Angiotensin converting enzyme (ACE) gene expression in experimentally induced liver cirrhosis in rats

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Abstract: Angiotensin converting enzyme (ACE) is a key player of Renin Angiotensin System (RAS), involved in conversion of active product, angiotensin-II. Alterations in RAS have been implicated in the pathophysiology of various diseases involving heart, kidney, lung and liver. This study is designed to investigate the association of ACE gene expression in induction of liver cirrhosis in rats. Total 12 male albino Wistar rats were selected and divided in two groups. Control group received 0.9% NaCl, where as Test group received thioacidamide (TAA), dissolved in 0.9%NaCl, injected intraperitoneally at a dosage of 200mg/Kg of body weight, twice a week for 12 weeks. The rats were decapitated and blood sample was collected at the end of experimental period and used for liver functions, enzyme activity, antioxidant enzymes and lipid peroxidation estimations. Genomic DNA was isolated from excised tissue determine the ACE genotypes using specific primers. The ACE gene expression in liver tissue was assessed using the quantitative RT-PCR method. The activity of ALT, total and direct bilirubin, SOD and CAT levels were significantly high ($p<0.05$) and level of MDA was significantly low ($p<0.05$) in TAA treated rats as compared to control rats. The ACE gene expression after 12 weeks TAA treatment in cirrhotic rats was significantly increased ($p<0.05$) in comparison to controls. This study describes the importance of RAS in the development of hepatic fibrosis and the benefits of modulation of this system ACE gene expression. The finding of major up-regulation of ACE in the experimental rat liver provides further insight into the complexities of the RAS and its regulation in liver injury. The development of specific modulators of ACE activity and function, in future, will help determine the role of ACE and its genetic variants in the pathophysiology of liver disease.

Keywords: ACE gene, liver cirrhosis, thiacetamide, antioxidant, lipid peroxidation.

INTRODUCTION

Angiotensin converting enzyme (ACE) is a zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells. Several different names refer to this enzyme in the scientific literature including Peptidyl-dipeptidase A, Dipeptidyl carboxypeptidase I and Dipeptide hydrolases (Sayed-Tabatabaei *et al.,* 2006). ACE converts the inactive decapeptide, angiotensin I (Ang I or Ang 1-10), to the active octapeptide and potent vasoconstrictor angiotensin II (Ang II or Ang 1-8), which is the main active product of the rennin-angiotensin system (RAS). Alterations in RAS have been implicated in the pathophysiology of various diseases (Oruc *et al.,* 2008). The renin-angiotensin system (RAS) is a circulatory cascade system primarily involved in the regulation of blood pressure and serum electrolytes (Shahid and Tabassum, 2005; Ruiz-Ortega *et al.,* 2003). The key enzyme in this system is the angiotensin converting enzyme (ACE), which converts angiotensin I to the potent vasoconstrictor angiotensin II (Stroth and Unger, 1999). The RAS has been shown to play a role in the pathogenesis of several diseases including fibrosis in the heart, kidney, lung and liver during chronic inflammation through the regulation of cell growth, inflammation, oxidative stress, angiogenesis and fibrosis (Marshall *et al.,* 2000; Bataller *et al.,* 2000). ACE mediates regulation of blood volume, arterial pressure, cardiac and vascular function, and electrolyte metabolism (Jayapalan *et al.,* 2010). In human, the insertion/deletion (I/D) polymorphism of the ACE gene accounts for the half of the variance of circulating and tissue ACE levels,with the highest ACE levels in DD homozygotes, the lowest in II homozygotes, and intermediate values in heterozygotes (Rigat *et al.,* 1990). The D allele of the ACE genotype has been associated with cardiovascular and renal disease (Kunz *et al.,* 1998; Ruggenenti *et al.,* 2008). However, the results are not uniform and analysis of both its pathophysiologic significance and the underlying mechanisms is complicated by multiple interactions with other genetic and environmental factors (Boonstra *et al.,* 2001; van der Kleij *et al.,* 2002). The ACE gene insertion/deletion (I/D) polymorphism was first identified in 1990. The ACE-D, a deletion polymorphism of a 287-bp fragment of intron 16 of the ACE gene allele, has been shown to result in higher levels of circulating enzyme in a dose-dependent manner (Rigat *et al.,* 1990).

The liver cirrhosis is an autoimmune disorder which is strongly influenced by poorly defined, complex genetic factors (Barlett *et al.,* 2009). This condition is characterized by abnormal accumulation of fibrous tissues and by degeneration of hepatic cells (Yashiro *et al.,* 1998).

