Angiotensin-Converting Enzyme and Angiotensin-Converting Enzyme 2 Are Involved in Sinoaortic Denervation-Induced Cardiovascular Hypertrophy in Rats

Li-Chao Zhang, Zhen-Zhen Li, Yong-Sheng Yu, Zhi-Bin Wang, Xin Wei, Ding-Feng Su, Li-Chao Zhang, Yong-Sheng Yu, Zhi-Bin Wang, Xin Wei, Ding-Feng Su, # and Ling Li

Department of Pharmacology, Second Military Medical University; Shanghai 200433, China; and Department of Pharmacy, Shanghai Seventh People’s Hospital; Shanghai 200137, China.

Received January 18, 2011; accepted May 26, 2011; published online June 17, 2011

The balance of angiotensin-converting enzyme (ACE) and angiotensin-converting enzyme 2 (ACE2) in high blood pressure variability (BPV) induced cardiovascular hypertrophy remains elusive. The aim of the present work was to investigate expression and activity of ACE and ACE2 in the heart and aorta of sinoaortic denervation (SAD) rats with high BPV and normal BP, and explore the potential role of ACE and ACE2 in high BPV-induced cardiovascular damage. Hemodynamics, cardiovascular hypertrophy, angiotensin II (Ang II) concentrations, ACE and ACE2 activity were determined. Cardiac-tissue ACE and ACE2 expression were assayed by real-time polymerase chain reaction and Western blot. Compared with sham-operated rats, systolic BPV and diastolic BPV increased and baroreflex sensitivity decreased significantly in SAD rats. SAD rats presented with obvious cardiovascular hypertrophy characterized by increased ratio of left ventricle weight to body weight and aortic weight to the length of aorta. There was no difference in plasma Ang II concentration between sham-operated and SAD rats. The cardiac and aortic ACE expression, aortic ACE2 expression and ACE activity were elevated in SAD rats. There was no significant difference in cardiac ACE2 expressions between sham-operated and SAD rats. The present work demonstrated that cardiac and aortic ACE expression, aortic ACE2 expression and ACE activity were increased in SAD rats. It is the tissue rather than the circulating renin–angiotensin system that contributes to high BPV-induced cardiovascular hypertrophy.

Key words angiotensin-converting enzyme; angiotensin-converting enzyme 2; blood pressure; hypertrophy; baroreflex

Hypertension is a major risk factor for cardiovascular diseases through its effects on target organs like the heart and aorta. Many studies have demonstrated that other than blood pressure (BP), blood pressure variability (BPV) is an also important factor contributing to cardiac damage and aortic hypertrophy in rats. However the exact biological mechanism responsible for high-BPV induced end-organ damage remains elusive. Our previous study demonstrated that the renin–angiotensin system (RAS) was involved in organ damage in sinoaortic denervation (SAD) rats with high BPV but normal BP.

Angiotensin-converting enzyme (ACE) is a crucial regulator of RAS, converting inactive decapeptide angiotensin I (Ang I) to potent vasoconstrictor angiotensin II (Ang II). Accumulating evidence from large clinical trials suggests that inhibition of ACE is effective in preventing cardiovascular diseases and target organ damage. On the other hand, angiotensin-converting enzyme 2 (ACE2), as a novel component of RAS and a homologue of ACE, cleaves only a single amino acid from the C-terminal of Ang I to form Ang (1–9) or directly converts Ang II to Ang (1–7), which has a vasodilatory action. ACE2 may be therefore a potent counter-regulator against ACE and play a significant role in regulating cardiovascular diseases. The balance between ACE and ACE2 is believed to play an important role in cardiovascular damage.

MATERIALS AND METHODS

Animals Male Sprague-Dawley (SD) rats (Experimental Animal Central of the Second Military Medical University, Shanghai, China) were housed at controlled temperature (23–25 °C), 12-h light and 12-h dark cycles (light, 08:00—20:00 h, darkness, 20:00—08:00 h), with free access to food and tap water. All animals received humane care in compliance with the institutional animal care guidelines.

