Effect of Chronic Neutral Endopeptidase Inhibition on Cardiac Hypertrophy after Experimental Myocardial Infarction

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Candoxatril is an inhibitor of neutral endopeptidase, a membrane-bound enzyme that degrades atrial natriuretic peptide. The effects of candoxatril on hemodynamic parameters and cardiovascular hypertrophy were evaluated in the rat model of myocardial infarction. Myocardial infarction was induced by left coronary artery ligation in rats and they were treated either with candoxatril (10 mg/kg per day) or a vehicle for up to 4 weeks. Systolic blood pressure and body weight did not change for up to 4 weeks between the 2 groups. At the end of treatment, hemodynamic parameters were measured, and then plasma, heart, lungs and kidneys were collected. Kidney neutral endopeptidase, as measured by the quantitative autoradiographic method, was significantly inhibited in candoxatril-treated rats compared with that in controls ($66.6 \pm 3.2\%$ of control, $p < 0.001$). On the contrary, there were no significant differences in right atrial pressure, left ventricular end-diastolic pressure, systemic pressure, and plasma level of atrial natriuretic peptide between the 2 groups. There were also no significant differences in cardiac weight and lung weight. These data indicate that inhibition of neutral endopeptidase by candoxatril at a dose of 10 mg/kg per day did not oppose cardiac hypertrophy in the rat model of myocardial infarction in spite of significant neutral endopeptidase inhibition. (*Jpn Circ J* 1998; 62: 680–686)

Key Words: Autoradiography; Candoxatril; Cardiac hypertrophy; Myocardial infarction; Neutral endopeptidase

Congestive heart failure carries a grave prognosis, with a mortality rate of over 50% within 5 years of diagnosis!^{1,2} The progressive nature of the disease is due in part to a series of neurohumoral homeostatic responses that initially maintain cardiac output and tissue perfusion but ultimately prove deleterious by increasing cardiac work and further reducing cardiac function.^{3,4} These responses include enhancement of sympathetic tone, alteration in regional vascular resistance, activation of the renin-angiotensin-aldosterone system, and sodium retention.

Atrial natriuretic peptide (ANP) is a peptide hormone of cardiac origin with multiple biologic effects including diuresis, natriuresis, vasodilatation, and inhibition of renin and aldosterone secretion.^{5,6} Based on these properties, some studies have suggested a therapeutic role for ANP in congestive heart failure.^{7,8} However, the therapeutic potential of ANP is limited by its peptidic nature and rapid elimination from circulation. Long-term therapy requires either an orally active analogue or an inhibitor of ANP metabo-

lism. Neutral endopeptidase (NEP) is a major ANP-degrading enzyme^{9–11} and a new therapeutic approach has been developed by inhibiting the enzyme and potentiating the effects of endogenous ANP.^{12–15} Beneficial acute hemodynamic and renal effects of ($\pm$)-candoxatrilat (UK69578), an NEP inhibitor, have been reported in patients with congestive heart failure.^{12,13} Furthermore, reports about the effects of NEP inhibitors on cardiovascular remodeling have been published recently.^{16,17}

In patients with myocardial infarction, progressive left ventricular dilatation begins soon after myocardial infarction¹⁸ and left ventricular volume is the most powerful predictor of prognosis.^{19,20} In the rat model of chronic heart failure with coronary ligation, a time-dependent increase in ventricular volume was attenuated by chronic therapy with captopril, an angiotensin converting enzyme (ACE) inhibitor²¹ and, moreover, therapy with captopril had a pronounced effect on survival.²² The aim of this study was to examine whether chronic treatment with the NEP inhibitor candoxatril (UK79300) would modify cardiac hypertrophy in the rat model of myocardial infarction. Candoxatril is an orally active indanyl ester prodrug of the compound candoxatrilat (UK73967), the active isomer of the racemic mixture known as ($\pm$)-candoxatrilat (UK69578).^{12,13}

Methods

Adult male Sprague-Dawley rats (Biological Research Laboratories, Austin Hospital, Victoria, Australia) weighing about 300 g were used for this study. These animal studies conformed to the guiding principles of the NHMRC/CSIRO code of conduct and were approved by