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The mechanisms of liver cirrhosis have been investigated in experimental models using carbon tetrachloride, bile duct ligation, alcohol and thioacetamide (TAA) administration (Teck *et al.,* 2004). It is evident that the TAA administration induced liver cirrhosis models represent more prominent fibrosis as well as regenerative hepatic nodules, therefore, TAA, which was originally used as a fungicide, is very widely used to induce liver cirrhosis in animal models (Qudsia *et al.,* 2011). The role of the ACE gene expression as a risk factor has been investigated in several diseases (Baudin, 2002). However, the prevalence of the ACE gene expression in liver cirrhosis and its contribution to the course of the disease has not yet been defined.

The animal model studies have not been satisfactorily carried out to observe the involvement of ACE gene expression in experimentally induced cirrhotic animals. The availability of an animal model to study the effects of different ACE expressions would therefore be useful. Therefore, this study is designed to investigate the association of ACE gene expression in induction of liver cirrhosis in rats and how it plays an important role in the progression of liver cirrhosis.

MATERIALS AND METHODS

Experimental animals

Twelve (12) male albino rats weighing 200-250 gm were purchased from the animal house, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan for the study. Animals were acclimatized to the laboratory conditions before the start of experiment and caged in a quiet temperature controlled animal room at 23±4°C. Rats had free access to water and standard rat diet. The experiments were conducted in accordance with ethical guidelines for investigations on laboratory animals.

Study protocols

The rats were randomly divided into two groups, 6 rats in each group as follows: The duration of the treatment was 12 weeks:

Group-I:Normal, untreated rats (controls)

The control rats received 0.9% NaCl, injected intraperitoneally at dosage of 200mg/Kg of body weight, twice a week for 12 weeks.

Group-II: TAA treated, cirrhotic rats (tests)

The TAA treated rats received TAA, dissolved in 0.9%NaCl, injected intraperitoneally at a dosage of 200mg/Kg of body weight, twice a week for 12 weeks.

Sample collection

The rats were decapitated and the blood sample was collected from the head wound in the lithium heparin coated tubes, at the end of experimental period. A portion of blood was taken in separate tube to collect the plasma. Liver was perfused with deionized water and homogenized in chilled potassium chloride (1.17%). The homogenates were centrifuged at 800g for five minutes at 4°C. The supernatant so obtained was centrifuged at 10,500g for 20 minutes at 4°C to get postmitochondrial supernatant, which was then utilized to assay superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels.

Assessment of ALT, total and direct bilirubin

The commercially available readymade reagent kits from Randox® Corporation were used for the estimation of Plasma ALT, total and direct bilirubin as described earlier (Reitman and Franhel, 1957; Sherlock, 1951).

Post mitochondrial supernatant preparation (PMS)

Liver tissue was perfused with saline and homogenized in chilled potassium chloride (1.17%) using a homogenizer. The homogenates were centrifuged at 800 g for 5 minutes at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500/g for 20 minutes at 4°C to get the post mitochondrial supernatant which was used to assay SOD, CAT and MDA activity.

Estimation of lipid peroxidation

The MDA content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) by the previously described method (Okhawa *et al.,* 1979). Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium dodecyle sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10%(w/v) of PMS. The mixture was brought up to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm and compared with those obtained from MDA standards. The concentration values were calculated from absorption measurements as standard absorption.

Estimation of CAT activity

CAT activity was assayed as described earlier (Sinha *et al.,* 1972). Briefly, the assay mixture consisted of 1.96 ml phosphate buffer (0.01 M, pH 7.0), 1.0 ml hydrogen peroxide (0.2 M) and 0.04 ml PMS (10%) in a final volume of 3.0 ml. 2 ml dichromate acetic acid reagent was added in 1 ml of reaction mixture, boiled for 10 min, cooled. Changes in absorbance were recorded at 570 nm.

Estimation of SOD

Levels of SOD in the cell free supernatant were measured by previously described method (Kono *et al*., 1978). Briefly, 1.3/ml of solution A (0.1 mmol EDTA containing

50 mmol Na_2CO_3, pH 10.0), 0.5/ml of solution B (90 µm nitro blue tetrazolium-NBT dye) and 0.1/ml of solution C (0.6% TritonX-100 in solution A), 0.1/ml of solution D (20 mmol Hydroxylamine hydrochloride, pH 6.0) were mixed and the rate of NBT reduction was recorded for one minute at 560/nm. 0.1/ml of the supernatant was added to the test cuvette as well as reference cuvette, which do not contain solution D. Finally, the percentage inhibition in the rate of reduction of NBT was recorded as described above. One enzyme unit was expressed as inverse of the amount of protein (mg) required inhibiting the reduction rate by 50% in one minute.

