Original Article

Time-Dependent Expression of Chymase and Angiotensin Converting Enzyme in the Hamster Heart under Pressure Overload

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The role of a dual angiotensin (Ang) II-forming pathway from the local renin angiotensin system (RAS) of the cardiac tissue was determined in a hamster model of cardiac hypertrophy. Time-dependent expressions of chymase and angiotensin converting enzyme (ACE) genes and their enzymes activities, and Ang II levels were measured in the hamster heart at 3 days, and at 4 and 8 weeks after pressure overload. Cardiac hypertrophy was induced by an operation to constrict the abdominal aorta. Compared to the sham-operated group, the cardiomyocyte diameters of hamster hearts at 3 days after overload underwent no obvious changes, while those at 4 and 8 weeks after overload increased markedly ($p < 0.01$), and both transcriptional expressions of chymase and ACE genes gradually increased in the hamster hearts at 3 days, and at 4 and 8 weeks after overload, but the transcriptional expressions of angiotensin II type 1 receptor (AT1R) gene gradually decreased. Chymase and ACE activities (U/mg) (0.441 ± 0.040 vs. 0.175 ± 0.014 , 0.446 ± 0.036 vs. 0.160 ± 0.016 and 0.522 ± 0.014 vs. 0.148 ± 0.038) ($p < 0.01$) and (0.142 ± 0.023 vs. 0.056 ± 0.038 , 0.317 ± 0.017 vs. 0.079 ± 0.016 and 0.466 ± 0.010 vs. 0.098 ± 0.003) ($p < 0.01$), respectively and Ang II levels (pg/g) (98.7 ± 4.5 vs. 71.2 ± 4.9 , 134.4 ± 7.8 vs. 71.9 ± 12.8 and 151.6 ± 10.1 vs. 80.7 ± 3.0) gradually increased in the hamster hearts, vs. sham treatment, respectively, at 3 days, and at 4 and 8 weeks after overload. However, the increases in chymase and ACE activities were much higher than those in their respective mRNA levels, and the levels of chymase activities were also higher than those of ACE activities during the development of cardiac hypertrophy. The results suggested that the increase in Ang II levels *via* the dual pathway of Ang II formation by chymase and ACE plays an important role in the cardiac hypertrophy of hamsters caused by the overloaded state. Importantly, in the non-hypertrophied hamster heart in the early stage after overload (at 3 days), chymase could be activated by mechanical stress in advance of an increase in its mRNA, and the Ang II level increased significantly. (*Hypertens Res* 2002; 25: 757–762)

Key Words: chymase, angiotensin converting enzyme, angiotensin II formation, time-dependent expression, pressure-overloaded hamster heart

Introduction

It has been demonstrated that the local renin angiotensin sys-

tem (RAS) in the cardiac tissue is closely related to the occurrence and development of cardiac hypertrophy (1). And increased angiotensin II (Ang II) levels in the cardiac tissue have been shown to be the result of left ventricular hypertro-

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phy produced by pressure overload (2). Recent studies in animal hearts have shown that Ang II can be formed *via* a dual pathway: through chymase and through angiotensin converting enzyme (ACE) in the cardiac tissue. The expressions of chymase and ACE genes reported in the literature were different during the occurrence and development of cardiac hypertrophy or the occurrence and development of heart failure. In one study, for example, chymase mRNA levels were increased markedly in the hypertrophied heart in congenital cardiomyopathy, while ACE mRNA levels did not change (3). And in an earlier study on the hypertrophied heart following human myocardial infarction associated with heart failure, ACE mRNA levels increased markedly, but chymase mRNA levels did not change (4). These findings suggested that chymase and ACE may be regulated during the occurrence and development of cardiac hypertrophy by external environmental stimulation or by time-dependent differences. The hamster is an ideal animal for research into the pathologic mechanisms of human disease, particularly in this case, since the hamster model of cardiovascular disease is close to that of humans (5). Moreover, because of important species differences in the pathways of vascular and intracardiac Ang II generation, it is important to choose the monkey, hamster or dog rather than the rat as an animal model when investigating the role of chymase-dependent Ang II formation (6, 7). In this study, we established a hamster model of cardiac hypertrophy caused by pressure overload and observed the time-dependent changes of chymase, ACE and angiotensin II type 1 receptor (AT1R) gene expressions, enzyme activities and Ang II levels in the overloaded hamster hearts in order to analyze the role of a dual Ang II formation pathway of the heart and to address the functional role of Ang II produced by chymase.

