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Effects of Endothelin-1_{1-31} on Cell Viabililty and $[Ca^{2+}]_i$ in Cultured Neonatal Rat Cardiomyocytes

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Summary

We previously found that Endothelin-1_{1-31} (ET-1_{1-31}) exhibited a pro-arrhythmogenic effect in isolated rat hearts. In this study, we further investigated the effects of ET-1_{1-31} on a cell viability and observed $[Ca^{2+}]_i$ in cultured cardiomyocytes. Cultured neonatal rat cardiomyocytes were treated with 0.1, 1, and 10 nM ET-1_{1-31} for 24h in the presence or absence of ET_A receptor antagonist (BQ_{123}) or phosphoramidon, a NEP/ECE inhibitor. Cell injury was evaluated by supernatant lactate dehydrogenase (LDH) assay, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) content. Cell viability was assessed by MTT assay. $[Ca^{2+}]_i$ was measured with Fluo-3/AM under a laser confocal microscope. 1) ET-1_{1-31} dose-dependently increased LDH release and decreased cell viability. 2) LDH and MDA levels were significantly elevated and SOD activity decreased after administration of 1 nM ET-1_{1-31} for 24h, and these changes were markedly attenuated by 1 uM BQ_{123}. 3) Exposure to 10 nM ET-1_{1-31} caused a continuous increase in $[Ca^{2+}]_i$ to cultured beating cardiomyocytes and termination of $[Ca^{2+}]_i$ transient within 6 min, and this change was reversed by 1 uM BQ_{123} and attenuated by 0.5 mM phosphoramidon. These results suggest that ET-1_{1-31} could cause cell injury, and that the effect of ET-1_{1-31} on $[Ca^{2+}]_i$ transients is mainly mediated by ET_A receptor and partially attributed to the conversion of ET-1_{1-31} to ET-1_{1-21}.

Key words

Endothelin-1_{1-31} • Rats • Cardiomyocytes • Calcium

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Introduction

Endothelin-1 (ET-1) is the prototype of a family of 21 amino acid peptides acting through two main receptor subtypes, called ET_A and ET_B. It is generated from big endothelin-1 through cleavage of the Trp^{22}-Val^{23} bond by endothelin converting enzyme-1 (ECE-1). However, it has been reported recently that bigET-1 may be also selectively cleaved at the Tyr^{31}-Gly^{32} bond by a chymase to produce a novel peptide, named ET-1_{1-31} (Nussdorfer 1999). In addition, it may transiently be generated by other chymotrypsin-type proteases, such as human cathepsin G in granulocytes and rat mast cell chymases.

ET-1_{1-31} has been found to reproduce many of the ET_A receptor-mediated vascular effects of ET-1_{1-21} (Miyauchi 1999), including contraction of porcine and rat aorta (Kishi 1998), raising of intracellular Ca^{2+} concentration in cultured human vascular smooth muscle cells (VSMC) (Yoshizumi 1998, Inui 1999, Yoshizumi 1999), and stimulation of VSMC proliferation (Yoshizumi 1998). However, the effects of ET-1_{1-31} on the heart or ventricular myocytes have not been observed. Previously, we have found for the first time that ET-1_{1-31} exhibited pro-arrhythmic effect in isolated rat hearts. This arrhythmogenic action is mediated by ET_A receptor and may be attributed mainly to the conversion of ET-1_{1-31} to ET-1_{1-21} (Ren 2006). The intracellular mechanisms underlying arrhythmogenic action of ET-1_{1-21} involve mobilization of intracellular calcium ($[Ca^{2+}]_i$) and injury on ventricular myocytes. In cultured neonatal rat

Table 1. Effects of the different concentration of ET-1_{1-31} on LDH and cell viability in the neonatal rat cardiomyocytes (mean±SD)

Group	n	LDH (U/l) Pre-treatment	24 h	cell viability
Control	6	20.7±2.7	24.2±2.9	0.288±0.013
ET-1_{1-31} 0.1 nM	6	21.2±2.3	29.8±3.2[ab]	0.242±0.027[a]
ET-1_{1-31} 1 nM	6	21.2±2.6	40.2±2.6[ab]	0.183±0.034[a]
ET-1_{1-31} 10 nM	6	20.1±2.7	48.7±2.3[ab]	0.150±0.028[a]

a: $p<0.01$ compared with normal control; b: $p<0.01$ compared with pre-treatment.

cardiomyocytes, ET-1_{1-21} significantly increased LDH release and decreased SOD activity and elevated diastolic $[Ca^{2+}]_i$ in myocytes (Lin 2003).

