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Endothelin-1 stimulates cardiomyocyte injury during mitochondrial dysfunction in culture

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Abstract

To understand the pathophysiological role of endothelin-1 in the failing heart, we constructed a cellular mitochondrial impairment model and demonstrated the effect of endothelin-1. Primary cultured cardiomyocytes from neonatal rats were pretreated with rotenone, a mitochondrial complex I inhibitor, and the cytotoxic effect of endothelin-1 on the cardiomyocytes was demonstrated. Rotenone gradually decreased the pH of the culture medium with incubation time and caused slight cell injury. Endothelin-1 markedly enhanced the effect of rotenone that decreased the pH of the medium and enhanced cellular injury. The enhancement of the decrease in pH and cell injury induced by endothelin-1 was counteracted by the endothelin ET_A receptor antagonist BQ123 or by maintaining the pH of the medium by the addition of 50 mM HEPES. Endothelin-1 markedly increased the uptake of 2-deoxyglucose and lactic acid production when the cardiomyocytes were pretreated with rotenone. These findings suggest that the stimulation of glucose uptake and anaerobic glycolysis followed by the increase in lactic acid accumulation in cardiomyocytes under the condition of mitochondrial impairment may be involved, at least in part, in the cellular injury by endothelin-1. Moreover, these findings suggest the possibility that the effect of endothelin-1 on myocardium is reversed by the condition of the mitochondria, and endogenous endothelin-1 may deteriorate cardiac failure with mitochondrial dysfunction. This may contribute to clarify the beneficial effect of endothelin receptor blockade in improving heart failures.

Keywords: Cardiomyocyte; Endothelin; Rotenone; Cytotoxicity; Anaerobic glycolysis; Mitochondria

1. Introduction

Endothelin-1 is a potent vasoconstrictor peptide first identified from the conditioned medium of vascular endothelial cells (Yanagisawa et al., 1988). Endothelin-1 is synthesized and secreted not only by endothelial cells but also by cardiac myocytes (Suzuki et al., 1993). Endothelin-1 exerts diverse physiological effects on the myocardium, including a positive inotropic effect (Hu et al., 1988; Ishikawa et al., 1988; Moravec et al., 1989; Shah et al., 1989; Takanashi and Endoh, 1991) and growth promotion (Shubeita et al., 1990). Recently, it has been reported that endothelin-1 is not only an inotropic or growth-promoting peptide but also a survival factor in cardiomyocytes (Araki et al., 2000; Kakita et al., 2001). On the other hand, there are reports that myocardial endothelin-1 may have a deteriorative role in some heart diseases. The levels of endothelin-1 in plasma and in ventricular myocardium markedly increase in close association with systolic dysfunction (Hiroe et al., 1991; Cody et al., 1992; Lerman et al., 1992; Rodeheffer et al., 1992; Ishikawa et al., 1995; Sakai et al., 1996a,b). Moreover, endothelin-1 receptor antagonists has been shown to improve cardiac function and survival in experimental animal models of heart failure (Sakai et al., 1996a; Mulder et al., 1997; Wada et al., 1997, 1999;

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Iwanaga et al., 1998; Borgeson et al., 1988; Nguyen et al., 1998; Yamauchi-Kohno et al., 1999). Thus, the pathophysiological role(s) of endothelin-1 in myocardium is controversial at this time.

Mitochondria are the organelles that generate most of the cellular energy by oxidative phosphorylation. It is known that genetical mitochondrial impairment results in cardiac failure termed cardiomyopathy (Wallace, 2000). In the ischemic heart, mitochondrial oxidative phosphorylation is inhibited (Allen and Orchard, 1987). Moreover, mitochondrial complex I activity was inhibited in heart-failing animals (Ide et al., 1999, 2001). Interestingly, endothelin-1 is increased in the failing hearts in hamsters with cardiomyopathy (Yamauchi-Kohno et al., 1999). Moreover, endothelin-1 production is reported to be stimulated in the ischemic heart (Tonnessen et al., 1997). Very recently, we reported that mitochondrial impairment caused by rotenone, a mitochondrial complex I inhibitor, increased endothelin-1 mRNA and peptide in primary cultured cardiomyocytes (Yuki et al., 2001, in press). Thus, mitochondrial dysfunction may relate to the progression of heart failures and production of endothelin-1.

