCR), Western blot analysis and a specific assay for angiotensin-converting enzyme (ACE) activity. Results from SC-QC-RT-PCR showed that the upregulated expression of angiotensin II type 1 (AT₁) receptor mRNA following chronic hypoxia could be completely restored to the control level after the rats were returned to the normoxic condition for 3 weeks. The reversibility of mRNA expression for angiotensin II type 2 (AT₂) receptor and angiotensinogen was observed after the return to normoxic conditions for 2 and 3 weeks respectively when compared with that of their respective controls. Results from Western blot analysis further confirmed that the expression of AT₁ receptor protein was also reversible after return to normoxic conditions for 4 weeks. In addition, the activation of ACE activity returned to its normal level in a time-dependent manner. These data indicate that the upregulation of a local pancreatic RAS affected by chronic hypoxia could be recoverable. The significance of its reversibility and adaptability following chronic hypoxia may be of physiological relevance to the pancreas.

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Introduction

The classical concept of the renin–angiotensin system (RAS) has been considered a circulating hormonal system that plays a crucial role in the maintenance of blood pressure. The physiologically active component of this system is angiotensin II. It originates from hepatic angiotensinogen acted upon by sequential processing of renin and angiotensin-converting enzyme (ACE), which are derived from the kidney and lung respectively (Menard *et al.* 1993). The action of angiotensin II is mediated through its interaction with two pharmacologically defined receptors, namely angiotensin II type 1 and type 2 (AT₁ and AT₂) receptors (De Gasparo *et al.* 2000).

Recently, the expression of RAS components was detected in multiple tissues and organs (Vinson *et al.* 1998, De Mello & Danser 2000, Leung 2002). Among these tissues and organs, our previous studies have demonstrated the existence of several key RAS components in the pancreas of rodents (Leung *et al.* 1997, 1999). Such a local pancreatic RAS plays a pivotal role in regulating the endocrine and exocrine functions of the pancreas (see review by Leung & Chappell 2003). Interestingly, the components of this local system are responsive to various physiological and pathophysiological stimuli (see review by Leung & Carlsson 2001). For example, our recent studies showed that the pancreatic RAS components were upregulated by experimental models of acute

Table 1 Sequences of primers used and length of PCR products amplified

Gene	Primer	Sequence	GeneBank no /PCR product (bp)
Ao	Sense	5'TTCAGGCCAAGACCTCCC3'	NM_134432
	Antisense	5'CCAGCCGGGAGGTGCAGT3'	Native: 309
	Ao-IR	5'CCAGCCGGGAGGTGCAGTAGAGACCAGGGTGCCAAAG3'	Competitor: 236
AT ₁	Sense	5'GCCTGCAAGTGAAGTGATT3'	X64052
	Antisense	5'TTTAACAGTGGCTTTGCTCC3'	Native: 204
	AT ₁ -IR	5'TTTAACAGTGGCTTTGCTCCTGAGTGCTTTCTCTGC3'	Competitor: 148
AT ₂	Sense	5'TCTGGCTGTGGCTGACTT3'	X62295
	Antisense	5'CAAGACTTGGTCACGGGT3'	Native: 511
	AT ₂ -IR	5'CAAGACTTGGTCACGGGTCAATCCCAGCAGACCACT3'	Competitor: 398

Ao, angiotensinogen; AT₁, angiotensin II type 1 receptor; AT₂, angiotensin II type 2 receptor; IR, internal reverse primer; IF, internal forward primer.

pancreatitis and chronic hypoxia (Chan *et al.* 2000, Leung *et al.* 2000, Ip *et al.* 2003). The activation of RAS components by chronic hypoxia may represent either a pathological change or a safeguard mechanism of the biological systems in order to adapt to these changes (Ip *et al.* 2002). The main aim of the present study was designed to elucidate the nature of RAS activation in the pancreas. In this respect, the most typical characteristic of adaptation is the reversibility of its biological systems. Accordingly, we hypothesized that the upregulation of a local pancreatic RAS and its adaptability under chronic hypoxic stress should be of physiological relevance to the pancreas. The present study, therefore, aimed to examine the reversibility of some major components of a pancreatic RAS following chronic hypoxia. After one month of hypoxic exposure, rats were returned to normoxic conditions for a varying period of time and the reversibility of the major RAS components was examined in the pancreas.