To shed light on the mechanism of arrhythmogenic action of ET-1_{1-31}, we investigated the effects of ET-1_{1-31} on cell viability and $[Ca^{2+}]_i$ in cultured neonatal rat cardiomyocytes and the effects of selective ET_A receptor antagonist BQ_{123}, ET_B receptor antagonist IRL_{1038} and phosphoramidon, an inhibitor of endothelin-converting enzyme on these biological activities of ET-$1_{1–31}$.

Methods

Preparation of cultured neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were cultured from 1–3-day-old Sprague-Dawley rats with a modification of the method described previously (Simpson 1982). In brief, the pups were decapitated and their ventricles were removed aseptically and minced in chilled Ca^{2+}- and Mg^{2+}-free Hanks' solution. The ventricles were cut into small pieces and digested with 0.2 % trypsin (Amresco, Solon, OH, USA) with constant agitation (100 cycles/min) at 37 °C. After digestion each supernatant except the first was collected every 10 min and centrifuged immediately at 1000 rpm/min for 10 min. The pellet was dispersed, filtered through a nylon mesh, and suspended in Dulbecco's Modified Eagle Medium (DMEM; GIBCO, Grand Island, NY, USA) containing 20 % neonatal bovine serum. After a 30 min period of preplating allowing noncardiomyocytes to attach, the nonattached cells were seeded at a concentration of 3×10^5, and cultured in a 5 % CO_2, 95 % air humidified incubator at 37 °C. The medium was added to 0.1mM 5-bromo-2'-deoxyuridine (Sigma, St. Louis, USA) that presumably prevented noncardiomyocytes proliferation without cardiomyocyte toxicity (Simpson 1982). Studies were performed after 4–5 days of culture in cells that had been serum-deprived for 24 h.

Assessment of endothelin-1_{1-31}-induced injury

To assess ET-1_{1-31}-induced injury to cultured cardiomyocytes, levels of lactate dehydrogenase (LDH), malondiadehyde (MDA) and superoxide dismutase (SOD) were determined after administration of ET-1_{1-31} for 24h. LDH was determined with an automatic biochemical analyzer (Hitachi 7150, Japan), MDA was determined by an MDA assay kit (Jian-Cheng Biomedical Engineering Co., Nanjing, China) and SOD was determined by the method described by Marklund (Marklund 1974).

Measurement of intracellular free calcium

Intracellular free calcium was measured with the Ca^{2+}-sensitive fluorescent dye Fluo-3. To be loaded with Fluo-3, a glass coverslip with cultured cells was incubated with 5 μM Fluo-3/acetoxymethyl ester (Fluo-3/AM; Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C and then rinsed with fresh DMEM. Afterwards, the coverslip was mounted in a chamber on the stage of a laser scanning confocal microscope (Zeiss LSM510, Thornwood, NY, USA), and continuously perfused with DMEM solution. Fluo-3 in cells was excited with light at 488 nm and emitted fluorescence was detected at Lp505 nm. Fluorescence images were acquired at 200-ms intervals. Since Fluo-3 is not ratiometric, absolute $[Ca^{2+}]_i$ was not calculated.

To observe the effects of the ET-1_{1-31} on intracellular calcium, ET-1_{1-31} was perfused for 15 min after a stabilization period of 10 min. ET_A receptor antagonist BQ_{123} or phosphoramidon, a NEP/ECE inhibitor was administrated 5 min before the perfusion of ET-1_{1-31}.