In the present study, therefore, to understand the pathophysiological role of endothelin-1 in the failing heart, we investigated the effect of endothelin-1 on the primary cultured cardiomyocytes under mitochondrial impairment caused by rotenone.

2. Methods

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Animal Research Committee of the School of Medicine, University of Tsukuba.

2.1. Isolation and primary culture of cardiomyocytes

Ventricular cardiomyocytes were isolated from 2- to 3-day-old Sprague–Dawley rats as described previously (Suzuki et al., 1997b). For the purification of myocytes, the isolated heart cells were suspended in a culture medium [Dulbecco's modified Eagle's minimum essential medium/Ham's F-12 medium (1:1) supplemented with 5 μg/ml of insulin and transferrin] supplemented with 10% fetal bovine serum and pre-incubated twice in tissue culture flasks for 20 min to separate non-adhering myocytes from adhering non-myocytes. The purified myocytes (greater than 90%) were suspended in the serum-free culture medium containing 0.1% bovine serum albumin and seeded into fibronectin-coated culture plates, and were incubated in a humidified 5% CO_2-air incubator. The medium was replaced with fresh medium on the first day. The experiment was started on the third day.

2.2. Cytotoxicity assay (MTT assay)

Cytotoxicity was assessed using a colorimetric assay system (Boehringer Mannheim, Mannheim, Germany). Briefly, cardiac myocytes were cultured in 48-well culture plates at a density of 10^5 cells/well. Seventy-two hours after the addition of rotenone to the myocytes, yellow labeling reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution, was added to each well and incubated for 4 h. Then, the culture medium was removed, and the cells were dissolved with a solubilizing solution (10% SDS in 0.01 M HCl). The purple formazan crystals were formed from yellow MTT by succinate dehydrogenase in living cells, and the optical density of the purple formazan solution was measured at 584 nm with a spectrophotometer.

2.3. 2-Deoxyglucose uptake assay

To estimate the activity of glucose uptake to the cardiomyocytes, 2-deoxy-D-[1-^{3}H]glucose (2-deoxyglucose; $<$ 1.1 TBq/mmol, Amersham, Little Chalfont, UK) was used as described previously (Suzuki et al., 1997a). Cardiomyocytes were cultured in 24-well culture plates. Twelve hours after the addition of rotenone (1 μM), endothelin-1 (10 nM) or vehicle was added to the medium. Concomitantly, 37 kBq of 2-deoxyglucose solution was added to the culture medium. The cells were incubated for 7 h. Then, the medium was aspirated, and the cells were washed twice with ice-cold phosphate-buffered saline. Then, the cells in each well were dissolved with 0.5 N NaOH solution. A part of each solution was used for the protein assay, and the remaining solution of each well was transferred to a scintillation vial, and the radioactivity was measured using a liquid scintillation counter (Beckman LS6500).

2.4. Measurement of lactic acid

The L-lactic acid concentrations in the culture media were quantified using a commercially available assay kit (F-Kit L-Lactate, JK International, Tokyo, Japan) 72 h after the addition of rotenone. Endothelin-1 was added to the cultures 12 h after the addition of rotenone.

2.5. Protein assay

Protein was determined using a BCA® protein assay system (Pierce, Rockford, IL) with bovine serum albumin as a standard.

2.6. Materials

Endothelin-1 was purchased from Peptide Institute (Osaka, Japan). Selective endothelin receptor antagonists, BQ123 for type A receptors and BQ788 for type B receptors (Osada et al., 1997) were from Banyu Pharmaceutical (Tokyo, Japan). Rotenone was purchased from Sigma (St. Louis, MO).

2.7. Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) followed by Scheffe's *F*-test for multiple comparisons.

