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Protective Effect of Coenzyme Q_{10} against Hypoxic Cellular Damage

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Heart cells from mouse embryos were cultured in either 5% CO_2 and 95% air or 5% CO_2 and 95 % N_2. Single myocardial cells showed frequent irregular beating under both conditions. Using this experimental system, we studied the effect of coenzyme Q_{10} (CoQ_{10}) on the cellular functions of myocardial cells. Under the hypoxic conditions, CoQ_{10} decreased the irregular beating frequency without changing the beating rate. CoQ_{10} increased the cardiac adenosine 3′,5′-cyclic monophosphate (cAMP) level under the normal conditions and tended to decrease cardiac cAMP level under the hypoxic conditions. After incubation of deuterium-labeled CoQ_{10} (CoQ_{10}-d_5), the presence and levels of endogenous CoQ_9, CoQ_{10} and exogenous CoQ_{10} were determined. Endogenous CoQ_9 and CoQ_{10} were found in the mitochondrial fraction. Exogenous CoQ_{10} was found in the plasma membrane, cytosol and mitochondrial fractions. These findings confirm the protective effect of CoQ_{10} in ischemic heart disease. The exogeneous CoQ_{10} may provide protection against ischemic cellular damage by modulating cell functions.

Keywords——deuterium labeled coenzyme Q_{10}; endogenous and exogenous coenzyme Q; cultured single myocardial cell; beating rate; irregular beating frequency; ischemic model; cyclic AMP

Introduction

Coenzyme Q_{10} (CoQ_{10}), which has been isolated from the heart mitochondrial fractions,[1] functions as an electron carrier in the electron transport chain of mitochondria. CoQ_{10} is known to have a protective action on the heart under conditions of myocardial metabolic disturbance[2] as well as a beneficial effect in heart failure.[3] Studies on CoQ_{10} and its effect on the heart have traditionally been done *in vivo*[4,5] or on isolated perfused hearts.[6] In recent years, progress has been made in heart cell culture methods, stimulating studies on the structure and function of the myocardium at the cellular level.[7–10] Electrophysiological studies on myocardial beating have advanced particularly well, enabling the action current of cells to be monitored without resorting to nervous or hormonal stimulation.[11,12] Recent studies by Goshima[8] and by Yoneda and Toyama[13] were designed to clarify the mechanisms involved in the development of arrhythmia in cultured myocardial cells. These studies were based on such findings as the appearance of irregular beating due to a change in the inorganic ion concentration within the culture medium, and the subsequent ionic change in the microstructure of the myocardial cells.

Recently, an ischemic myocardial injury model has been developed and several workers have reported that CoQ_{10} has a protective effect against ischemic myocardial damage.[5,6] In these studies, the myocardial oxygen supply was descreased and cellular respiration was inhibited. The cellular metabolism shifted from aerobic to anaerobic and the adenosine triphosphate (ATP) content decreased. Because the cellular damage in these cases appeared to

occur within the plasma membrane in the initial stage, we studied the protective effects of CoQ_{10} during this period. After incubation in an atmosphere of 5% CO_2 and 95% air the myocardial cells were exposed to an atmosphere of 5% CO_2 and 95% N_2 gas. The resulting hypoxic state served as an ischemic myocardial model in which we were able to determine the protective effects of CoQ_{10} in the initial stage of ischemic cellular damage. To determine the action of CoQ_{10} on the plasma membrane, adenosine 3′,5′-cyclic monophosphate (cAMP) levels of the cell were measured and compared with the regular beating or irregular beating rate of myocardial cells under control and hypoxic conditions. Furthermore, the intracellular distribution of endogenous and exogenous CoQ in the cultured myocardial cells was determined.