Sinoaortic Denervation Sinoaortic denervation was performed in male SD rats aged 10 weeks according to the procedure described by Krieger with minor modification. Briefly, after anesthesia induced by intraperitoneal (i.p.) administration of 50 mg/kg ketamine i.p. and 5 mg/kg diazepam i.p., all animals were treated with 0.5 mg/kg atropine sulfate i.p. and 60000 U procaine benzylpenicillin i.p. A midline cervical incision was made to isolate the bilateral neck muscles. The aortic baroreceptor pathways on both sides were interrupted by removing a 1-cm segment of the aortic nerve, cutting the superior laryngeal nerve at its origin from the nodose ganglion, and removing a 1-cm segment of the cervical sympathetic trunk. The region of the carotid bifurcation was denervated bilaterally by careful mechanical stripping of the adventitia from the vessel, extending about 5 mm along the internal, external and common carotid artery. The region was then painted with 10% phenol in absolute ethanol. Sham operation was performed under the same conditions, without cutting and removing the nerves and carotid adventitia, while the common carotid artery was painted with 10% phenol.

© 2011 Pharmaceutical Society of Japan

* To whom correspondence should be addressed. e-mail: dfsu2008@gmail.com; lingli_z163@163.com
# These authors contributed equally to this work.
Animals were allowed to recover spontaneously after derivation. Arterial baroreflex function was assessed in conscious rats by intravenous injection of 5 g/kg phenylephrine via the left femoral vein. If phenylephrine induced a 50 mmHg increase in blood pressure with less than 20 beats/min decrease in heart rate, SAD was considered complete. After operation, the rats were allowed to survive for 32 weeks.

**BP Measurement** Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart period (HP) were measured continuously according to the method described previously. Briefly, rats were anesthetized with 40 mg/kg ketamine i.p. and 6 mg/kg diazepam i.p. A floating polyethylene catheter was inserted into the lower abdominal aorta via the left femoral artery for BP measurement, and another catheter was brought into the left femoral vein for intravenous injection. The catheters were exteriorized through the interscapular skin. After a 2-d recovery period, the animals were placed for BP recording in individual cylindrical cages with food and water. The aortic catheter was connected to a BP transducer via a rotating swivel allowing the animals to move freely in the cage. After about 14-h habituation, BP signals were digitized by a microcomputer, and beat-to-beat SBP, DBP and HP values were determined on line. The mean values of these parameters during the 24-h period were calculated and served as SBP, DBP and HP. Standard deviation over the mean was calculated and defined as the quantitative parameter of BPV and HPV.

**Baroreflex Sensitivity (BRS) Measurement** Under the above-mentioned BP recording condition, BRS was measured in conscious rats by using our previously described method. A bolus injection of phenylephrine was used to induce a BP elevation. The dose of phenylephrine was adjusted to raise SBP between 20 and 40 mmHg. There existed a delay about 1 s between the elevation of BP (stimulus) and the prolongation of HP (response) for arterial baroreflex. The rat heart rate is about 6 s⁻¹. So HP was plotted against SBP for linear regression analysis for 2—10 shifts (calculated by computer); the slope with the largest correlation coefficient for linear regression analysis was used as the quantitative parameter of BRS (ms/mmHg). The mean of the two measurements with a proper dose was used as the final result.

**Measurement of Aortic and Left Ventricular Weight and Tissue Preparation** After BP monitoring and BRS determination, the animals were weighed, anesthetized, and killed by decapitation. The aorta and heart were immediately excised and rinsed in cold physiological saline. Then, the atria and vessels were removed from the ventricles. The right ventricular (RV) free wall was separated from the left ventricle (LV) and septum, and they were blotted and weighed separately. At the same time, the aorta was cleaned of the adhering fat and connective tissue. A 22 mm-long segment of the thoracic aorta harvested just below the branch of the left subclavicular artery was blotted and weighed. The ratio of LV weight to body weight (LVW/BW) and aortic weight to the length of aorta (AW/length) were calculated. Immediately after measurement of ventricular and aortic weight, 50—100 mg of each ventricle (near the apex of heart) and the abdominal aorta were frozen separately in liquid nitrogen and stored at −80 °C until use.