Materials and Methods

Reagents

TRIzol reagent, dNTP, Oligo(dT)₁₅, M-MLV and RNasin were purchased from Gibco BRL (Gaithersburg, USA). Taq DNA polymerase was the product of Huamei Co. (Beijing, P.R. China). All the primers used in this study were synthesized by Shanghai Sangong Biological Engineering Technology and Service Co. Ltd. (Shanghai, P.R. China). Angiotensin I (Ang I), Ang II and aprotinin were the products of Sigma Co. (St. Louis, USA). Lisinopril was the gift of ZENECA Ltd., (Maccles Field, UK). The Ang I and Ang II radio-immunoassay (RIA) kits were purchased from Beijing North Biotech. Co. (Beijing, P.R. China). All other reagents were local products of analytical grade.

Establishment of a Hamster Model with Cardiac Hypertrophy

The hamster model of cardiac hypertrophy was produced un-

der pressure overload caused by abdominal aorta constriction. Eight-week-old hamsters (male) were anesthetized with 5% hydration chloroaldehyde injected intraperitoneally and were opened at the abdominal central region after disinfecting with 70% ethanol. Non-traumatic ligation was made using a No. 4.5 needle inserted into the abdominal aorta. The needle was promptly removed, yielding a constriction similar in size to the outer diameter of the needle, and the wound was sutured. The sham-operated hamsters of the control groups were opened at the same region of the abdominal cavity as the model groups, but without abdominal aorta constriction. Hamsters were sacrificed at 3 days, and at 4 and 8 weeks after overload operation (model group) or sham operation (control group).

Measurement of Cardiomyocyte Diameter

Hamster hearts were sectioned and stained with hematoxylin eosin according to the conventional techniques (including fixation in 10% neutral formalin and paraffinization). The diameters of 200 cardiomyocytes were measured in each section using a BHEC microscope-computer image system.

Reverse Transcription-PCR

Total RNA in each hamster heart was extracted with TRIzol reagent (ZENECA Ltd.) according to the manufacturer's instructions. RNA concentration was assessed by spectrophotometry at 260 nm. For reverse transcription (RT), 2 µg of total RNA from each RNA preparation was reverse transcribed in a 20 µl reaction mixture containing 0.05 mol/l Oligo(dT)₁₅, 20 U RNasin, 10 mmol/l DTT, 0.5 mmol/l dNTP, 200 U M-MLV, 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, and 3 mmol/l MgCl₂ at 42°C for 1 h. The reaction was terminated by heating at 95°C for 5 min, and 2 µl of the RT reaction solution was amplified in 50 µl of PCR reaction mixture containing 0.5 µmol/l of each primer, 0.2 mmol/l dNTP, 1.5 U Taq E, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.001% gelatin, 0.05% Triton X-100 and 1.5 mmol/l MgCl₂. Cycling conditions consisted of denaturation at 94°C for 3 min, 30 cycles of 94°C for 50 s, 61°C for 30 s and 72°C for 1 min, and a final elongation at 72°C for 10 min. Ten microliter of the RT-PCR solution was used for gel electrophoresis. The RT-PCR product was quantified by scanning with an optical densitometer (Bior-Rad Gel Doc 1000; Bio-Rad, Hercules, USA) and the results were expressed as the ratio of ACE, chymase and AT1R to GAPDH mRNA levels, respectively. The primers used in the current study were as follows: ACE (P1: 5'-GCTTGCCCAACAAGACTGCCA-3', P2: 5'-CCA CATGTCTCCCAGCAGATG-3'); chymase (P3: 5'-ATCA TGAGCTCCAAGAGTGAC-3', P4: 5'-ACGGGAAATGCTGGCCCTAC-3'); GAPDH (P5: 5'-TCACTGCCACCC, AGAAGA-C3', P6: 5'-CTGTTGAAGTCGCAGGAGAC-3') and AT1R (P7: 5'-GCCCTTAACCTCTTGCTGA-3', P8: 5'-TCGATGCTGAGACACGTGAG-3').