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Experimental Myocardial Infarction

Myocardial infarction was induced by left coronary artery ligation using the method of Pfeffer et al^{21,22} and this method is well established in our laboratory^{23,24}. Briefly, rats were anesthetised with ether, intubated and then ventilated using a small rodent respirator. Anesthesia was maintained by a mixture of oxygen and enflurane. A left thoracotomy was performed, the heart exteriorized, and a 6-0 Prolene ligature placed between the pulmonary artery outflow tract and the left atrium. The heart was returned to the normal position, then the lungs were reinflated using positive end-expiratory pressure. The ribs were pulled together with 3-0 silk, and muscles and skin were sutured separately by 3-0 silk. Thoracotomy without left coronary artery ligation was performed on sham-operated rats ($n=6$). After surgery the rats were allowed free access to tap water and a normal diet containing 20% protein, 0.8% potassium, and 0.3% sodium by weight (Clark King & Co, Melbourne, Victoria, Australia).

Treatment

One week after coronary ligation or sham-operation, the rats were weighed and systolic blood pressure was measured by the tail-cuff method with preheating. Then the rats with coronary ligation were assigned to one of two treatments. The first group received 10 mmol/L TRIS (Sigma Chemical Co, St Louis, MO, USA) as drinking fluid (control group), and the second group received candoxatril dissolved in 10 mmol/L TRIS as drinking fluid. Body weight was measured weekly and the concentration of candoxatril in the drinking fluid was adjusted weekly to a daily dose of 10 mg/kg per day. Sham-operated rats received 10 mmol/L TRIS as drinking fluid. The treatment was continued for 4 weeks.

Hemodynamic Measurement and Organ Preparation

Systolic blood pressure was measured weekly by the tail-cuff method with preheating during the experimental period. At the end of the 4-week treatment period, by which time the necrotic myocardium had been completely replaced by fibrous scar tissue²⁵ rats were anesthetised and placed on a temperature-controlled table (37°C) for hemodynamic measurement. Anesthesia was induced by ether, then maintained with oxygen and 2% enflurane mixture under spontaneous respiration. The right carotid artery and the right jugular vein were cannulated with a polyethylene tube (PE-30). The catheters were connected via a stop cock to a Statham transducer (Model P23 G6), and pressure was recorded on a polygraph (Neotrace 400 ZEF, Neomedix System, Victoria, Australia). The arterial and venous catheters were then advanced into the left ventricle and right atrium, respectively, and were then withdrawn to the aorta and superior vena cava for recording of phasic and mean left ventricular, ascending aortic and right atrial pressure and heart rate. After hemodynamic measurement, blood was drawn from the arterial catheter for hormonal measurement. The rats were then killed by decapitation for organ collection.

The heart and lungs were removed, washed in saline, and weighed. The cardiac chambers were then individually dissected, separated, and weighed. Organ weights were corrected for body weight. Infarct size was determined by planimetry and expressed as the percentage of fibrous scar

to total left ventricular circumference^{23,24}. The kidneys were rapidly removed, washed in saline, snap-frozen in isopentane at -40°C, and used for autoradiographic localization of NEP.