Materials and Methods

Preparation of Single Myocardial Cells——Heart cell cultures were prepared by the methods previously reported,[9] which were modified from the methods of Harary and Farley.[14] Sixteen hearts were dissected from 18-d-old mouse embryos (ICR strain). The atria and vessels were removed, and the ventricles were minced into approximately 0.5 mm cubes and placed into a Ca^{2+}- and Mg^{2+}-free[15] saline A solution containing 137 mM NaCl, 5.4 mM KCl, 5.6 mM glucose and 4.2 mM $NaHCO_3$. The cubes were trypsinized for 10 min at 37 °C with 10 ml of saline A containing 0.03% (w/v) trypsin (1 : 250, DIFCO Laboratories, Inc., U.S.A.) and 0.03% (w/v) collagenase (CLS II, Worthington Biochemical Corp., U.S.A.) in a low speed magnetic stirrer. This treatment was repeated 3 to 4 times. The supernatants from the second to the fourth trypsin incubation were combined and centrifuged at $200 \times g$ for 10 min. The precipitated myocardial cells were resuspended in Ham's F-12 (Nissui Seiyaku Co., Ltd., Japan) containing 10% fetal bovine serum (Grand Island Biological Company, U.S.A.). The culture medium was then filtered with lens paper to remove large clumps of cells. To confirm the integrity of the cells after treatment with trypsin and collagenase in the calcium-free saline solution, the ultrastructural morphology and beating functions of random samples were observed. There was no evidence of either morphological or physiological damage.

For the pulsation experiments, the filtered cell suspension was adjusted to $3—5 \times 10^3$ single cells/ml by means of a Coulter counter. For the other experiments, the cell number was diluted to $3—5 \times 10^5$ cells/ml. A 2 ml aliquot of the myocardial cell suspension was pipetted into Falcon dishes (35 mm in diameter; Becton, Dickinson Overseas Inc., U.S.A.) fitted with a 1-mm grid glass plate. The myocardial cell suspension was then placed in a CO_2 incubator (model 7341, Heiniche/National Company, U.S.A.) at 37 °C for 48 h in an atmosphere of 5% CO_2 and 95% air.

To produce the ischemic injury model, the myocardial cells after incubation under the above conditions, were placed in a CO_2 incubator in an atmosphere of 5% CO_2 and 95% N_2 gas for 60 min.

Beating Rate and Irregular Beating Frequency——The myocardial cell suspension was cultured on 1 mm grid glass plates placed in dishes to facilitate the identification of single myocardial cells, and CoQ_{10} solution[6] containing 4.0 mg of CoQ_{10}, 1.8 mg of phospholipids, 30.0 mg of polyglycols and other solvents and 45.0 mg of D-sorbitol in 1.0 ml, pH 6.5 (Eisai Co., Ltd., Japan), or the drug solution containing no CoQ_{10} (referred to as the vehicle) was added to determine the effects of each on the beating of myocardial cells. The beating of single myocardial cells was monitored for 5 min, then CoQ_{10} was added, and the monitoring was continued for 60 additional min on the same cell. Monitoring was carried out with a television set (Hitachi Denshi, Ltd., Japan) connected to an inverted microscope (MD, Nippon Kogaku K.K., Japan) in order to observe any change in beating rate or irregular beating. Changes in light transmission arising from the contraction and relaxation of myocardial cells were recorded by a phototransistor (Nippon Kohden Kogyo Co., Ltd., Japan). The contraction interval was compared to the mean contraction interval for a period of two or three min. We defined irregular beating as any interval with a change of more than 20% (elongation or reduction) in the contraction interval. Irregular beats were expressed as numbers per minute or percentages of the control.

Determination of Enzyme Activity and Other Substances——Thymidine 5′-monophosphate *p*-nitrophenyl ester was used as a substrate to measure phosphodiesterase I. The reaction was performed in 0.5 M glycine buffer (pH 9.6) at 37 °C. The isolated *p*-nitrophenol was determined by measuring the absorbance at 400 nm.[16] Cytochrome c reductase was measured according to the method of Azzi *et al.*[17] The amount of K^+ in the medium was determined by the ion electrode method. cAMP concentrations were determined by radioimmunoassay (cAMP assay kit; Yamasa

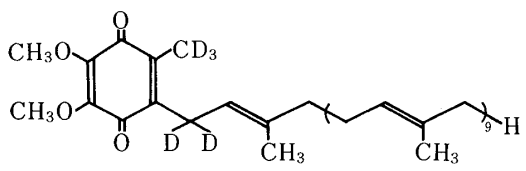

Fig. 1. Structure and Labeled Sites of Coenzyme Q_{10}-d_5

Co., Ltd., Japan). The amount of protein was determined using the method of Lowry *et al.* with bovine serum albumin as a standard.[18] The cell homogenate was boiled for 2 min and then cooled in iced water for 10 min. To determine ATP, the chilled homogenate was centrifuged at 3000 rpm for 10 min. The ATP content of the supernatant was measured with a CHEM-GLOW photometer (Amicon, U.S.A.) using luciferin as a substrate.[19] The pO_2 was measured with an automatic blood gas analyzer (Corning 175).