Materials and methods

Chronic hypoxia rat model and reversibility study

For the exposure of rats to chronic hypoxia, Sprague–Dawley rats aged 28 days were raised inside an acrylic chamber filled with 10% ± 0.5 oxygen for isobaric hypoxia for 4 weeks, as described previously in our laboratory (Chan *et al.*

2000, Ip *et al.* 2002). After 4 weeks of hypoxia, rats were returned to normoxic conditions for a varying period of time, i.e. 1, 2, 3 and 4 weeks. The degree of recovery in the altered expression for each of the RAS components was examined accordingly. For controls, time- and age-matched rats were kept in the same housing and allowed to breathe room air. Ethical approval of the animal model was obtained from the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Preparation of native and competitor template

The strategy and procedure used for the construction of plasmids containing native and competitor products for angiotensinogen, AT₁, and AT₂ were similar to previously described methods (Tsai *et al.* 2001, 2002, Wu *et al.* 2002). Briefly, specific primer pairs were designed to amplify a fragment of DNA from mRNA transcript, and their brief details are shown in Table 1. These DNA fragments were cloned into PCR cloning vectors (TA cloning vector, Invitrogen, Carlsbad, CA, USA) and positive clones were identified. The internal primer was paired with the upstream primer to amplify a shorter fragment that also contains the downstream primer sequences (angiotensinogen and AT₂) or vice versa (AT₁). The amplified fragment was again cloned into a PCR cloning vector and a positive clone was identified. This clone served as the competitor plasmid for angiotensinogen, AT₁, and

AT₂ respectively. All amplified DNA fragments were sequenced by an ABI Prism terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, USA) according to the manufacturer's protocol for verification. Plasmids containing native or competitor DNA were linearized by proper restriction enzymes and transcribed *in vitro* using T7 RNA polymerase (Promega, Madison, WI, USA). The transcribed RNAs were precipitated twice using sodium acetate (0.3 M, pH 4.2) and 100% ethanol after DNase treatment and phenol-chloroform extraction. The concentrations of RNAs were quantified by OD 260 absorbance, aliquoted, and stored at -80°C . Each RNA aliquot was used only once to reduce variation due to potential degradation of RNA after freezing and thawing.

Standard curve-quantitative competitive-RT-PCR

The detailed procedure of standard curve-quantitative competitive-RT-PCR was described previously (Tsai & Wiltbank 1996). In brief, a constant amount of competitor RNA (3.2 attomoles) was added into the reverse transcription master mix (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3, 10 mM dithiothreitol, 100 pmol random primer, 4 mM dNTPs and 50 U SuperScriptII RNase H-reverse transcriptase). This mix was then dispensed into 0.2 ml thin wall PCR tubes, and serially diluted native RNA (25.6–0.4 attomoles) in 2 μl diethyl pyrocarbonate-treated water or 2 μl unknown mRNA samples were added individually to each tube. The reverse transcription was performed at 42°C for 60 min followed by incubation at 95°C for 10 min, and quick chilled to 4°C in a programmable thermocycler (MJ Research PTC-200). Five microliters RT products were added to 15 μl PCR mix (final concentration: 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPs, 0.5 U Taq polymerase, and 0.4 μM of primers). This was subjected to 30 cycles of amplification (30 s denaturation at 95°C , 30 s annealing at 57°C , and 30 s elongation at 72°C) followed by final elongation at 72°C for 5 min. Ten microliters PCR products were directly separated on a 5% acrylamide gel with $1 \times$ TBE buffer (0.09 M Tris, 0.09 M boric acid, 0.001 M EDTA, pH 8.0) at 110 V for 40 min using a

Mini-protein II electrophoresis system (BioRad, Richmond, CA, USA). The gel was then stained with ethidium bromide and placed on a UV illuminator equipped with a camera connected to a computer. The gel image was analyzed using an image analyzer (Molecular Dynamics Image Quant, Sunnyvale, CA, USA). In each lane of the gel, the intensity of the native and competitor bands was quantified and the ratio of this intensity was calculated. The logarithmic ratio of native to competitor product was plotted against the logarithmic initial amounts of native product to produce the standard curve (see Figs 1, 3, and 5). Concentrations of specific mRNA transcripts in samples were calculated by comparison with the standard curve as previously described (Tsai & Wiltbank 1996).