Genotyping

Genomic DNA was isolated from excised tissue as described previously (Korstanje *et al.,* 2004). To determine the ACE genotypes, primers were used as described by Hilbert *et al.,* (1991) to amplify the microsatellite located at the 50 end of the intron between exons 13 and 14.

ACE gene expression in liver

The expression of ACE gene in liver tissue was assessed using the quantitative RT-PCR method. RNA was isolated using Total RNA Prep Plus (A and A Biotechnology, Gdansk, Poland). In brief, amplification reaction was performed in 12.5 µl total volume, containing a pair of specific primer: 5'CAGCTTCATCATCCAGTTCC3' and 5'CTAGGAAGAGCAGCACCCAC. PCR program consisted 30 cycles at an annealing temperature of 52-64°C. Restriction fragments were subsequently analyzed in 2% agarose gel stained with the help of ethidium bromide (Sulikowski *et al*., 2011) (fig. 1).

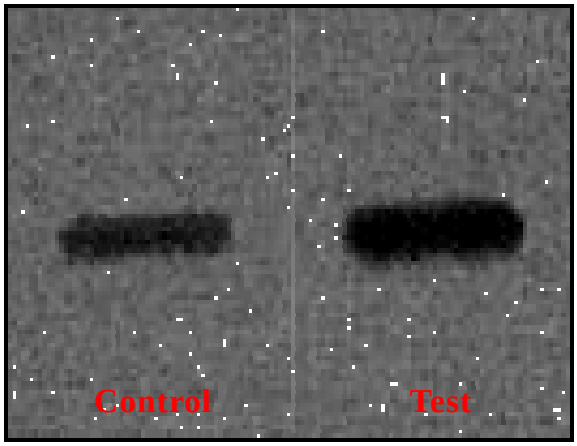

Fig. 1: Agarose gel electrophoresis showing the amplification for ACE gene in controls and TAA-induced cirrhotic rats.

STATISTICAL ANALYSES

Data are expressed as mean±SEM and were analyzed using the statistical program SPSS v17.0 for Windows (SPSS Inc., Chicago, IL). Statistical differences were determined using the unpaired Student's *t*-test. Significance was accepted at the level of $p<0.05$ and $p<0.01$.

RESULTS

This study finds a significant rise in the body and liver weights of TAA-induced cirrhotic rats as compared to control rats (table 1).

The liver functions and enzyme activity was measured by means of ALT, total and direct bilirubin levels and presented in table 2. The activity of ALT, total and direct bilirubin levels were found to be high significantly in TAA treated rats as compared to control rats. This shows the degree of hepatic damage caused by the treatment with TAA.

Table 1: Body and liver weights in controls and TAA-induced cirrhotic rats

Liver Weights	Controls	TAA-induced cirrhotic rats	Statistical Significance
Mean body weight (gm)	235.6 ±7.43	330± 10.05	p<0.05
Mean liver weight (gm)	2.68± 0.34	7.89± 1.12	p<0.01
Relative liver weight (gm)	0.02± 0.002	0.34± 0.006	p<0.01

Table 2: Liver ALT, total and direct bilirubin in coltrols and TAA-induced cirrhotic rats

Parameters	Controls	TAA-induced cirrhotic rats	Statistical Significance
ALT(U/L)	10.12±0.22	51.14±4.37	p<0.01
Total Bilirubin (U/L)	0.65±0.03	4.25±0.19	p<0.01
Direct Bilirubin (U/L)	1.88±0.41	5.12±0.95	p<0.01

The levels of antioxidant enzymes (SOD and CAT) and lipid peroxidation (MDA) are given in table 3. The activities of SOD and CAT were found to be significantly low in TAA treated cirrhotic rats where as the level of MDA was observed significantly high in TAA treated cirrhotic rats as compared to controls (table 3).