Table 2. Effects of ET-1_{1-31} and ET_A and ET_B receptor antagonist on supernatant LDH, SOD and MDA activity in neonatal rat cardiomyocytes (mean±SD)

Group	n	LDH (U/ l)		SOD (U/ ml)		MDA (nmol/ ml)	
		Pre-treatment	24 h	Pre-treatment	24 h	Pre-treatment	24 h
Control	5	22.6±3.2	25.8±2.8	34.1±2.8	30.3±5.5	1.1±0.1	1.4±0.2[d]
ET-1_{1-31} 1nM	6	23.5±2.9	41.8±2.6[bd]	31.4±3.7	17.5±4.4[bd]	1.3±0.2	2.6±0.4[bd]
BQ_{123} 1μM +ET-1_{1-31} 1nM	6	21.7±3.0	30.7±2.1[adf]	34.9±3.6	22.4±3.5[bdf]	1.1±0.1	2.2±0.2[bde]
IRL_{1038} 1μM +ET-1_{1-31} 1nM	5	22.2±2.5	31.4±4.0[adf]	31.7±2.2	17.2±2.4[bd]	1.2±0.1	2.4±0.2[bd]

a: p<0.05, b: p<0.01 compared with normal control; c: p<0.05, d: p<0.01 compared with pre-treatment; e: p<0.05, f: p<0.01 compared with ET-1_{1-31} group.

Data and statistics

All data are expressed as mean ±SD. The LDH, SOD activity, and MDA content before and after administration of ET-1_{1-31} were compared by Student's paired t-test, since samples were taken from the same culture. $[Ca^{2+}]_i$ in each group was assessed by one-way ANOVA. P<0.05 was considered statistically significant.

Results

Effects of ET-1_{1-31} on characterization of cultured cardiomyocytes

The neonatal rat cardiomyocytes were observed to contract spontaneously after 1–2 days in culture and reached confluence after 4–5 days. The confluent cells contracted synchronously and rhythmically at a frequency of 60–90 beats/min. After 24 h exposure to 1 nM ET-1_{1-31} almost no visible contraction was observed. However, in 1 μM BQ_{123}-incubated cells the contraction was maintained and faster than control.

Changes in supernatant LDH activity and cell viability induced by ET-1_{1-31}

Table 1 shows ET-1_{1-31} (0.1 nM, 1 nM, 10 nM) dose-dependently increased LDH release and decreased cell viability. LDH levels in ET-1_{1-31} treated groups at the three concentrations were 29.8±3.2, 40.2±2.6 and 48.7±2.3 U/l, which were all significantly higher than that of control group (24.2±2.9U/l, n=6, p<0.01); cell viability in ET-1_{1-31} treated groups at the three concentrations were 0.242±0.027, 0.183±0.034 and 0.150±0.028, which were all lower than that of the control group (0.288±0.013, n=6, p<0.01).

Effects of BQ_{123} and IRL_{1038} on ET-1_{1-31}-induced changes in supernatant LDH, SOD activity and MDA content

To assess ET-1_{1-31}-induced injury to cultured cardiomyocytes, levels of LDH, MDA and SOD were determined. The LDH activity in the medium reflects the damage of cells and LDH leakage from the damaged cells. Evaluation of MDA levels was performed to estimate the degree of lipid peroxidation. SOD, an important anti-oxidant enzyme, plays a pivotal role in preventing cellular damage caused by ROS. In normal cultures, supernatant LDH, SOD activity and MDA remained stable after 24 h of incubation (Table 2). When exposed to 1 nM ET-1_{1-31} for 24 h, LDH activity and MDA significantly increased from 23.5±2.9 U/l to 41.8±2.6 U/l (n=6, P<0.01) and from 1.3±0.2 nmol/ml to 2.6±0.4 nmol/ml (n=6, P<0.01) respectively, while SOD activity significantly decreased from 31.4±3.7 U/ml to 17.5±4.4 U/ml (n=6, P<0.01). BQ_{123} significantly reduced LDH release and MDA content and enhanced SOD activity. In the presence of 1 uM BQ_{123}, LDH and MDA were 30.7±2.1 U/L and 2.2±0.2 nmol/ml, respectively, both significantly lower than that in the ET-1_{1-31} group (n=6, P<0.05); SOD was 22.4±3.5 U/ml, significantly higher than the ET-1_{1-31} group (n=6, P<0.01) (Table 2). However, IRL_{1038} did not significantly change MDA content or SOD activity, although significantly reduced LDH release.