**Measurement of Angiotensin II Concentration** Immediately after decapitation, a 2 ml blood sample was collected in a prechilled tube containing a mixture of peptidase inhibitors (ethylenediamine tetra-acetic acid, o-phenanthroline, p-chloromercuribenzoic acid, pepstatin A), cooled in ice-water bath, and centrifuged at 4 °C. The aliquots of plasma were stored at −80 °C before assay. The Ang II concentration was determined using a radioimmunoassay kit (China Institute of Atomic Energy, Beijing, China). All samples were assayed in duplicate according to the manufacturer's instructions.

**Measurement of Tissue ACE and ACE2 Activity** Tissue were homogenized at 4 °C in cold Tris–HCl buffer (pH=7.8) containing 30 mM KCl, 5 mM magnesium acetate, 0.25 mM sucrose, and protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 3000 g for 10 min at 4 °C. The supernatants were collected and centrifuged at 30000 g for 30 min at 4 °C. The supernatant was removed and the pellet was resuspended in the above buffer containing 0.5% Triton X-100 and incubated overnight on ice at 4 °C. After centrifugation, the soluble samples were kept frozen at −80 °C until assayed. Protein concentration was determined by the bicinchoninic acid method (Sigma), using bovine serum albumin as standard.

ACE activity was determined with Hip-His-Leu (5 mmol/l) as a substrate by reverse-phase high performance liquid chromatography (HPLC) as described previously. The specificity of the reaction was confirmed by a control incubation in the presence of a specific ACE inhibitor, perindoprilat (10 μmol/l).

ACE2 activity studies were performed with AngII as a substrate in the presence of protease inhibitors (perindoprilat, amastatin, and bestatin; 10, 100, 100 μmol/l, respectively). The specificity of the reaction was confirmed by control incubation in the presence of a specific ACE2 inhibitor: DX600. After incubation, conversion of AngII to Ang (1—7) was measured quantitatively by reverse-phase high-performance liquid chromatography.

**Isolation of Total RNA, Synthesis of cDNA, and Quantitative Real-Time Polymerase Chain Reaction** Total RNA was extracted from the heart and aorta by using TRizol (Life Technologies Inc., Gaithersburg, MD, U.S.A.) according to the manufacturer's protocol. First-strand cDNA was prepared from total RNA by using SuperScript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, U.S.A.). To assess genomic DNA contamination, controls without reverse transcriptase were included. Oligonucleotide primers were designed based on the cDNA sequences reported in the GenBank database. The sequences of primers are listed in Table 1. The real-time PCR analysis was performed with a QuantiTect™ SYBR® Green PCR QIAGEN Inc., China) according to the manufacturer's instructions. The highly specific measurement of mRNA was carried out for ACE and ACE2 using the LightCycler system (Bio Rad Inc., U.S.A.). Each sample was run and analyzed in duplicate. The quantitation was performed using the samples of known concentrations prepared from amplified DNA fragments extracted and purified from agarose gel for electrophoresis for ACE and ACE2. By this method, 6-order linearity was attained in serial dilutions of the sample. The mRNA level of ACE and ACE2 was adjusted for as the values relative to those of GAPDH. GAPDH was used as the endogenous control to ensure equal starting
tical analysis was performed using SPSS 11.0.0 software.

Aorta from SAD operated group. Each value represents mean ± S.D. (n=10 in each group).

