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Cardioprotective Effect of SEA0400, a Selective Inhibitor of the Na^+/Ca^{2+} Exchanger, on Myocardial Ischemia-Reperfusion Injury in Rats

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Abstract. In this study, we investigated whether the Na^+/Ca^{2+} exchanger (NCX) inhibitor SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy-5-ethoxyaniline) might have a protective effect against myocardial ischemia-reperfusion injury in rats. In particular, we focused on cardiac function using Doppler echocardiography and cardiac gene expression. We intravenously administered either SEA0400 and delivery vehicle or only the vehicle (as a control) to Wistar rats 5 min before ischemia was induced. Reperfusion was performed after 30 min of ischemia. At 1 week after ischemia-reperfusion injury, we assessed hemodynamics by inserting a polyethylene-tubing catheter, cardiac function by Doppler echocardiography, and myocardial mRNA expression was determined by Northern blot analysis. Left ventricular (LV) end-diastolic dimensions (LVDd) and LV end-diastolic volume (LVEDV) were significantly increased in the ischemia-reperfusion rat model group compared to the control group. The SEA0400-treated group had a significantly attenuated LVDd ($P<0.05$) and LVEDV ($P<0.01$) increase, compared to the vehicle-treated group. A decrease in the LV ejection fraction ($P<0.05$) was significantly prevented in the SEA0400-treated group compared to the vehicle-treated group. Moreover, mRNA expression of plasminogen activator inhibitor-1 in the non-infarcted LV of the SEA0400-treated group was significantly lower than in the vehicle-treated group ($P<0.05$). This study demonstrates that the NCX is an important mechanism for cell death in myocardial ischemia and reperfusion in rats. SEA0400 may prove to be a promising new drug in the clinical treatment of myocardial ischemia and reperfusion.

Keywords: myocardial infarction, echocardiography, gene expression, ischemia-reperfusion injury, Na^+/Ca^{2+} exchanger

Introduction

Thrombolytic therapy and percutaneous coronary intervention have become the standard treatment for patients with acute myocardial infarction (MI) (1, 2). Although early reperfusion is the goal of therapy, reperfusion itself may contribute to additional tissue damage called reperfusion injury (3). It has been suggested that activation of the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) contributes to myocardial ischemia-reperfusion injury (4, 5). Previous reports have demonstrated the beneficial effects of NCX inhibitors on myocardial ischemia-reperfusion injuries (6, 7). However, as the NCX inhibitors used in those studies have other non-specific actions including Na^+/H^+ exchanger (NHE) inhibition and Ca^{2+} channel blockade (8), the question remains as to whether the benefits seen were actually due to inhibition of the cardiac NCX. Therefore, a more selective NCX inhibitor is required for studies on the pharmacological roles of the NCX. Recent studies have reported that a newly synthesized compound, 2-[4-[(2,5-difluorophenyl)methoxy] phenoxy]-5-ethoxyaniline (SEA0400), is the most potent and selective inhibitor of the NCX currently

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available (9). However, whether SEA0400 has any beneficial effects on the myocardial ischemia-reperfusion injury model has yet to be fully evaluated.

In this study, we investigated whether the NCX inhibitor SEA0400 might be protective against myocardial ischemia-reperfusion injury in rats. In particular, we focused on cardiac function using Doppler echocardiography and cardiac gene expression.

Materials and Methods

Myocardial ischemia and reperfusion production

Male Wistar rats weighing 250 to 270 g were purchased from Clea Japan, Inc. (Osaka). Rats were anesthetized with pentobarbital sodium (35 mg/kg, intraperitoneally). The animals were intubated and artificially ventilated with room air. The femoral vein was cannulated for drug administration. The electrocardiogram (ECG) was recorded from standard limb lead II and was monitored on the ECG recorder. The chest was opened by a left thoracotomy and a 6-0 prolene suture was placed around the left coronary artery (LCA). After stabilization, ischemia was induced by ligation of the LCA for 30 min. SEA0400 (1.0 mg/kg) or the control vehicle (0.5% carboxymethylcellulose solution) was injected intravenously 5 min before ligation. Successful occlusion of the LCA was confirmed by an immediate ST segment elevation on the ECG. Reperfusion was performed by cutting and removing the suture from around the coronary artery, followed by chest closure.