Effects of exogenous ET-1_{1-31} on $[Ca^{2+}]_i$

Spontaneous $[Ca^{2+}]_i$ transient synchronous to cell contraction was observed in normal cultured cardiomyocytes (Fig. 1A), while no $[Ca^{2+}]_i$ oscillation was observed in noncardiomyocytes (fibroblasts).

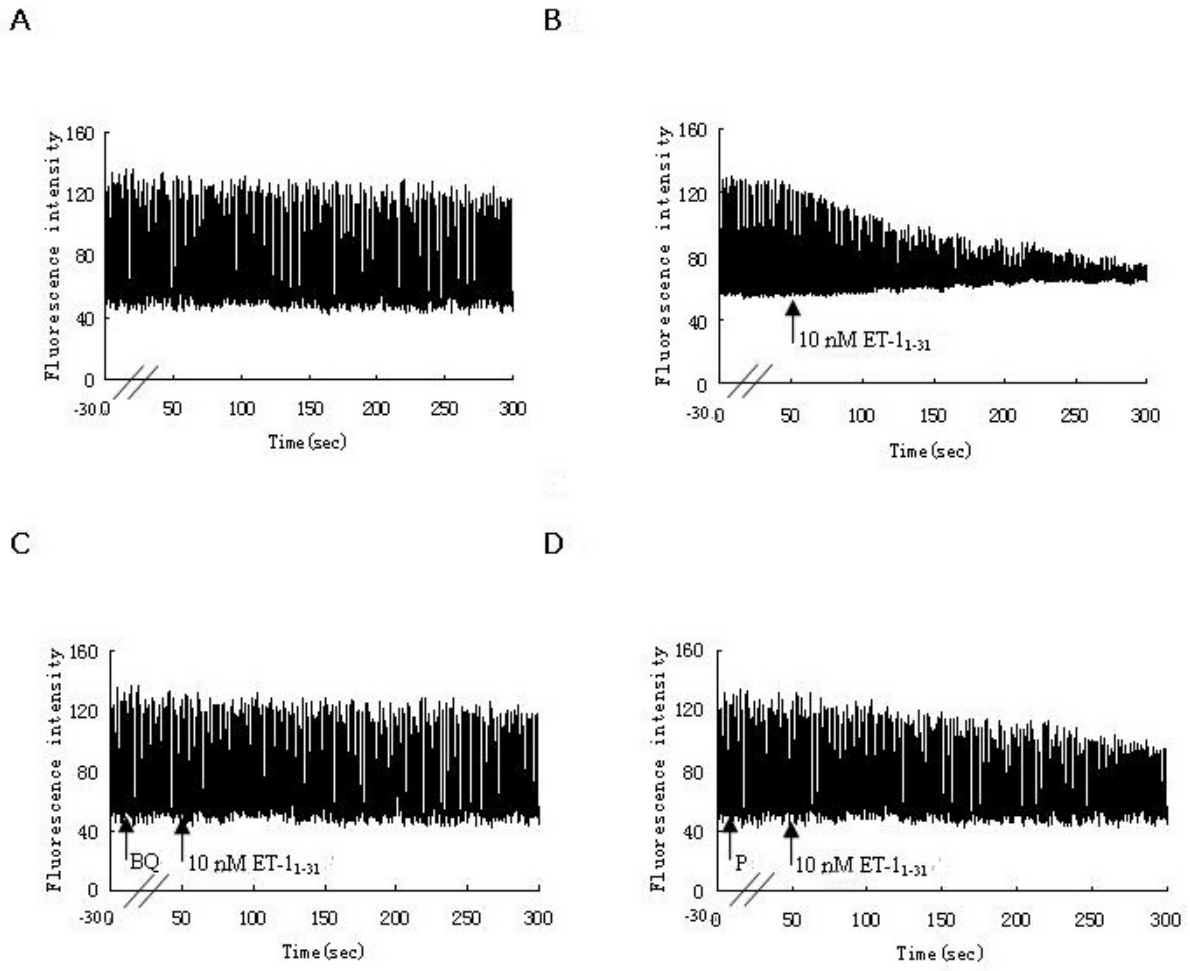

Fig. 1. Effects of 10 nM ET-1_{1-31} on $[Ca^{2+}]_i$ transients in spontaneously beating cultured neonatal rat cardiomyocytes. (A) control, (B) 10 nM ET-1_{1-31}, (C) 1μM BQ_{123} (BQ)+10 nM ET-1_{1-31}, (D) 0.5mM phosphoramidon (P) +10 nM ET-1_{1-31}.

Exposure of cardiomyocytes to 10 nM ET-1_{1-31} resulted in a significant increase in diastolic $[Ca^{2+}]_i$ and a significant decrease in systolic $[Ca^{2+}]_i$ (Table 3, Figure 1B). $[Ca^{2+}]_i$ transients terminated in 6 min. This may be the important mechanism of arrhythmogenic action of ET-1_{1-31}.

Effects of BQ_{123} and phosphoramidon on ET-1_{1-31}-induced $[Ca^{2+}]_i$ changes

To determine whether the effect of ET-1_{1-31} is a receptor-mediated pheomenon, we examined the effects of endothelin receptor antagonists on the changes in $[Ca^{2+}]_i$ evoked by ET-1_{1-31}. As shown in Table 3 and Figure 1C, BQ_{123} at 1uM almost abolished the changes in $[Ca^{2+}]_i$ induced by 10 nM ET-1_{1-31}. To investigate the possibility that changes induced by ET-1_{1-31} may be due to further degradation of ET-1_{1-31} to ET-1_{1-21} by endothelin-converting enzyme in the medium or in the cells, we examined the effect of an inhibitor of endothelin-converting enzyme, phosphoramidon, on the ET-1_{1-31} induced $[Ca^{2+}]_i$ Changes. We found that phosphoramidon attenuated but not abolished ET-1_{1-31}–induced changes in $[Ca^{2+}]_i$ transients (Table 3, Figure 1D).