In Vitro Autoradiography of NEP

Serial frozen sections (20 μ m) of the kidney were cut in a cryostat maintained at -20°C, thaw-mounted on gelatin-coated glass slides, dried in a desiccator for 2 h at 4°C, and then stored at -80°C until use. SCH47896 (Schering-Plough Research, NJ, USA) was radioiodinated using ¹²⁵I and an iodogen (Pierce Chemicals Co, Rochford, ILL, USA) as mentioned previously²⁶. SCH47896 is a phenolic analogue of SCH39370 and is a suitable substrate for iodination. SCH48446, the di-iodo analogue of SCH47896, is the major product of iodination and also an excellent inhibitor of NEP, with potency comparable to that of SCH39370²⁶. The sections were incubated by a modification of the previously reported technique²⁶. All sections were warmed to 20°C just before incubation. The sections were incubated with approximately 0.2 nmol/L (500000 cpm/ml) of ¹²⁵I-SCH47896 in 150 μ l of 10 mmol/L Na₂HPO₄ buffer, pH 7.4, containing 150 mmol/L NaCl and 0.2% bovine serum albumin (CIS Ltd, Victoria, Australia) for 40 min at 20°C. Nonspecific binding was determined in the presence of 10 μ mol/L SCH39370 (Schering-Plough Research). After incubation, the slide-mounted sections were transferred through four successive 1-min washes of 10 mmol/L Na₂HPO₄ buffer (pH 7.4), containing 150 mmol/L NaCl, at 0°C. The slides were then rapidly dried under a stream of cold air, loaded in X-ray cassettes, and exposed to Agfa Scopix X-ray film (Agfa Gevaert, Australia) for 5 days at room temperature. The X-ray films were processed in a Kodak RPX-OMAT automatic developer, and optical density was quantitated by computerized densitometry using an Eye Com Model 850 image analysis system (Spatial Data System, Springfield, VA, USA) coupled to a DEC 11/23 LSI computer (Digital Equipment Corp, Maynard, MA, USA). Standards of ¹²⁵I radioactivity were applied to 5-mm diameter discs of 20- μ m rat brain sections and mounted on slides. One set of the radioactivity standards was exposed to the same film in parallel with the tissue sections. These standards were fitted to a calibration curve by computer to convert optical density values of each pixel into dpm/mm². Four sections of each rat were incubated for total binding, and 2 sections of each rat were incubated for nonspecific binding. Specific binding for each rat was calculated as the average of total binding minus nonspecific binding.

Plasma ANP Measurement

Plasma ANP (determined as immunoreactive atrial natriuretic factor) was measured by a modified method of the previously reported technique, without extraction²³. Briefly, blood samples were put into plastic tubes containing the following inhibitors: disodium-EDTA (EDTA-Na₂, AJAX Chemicals Ltd, Sydney, NSW, Australia; 1 mg/ml), aprotinin (Sigma Chemical Co; 100 KIU/ml), and soy bean trypsin inhibitor (SBTI, Sigma Chemical Co; 50 BAEE/ml). Plasma was separated by centrifugation at 3000 rpm for 15 min at 4°C and stored at -70°C until assay. A phosphate buffer (100 mmol/L), pH 7.4, containing 0.1% bovine serum albumin, aprotinin (500 KIU/ml), SBTI (50 BAEE/ml), 0.1% TritonX-100 (Packard Inst, Downers Grove, ILL, USA), EDTA-Na₂ (10 mmol/L), and 0.1%

Table 1 Effects of 4 Weeks of Treatment on Hemodynamic Parameters in Sham-Operated Rats and Rats With Myocardial Infarction

	<i>n</i>	HR (beats/min)	SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	LVSP (mmHg)	LVEDP (mmHg)	RAP (mmHg)
Sham	6	320.0±5.8	114.4±4.0	93.3±2.7	79.0±2.0	122.7±3.0	5.8±0.7	1.0±0.1
Control	6	316.0±5.7	110.7±3.6	92.7±3.5	79.7±3.6	120.3±1.9	11.7±1.3 [†]	2.6±0.5*
Candoxatril	7	348.6±9.7**	112.6±1.7	98.6±1.6	82.7±1.8	124.6±1.9	12.9±1.1 [†]	2.9±0.5*

Values are expressed as means ± SEM. Sham, sham-operated rats; Control, vehicle-treated rats with myocardial infarction; Candoxatril, candoxatril-treated rats with myocardial infarction; HR, heart rate; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; RAP, right atrial pressure. **p*<0.05, [†]*p*<0.01 compared with sham, [‡]*p*<0.05 compared with control.