Western blot analysis

The procedures for immunoblotting of AT₁ receptor were reported previously (Leung *et al.* 2001). Briefly, pancreatic tissues from the control and experimental rats were homogenized at 4°C in water, 1:9 (wt/vol) containing 10 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The resultant protein was determined (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins (5 μg /lane) were subjected to electrophoresis on a 12% (vol/vol) polyacrylamide gel in SDS and the gel was then processed for electroblotting to a polyvinylidene difluoride membrane. The blotted membrane was saturated with 5% (wt/vol) skimmed milk in PBS, pH 7.4 and 0.1% (vol/vol) Tween 20 for 1 h at room temperature. The membrane was then incubated in AT₁ receptor antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, 1/1000) overnight at 4°C and peroxidase-labeled IgG serum (1/500) for 1 h at room temperature. The positive band was revealed using an ECL detection kit (Amersham Biosciences, Uppsala, Sweden) and quantified with an image analyzer.

Specific assay for ACE activity

The method for the measurement of ACE activity in rat pancreas has been described in detail from our previous publication (Ip *et al.* 2003). Briefly, the protein fraction was isolated from

Figure 1 Quantification of angiotensinogen mRNA by quantitative competitive-RT-PCR. (A) Representative gel image of ethidium bromide-stained PCR products (0.4–25.6 attomoles) of native RNA co-amplified with 3.2 attomoles of competitor RNA. (B) Standard curve produced by analyzing the intensity of the bands shown in panel A. The log ratio of native to competitor product was plotted against the log amount of initial native RNA added to the RT-PCR.

pancreatic homogenate by the CytoBuster Protein Extraction Assay kit (Novagen, Madison, WI, USA) and incubated with 20 μ M fluorogenic substrate (2-amino-3-(7-methoxy-4-coumaryl) propanoic acid) at 37 °C for 60 min. The fluorescence of the supernatant was measured using an excitation wavelength at 328 nm and an emission wavelength at 392 nm. ACE activity was calculated and expressed as μ mol/min/ μ g protein.

Data analysis

Data were expressed as means $\pm$ S.E.M. and analyzed by one-way ANOVA followed by Duncan's multiple range test in order to detect inter-group differences. Significant difference from the control was set at $P < 0.05$.

Figure 2 Time-dependent recovery of pancreatic angiotensinogen mRNA from the effects of one month of hypoxia. Values given are means $\pm$ S.E.M., $n=4$. *Significantly different from the normoxic control (0 week). †Significantly different from the hypoxia-treated control (0 week). The level of angiotensinogen mRNA (attomoles/ μ g RNA) was plotted against the time (week) after one month of hypoxia

Results

Expression and reversibility of angiotensinogen mRNA

RT-PCR with internal RNA competitive standards provides a convenient way of measuring absolute amounts of mRNA transcripts. A standard curve was produced with serially diluted native angiotensinogen RNA (25.6 to 0.4 attomoles) and a constant amount (3.2 attomoles) of competitive RNA (Fig. 1). The amount of angiotensinogen mRNA in an unknown sample was then determined directly by comparison with the ratio of PCR products to the standard curve. This method has been shown to be sensitive, repeatable, and quantitative (Tsai & Wiltbank 1996). By this method, the amount of angiotensinogen mRNA in rat pancreas was measured. The data showed that angiotensinogen mRNA was decreased time-dependently when the rats were returned to normoxic conditions for 1 to 4 weeks after a one-month hypoxic exposure. The expression of angiotensinogen mRNA completely recovered after 2 weeks (Fig. 2).

Expression and reversibility of AT₁ receptor mRNA

A competitive RT-PCR standard curve was produced with different amounts of native AT₁

Figure 3 Quantification of AT_1 receptor mRNA by quantitative competitive-RT-PCR. (A) Representative gel image of ethidium bromide-stained PCR products (0.4–25.6 attomoles) of native RNA co-amplified with 3.2 attomoles of competitor RNA. (B) Standard curve produced by analyzing the intensity of the bands shown in panel A. The log ratio of native to competitor product was plotted against the log amount of initial native RNA added to the RT-PCR.

receptor RNA and a constant amount of competitive RNA (Fig. 3). AT_1 receptor mRNA was decreased time-dependently when the rats were returned to normoxic conditions for 1 to 4 weeks. The recovery of angiotensinogen mRNA was faster than that of AT_1 receptor mRNA, which was restored to the level of the control after 3 weeks (Fig. 4).