Discussion

Since endothelin-1_{1-21} posses a wide variety of biological activities, endothelin-1_{1-31} may also be a novel vasoactive peptide in endothelin family. In our previous study, we have found that ET-1_{1-31} exhibited pro-arrhythmic effect in isolated rat hearts (Ren 2006). It is very important for elucidating ET-1_{1-31} proarrhythmic effect to study the direct effect of ET-1_{1-31} on cardiomyocytes. In the present study, we investigated the effect of exogenous ET-1_{1-31} on cultured cardiomyocytes

In the present study, cell injury was evaluated by LDH assay, SOD activity and MDA content. We found

Table 3. Effects of exogenous ET-1_{1-31} on systolic $[Ca^{2+}]_i$ (A) and diastolic $[Ca^{2+}]_i$ (B) and effects of BQ_{123} and phosphoramidon on ET-1_{1-31}-induced $[Ca^{2+}]_i$ changes

A

Group	n	Time (s)						
		-300	50	100	150	200	250	300
Control	6	139.17±10.26	138.00±10.12	141.33±11.20	138.17±8.01	134.33±9.71	133.17±11.11	133.50±9.97
ET-1_{1-31} 10nM	6	137.33±13.05	135.67±15.10	129.50±13.78	120.00±12.23[a]	109.00±9.96[b]	99.17±11.97[b]	87.33±13.29[b]
BQ_{123} 1μM +ET-1_{1-31} 10nM	6	143.17±10.94	143.83±9.39	143.00±10.04	139.83±10.93[c]	136.00±9.21[d]	133.50±11.15[d]	129.17±13.47[d]
Phosphoramidon 0.5mM +10 nM ET-1_{1-31}.	6	140.00±10.20	140.33±9.48	135.00±8.00	129.50±7.37	122.67±8.76[c]	113.33±7.00[bc]	105.00±7.67[bc]