Drugs

SEA0400 was synthesized by Taisho Pharmaceutical (Saitama). SEA0400 was prepared and administered as a lipid emulsion containing 10% soybean oil. The dosage of SEA0400 was based upon previous studies (9). No antiarrhythmic treatment was used in this study.

Doppler echocardiographic studies and physiological studies

Transthoracic echocardiographic studies were performed, as previously described in detail (10), 1 week after myocardial reperfusion. In brief, rats were lightly anesthetized with an intraperitoneal injection of ketamine HCl and xylazine. Echocardiograms were performed using an echocardiographic system equipped with a 12-MHz phased-array transducer (SONOS 5500; Philips Medical System, Best, The Netherlands). Two-dimensional short-axis views of the left ventricle (LV) and M-mode tracings were recorded through the anterior and posterior LV walls at the papillary muscle level, in order to measure LV end-diastolic dimension (Dd). LV ejection fraction (EF) was measured using a modified Simpson's method that utilized a 4-chamber view. Pulsed wave Doppler spectra (E and A wave velocities) of the mitral inflow were recorded from the apical 4-chamber view, with the sample volume situated near the tips of the mitral valve leaflets. All Doppler spectra were recorded on paper at 100 mm/s and analyzed off-line.

Hemodynamics were measured as previously described in detail (11). In brief, LV pressure was recorded by inserting a polyethylene-tubing catheter (0.58-mm internal diameter, PE-50) into the right carotid artery and advancing it into the LV. Catheters were water-filled and connected via tubing to a water-filled pressure transducer. The pressures were recorded on a physiological recorder, while rats were allowed to breathe spontaneously. LV end-diastolic pressure (LVEDP) was obtained by averaging the values over 10 cardiac cycles. Infarct size was calculated and expressed as a percentage of LV surface area as previously described (12).

After determination of infarct size, the heart was immediately excised and the adjacent septal myocardium was separated for use as a non-infarcted sample. The specimens were immediately frozen and stored at −80°C until use.

RNA preparation and Northern blot analysis

All procedures were performed as previously described in detail by the authors (10). In brief, total RNA was isolated from the septal myocardium by the guanidium thiocyanate-phenol-chloroform method and 20 μg of total RNA samples were subjected to 1% agarose gel electrophoresis. RNA bands were transferred onto nylon membrane and hybridization was carried out with (^{32}P)-dCTP-labeled cDNA probes for transforming growth factor β1 (TGF-β1), plasminogen activator inhibitor-1 (PAI-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The densities of the individual mRNA bands were measured by using a bioimaging analyzer (BAS-2000; Fuji Photo Film Co., Tokyo).

Light microscopy

For light microscopy, specimens were fixed in 10% formaldehyde, embedded in paraffin and cut into 4-μm-thick sections. The tissue sections were stained with haematoxylin and eosin and examined under a light microscope.

Statistics

Results were expressed as the mean ± S.E.M. Statistical significance was determined using an unpaired *t*-

test and ANOVA. Differences were considered statistically significant at $P<0.05$.

Results

Changes in hemodynamics and ventricular weights

As shown in Table 1, there were no significant differences in hemodynamics and ventricular weights between the SEA0400-treated group and the control vehicle-treated group in the ischemia-reperfusion rat model. Infarct sizes were 21% in the vehicle-treated group and 16% in the SEA0400-treated group ($P<0.05$).

Doppler echocardiographic assessment

Echocardiographic assessments of LV geometry and function at 1 week after ischemia-reperfusion are shown in Table 2 and Fig. 1. SEA0400 treatment significantly prevented increases in LVDd and LV end-diastolic volume (EDV) compared to the vehicle-treated group. Decrease of LVEF in the SEA0400-treated group was significantly prevented compared to the vehicle-treated group.

Examples of pulsed wave Doppler recordings of mitral inflow from the 2 groups are shown in Fig. 2. The ischemia-reperfusion rat model group had significant diastolic dysfunction, as shown by an increased early rapid filling wave (E wave) velocity, a decreased late filling wave due to atrial contraction (A wave) velocity, an increased ratio of E wave to A wave (E/A ratio), and a decelerated E wave rate. There was no significant difference in E wave velocity and A wave velocity between the SEA0400-treated group and the vehicle-treated group. However, SEA0400-treatment significantly prevented the increase in E/A ratio compared to vehicle treatment.