Enzyme Activity Assay

Chymase and ACE activities in the hamster heart were determined by RIA. About 0.1g mouse heart tissue was washed with cold saline, minced in 1 ml of 20 mmol/l cold Tris-HCl, pH 7.4, and homogenized. The protein concentrations were measured by Lowry's method (8). Aprotinin (protease inhibitor) and lisinopril (ACE inhibitor) were used to inhibit proteases other than chymase. The reactions were divided into four aliquots of 500 μ l volumes each containing enzyme preparation and 1) 20 mmol/l Tris-HCl, pH 7.4, 6 ng Ang I; 2) the ingredients in 1) plus 50 μ mol/l lisinopril; 3) the ingredients in 2) plus 20 μ mol/l aprotinin and 20 mmol/l EDTA; and 4) the ingredients in 3) but without enzyme preparation as a control. The reaction was initiated by adding the enzyme preparation, continued at 37°C for 15 min, and terminated by adding 2.5 volumes of ethanol. Following centrifugation at 15,000 $\times g$ for 30 min, the supernatant was lyophilized and re-dissolved in 400 μ l of buffer provided in the Ang I RIA kit, and then all samples were assayed using this kit. Chymase and ACE activities were determined based on the decrease of Ang I. One unit (U) of activity was defined as the amount of enzyme producing a decrease of 1 ng Ang I per min. The activity not inhibited in the presence of both inhibitors (lisinopril and aprotinin) was considered to be chymase-like activity and the activity inhibited by lisinopril was considered to be ACE activity.

Ang II Level Assay

Myocardial tissue (0.15 g) was washed with 0.9% cold NaCl solution and minced. The tissue was transferred into a tube containing 1 ml of 0.1 mol/l HCl and then incubated in boiled water for 10 min. The tissue suspension was then homogenized with Polytron and centrifuged at 15,000 $\times g$ for 30 min. The supernatant was collected and mixed in 2.5 volumes of cold ethanol and then incubated in an ice bath for 30 min. It was then centrifuged again at 15,000 $\times g$ for 30 min. The supernatant was freeze-dried and re-suspended in 400 μ l of buffer provided in the RIA kit. The amount of Ang II was determined using the RIA kit according to the manufacturer's instruction.

Statistical Analysis

All data were presented as the mean $\pm$ SD. Values of $p < 0.05$ by two-tailed Student's *t*-test were considered to indicate statistical significance.

Results

Cardiomyocyte Diameters in the Overloaded Heart

Cardiomyocyte diameters were measured in each section using a BHEC microscope-computer image system (40 $\times$ mag-

Fig. 1. Cardiomyocyte diameters of sham-operated and operated hamsters. The abscissa represents the three groups, which were respectively sacrificed at 3 days (3d), 4 weeks (4w) and 8 weeks (8w) after operation to constrict the abdominal aorta. Data are expressed as the means $\pm$ SD (μ m). ** $p < 0.01$ vs. the sham-operated group, $n = 4$.

nification). Hamster cardiomyocyte diameters, used as a reflection of cardiomyocyte size, were increased with the prolongation of interval after overload, but there was no marked difference among the three sham-operated groups. Cardiomyocyte diameters of hamster hearts at 3 days after overload showed no obvious change, while those at 4 and 8 weeks after overload increased markedly ($p < 0.01$), demonstrating that a hamster model of cardiac hypertrophy had been successfully established (Fig. 1).

Transcriptional Expression of the Chymase, ACE and AT1R Genes in the Heart

Transcriptional expression of chymase (0.297 ± 0.011 vs. 0.297 ± 0.030 , ns; 0.334 ± 0.008 vs. 0.267 ± 0.009 , $p < 0.05$; and 0.361 ± 0.006 vs. 0.285 ± 0.013 , $p < 0.05$; respectively) and ACE (0.183 ± 0.005 vs. 0.155 ± 0.004 , $p < 0.05$; 0.200 ± 0.015 vs. 0.190 ± 0.001 , ns; and 0.216 ± 0.017 vs. 0.189 ± 0.001 , ns; respectively) genes gradually increased in the hamster hearts at 3 days, 4 weeks, and 8 weeks after overload. Such an increase was not seen for transcriptional expression of the AT1R gene; instead, the AT1R gene tended to gradually decrease (0.574 ± 0.093 vs. 0.705 ± 0.083 ; 0.516 ± 0.088 vs. 0.606 ± 0.003 ; and 0.472 ± 0.053 vs. 0.537 ± 0.004 ; respectively) (Fig. 2).