B

Group	n	Time (s)						
		-300	50	100	150	200	250	300
Control	6	53.17±5.00	52.17±5.85	51.00±4.05	51.67±5.72	50.83±6.05	51.00±6.32	51.33±7.50
ET-1_{1-31} 10nM	6	52.17±6.79	52.00±7.87	54.83±8.11	58.00±8.65	61.17±9.06[a]	65.00±8.92[a]	68.33±9.52[b]
BQ_{123} 1μM +ET-1_{1-31} 10nM	6	54.00±6.84	54.50±7.04	55.17±6.65	55.83±7.17	54.67±6.44	54.33±7.39[c]	54.83±8.04[c]
Phosphoramidon 0.5mM +10 nM ET-1_{1-31}.	6	50.50±6.32	51.33±6.50	51.67±6.62	52.50±6.16	53.83±6.08	54.83±6.08[c]	56.33±5.68[c]

a: $p<0.05$, b: $p<0.01$ compared with normal control, c: $p<0.05$, d: $p<0.01$ compared with ET-1_{1-31} group.

that ET-1_{1-31} increased LDH release and decreased SOD activity in cultured neonatal rat cardiomyocytes. To date, two subtypes of ET receptors have been identified in cardiomyocytes: ET_A receptor and ET_B receptor. To determine whether the effects of ET-1_{1-31} are a receptor-mediated phenomenon, we used the ET_A receptor antagonist BQ_{123} and ET_B receptor antagonist IRL_{1038}. We found that BQ_{123} alleviated the changes induced by ET-1_{1-31}. However, IRL_{1038} did not significantly change MDA content or SOD activity. These results suggest that ET-1_{1-31} induce injury is mediated through ET_A receptor of the cells.

Therefore, it is important to elucidate whether the response to ET-1_{1-31} is mediated by ET-1_{1-21} after hydrolysis of ET-1_{1-31} or whether ET-1_{1-31} itself acts directly on the receptors of the cells. ECE is a membrane-bound neutral metalloprotease and ECE activity is inhibited by phosphoramidon, a dual neutral endopeptidase/ECE inhibitor (Matsumura 1990). Yoko *et al*. reported that ET-1_{1-31} could increase in $[Ca^{2+}]_i$ of bronchial smooth muscle cells. Treatment with phosphoramidon almost completely inhibited the increase in $[Ca^{2+}]_i$ caused by ET-1_{1-31} and phosphoramidon itself did not change $[Ca^{2+}]_i$ (Yoko 1999). These results suggest that ET-1_{1-31} cause an increase in $[Ca^{2+}]_i$ by being converted into ET-1_{1-21} by neutral endopeptidase or ECE in the medium. In the present study, phosphoramidon did not directly affect the $[Ca^{2+}]_i$ but partially inhibited the changes in $[Ca^{2+}]_i$ induced by ET-1_{1-31} (Figure 1D), which suggest that the ability of ET-1_{1-31} to change $[Ca^{2+}]_i$ of cardiomyocytes is partially the consequence of its conversion to ET-1_{1-21}.

In conclusion, ET-1_{1-31} is a novel putative bioactive peptide in the ET family and may be deeply involved in the chymase-related pathophysiological progress. ET-1_{1-31} cause cardiomyocytes injury and the effects of ET-1_{1-31} on $[Ca^{2+}]_i$ transients is partially mediated by ET_A receptor and mainly due to the conversion of ET-1_{1-31} to ET-1_{1-21}. However, the mechanisms of transsarcolemmal Ca^{2+} movement and SR Ca^{2+} handling underlying the effects of ET-1_{1-31} on $[Ca^{2+}]_i$ remain to be clarified.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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