3. Results

3.1. Cell toxicity of rotenone and endothelin-1

Morphological changes of cardiomyocytes by the treatment with rotenone (1 μM), endothelin-1 (10 nM) and combination of rotenone and endothelin-1 are shown in Fig. 1. Endothelin-1 induced arrangement of contractile proteins in the cells compared with the control cells. No cytotoxic effect of endothelin-1 was observed in the car-

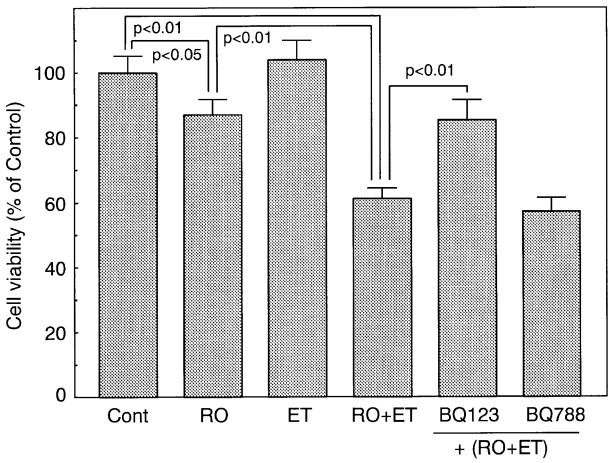

Fig. 2. Effect on the cell viability of rotenone (1 μM, RO), endothelin-1 (10 nM, ET), and the combination of rotenone and endothelin-1, and the counteracting effect of the endothelin ET_A receptor antagonist BQ123 on the endothelin-1 induced enhancement of cell injury at 72 h after the addition of rotenone. Cell viability was estimated with MTT reduction activities. Endothelin-1 was added to cultures 12 h after rotenone. BQ123 (10 μM) and BQ788 (10 μM) were added 1 h before endothelin-1. Each value is the mean and S.E.M. from nine samples of three different cultures. Statistical analysis was performed on the values of the cell viability. $P < 0.05$ and $P < 0.01$ are statistically significant.