Chymase and ACE Activities in the Heart

The activity of chymase in the hamster hearts at 3 days after overload (U/mg) (0.441 ± 0.040) increased markedly to a level 2.5-fold that of the sham-operated animals (0.175 ± 0.014) ($p < 0.01$), while the activities of chymase at weeks 4 and 8 (0.446 ± 0.036 vs. 0.160 ± 0.016 and 0.522 ± 0.014 vs. 0.148 ± 0.038 , respectively) were slightly higher than that at 3 days (Fig. 3), which trend was slightly different from the

Fig. 2. Transcriptional expression of the chymase, ACE and AT1R genes in the hamster heart. A: Electrophoretic analysis of RT-PCR products. M: molecular weight marker (100 bp ladder: 1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp) (lane 1); S, sham-operated hamsters (lanes 2, 3, 8, 9, 14, 15); O, operated hamsters (lanes 4, 5, 6, 7, 10, 11, 12, 13, 16, 17, 18, 19). B: Semi-quantitative analysis. The abscissa represents the assays for the chymase, angiotensin converting enzyme (ACE) and angiotensin II type 1 receptor (AT1R) mRNA levels in the three groups at 3 days (3d), 4 weeks (4w) and 8 weeks (8w) after operation to constrict the abdominal aorta. mRNA levels (glyceraldehyde-3-phosphate dehydrogenase: GAPDH) as shown in the ordinate represent the ratios of the chymase, ACE and AT1R mRNA levels to the GAPDH mRNA levels. Data are expressed as the means $\pm$ SD. * $p < 0.05$ vs. the sham-operated group, ** $p < 0.01$ vs. the sham group, $n = 4$.

upward trend in chymase mRNA level (Fig. 2). The activity of ACE was also gradually increased in the hamster hearts, vs. the sham-operated group, at each of 3 days, 4 weeks, and 8 weeks after overload (0.142 ± 0.023 vs. 0.056 ± 0.038 ; 0.317 ± 0.017 vs. 0.079 ± 0.016 ; and 0.466 ± 0.010 vs. 0.098 ± 0.003 , respectively) ($p < 0.01$) (Fig. 3), which was much faster and larger than the upward trend in ACE mRNA level (Fig. 2).

Fig. 3. Activities of chymase and ACE in the hamster heart. The abscissa represents the assays for the activities of chymase and angiotensin converting enzyme (ACE) at 3 days (3d), 4 weeks (4w) and 8 weeks (8w) after operation to constrict the abdominal aorta. Data are expressed as the means $\pm$ SD (U/mg.). ** $p < 0.01$ vs. the sham-operated group, $n = 3$.

Fig. 4. Ang II level in the hamster heart. The abscissa represents the three groups respectively sacrificed at 3 days (3d), 4 weeks (4w) and 8 weeks (8w) after operation to constrict the abdominal aorta. Data are expressed as the means $\pm$ SD (pg/g). * $p < 0.05$ vs. the sham-operated group, $n = 3$.

Angiotensin II Level in the Heart

As the overloaded interval was prolonged, Ang II levels in the hamster hearts gradually increased (Fig. 4). The cardiac Ang II levels (pg/g) were 98.7 ± 4.5 , 134.4 ± 7.8 and 151.6 ± 10.1 , respectively, at 3 days, 4 weeks, and 8 weeks after overload and 71.2 ± 4.9 , 71.9 ± 12.8 and 80.0 ± 3.7 , respectively, in each group after sham operation ($p < 0.05$).

Discussion

Pressure overload is one of the factors leading to cardiac hypertrophy, but it remains unknown how this mechanical stress converts into a biological signal. In this study, there are three findings. First, we demonstrated that an increase in Ang II is an important pathway by which mechanical stress stimulates cardiac hypertrophy. Our results showed that the