Cardiac gene expression after ischemia-reperfusion rat model

The results of cardiac gene expression at 1 week after ischemia-reperfusion are shown in Table 3 and Fig. 3. PAI-1 mRNA expression significantly increased in the non-infarcted myocardium. SEA0400 significantly attenuated the increase in expression of PAI-1 mRNA compared to the vehicle-treated group ($P<0.05$). However, there was no significant difference in TGF-β1 mRNA expression between the SEA0400-treated group and the vehicle-treated group.

Light microscopy

Figure 4 shows myocardium from the septum (adjacent to an infarction) in vehicle-treated tissue and in SEA0400-treated rats. In myocardial infarcted rats

Table 1. Hemodynamics and ventricular weights

	Control	Vehicle	SEA0400
n	8	7	7
Heart rate, bpm	393 ± 17	394 ± 7	423 ± 13
LVSP, mmHg	96 ± 10	88 ± 45	89 ± 5
LVEDP, mmHg	4.5 ± 0.5	7.6 ± 1.3	6.7 ± 1.0
Body weight, g	286 ± 2	293 ± 4	284 ± 8
LV/BW weight, g/kg	1.86 ± 0.06	1.62 ± 0.03**	1.59 ± 0.06**
RV/BW weight, g/kg	0.44 ± 0.02	0.46 ± 0.01	0.49 ± 0.01
MI size, %		21 ± 1	16 ± 1†

**$P<0.01$ *vs* Control, $^{\dagger}P<0.05$ *vs* Vehicle.

Table 2. Doppler echocardiographic assessment of left ventricular geometry and function

	Control	Vehicle	SEA0400
LVDd, mm	7.0 ± 0.2	8.9 ± 0.2**	8.1 ± 0.2**,††
LVEDV, μl	335 ± 14	532 ± 45**	376 ± 23††
LVEF, %	63 ± 2	36 ± 2**	44 ± 3**,†
E wave, cm/s	65 ± 4	89 ± 4**	96 ± 5**
A wave, cm/s	49 ± 2	33 ± 4**	41 ± 2*
E/A ratio	1.4 ± 0.1	3.1 ± 0.3**	2.3 ± 0.2**,†
Dct, m/s	19 ± 1	23 ± 4	23 ± 1

*$P<0.05$, **$P<0.01$ *vs* Control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *vs* Vehicle.

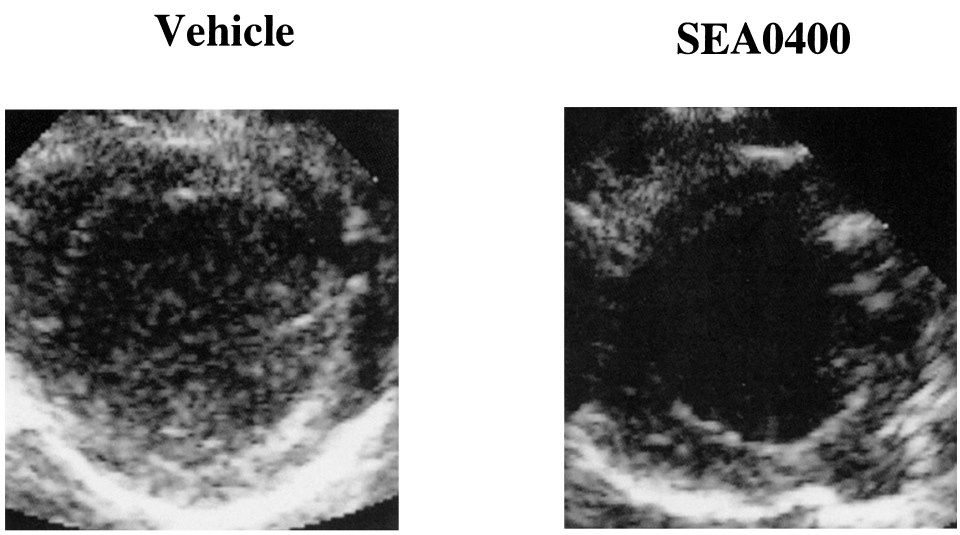

Fig. 1. Examples of two-dimensional echocardiograms (parasternal short-axis view) from a non-treated myocardial infarcted rat and a myocardial infarcted rat treated with SEA0400 at diastolic dimension, 1 week after myocardial infarction. Short-axis images were obtained at the level of the papillary muscles for consistency. SEA0400 decreased the left ventricular diastolic dimension in a myocardial infarcted rat.