Intracellular Distribution of Endogenous and Exogenous CoQ in Cultured Myocardial Cells——To determine simultaneously the amounts of endogenous and exogenous CoQ, the added CoQ_{10} was labeled with deuterium (CoQ_{10}-d_5).[20] Figure 1 shows the structure of CoQ_{10}-d_5. After incubation with 200 μg/ml of CoQ_{10}-d_5, the myocardial cells were washed 3 times in a saline solution to remove any unincorporated CoQ_{10}-d_5. Washed cells were then scraped from the plate and placed in 0.5 ml of 0.25 M sucrose solution. The suspension was homogenized gently at 800—1000 rpm with a Teflon homogenizer for 3 strokes. The homogenate was layered onto 4.5 ml of sucrose solution (linear density gradient 0.25—2.1 M). Continuous density gradient centrifugation was performed at 50000 rpm for 180 min using a Hitachi 55P-2 automatic preparative ultracentrifuge (RP-50 rotor). One-half ml fractions were collected for analysis and the lipids in each fraction were extracted. Endogenous CoQ_9, CoQ_{10}, and exogenous CoQ_{10} were determined simultaneously by the method described in a previous report[20] with only minor modifications. The extracts were then hydrogenated in a hydrogen atmosphere using a platinum catalyst and subjected to direct-inlet integrated mass fragmentography (DI-IMF).

Results

Pulsation of Single Myocardial Cells

After 6 to 8 h in culture, the myocardial cells adhered to the bottom of the dish and began to pulsate. Forty-eight hours after the start of incubation the pulsation characteristics of single myocardial cells were observed under a phase contrast microscope. After the addition of the vehicle, the mean beating rate decreased to $83 \pm 3\%$ for 5 min and then increased to $102 \pm 7\%$ for 60 min (mean $\pm$ SE) relative to the original level, whereas it increased to 131 ± 18, 129 ± 13, 144 ± 22, and $161 \pm 7\%$ after the addition of CoQ_{10} for 5 min at concentrations of 50, 100, 200 and 400 μg/ml, respectively. The difference in the beating rate between the vehicle- and CoQ_{10}-treated myocardial cells was significant ($p < 0.01$). Furthermore, the vehicle did

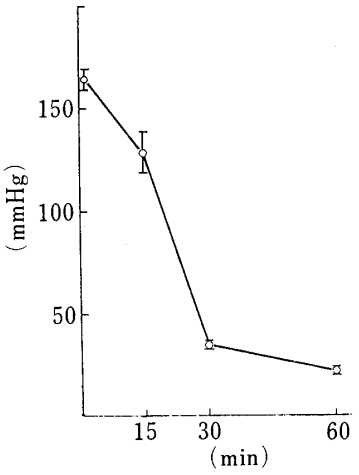

Fig. 2. pO_2 Levels in the Culture Medium During Hypoxia

The values represent the mean $\pm$ S.E. of 8 experiments.

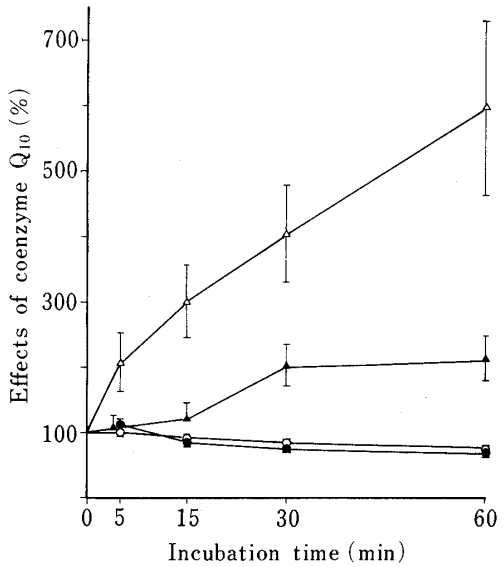

Fig. 3. Effects of Coenzyme Q_{10} on Beating Rate and Irregular Beating Frequency of Single Myocardial Cells under Hypoxic Conditions

—○—, beating rate during hypoxia; —●—, beating rate after the addition of CoQ_{10} (200 μg/ml) during hypoxia; —△—, irregular beating frequency during hypoxia; —▲—, irregular beating frequency after the addition of CoQ_{10} during hypoxia. The values represent the mean $\pm$ S.E. of 12 experiments.

not cause significant changes in the irregular beating frequency, and the presence of vehicle did not affect the beating rate or irregular beating of the myocardial cells. However, after the addition of CoQ_{10} for 5 min, the irregular beating frequency decreased to 79 ± 7, 59 ± 9 and $67\pm10\%$ of the original frequency at concentration of 100, 200 and 400 μg/ml, respectively. At these concentrations, the decrease in irregular beating frequency was statistically significant ($p<0.02$).