Ang II level increased significantly in hamster hearts at 3 days after overload in the absence of cardiomyocyte hypertrophy, although previous experiments on cellular level indicated that Ang II was not involved in the development of cardiac hypertrophy produced by mechanical stress (9). In the present study, when cardiomyocytes hypertrophied after prolonged cardiac overload of from 3 days to 8 weeks, the increase in Ang II level (Fig. 4) was parallel to the extent of cardiomyocyte hypertrophy (at 4 and 8 weeks after overload) (Fig. 1). This indicated that RAS, through Ang II formation, participated in the development of cardiac hypertrophy produced by mechanical stress, and that an increase of Ang II may be one pathway by which mechanical stress is converted into a biological signal. Second, the results of this study indicate that the dual pathway hypothesis of Ang II formation is extensible to the hamster heart. The levels of chymase and ACE activities were markedly increased in the hamster hearts after abdominal aorta constriction. The levels of chymase activity in hamster hearts were much higher than those of ACE within 4 weeks after overload, with both activities being nearly equal at 8 weeks after overload. This suggested that Ang II formation in the hamster heart initially originated from primarily the chymase pathway in the early stage of overload, and then from the ACE pathway as cardiac hypertrophy progressed. Therefore, the increased Ang II level was due to catalytic reactions of both enzymes, chymase and ACE, indicating that a dual pathway of Ang II formation exists in the hamster heart. Third, this study demonstrated that chymase could be activated by mechanical stress before its mRNA level increased, because the chymase activity in the hamster hearts at 3 days after overload was markedly increased, but the chymase mRNA level was unchanged. Moreover, the increase in Ang II level was due to pathways originated from chymase and ACE in the non-hypertrophied hamster hearts suffering from mechanical stress, since activities of both chymase and ACE were also significantly increased in the non-hypertrophied heart at 3 days after overload, with the chymase activity level showing a particularly marked increase within 4 weeks after overload (Fig. 3).

ACE, a membrane-bound enzyme, is distributed in cardiac vascular endothelial cells, while chymase is stored in the cytoplasmic secretory granules of mast cells, endothelial cells and interstitial cells in the cardiac interstitium and vascular tissues (6, 7, 10). Chymase shows maximal activity immediately upon release into the extracellular matrix in vascular tissues after mast cells have been activated by a strong stimulus, such as injury by catheter (7, 11). In this study, the level and the increased extent of chymase activity were higher than those of ACE activity in the hamster heart at 3 days after overload. This suggested that mast cells in the cardiac interstitium were activated in the overloaded state like vascular tissues activated by stimulus and chymase may be released into the extracellular matrix after degranulation, resulting in the increases of chymase activity and Ang II level. These results indicated that mechanical stress may regulate chymase

and ACE activity in many ways, such as through the release and activation of chymase in the early stage and stimulation of the gene expression of chymase in the later stage. There are two possible explanations for the finding that the increase in enzyme activity was significantly higher than the increase in mRNA level. The first is that regulation of the increase in the activity of both enzymes in response to overload may occur at various levels, such as the gene-transcriptional level (including an increase of the mRNA level and its stability), the translational level (including increase of translation efficiency), the post-translational level, or during the enzyme's activation and release. The initial response of chymase to overload is modification of enzyme protein activity rather than slower gene regulation. The second possibility is that RIA may be more sensitive at detecting the activities of the two enzyme than semi-quantitative RT-PCR at detecting their mRNA levels. In addition, the time-dependent expression of cardiac chymase and ACE induced by overload may be affected by the use of differently established animal models. Shiota *et al.* (12) reported that cardiac chymase-like activity increased only in the late phase of hypertrophy in the pressure-overloaded hamster heart induced by a two-kidney, one-clip (2K1C) hypertension procedure, while angiotensin-converting enzyme was not activated. This suggests that chymase might be involved in cardiac tissue remodeling during the chronic stage of hypertension.

In this study, the AT1R mRNA level gradually decreased, while Ang II levels increased in the overloaded hamster hearts. Previous studies have reported that up-regulation of Ang II levels occurred in the human myocardium with heart failure, while both the AT1R mRNA level and AT1R density were down-regulated, suggesting that transcriptional expression of the AT1R gene in the hypertrophied or failure heart was regulated by the negative feed back of Ang II level in the local RAS of the heart tissue (13, 14).

In the past, research into the role of the Ang II formation pathway in the heart has focused on the later stage of cardiac hypertrophy or the stage of heart failure. In the present study, increased chymase and ACE activities in the hamster heart were parallel to the increase of Ang II level not only in the later stage with cardiac hypertrophy, but also in the early stage without cardiac hypertrophy under overloaded state (Figs. 3 and 4), supporting the dual pathway hypothesis of Ang II formation *in vivo*. Moreover, the increases of chymase and ACE activities induced by mechanical stress may be regulated in diversified ways, whereas Ang II formation originated mainly from the higher chymase activity in the hamster heart at 3 days after overload.

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