Fig 1. Line graphs showing the sequential value for body weight (panel A) and systolic blood pressure (panel B) for vehicle-treated control rats and candoxatril-treated rats. There were no statistically significant differences in body weight and systolic blood pressure between the 2 groups during the experimental period.

sodium azide, was used to dissolve all reagents and to perform the radioimmunoassay. Fifty microliters of a standard solution of synthetic α -human ANP (Peninsula Laboratories Inc, CA, USA) or plasma samples was incubated for 24 h at 4°C with 150 μ l of buffer and 100 μ l of antiserum (1:75,000, sheep anti- α -human ANP). Then 100 μ l of 125 I-labeled ANP (approx. 5,500 cpm) and 300 μ l of buffer were added and incubated for 16 h. The separation of antibody-bound and free peptides was performed using goat anti-sheep γ -globulin in the presence of normal sheep plasma. The anti- α -human ANP antibody was directed against the COOH-terminal portion of ANP and cross-

Fig 2. Bar graphs showing total heart weight and lung weight (panel A) and 4 cardiac chamber weight (panel B) for vehicle-treated control rats and candoxatril-treated rats. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. Values are expressed as mg/100 g body weight. There was no statistically significant difference in any of the parameters between the 2 groups.

reacted 100% with α -rat ANP.

Statistics

Data are shown as mean ± SEM. The values were analyzed by analysis of variance, and significant difference was determined by the multiple comparison test (Scheffé test). Statistical significance was defined as *p*<0.05.

Results

After surgery, 10 of the original 30 rats with coronary artery ligation died within 1 week, and most of these 10

rats died within 24 h after surgery. Two vehicle-treated rats and one candoxatril-treated rat died during the subsequent 4-week treatment period, and these rats were excluded from analysis. Two vehicle-treated rats and 2 candoxatril-treated rats did not have any apparent myocardial infarction at the end of the experiment. These rats were also excluded from analysis. All sham-operated rats survived through the experimental period.

Comparison Between Infarct Rats and Sham-Operated Rats

There were no significant differences in body weight and systolic blood pressure during the study period between control infarct rats and sham-operated rats (data not shown). Control infarct rats had a higher heart weight (301.2 ± 4.9 vs 288.3 ± 1.0 mg/100 g body weight, $p < 0.05$) and a higher plasma ANP level (507.5 ± 102.3 vs 187.3 ± 13.4 pg/ml, $p < 0.05$) compared with the sham-operated rats. Hemodynamic measurements demonstrated that control infarct rats had higher left ventricular end-diastolic pressure (LVEDP) and higher right atrial pressure (RAP) than those of the sham-operated rats (Table 1).

Fig 3. Plasma atrial natriuretic peptide (ANP) concentration determined as immunoreactive atrial natriuretic factor in vehicle-treated control rats and candoxatril-treated rats. Both groups showed high ANP concentration, but there was no significant difference between the groups.

Effects of Chronic Candoxatril Treatment in Rats With Infarction

There was no significant difference in the mean infarct size between vehicle-treated control rats ($n=6$, $30.2 \pm 4.2\%$) and candoxatril-treated rats ($n=7$, $33.7 \pm 5.1\%$). Body weight change during the experimental period is shown in Fig 1A. There was no significant difference in basal body weight between the 2 groups. Treatment with candoxatril induced no significant difference in body weight gain compared with vehicle treatment for up to 4 weeks. Systolic blood pressures of the 2 groups are shown in Fig 1B. There was no significant difference in systolic blood pressure between the 2 groups at the beginning of treatment. Again, treatment with candoxatril induced no significant difference in blood pressure compared with controls during the experimental period. Table 1 shows hemodynamic parameters at the end of treatment. Candoxatril-treated rats had a higher heart rate compared with control infarct rats and sham-operated rats. LVEDP and RAP of candoxatril-treated rats were higher than those of sham-operated rats, but they were not significantly different from those of control infarct rats. Fig 2 shows the total heart weight and lung weight (panel A) and the four cardiac chamber weight (panel B) of the 2 groups. There were no significant differences in total heart weight and lung weight or in four cardiac chamber weights between the controls and candoxatril-treated rats. Fig 3 shows the plasma ANP concentrations of the 2 groups. There was no significant difference in the plasma ANP level between the 2 groups. Autoradiographic visualization of NEP in the kidney is shown in Fig 4. Nonspecific binding was less than 10% of total binding in control rats, and there was no significant difference in nonspecific binding between the 2 groups. Thus, only pictures of total binding are shown in the Figure. In control rats, ^{125}I -SCH47896 bound to regions of the outer stripe of the outer medulla and to the inner cortex, consistent with binding to the deep proximal tubules. There was no binding detectable in the glomeruli. NEP binding in the kidney was markedly inhibited by candoxatril treatment. Fig 5 shows quantitative values of NEP binding obtained from computer analysis. NEP activity in candoxatril-treated rats was $66.6 \pm 3.2\%$ of the control