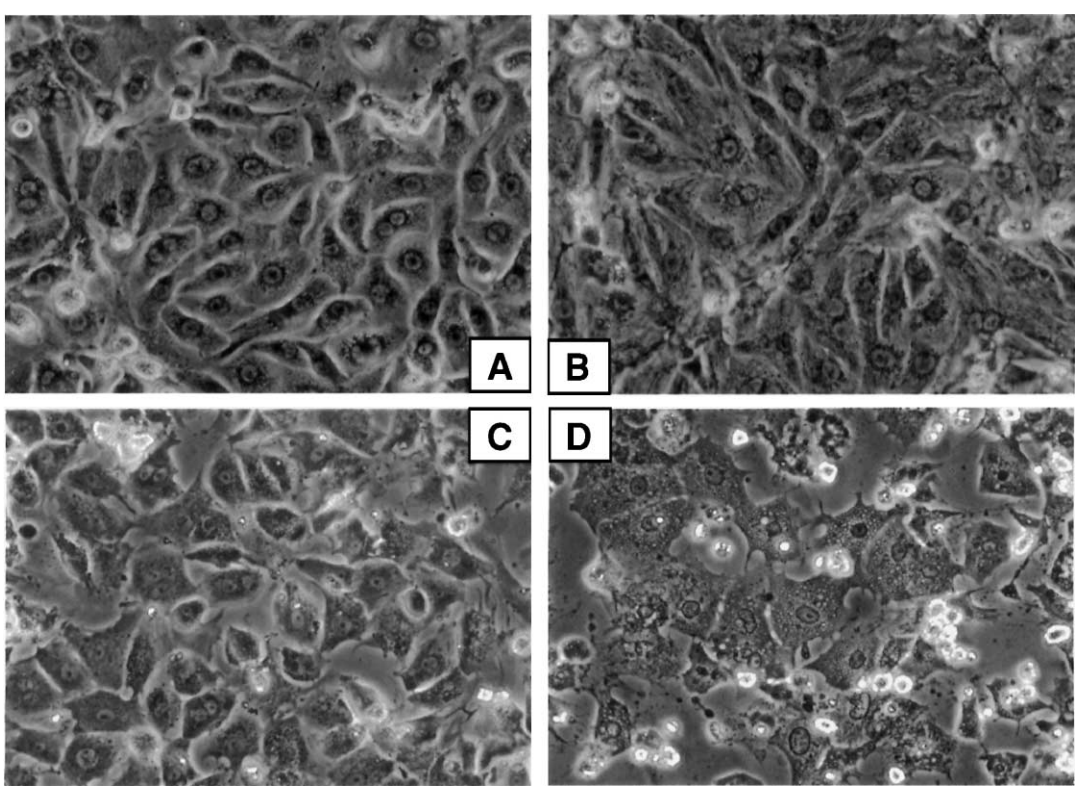

Fig. 1. Photographs show the morphological changes of the primary cultured cardiomyocytes treated with rotenone, endothelin-1 and the combination of rotenone and endothelin-1. Photographs were taken at 72 h after rotenone. Endothelin-1 was added to the cultures 12 h after rotenone. A: control, B: endothelin-1 (10 nM), C: rotenone (1 μM), and D: the combination of rotenone and endothelin-1.

diomyocytes. Rotenone induced shrinking of the myocytes. The combination of rotenone and endothelin-1 induced nuclear condensation and produced many vacuoles in the cytoplasm of the myocytes. Moreover, many floating cells were observed in the culture dishes (Fig. 1).

After 3 days from the treatment with rotenone, cell viabilities were assessed with MTT assay. Endothelin-1 did not affect the cell viability when compared with the control. Rotenone slightly decreased the viability of cardiomyocytes. However, the combination of rotenone and endothelin-1, which was added to the cells 12 h after rotenone, markedly decreased the cell viability. BQ123, an endothelin ET_A receptor antagonist, antagonized the effect of endothelin-1, whereas the same dose of the endothelin ET_B receptor antagonist BQ788 did not (Fig. 2). BQ123 and BQ788 themselves did not affect the cell viabilities (data not shown).

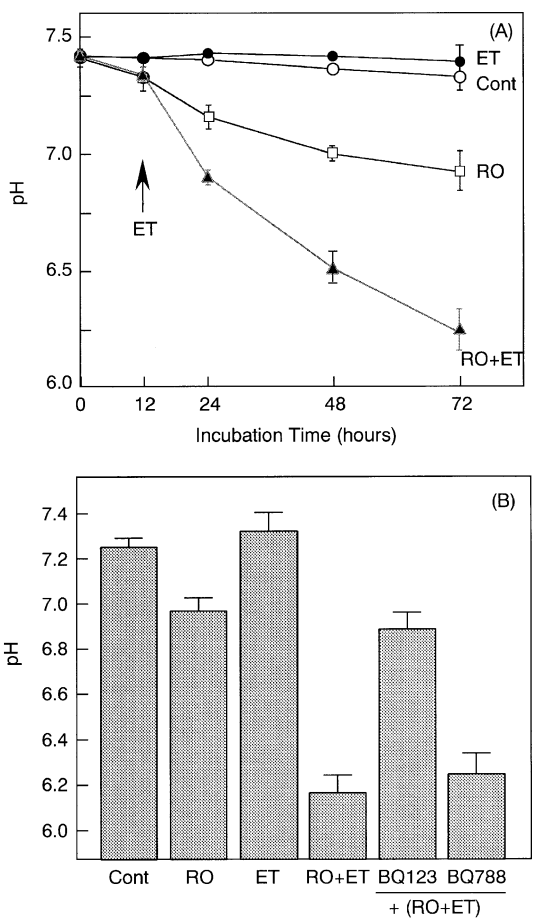

Fig. 3. (A) Effect of rotenone (1 μM, RO), endothelin-1 (10 nM, ET) and the combination of rotenone and endothelin-1 on the pH changes of the culture medium. Endothelin-1 was added to the cultures 12 h after rotenone. (B) Antagonistic effect of the endothelin type A receptor antagonist BQ123 (10 μM) and the type B receptor antagonist BQ788 (10 μM) on the decrease in pH induced by the treatment with the combination of rotenone and endothelin-1 at 72 h after rotenone. Each value is the mean and S.E.M. from nine samples of three different cultures.