Table 2. General Information: Hemodynamic Parameters, Plasma Ang II, and Heart and Aortic Damage Parameters in 32-Week SAD Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n=7)</th>
<th>SAD (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart period</td>
<td>152±13.3</td>
<td>21.90±5.38</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>132±8.9</td>
<td>140±11</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>86±7.5</td>
<td>90±8.6</td>
</tr>
<tr>
<td>BPV (mmHg)</td>
<td>8.16±0.71</td>
<td>16.36±1.90**</td>
</tr>
<tr>
<td>DBPV (mmHg)</td>
<td>6.79±1.25</td>
<td>13.45±2.15**</td>
</tr>
<tr>
<td>HPV (ms)</td>
<td>20.02±5.55</td>
<td>13.50±2.15**</td>
</tr>
<tr>
<td>R VW/BW (mg/g)</td>
<td>1.85±0.15</td>
<td>2.04±0.19*</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>1.74±0.15</td>
<td>1.91±0.17*</td>
</tr>
<tr>
<td>VV/BW (mg/g)</td>
<td>0.507±0.056</td>
<td>0.593±0.061**</td>
</tr>
<tr>
<td>AW/length (mg/mm)</td>
<td>181.1±47.2</td>
<td>157.1±91.2</td>
</tr>
<tr>
<td>ACE mRNA (%)</td>
<td>0.15 1.91</td>
<td>2.04±0.19*</td>
</tr>
<tr>
<td>ACE2 mRNA (%)</td>
<td>0.17 1.91</td>
<td>2.04±0.19*</td>
</tr>
<tr>
<td>BRS</td>
<td>0.620±0.146</td>
<td>0.156±0.070**</td>
</tr>
<tr>
<td>Ang II (pg/ml)</td>
<td>0.17 1.91</td>
<td>2.04±0.19*</td>
</tr>
<tr>
<td>Ang III (pg/ml)</td>
<td>0.620±0.146</td>
<td>0.156±0.070**</td>
</tr>
<tr>
<td>Ang IV (pg/ml)</td>
<td>0.17 1.91</td>
<td>2.04±0.19*</td>
</tr>
<tr>
<td>Ang V (pg/ml)</td>
<td>0.620±0.146</td>
<td>0.156±0.070**</td>
</tr>
<tr>
<td>Ang VI (pg/ml)</td>
<td>0.17 1.91</td>
<td>2.04±0.19*</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. SBP: systolic blood pressure; DBP: diastolic blood pressure; HP: heart period; SBPV: systolic blood pressure variability; DBPV: diastolic blood pressure variability; HPV: heart period variability; LVW: left ventricular weight; BW: body weight; RVW: right ventricular weight; AW: aortic weight. * p<0.05; ** p<0.01.

Fig. 1. Real-Time PCR Analysis of ACE and ACE2 mRNA Expression in the Heart and Aorta of Sham-Operated and SAD Rats 32 Weeks after Operation (A) ACE mRNA expression in the heart and aorta. (B) ACE2 mRNA expression in the heart and aorta. Total RNA was extracted from tissue, and real-time PCR was performed. Levels of ACE or ACE2 mRNA were corrected using GAPDH as an internal control. Each value represents mean ± S.D. (n=10 in each group). * p<0.05 vs. sham-operated group.

RESULTS

BP, Plasma AngII Concentration, and End Organ Damage in SAD Systolic BPV and diastolic BPV were significantly higher in SAD rats than that in sham-operated rats. BRS was evaluated to confirm the denervation and significantly lower in SAD rats than that in sham-operated rats. SAD rats presented with obvious cardiovascular hypertrophy characterized by increased LVW/BW (reflecting LV hypertrophy) and AW/length (reflecting aortic hypertrophy). There was no significant difference in plasma Ang II concentration between sham-operated and SAD rats (Table 2). Expression of ACE and ACE2 mRNA in the Heart and Aorta from SAD Compared with sham-operated rats, mRNA levels of ACE in the heart and aorta of SAD rats increased by 83% and 68% respectively (Fig. 1A), and mRNA levels of ACE2 in the aorta increased by 62%. There was no significant difference in heart ACE2 mRNA between sham-operated and SAD rats (Fig. 1B).
Expression of ACE and ACE2 Protein in the Heart and Aorta of SAD Rats

Compared with sham-operated rats, the protein levels of ACE in the heart and aorta of SAD rats increased by 103% and 69%, respectively (Fig. 2A), and the protein levels of ACE2 in the aorta increased by 93%. There was no significant difference in heart ACE2 protein expression between SAD and sham-operated rats (Fig. 2B).

ACE and ACE2 Activity in the Heart and Aorta of SAD Rats

Compared with sham-operated rats, ACE activity in the aorta of SAD rats increased by 101%, whereas ACE2 activity was not significantly altered in the aorta. ACE and ACE2 activity in heart were not different between SAD and sham-operated rats (Table 3).

DISCUSSION

It was found in the present study that ACE expression in the heart and aorta, ACE activity in aorta of SAD rats with high BPV was significantly higher than that in control rats. SAD increased ACE2 expression in the aorta, but not in the heart.