Fig 4. Computer-generated pseudo-color image of autoradiographs of ^{125}I -SCH47896 binding to rat kidney. Panel A: section from vehicle-treated control rat. Panel B: section from candoxatril-treated rat. Red represents high levels of binding and blue represents low-to-undetectable levels of binding. High densities of binding sites are seen in the outer stripe of the outer medulla and in the inner cortex. Note the marked suppression of binding sites in the candoxatril-treated rat.

Fig 5. Quantitative value of kidney neutral endopeptidase (NEP) binding obtained from computer analysis. NEP binding in candoxatril-treated rats was significantly inhibited compared with that in control rats. * $p < 0.001$ vs control.

rats ($p < 0.001$).

Discussion

In our present experiment, chronic candoxatril treatment of rats with myocardial infarction failed to exert any significant changes in hemodynamic parameters other than heart rate or any significant changes in heart weight and lung weight (or in other words, lung congestion). As NEP is most prominent in brush border vesicles of proximal tubular cells⁹⁻¹¹ we measured kidney NEP by the quantitative autoradiographic method in the present study. Kidney NEP binding in candoxatril-treated rats was significantly inhibited compared with that in vehicle-treated rats.

As far as we know, there are only 3 reports concerning the effects of chronic NEP inhibition on cardiac hypertrophy in rats with myocardial infarction. One of these reports is on the effects of 4 weeks of treatment with TM1, a prodrug of the NEP inhibitor SQ28603²⁷ and the other 2 reports describe the effects of 4 weeks of treatment with alatriopril or aladotril, mixed inhibitors of NEP and ACE^{28,29}. Treatment with TM1 reduced the development of right ventricular and atrial hypertrophy but had no effect on the left ventricle and septum weights²⁷. Alatriopril showed a greater potency than captopril in reducing cardiac hypertrophy, suggesting that enhancement of ANP levels via NEP inhibition magnifies the capacity of ACE inhibition to prevent cardiac hypertrophy²⁸. Aladotril decreased LVEDP to approximately the same extent as captopril and reduced cardiac hypertrophy without any hypotensive effect²⁹.

In the present study, we examined the effects of only one dose of candoxatril. In our preliminary experiment using quantitative in vitro autoradiography, a single gavage of candoxatril at a dose of 10 mg/kg inhibited kidney NEP binding to 13% of that of the controls at 1 h, and this inhibition was significant for up to 24 h. As demonstrated in the present study by quantitative in vitro autoradiography, NEP binding was significantly inhibited compared with that of the controls, even after the chronic 4-week treatment period. Although the plasma ANP level of candoxatril-treated rats was not significantly different from that in controls, reports about the relationship between the effects of NEP inhibition and plasma ANP level are not consistent. In some reports, effects of NEP inhibition were in parallel with the elevation of plasma ANP level^{12,15} while in other reports, NEP inhibitors exerted effects without any change

in the plasma ANP level^{30,31} or their effects did not parallel increases in plasma ANP³². In the present experiment, candoxatril was administered to the rats by dissolving it in drinking water. This method might have caused the difference in the relationship among plasma ANP level, kidney NEP binding and cardiovascular actions, because in most studies, NEP inhibitors were administered using a bolus method. Helin et al reported that following injection of SCH39370 in rats with heart failure and in the controls, diuresis tended to increase during the first 6 h and then decreased significantly during the next 6 h¹⁴. Electrolyte excretion also decreased significantly during the latter 6 h in SCH39370-treated rats. The administration of candoxatril to rats by dissolving it in drinking water may have resulted in a distinct pattern of hemodynamic and renal effects. Because rats drink more water during the night, significant cardiorenal action might have occurred during the night and significantly decreased diuresis and natriuresis due to rebound occurring during the daytime. However, this is not clear from our study because we did not complete a metabolic study. It remains to be examined whether higher doses of candoxatril and structurally different NEP inhibitors may oppose cardiac hypertrophy after myocardial infarction or not.