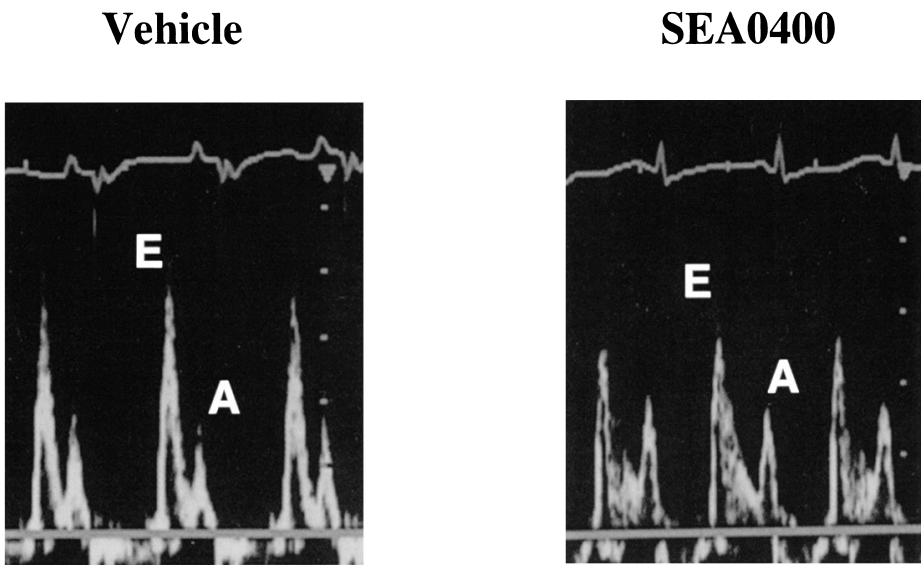

Fig. 2. Examples of pulsed wave Doppler spectra of mitral inflow from a non-treated myocardial infarcted rat and a myocardial infracted rat treated with SEA0400 at 1 week after myocardial infarction. The mitral inflow pattern shows an early rapid filling wave (E wave) velocity and a late filling wave due to atrial contraction (A wave) velocity. SEA0400 decreased the E/A ratio in a myocardial infarcted rat.

without SEA0400, replacement fibrosis and interstitial cells were prominent. In the SEA0400-treated group, however, the extent of fibrosis tended to be small.

Discussion

In the present study, we investigated the effects of SEA0400 using Doppler echocardiography and cardiac gene expression. Doppler echocardiography is currently the primary technique for evaluating LV geometry change and cardiac functions such as systolic and diastolic function. We found that the SEA0400-treated group had a significantly decreased LVDd, decreased LVEDV, increased LVEF, and an attenuated transmitral inflow pattern compared to the vehicle-treated group. This data suggests that SEA0400 attenuates myocardial

Table 3. Gene expression after myocardial ischemia-reperfusion model

	Control	Vehicle	SEA0400
PAI-1			
MI site	1.00 ± 0.12	2.72 ± 0.50*	2.93 ± 0.63*
non-MI site	1.00 ± 0.11	4.85 ± 1.03**	1.88 ± 0.39††
TGF-β1			
MI site	1.00 ± 0.06	1.35 ± 0.16*	1.60 ± 0.13**
non-MI site	1.00 ± 0.07	0.94 ± 0.11	1.10 ± 0.08

*P<0.05, **P<0.01 *vs* Control; P<0.01 *vs* Vehicle.