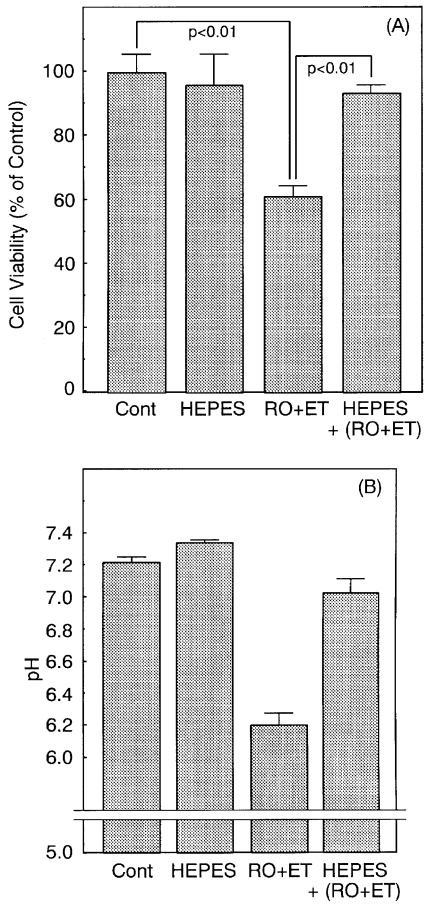

Fig. 4. Counteracting effect of HEPES (50 mM) on the cell viability (A) and on the decrease in pH (B) induced by the treatment of the cardiomyocytes with the combination of rotenone (1 μM, RO) and endothelin-1 (10 nM, ET) at 72 h after the rotenone. HEPES was added to the cultures at the same time with rotenone, and endothelin-1 was added to the cultures 12 h after the rotenone. Each value is the mean and S.E.M. from nine samples of three different cultures. Statistical analysis was performed on the values of the cell viability. $P < 0.01$ is statistically significant.

3.2. Changes in pH of culture medium by rotenone and endothelin-1

Fig. 3A shows a time course of pH changes of the culture media of myocytes treated with rotenone, endothelin-1 and the combination of rotenone and endothelin-1. The control culture medium of cardiomyocytes slightly and gradually decreased the pH with incubation time. Endothelin-1 (10 nM) alone did not decrease the pH of the medium. In contrast, the culture medium of myocytes treated with rotenone (1 μM) gradually decreased the pH of the culture medium with incubation time. Endothelin-1 markedly enhanced the decrease in pH of the culture medium when endothelin-1 was added 12 h after the addition of rotenone. Fig. 3B shows the pH values at 72 h after the addition of rotenone. BQ123 counteracted the

enhancement of the decrease in pH caused by endothelin-1 observed in the combination with rotenone, whereas BQ788 did not.

3.3. Effect of HEPES buffer to the pH change and cell injury

The addition of 50 mM of HEPES (pH 7.4) into the medium counteracted the decrease in pH induced by the combination of rotenone and endothelin-1 (Fig. 4B). Meantime, HEPES inhibited the cardiomyocyte injury caused by the combination of rotenone and endothelin-1. HEPES itself did not affect the cell viability (Fig. 4A).

3.4. 2-Deoxyglucose uptake and lactic acid production

To determine the glucose uptake activity to the cardiomyocytes, the uptake ratio of radiolabeled 2-deoxyglucose to the cells was measured. Pretreatment with rotenone increased about 2.3-fold the uptake of 2-deoxyglucose when compared with the control cultures. Endothelin-1 also increased uptake of 2-deoxyglucose about twofold; however, endothelin-1 markedly increased the uptake of 2-deoxyglucose when the cardiomyocytes were pretreated with rotenone. This enhancement of the uptake of 2-deoxyglucose by endothelin-1 was antagonized by BQ123 but not by BQ788 (Fig. 5).