Degradation of ANP by NEP would block the potential effect of plasma-derived peptides on renal function. The presence of NEP on the brush border on the proximal tubules and its inhibition by NEP inhibitors may allow delivery of intact filtered ANP to ANP receptor sites on the inner medullary collecting duct^{33,34}. However, as demonstrated by Tsunoda et al, ANP binding sites of the inner medulla in rats with chronic heart failure are reduced²³. It is not clear whether these binding sites are type-A receptors (NPR-A) or type-C receptors (NPR-C), because both types of receptor bind ANP with high affinity^{35,36}. By in vivo microperfusion of the inner medullary collecting duct in rats, Sonnenberg et al showed that administration of ANP intraluminally resulted in reduced sodium efflux or decreased sodium reabsorption³⁷. This observation suggests the possibility that ANP receptors are located on the luminal site. If luminal receptors for ANP are present, they would not be expected to downregulate in heart failure, because they would not be exposed to the high circulatory levels of ANP. Therefore, the putative intraluminal ANP receptors would maintain responsiveness to any ANP protected by NEP inhibition. If intraluminal receptors for ANP in the inner medullary collecting duct are exposed to a high concentration of ANP by chronic NEP inhibition, then it is possible that these receptors might be downregulated during long-term NEP inhibition.

Short-term beneficial cardiovascular and renal effects of NEP inhibition have been reported in humans and in animals. On the other hand, Richards et al demonstrated differences between acute and chronic studies. In their chronic study, they administered candoxatril to normal volunteers over a period of 4 days³⁰. This treatment caused a range of effects that differed from those documented in single-dose studies using similar subjects studied under similar controlled conditions³⁸. Similar phenomena, including lack of a significant increase in plasma ANP level³⁰ might occur in a model of myocardial infarction, probably due to changes in the interaction between ANP and its receptors.

ANP is not the sole vasoactive substrate for NEP. Others include angiotensin I and II, bradykinin, substance P, big

endothelin and endothelin³⁹⁻⁴¹ Richards et al reported that inhibition of NEP significantly reduced clearance of infused angiotensin II (Ang II) in association with enhanced pressor response to exogenous Ang II⁴² Abassi et al reported that inhibition of NEP by SQ29072 increased the urinary excretion of endothelin (ET) and plasma concentration of ET, and they also demonstrated that a similar NEP inhibitor, SQ28603, increased the urinary excretion of ET and increased the accumulation of exogenous ET in the lung and kidney.⁴³ Many reports support the importance of the cardiac renin-angiotensin system in the pathophysiology of cardiac remodeling.^{21,22} The role of ET in the pathophysiology of chronic congestive heart failure is now supported by reports of its plasma level elevation in human and experimental heart failure models.⁴⁴ The long-term effects of ET activation, including the peptide's action as a growth factor, which may contribute to cardiovascular remodeling, may become excessive and may account for a continuing deterioration in cardiac function. Therefore, the net hemodynamic and structural effects of NEP inhibition may be dependent on the proportional effects of vasopressor and vasodepressor substrates or growth promoter and growth inhibitor substrates. Raised levels of substrates other than ANP may counteract any favorable cardiovascular effects of increased plasma and tissue level ANP.

In summary, chronic treatment with candoxatril in a rat model of myocardial infarction significantly inhibited tissue NEP activity but it did not exert any favorable changes in hemodynamic parameters or in heart and lung weights. Although many reports have shown that NEP inhibition potentiates the action of ANP, other substrates for NEP might be potentiated simultaneously. Interaction among these substrates and perhaps modulation at receptor sites might have caused the present results. Further study is needed to elucidate the effects of chronic NEP inhibition on cardiac hypertrophy after myocardial infarction.

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