Expression and reversibility of AT_2 receptor mRNA

A competitive RT-PCR standard curve was produced with different amounts of native AT_2 receptor RNA and a constant amount of competitive RNA (Fig. 5). After one month of hypoxic exposure, the increased level of AT_2 receptor mRNA was reversibly regulated when the animals were returned to normoxic conditions. The

Figure 4 Time-dependent recovery of pancreatic AT_1 receptor mRNA from the effects of one month of hypoxia. Values given are means $\pm$ S.E.M., $n=4$. *Significantly different from the normoxic control (0 week). †Significantly different from the hypoxia-treated control (0 week). The level of AT_1 receptor mRNA (attomoles/μg RNA) was plotted against the time (week) after one month of hypoxia.

recovery of AT_2 receptor mRNA was much faster than that of AT_1 receptor and angiotensinogen mRNAs. Its level was completely recovered when the animals were returned to a normoxic environment for 2 weeks (Fig. 6).

Expression and reversibility of AT_1 receptor protein

In parallel with the expression of AT_1 receptor mRNA, the level of AT_1 receptor protein was determined by Western blot analysis. A standard curve was produced with serial dilutions of AT_1 receptor protein (Fig. 7). After one month of hypoxic exposure, there was a time lag in the recovery of AT_1 receptor protein when compared with AT_1 receptor mRNA (Fig. 4). AT_1 receptor protein was not significantly changed when the rats were returned to normoxic conditions for the first 3 weeks. However, its expression level completely recovered after 4 weeks (Fig. 8).

Reversibility of pancreatic ACE activity

ACE activity was measured by a specific fluorometric assay. ACE activity in the rat pancreas was decreased time-dependently when the rats were returned to normoxic conditions for 1 to 4 weeks.

Figure 5 Quantification of AT₂ receptor mRNA by quantitative competitive-RT-PCR. (A) Representative gel image of ethidium bromide-stained PCR products (0.4–25.6 attomoles) of native RNA co-amplified with 3.2 attomoles of competitor RNA. (B) Standard curve produced by analyzing the intensity of the bands shown in panel A. The log ratio of native to competitor product was plotted against the log amount of initial native RNA added to the RT-PCR.

The ACE activity was restored to the level of the control after returning to normoxic conditions for 3 weeks (Fig. 9).

Discussion

Hypoxia is a common stress that affects an organism's homeostasis. Although much information about the mechanisms of the cellular and biochemical responses to hypoxia have been provided, very little is known about the responses of the pancreas to chronic hypoxia. Our previous study showed that chronic hypoxia could upregulate the RAS components in the rat pancreas (Chan *et al.* 2000, Ip *et al.* 2003). The activation of pancreatic RAS components may represent a pathophysiological and a physiological phenomenon. A pathological condition is represented by a consistent upregulation of RAS components even when the subjects are returned to normoxic

Figure 6 Time-dependent recovery of pancreatic AT₂ receptor mRNA from the effects of one month of hypoxia. Values given are means $\pm$ S.E.M., $n=4$. *Significantly different from the normoxic control (0 week). †Significantly different from the hypoxia-treated control (0 week). The level of AT₁ receptor mRNA (attomoles/μg RNA) was plotted against the time (week) after one month of hypoxia.

conditions for a long time. In such a case, therapeutic intervention is required to avoid further deterioration of the biological system. It is well known that activation of the angiotensin II receptors is associated with hypertensive diseases (Berry *et al.* 2001, Wagenaar *et al.* 2002). On the other hand, a physiological condition is manifested with an adaptive response, in which the RAS components increase temporarily under adverse environmental conditions and recover when returned to normal conditions. The most typical characteristic of adaptation is its reversibility. The present study clearly demonstrated the reversibility of a pancreatic RAS in rats exposed to one month of hypoxia. Among the results, the time required for the recovery of the major pancreatic RAS components including mRNA of AT₁, AT₂ and angiotensinogen was 2–3 weeks as shown in Figs 2, 4 and 6. When compared with the two angiotensin II receptor subtypes, the recovery of AT₂ receptor mRNA (Fig. 6) preceded that of AT₁ receptor mRNA (Fig. 4). It was intriguing that there was a time lag in the recovery of AT₁ receptor protein (Fig. 8) when compared with that of AT₁ receptor mRNA. Nevertheless, all RAS components observed in the present study were completely reversible when the animals were returned to normoxic conditions for 4 weeks (Figs 2, 4, 6, 8, 9).

Figure 7 Quantification of AT₁ receptor protein by Western blot. (A) Representative gel image of Western analysis of AT₁ receptor protein. (B) Standard curve produced by analyzing the intensity of the bands shown in panel A.