Lactic acid was markedly produced by the cells treated with rotenone (1 μM). Endothelin-1 (10 nM) further enhanced the production of lactic acid whereas endothelin-1 alone did not affect the production of lactic acid (Fig. 6). BQ123 antagonized the effect of endothelin-1. However, BQ788 did not affect the production of lactic acid induced by the combination of rotenone and endothelin-1 (Fig. 6). BQ123 or BQ788 themselves did not affect the production of lactic acid (data not shown).

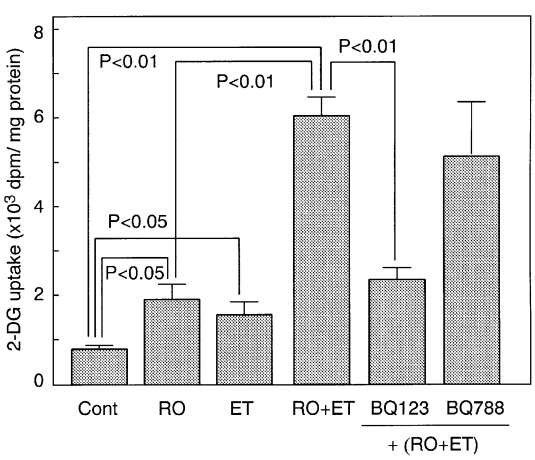

Fig. 5. Effect on the 2-deoxyglucose (2-DG) uptake to the cardiomyocytes of rotenone (1 μM, RO), endothelin-1 (10 nM, ET), and the combination of rotenone and endothelin-1, and the counteracting effect of the endothelin ET_A receptor antagonist BQ123 on the endothelin-1 induced enhancement of the 2-deoxyglucose uptake. BQ123 (10 μM) and BQ788 (10 μM) were added 1 h before endothelin-1. Each value is the mean and S.E.M. from nine samples of three different cultures. $P < 0.05$ and $P < 0.01$ are statistically significant.

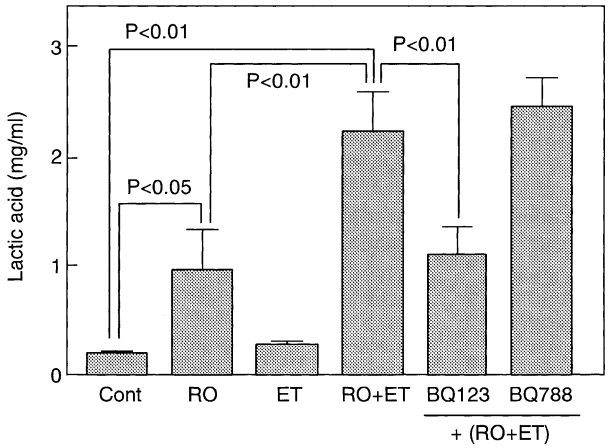

Fig. 6. Effect on the lactic acid production by the cardiomyocytes of rotenone (1 μM, RO), endothelin-1 (10 nM, ET), and the combination of rotenone and endothelin-1, and the counteracting effect of the endothelin ET_A receptor antagonist BQ123 on the endothelin-1 induced enhancement of the lactic acid production. BQ123 (10 μM) and BQ788 (10 μM) were added 1 h before endothelin-1. Each value is the mean and S.E.M. from nine samples of three different cultures. $P < 0.05$ and $P < 0.01$ are statistically significant.

4. Discussion

Endothelin-1 shows many physiological effects on cardiomyocytes through endothelin receptors such as hypertrophy, gene expression, activation of calcium mobility, etc. (Shubeita et al., 1990). Endothelin-1 stimulates intracellular kinases such as protein kinases, MAP kinases or Akt as well as many humoral factors (Douglas and Ohlstein, 1997). Those humoral factors activated in heart failure may possibly play positive and negative roles in the regulation of myocardial cell apoptosis. In many cases of heart failure, mitochondrial functions in myocytes are reduced. We therefore tried to construct a mitochondrial impairment model of cardiomyocytes in vitro and evaluate the effect of endothelin-1.