High BPV could induce organ damage such as cardiac hypertrophy and vascular remodeling. 15) Arterial baroreflex reflects arterial baroreflex function and is one of the most important physiological mechanisms controlling homeostasis of BP.16) Impaired BRS is a marker of autonomic dysfunction; it plays a major role in the long-term development of arterial hypertension and related complications.16,17) The decrease in BRS, as observed in the present study, has been proposed as an independent variable relate to cardiovascular end organ damage, i.e., cardiac hypertrophy and fibrosis.18,19) As SAD through interruption of the arterial baroreflex may lead to a substantial increase in BPV, chronic SAD in rats is considered an experimental model of high BPV without causing sustained hypertension. Our results of aortic and LV hypertrophy at 32 weeks after SAD are consistent with previous data reported by our lab and others.20,21) However, the mechanism of high BPV-induced cardiovascular hypertrophy remains unclear.

Local tissue RAS may play an important role in the development of cardiovascular hypertrophy by high BPV, independent of circulating RAS. Our results showed that the plasma Ang II concentration was similar in SAD and sham-operated rats 32 weeks after the operation. Our previous study found that the plasma Ang II concentration was not significantly changed at 2, 10 and 16 weeks after SAD.4) Take together, our results further demonstrated that cardiovascular hypertrophy induced by long-standing high BPV was independent of circulating RAS.

Our previous study showed that Ang II concentrations in LV and RV were not significantly changed at 2, 10 and 16 weeks after SAD.5) It is known that Ang II is mainly formed by ACE from Ang I and degraded by ACE2 to Ang (1—7). The interplay between ACE and ACE2 may govern the formation and metabolism of Ang II. Ang II may act as a growth factor to promote hypertrophy of myocardial cells and proliferation of vascular smooth muscle cells. Our results showed that from 32-weeks cardiac-tissue ACE expression increased more significantly in SAD rats as compared with that in sham-operated rats, while cardiac-tissue ACE2 activity and expression and ACE activity did not differ significantly between the two groups. In this study, although mRNA and protein levels of ACE in the heart are increase, the enzymatic activity does not increase. The discordance between ACE expression and activity in the heart may be attributed to post-translational proteolytic cleavage of ACE protein in heart.22) The exact mechanism need to be further studied. However,
the increase of cardiac-tissue ACE expression induced by high BPV is consistent with the previous study, which showed that cardiac-tissue ACE was up-regulated in cardiac disorders such as myocardial hypertrophy. The present data suggest that increased local production of Ang II in the heart may account, at least partially, for high BPV-induced cardiac hypertrophy. However, it's a pity that we did not detect concentration of angiotensin II and angiotensin (1—7) in heart or aortic tissue. Although local Ang II concentration was not measured in this study and cardiac Ang II was unchanged in previous study. Perhaps it is due to the different time points after SAD used in these two studies; 2, 10, 16 weeks in previous study and 32 weeks in the present study. To a great extent, increased local production of Ang II explains the antihypertrophic effect of ACE inhibitors due to local inhibition of this enzyme and afterwards local inhibition of both Ang II formation and bradykinin degradation on cardiac hypertrophy caused by either pressure- or volume-overload.

However, there are also controversial reports arguing that increased local production of Ang II by ACE over-expression in the heart is not sufficient to induce ventricular hypertrophy or fibrosis. In addition, there is also evidence that increased ACE was unable to cause cardiac disorders directly. A study reported that neither cardiac hypertrophy nor other morphological abnormalities were observed in transgenic rats with selective over-expression of human ACE in the cardiac ventricles under standard living conditions. However, after induction of hypertension by suprarenal aortic banding, the degree of cardiac hypertrophy in transgenic rats was significantly higher than that of banding control rats. The expressions of both atrial natriuretic factor and collagen III, molecular markers of cardiac hypertrophy, were also increased in banded transgenic rats compared with banding controls. These results suggest that increased cardiac ACE augmented rather triggered cardiac hypertrophy.