Figure 8 Time-dependent recovery of pancreatic AT₁ receptor protein from the effects of one month of hypoxia. Values given are means $\pm$ S.E.M., $n=5$. *Significantly different from the control at the same time point. The level of AT₁ receptor protein was plotted against the time (week) after one month of hypoxia.

Figure 9 Time-dependent recovery of pancreatic ACE activity from the effects of one month of hypoxia. Values given are means $\pm$ S.E.M., $n=5$. *Significantly different from the normoxic control (0 week). †Significantly different from the hypoxia-treated control (0 week). The activity of ACE ($\mu\text{mol}/\mu\text{g}$ total protein/min) was plotted against the time (week) after one month of hypoxia.

The reversible regulation of pancreatic RAS by chronic hypoxia could play an important role in the physiological aspect of the pancreas. In fact, a recent finding showed the pulmonary expression and functional role of AT₁ and AT₂ receptors during hypoxic pulmonary hypertension development and their subsequent reversal upon return to normoxic conditions (Chassagne *et al.* 2000). Under hypoxic conditions, the RAS has been suggested to play a pivotal role in the preservation of blood supply to organs (Achard *et al.* 2001). In addition to its pressor function, angiotensin II can exert its effect through two time-dependent events. The RAS controls a fast opening of the reserve collateral circulation and a slow response of angiogenesis to acute hypoxia and chronic hypoxia respectively (Achard *et al.* 2001). In recent years, much attention has been focused on the role of the RAS in regulating angiogenesis and rarefaction. Angiotensin II has been suggested as a regulator of microvessel density acting through the mediation of AT₁ and AT₂ receptors (Greene & Amaral 2002). It has been reported that the RAS stimulated the production of vascular endothelial growth factor (Chua *et al.* 1998, Lonchampt *et al.* 2001). A recent study demonstrated that the RAS promoted early angiogenesis by supporting inflammatory cell infiltration and angiogenic cytokine expression (Sasaki *et al.* 2002). However, other studies showed

that the RAS inhibited angiogenesis (Celerier *et al.* 2002, Silvestre *et al.* 2002). This discrepancy could be due to the distinct distribution of AT₁ and AT₂ receptors in different organs. The RAS may stimulate angiogenesis in vital organs but may inhibit the process in organs with higher tolerance to hypoxia when there is a shortage of oxygen supply.

The upregulation of a local RAS by chronic hypoxia may result in other undesirable and pathological changes such as inflammation and stimulation of free radical formation. Many studies have shown that the RAS may enhance an inflammatory response through the production of inflammatory cytokines (Schieffer *et al.* 2000) and the recruitment of inflammatory cells (Alvarez & Sanz 2001, Kintscher *et al.* 2001). Our recent study showed that experimental pancreatitis was associated with the activation of a local pancreatic RAS (Leung *et al.* 2000, Ip *et al.* 2003). Interestingly, administration of saralasin, a non-specific angiotensin II receptor antagonist of RAS, could be protective against the severity of acute pancreatitis, indicating that activation of a pancreatic RAS may play a role in pancreatic tissue injury (Tsang *et al.* 2003). Taken together, a pancreatic RAS may have a potential value in the manipulation of some pancreatic diseases (Leung 2003). In addition, many studies have shown that angiotensin II stimulated the formation of reactive oxygen species through the activation of NAD(P)H oxidase (Warnholtz *et al.* 1999, Wingler *et al.* 2001, Schieffer *et al.* 2000). Fortunately, the production of reactive oxygen species is limited under low oxygen tension. However, when the animals are returned to normoxic conditions, the production of oxygen radicals may be drastically enhanced. When the formation of reactive oxygen species exceeds the capacity of the antioxidant defence it results in oxidative damage to cellular molecules. The situation is similar to the well-known ischemia/reperfusion injury in which xanthine oxidase, instead of NAD(P)H oxidase, is activated (Anaya-Prado *et al.* 2002). Therefore, the reversible regulation of pancreatic RAS may represent a safeguard mechanism under the different insult of chronic hypoxia.

In conclusion, the present study clearly demonstrated the reversibility of upregulated pancreatic RAS due to chronic hypoxia. The reversible changes of a local pancreatic RAS imply a typical

characteristic of an adaptive response and represent different aspects of physiological modification in the pancreas under different oxygen tension.

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