In this study, we used 1 μM of rotenone, a mitochondrial complex I inhibitor, to reduce the mitochondrial activity. The dose we used (1 μM) partially but not completely inhibited the mitochondrial activity assessed by the measurement of the membrane potential of mitochondria (Yuki et al., 2001, in press). Rotenone induced cardiomyocyte injury as assessed by MTT bioreduction activity. Rotenone also decreased the pH of the culture medium of cardiomyocytes. Endothelin-1 itself did not show any cytotoxic effect, or decrease in pH of the culture medium. However, after pretreatment with rotenone, endothelin-1 markedly enhanced the cardiomyocyte injury and acceler-

ated the decrease in pH of the culture medium. Since the enhancement of cellular injury and pH decrease by endothelin-1 in the presence of rotenone was abolished by the endothelin ET_A receptor antagonist BQ123 but not by the endothelin ET_B receptor antagonist BQ788, the enhancement of the decrease in pH and cell injury by endothelin-1 was acting via the endothelin ET_A receptor system.

To understand the relationship between the decrease in pH and the cellular injury, we aimed to inhibit the decrease in pH by the addition of HEPES buffer. As a result, HEPES counteracted the decrease in pH induced by the combination of rotenone and endothelin-1. HEPES also counteracted the cellular injury of cardiomyocytes caused by the rotenone and endothelin-1. These findings suggested that the decrease in pH may be involved, at least in part, in the cause of the cellular injury induced by the combination of rotenone and endothelin-1. Webster et al. (1999) demonstrated that severe chronic hypoxia alone did not cause apoptosis of cardiac myocytes in culture, and the apoptosis occurred when there was a decrease in extracellular pH under hypoxia. They also reported that the apoptosis did not occur when extracellular pH was neutralized or maintained by NaOH or HEPES buffer, and that the addition of acidic medium or exogenous lactic acid stimulated apoptosis in myocytes under the hypoxic condition. This report may support our results that a decrease in extracellular pH is important for the appearance of cytotoxicity. The cellular ATP level is important for cell survival, and endothelin-1 stimulates ATP consumption through the stimulation of various cellular phenomena such as induction of hypertrophy, gene expression, calcium ion handling, etc. Therefore, it is possible that the acceleration of decrease in pH by endothelin-1 under mitochondrial impairment affect the cellular ATP level.

Excess protons produced in the cells are expelled through the three main transporters: the Na^+/H^+ exchanger, Na^+/HCO_3^- co-transporters, and the vacuolar proton ATPase (Karwatowska-Prokopczuk et al., 1998; Lagadic-Gossmann et al., 1992; Lazdunski et al., 1985). Protons accumulated in cells are exchanged with Na^+, and this results in Ca^{2+} overload through the Na^+/Ca^{2+} exchanger (Piece and Czubryt, 1995). Therefore, cellular acidosis is able to cause Ca^{2+} overload followed by cellular injury. However, it was reported that a severe pH decrease itself inhibits the Na^+/Ca^{2+} exchanger activity (Haworth et al., 1987). Endothelin-1 stimulates the Na^+/H^+ exchanger (Wang and Morgan, 1992). However, stimulation of the Na^+/H^+ exchanger induced by endothelin-1 may not have been the cause of cellular injury in the present study because: (1) the pH decrease itself inhibits the Na^+/Ca^{2+} exchanger activity (Haworth et al., 1987); (2) if the extracellular protons are buffered, the efflux of protons from the cells increases. This results in the increase of Na^+ influx and Ca^{2+} overload followed by cell injury. In other words, if the Na^+/H^+ exchanger is stimulated under the condition of the stimulation of H^+ production, the neutralization of extracellular H^+ by a buffer causes the stimulation of H^+ efflux and Na^+ influx, and causes Ca^{2+} overload of the cells. This may accelerate the cell injury. However, in this study, maintaining the medium pH by HEPES showed a cytoprotective action. Therefore, the stimulation of the Na^+/H^+ exchanger by endothelin-1 may not be the main mechanism of the myocyte injury under the mitochondrial impairment.