Functional Cellular Change Caused by Hypoxia and Protective Effects of CoQ_{10}

Figure 2 shows the pO_2 levels in the culture medium under these conditions. The pO_2 in the incubation medium was below 50 mmHg for 30 min. The level of ATP after incubation under hypoxic conditions was 36.7 ± 4.8 nmol/mg of protein. The level of ATP after the addition of 200 μg/ml of CoQ_{10} was 31.4 ± 7.8 nmol/mg of protein and that after addition of the vehicle was 35.7 ± 7.2 nmol/mg of protein.

Before hypoxic treatment, the beating rate of myocardial cells was 62.8 ± 22.6 beats/min and irregular beating was 2.3 ± 1.3 irregular beats/min. After the induction of hypoxic conditions, the beating rate in the myocardial cells decreased to 46.6 ± 22.1 beats/min, and the irregular beating frequency increased to 5.4 ± 2.9 irregular beats/min after 30 min and to 7.0 ± 4.1 irregular beats/min after 60 min (Fig. 3). After the addition of CoQ_{10} to the medium the beating rate and irregular beating frequency were measured for 60 min. The beating rate did not change as compared with the control value, but the rate of irregular beating dramaticaly decreased (Fig. 3).

The K^+ concentration in the culture medium was measured under these conditions. During hypoxia for 60 min the apparent release of K^+ increased from 2.88 ± 0.04 mM to 3.44 ± 0.05 mM. Upon the addition of 200 μg/ml of CoQ_{10} during hypoxic treatment for 60 min, the K^+ concentration decreased to 3.03 ± 0.05 mM. The concentration of K^+ in the medium of the myocardial cells after the addition of the vehicle remained unchanged. The apparent release of K^+ during hypoxia was statistically significant relative to the control ($p<0.01$) or the addition of CoQ_{10} ($p<0.01$).

cAMP concentrations in the myocardial cells were measured both before and after hypoxia, and the effect of CoQ_{10} on cardiac cAMP level was also measured (Fig. 4). The cardiac cAMP level was decreased in cells under hypoxic conditions during a 60 min period of

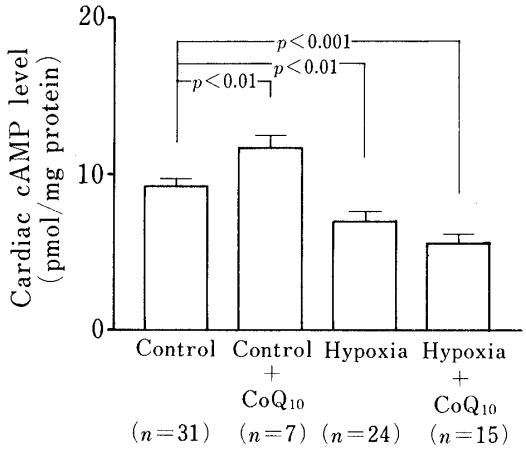

Fig. 4. Effects of CoQ_{10} on Cardiac cAMP Level under Normal and Hypoxic Conditions for 60 min

Cardiac cAMP content was measured by a radioimmunoassay method. The values represent the mean $\pm$S.E. and the number of experiments is indicated in parentheses.

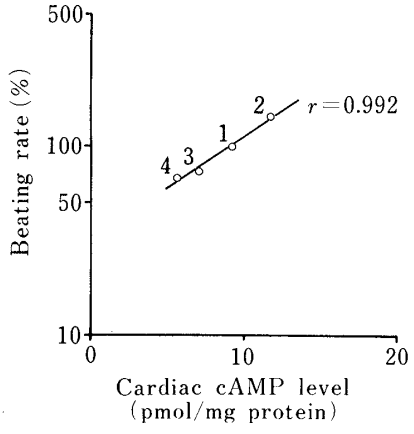

Fig. 5. Relationship of Cardiac cAMP Level and Beating Rate of Cultured Single Myocardial Cells for 60 min

The numbers indicate the conditions of the experiments. 1, normal conditions; 2, addition of CoQ_{10} under the normal conditions; 3, hypoxic conditions; 4, addition of CoQ_{10} under the hypoxic conditions.