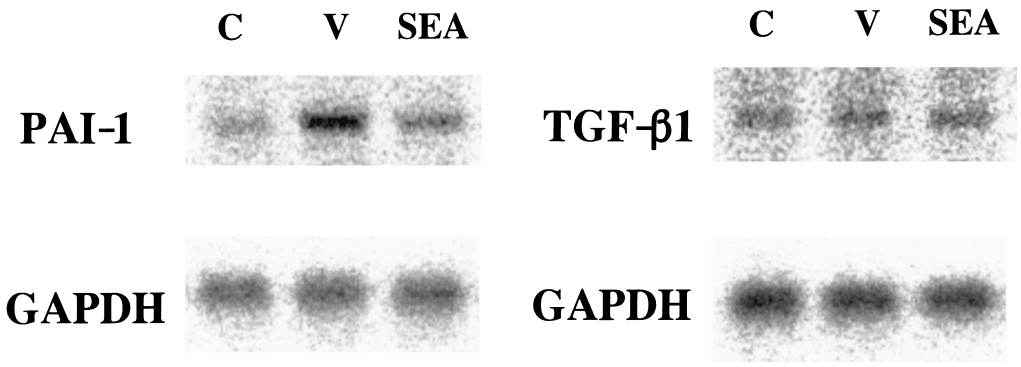

Fig. 3. Autoradiograms of Northern blot analysis showing mRNA expression levels of PAI-1, TGF-β1, and GAPDH in the LV non-infarcted region (septum) at 1 week after myocardial infarction. SEA0400 attenuated the increase of PAI-1 mRNA in a myocardial infarcted rat. C: Control, V: Vehicle, SEA: SEA0400.

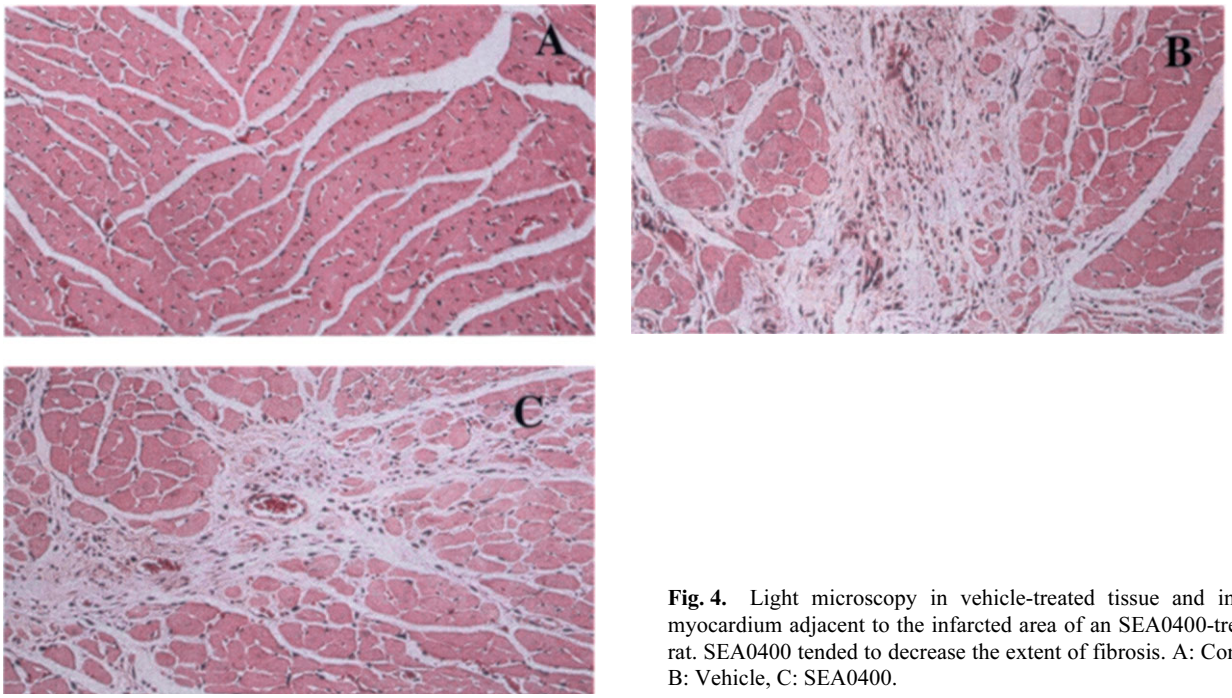

Fig. 4. Light microscopy in vehicle-treated tissue and in the myocardium adjacent to the infarcted area of an SEA0400-treated rat. SEA0400 tended to decrease the extent of fibrosis. A: Control, B: Vehicle, C: SEA0400.

ischemia-reperfusion injury in rats.

There were no changes in hemodynamics and ventricular weight between the SEA0400-treated and non-treated groups, while echocardiography demonstrated an improvement in cardiac function. MI can cause LV remodeling, which includes LV dilatation and hypertrophy. In general, hypertrophy had not occurred in non-infarcted myocardium 1 week after a MI affecting 20% of the LV wall. SEA0400 decreased the infarction size compared to the non-treated group. Infarct size was 21% in the vehicle-treated group and 16% in the SEA0400-treated group. While this change was not sufficient to cause a clear hemodynamic difference between the two groups, infarct size is nevertheless an important factor in determining cardiac function. Doppler echocardiography is a useful tool to analyze fine cardiac systolic and diastolic function.