Moreover, as well known not only ACE but also chymase plays an important role in the production of tissue Ang II in cardiovascular tissues. In a dog model, chymase activity was significantly augmented in the injured artery 1 month after balloon catheter injury, but ACE activity was not. In this model, an angiotensin receptor blocker candesartan or a chymase inhibitor NK3201 prevented vascular proliferation after balloon catheter injury, but an ACE inhibitor enalapril did not. The chymase inhibitor specifically inhibited the augmented chymase activity in the injured artery and did not affect on ACE activities in the plasma and injured artery. These studies suggest that chymase-dependent Ang II formation may play an important role in vascular proliferation after balloon catheter injury. Furthermore, chymase is also involved in cardiac dysfunction after myocardial infarction. Chymase inhibitors attenuated the chymase activity and the cardiac dysfunction, which extended survival. However, in these studies, it has been unclear whether chymase-dependent Ang II is directly involved in the cardiac fibrosis after myocardial infarction. The chymase inhibitor could not reduce the cardiac hypertrophy, in rats with hypertensive myocardial fibrosis. The angiotensin receptor blocker reduced not only the cardiac fibrosis but also the cardiac hypertrophy in the hamster model of cardiomyopathy. The difference between angiotensin receptor blockers and chymase inhibitors with respect to the effect on cardiac hypertrophy suggests different mechanisms are involved in their improvement of cardiac function. Increases in cardiac chymase activity in cardiomyopathy may contribute to transforming growth factor-β activation rather than Ang II formation, and this may play an important role in inducing cardiac fibrosis. Taken together, chymase may become an important target to prevent organ damage in cardiovascular diseases. Even though we did not evaluate the effect of chymase on circulating and local RAS after SAD in rats, we might hypothesize that it also alter and play a role in organ damage with high BPV.

ACE2 is identified as a critical negative modulator of Ang II bioactivity, counterbalancing the effect of ACE in determining net tissue Ang II levels. There is accumulating evidence that the heart to body weight ratio and cardiac morphology are virtually identical in ACE2-knockout mice and the wild type control animals. A study showed that after transverse aortic constriction, cardiac hypertrophy, as assessed by the heart-to-body weight ratio, was more severe in ACE2 knockout mice than that in wild-type control animals. Our present work showed that cardiac-tissue ACE2 expression and activity did not differ significantly between SAD and sham-operated rats. The result is not consistent with the previous reported that heart ACE2 mRNA and protein expressions were markedly decreased in spontaneously hypertensive rats when compared with WKY rats. However, Gallahger et al. showed that myocyte treatment with inhibitors of myocyte and cardiac fibroblast growth, such as Ang-(1—7) or atrial natriuretic peptide reversed the ACE2 mRNA downregulation by hypertrophic hormones like Ang II or ET-1, indicated that ACE2 expression in the heart was dependent upon the regulation and concentration of multiple hypertrophic and anti-hypertropic peptides. Thus the discrepancy may due to the different induced factor of cardiac hypertrophy; high BP in previous study and high BPV in the present study. Taken together, these studies suggest that ACE2 does not necessarily play a critical role in regulating normal cardiac function, but may be important in modulating responses to pressure overload and perhaps other stresses and injuries.

In addition, our results showed that ACE2 expression and ACE expression and activity in the aorta of SAD rats increased significantly as compared with sham-operated rats. Strong staining for ACE and ACE2 were observed in endothelial cells and vascular smooth muscle cells in the aorta by immunohistochemistry (our unpublished data). This correlates well with the demonstration of ACE and ACE2 mRNA and protein in aortic tissue. Because the previous study showed that the aortic hypertension occurred earlier than left ventricular hypertrophy and the aorta may be more sensitive to substantially high BPV in SAD rats. Taking into account the specific in vitro affinities of ACE2 and ACE for Ang I, Ang I would be preferentially cleaved to Ang II by ACE rather than to Ang(1—9) by ACE2. Thus, the increased ACE2 expression in the aorta seemed to be a compensatory reaction rather than a causative mechanism in high BPV-induced aortic hypertrophy. The precise mechanism of increased ACE2 expression in the aorta remains to be fully elucidated.

In conclusion, the present work demonstrated that cardiac and aortic ACE expression, aortic ACE2 expression and ACE activity were elevated in SAD rats. It is the tissue rather
than the circulating RAS that contributes to high BPV-induced cardiovascular hypertrophy.

Acknowledgement This study was supported by the Grant from the National Natural Science Foundation of China (30971427) and the National Science and Technology Major Project (2009ZX09303-002) and Academic Leader Grant of Shanghai Pudong New Area (PWRd2008-7) and Key Discipline Grant of Shanghai Pudong New Area health system (PWZXK2010-11).

REFERENCES