As demonstrated in the present study, endothelin-1 accelerated the glucose uptake into the myocytes during the treatment with rotenone. Furthermore, lactic acid production was also enhanced by endothelin-1. Therefore, these findings suggest that the acceleration of glucose uptake by endothelin-1 and anaerobic glycolysis followed by the accumulation of lactic acid in cardiomyocytes under the condition of mitochondrial impairment may be the cause of the stimulated decrease in pH and the cellular injury. Recently, it was reported that endothelin-1 stimulates glucose uptake by translocation of the glucose transporter (Glut) 4 in 3T3-L1 adipocytes and cardiomyocytes via endothelin ET_A receptors (Wu-Wong et al., 1999, 2001). Moreover, it is reported that Akt, a serine–threonine kinase, activation also enhanced sarcolemmal expression of Glut 4 in vivo and increased glucose uptake in vitro (Matsui et al., 2001). Akt exists downstream of PI-3 kinase, and PI-3 kinase is one of the stimulatory pathways of endothelin-1. Therefore, endothelin-1 may stimulate glucose uptake to the myocytes via Akt stimulation. In the present study, the enhancement of 2-deoxyglucose uptake was counteracted by the endothelin ET_A receptor antagonist BQ123. Therefore, it is possible that the stimulation of translocation of the glucose transporters to the cellular membranes by endothelin-1 is a mechanism of the stimulation of glucose uptake into cardiomyocytes. It is reported that rotenone itself also enhances the uptake of glucose into cardiomyocytes via the upregulation of recruitment of Glut 4 to the cellular membranes (Wheeler et al., 1994). Therefore, there may be an additive mechanism that endothelin-1 greatly accelerates glucose uptake under mitochondrial impairment by rotenone in cardiomyocytes.

The predominant energy metabolism shifts from fatty acid beta-oxidation to glycolysis in failing hearts. Increasing glucose use improves survival of the ischemic myocardium via several potential mechanisms, including increasing the rate of anaerobic glycolysis, and reducing the toxic effects of fatty acid accumulation (Apstein, 2000). Therefore, it is possible that the stimulated glucose uptake into cardiomyocytes is needed for the myocyte survival at the onset of myocardial ischemia. Akt activation is reported to exert a powerful cardioprotective effect after transient ischemia (Matsui et al., 2001). Moreover, Plas et al. (2001) recently reported that Akt-mediated survival is dependent on promoting glycolysis and maintaining a physiologic mitochondrial potential. These reports suggest that the stimulating glycolysis is important for the cell

survival against ischemic stimuli to the cardiomyocytes. Since, endothelin-1 production is reported to be stimulated in the ischemic myocardium, endothelin-1 produced in the myocardium may act as an emergency peptide through the stimulation of glucose uptake. However, if mitochondrial impairment occurs, severe acidosis may occur in the ischemic area of myocardium by the stimulation of glucose uptake, resulting in a detrimental effect on the myocardium as shown in this study. This suggests that the effect of endothelin-1 on the myocardium is reversed by the intactness of the mitochondria. In other words, mitochondrial function may be the key that controls the beneficial or deteriorative effect of endothelin-1 on cardiomyocytes.

There are several studies that show the effectiveness of the blockade of endothelin receptors in improving survival (Iwanaga et al., 1998; Mulder et al., 1997; Sakai et al., 1996a; Yamauchi-Kohno et al., 1999) and hemodynamic features (Borgeson et al., 1988; Nguyen et al., 1998; Sakai et al., 1996a; Wada et al., 1997, 1999; Yamauchi-Kohno et al., 1999) in heart failure animal models. One of the mechanisms of the beneficial effect of endothelin-1 blockade on heart-failing animals was suggested to be the reduction of the afterload of the heart. However, a direct pathophysiological role of endothelin-1 in the myocardium has not been reported. From the present study, we propose a possible mechanism for the beneficial effect of endothelin-1 blockade that antagonists of endothelin-1 counteract the stimulation of glucose uptake followed by the accumulation of lactic acid under the condition of mitochondrial impairment.

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