The NCX is one of the major mechanisms involved in regulating intracellular Ca^{2+} concentrations via the forward mode (Ca^{2+} extrusion) or the reverse mode (Ca^{2+} influx) in excitable cells (13). It has been suggested that activation of the reverse mode of the NCX contributes to myocardial ischemia-reperfusion injury (4, 5). Myocardial ischemia is characterized by ATP depletion and intracellular acidosis. These changes lead to inactivation of the Na^+/K^+-ATPase and to activation of the NHE, resulting in intracellular Na^+ accumulation (14). During the early phase of reperfusion, the Na^+ accumulation is further accelerated by the activation of the NHE, which follows washout of extracellular H^+ (15). The reverse mode of the NCX is then activated and intracellular Ca^{2+} overload takes place (16). The pathological increase in intracellular Ca^{2+} concentration leads to activation of cell injury pathways (17).

There have been reports demonstrating the beneficial effects of NCX inhibitors on myocardial ischemia-reperfusion injuries (6, 7). However, as the NCX inhibitors used in those studies have other non-specific actions including NHE inhibition and Ca^{2+} channel blockade (8), the question remains as to whether the benefits seen were actually due to inhibition of the cardiac NCX. Recently, 2-[2-[4-(4-nitrobenzyloxy) phenyl]isothiourea (KB-R7943) (18) and SEA0400 (9) have been introduced as potent and selective inhibitors of the NCX, and the pathophysiological roles of the NCX were investigated in the heart. In rat cardiomyocytes, SEA0400 was found to be approximately 100 times more potent than KB-R7943 at inhibiting Na^+-dependent $45Ca^{2+}$ uptake (19). The IC_{50} value for SEA0400 was 92 nM and that for KB-R7943 was 9.5 μM. SEA0400 has a highly selective profile for the NCX.

PAI-1 is known to contribute to thrombus formation and to the development and clinical course of acute and chronic cardiovascular disease, as well other arterial and venous thromboembolic diseases. It has already been reported that PAI-1 increased in infarcted regions (20). Interestingly, we found increased PAI-1 mRNA levels in non-infarcted myocardium and SEA0400 attenuated this increase. The reason for this is not clear, but we speculate that the underlying process of cardiac fibrosis may be involved. PAI-1 is a major physiological inhibitor of the plasminogen activator/plasmin system, a key regulator of extracellular matrix turnover and fibrinolysis (21). Katoh et al. has reported that angiotensin-converting enzyme inhibition prevented increases in PAI-1 levels in the LV and perivascular fibrosis induced by chronic inhibition of nitric oxide synthesis, which made the LV remodeling (22). PAI-1 may have contributed to the extracellular matrix turnover and fibrinolysis in the non-infarcted myocardium of the LV remodeling. In our study, SEA0400 decreased infarct size compared with the non-treated group, which means that the remodeling process may have been attenuated in the SEA0400-treated group. Infarct size is an important factor in determining the extent of LV remodeling after MI (23). However, further work is needed to elucidate the more detailed mechanisms responsible for the beneficial effects of SEA0400 in decreasing PAI-1 levels in non-infarcted myocardium after MI.

Cardioprotective strategies following reperfusion aim to limit reperfusion injury, which may contribute as much as 25% to 50% of final infarct size (24). Whereas cell death during reperfusion may be a continuation of the necrotic process initiated during the preceding ischemia, apoptosis (programmed cell death) is now recognized as an additional mechanism of cell death during ischemia-reperfusion and may be particularly activated during reperfusion. Inhibition of apoptosis by growth factors, such as TGF-β1, is currently being investigated (24). In this study, SEA0400 did not show any effect on TGF-β1 mRNA expression. The cardioprotective effect of SEA0400 is therefore not related to TGF-β1 in this model.

In conclusion, we found SEA0400 to have beneficial effects in in vivo models of myocardial ischemia-reperfusion injuries. Therefore, our results support the previous studies showing the contribution of the NCX to myocardial ischemia-reperfusion injuries.

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