Table 3: Antioxidant enzymes in controls and TAA-induced cirrhotic rats

Enzymes	Controls	TAA-induced cirrhotic rats	Statistical Significance
SOD (U/gm tissue)	43.45±8.23	28.11±3.84	p<0.01
CAT(mmol /gm tissue)	2.67±0.28	1.12±0.47	p<0.01
MDA(nmol /gm tissue)	0.68±0.09	0.81±0.02	p<0.05

ACE gene expression is presented in fig. 2. The expression of ACE gene after 12 weeks of TAA treatment in cirrhotic rats was significantly increased ($p<0.05$) in comparison with tissue samples from the control group (fig. 2).

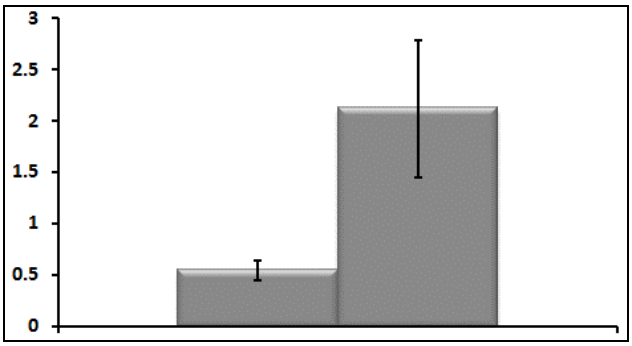

Fig. 2: ACE gene expression in controls and TAA-induced cirrhotic rats.
*****$p<0.05$ as compared to controls

DISCUSSION

Globally, in general and in Pakistan, in particular, the morbidity and mortality is increasing due to various diseases including liver cirrhosis. One of the basic reasons of rapid progression of liver diseases, cirrhosis for example, is the late diagnosis of disease and ignoring attitude of general physicians towards significant biochemical investigations to be done at the onset of disease.

The present study augments the implication of crucial demographic, physiological and biochemical parameters towards better control and management of the liver damage. Through proposed biochemical and genetic analyses the ACE gene expression and/or polymorphism as well as its association with the routine biochemical investigations will be documented in animal models with liver toxicities and its complications.

Several studies have been performed to identify the factors that contribute to the renal, hepatic, vascular and cardiac damage in experimental animals. At genetic level, deletion in intron 16 of the ACE gene results in an increased ACE activity in both plasma and tissue level (Danser *et al.*, 1995; Mizuiri *et al.*, 2001). Numerous studies have been performed analyzing the possible association of the ACE I/D genotype with cardiovascular and renal disease (Ng *et al.*, 2005; Staessen *et al.*, 1997). Whereas meta-analyses confirm a role for the D allele as a renal and cardiovascular risk factor (Samani *et al*., 1996), the significance of genetically high/low ACE is still controversial (Staessen *et al*., 1997). A possible explanation for such inconsistency is the presence of multiple interactions with both genetic and environmental factors, i.e., sodium status, disease duration, and sex. Animal models would be helpful to unravel these complex interactions in well-ontrolled experimental conditions. The present study indicates an over expression of ACE gene after 12 weeks of TAA treatment and induction of liver damage in experimental rats (fig. 2). The induction of liver damage was ascertained by means of hyper activity of ALT, total and direct bilirubin TAA treated rats as compared to control rats (table 2).

Although ACE gene expression is low in normal liver tissue, the results of this study show that in rat liver disease there is major up-regulation of hepatic ACE at the gene levels, accompanied by significant increases in ACE activity. These findings demonstrate that chronic liver injury causes a major increase in the hepatic expression of ACE and raise the possibility that it may play a role in disease pathogenesis.

Evidence to date has indicated that in normal physiology expression of ACE gene is more restricted and is localized to the heart, kidney, testis, and large and small intestine (Tipnis *et al.*, 2000; Donoghue *et al*., 2000). With the use of highly sensitive QRT-PCR techniques, low levels of ACE mRNA have been detected in the normal human liver and this is consistent with our own findings in control and cirrhotic rats. This study demonstrates the ACE gene is highly expressed in hepatocytes during in this experimental model of liver injury suggesting that a widespread increase in ACE gene expression may be a general feature of liver injury in both rats and humans.

In conclusion it is said that the recent research has been directed towards the importance of the RAS in the development of hepatic fibrosis and the potential benefits of modulation of this system either by ACE inhibition or Angiotensin receptor antagonism. The finding of major up-regulation of ACE in the experimental rat liver provides further insight into the complexities of the RAS and its regulation in liver injury. In the future, the development of specific modulators of ACE activity and function will help determine the role of ACE and its genetic variants in the pathophysiology of liver disease.

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