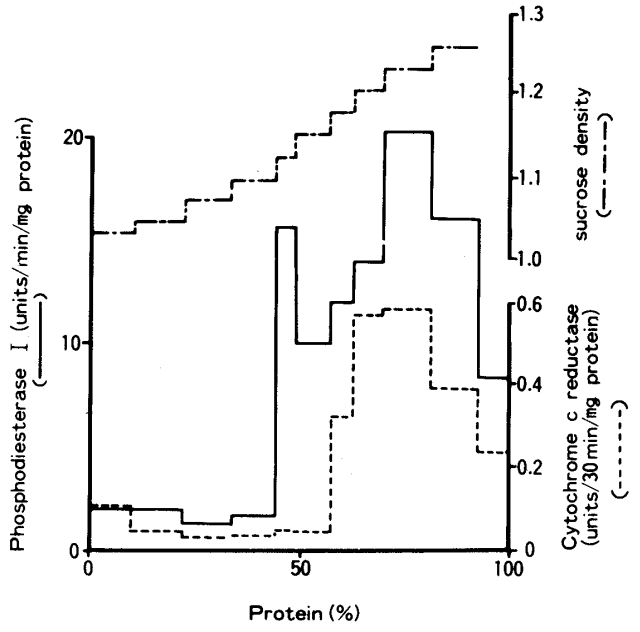

Fig. 6. Concentrations of Phosphodiesterase I and Cytochrome c Reductase in Subfractions Obtained from Cultured Myocardial Cells

Conditions are described in the text. The abscissa indicates percentage of total protein.

observation ($p<0.01$). It is interesting that during the observation period CoQ_{10} increased cardiac cAMP level under normal conditions but tended to decrease it in the presence of CoQ_{10} during the same time period in cells under hypoxic conditions. Figure 5 shows the relationship between the beating rate of cultured single myocardial cells and cardiac cAMP level. There is a good correlation, and the coefficient of correlation is 0.992.

Intracellular Distribution of Endogenous and Exogenous CoQ in Cultured Myocardial Cells

After fractionation of the cultured myocardial cells, both the quantity of protein and the specific activities of phosphodiesterase I and cytochrome c reductase were determined; the results are shown in Fig. 6. Phosphodiesterase I activity was found in 2 fractions with specific gravities of 1.13 and 1.23. Phosphodiesterase I was used as a marker enzyme for plasma membranes. The presence of this enzyme in lysosomes has also been reported.[21] The fraction with the specific gravity of 1.13 corresponds to the cell membranes and that with the specific gravity of 1.23 corresponds to the lysosomes. The finding of phosphodiesterase I activity in two fractions indicates that the cell membranes and lysosomes were separated. Cytochrome c reductase activity was found in fractions with specific gravities from 1.21 to 1.23. Cytochrome c reductase was measured as a marker enzyme for the mitochondria. Mitochondria of cultured myocardial cells were present in fractions with specific gravities from 1.21 to 1.23. Thus, the specific gravity of the lysosomal fractions did not differ from that of the mitochondrial fractions, and the fractions with specific gravities from 1.21 to 1.23 contained both mitochondria and lysosomes. Further separation of these organelles was difficult under our experimental conditions. The fractions with specific gravities from 1.21 to 1.23 were treated as mitochondrial fractions.

The presence of endogeneous CoQ_9, CoQ_{10}, and exogenous CoQ_{10} in various fractions was determined by DI-IMF (Fig. 7). Endogenous CoQ_9 and CoQ_{10} were mainly found in fractions containing mitochondria and lysosomes under both conditions. CoQ_{10}-d_5 was incorporated in amounts ranging from 0.86 to 1.2 μg/mg protein in 60 min under normal incubation, but the incorporated amount of CoQ_{10}-d_5 decreased to 0.56 μg/mg protein after 60 min of hypoxia. The decrease was apparently due to a decrease in cell function during hypoxia. However, these results indicate that CoQ_{10} was incorporated into the myocardial cells during hypoxia in an amount sufficient to be effective in reducing hypoxic damage.

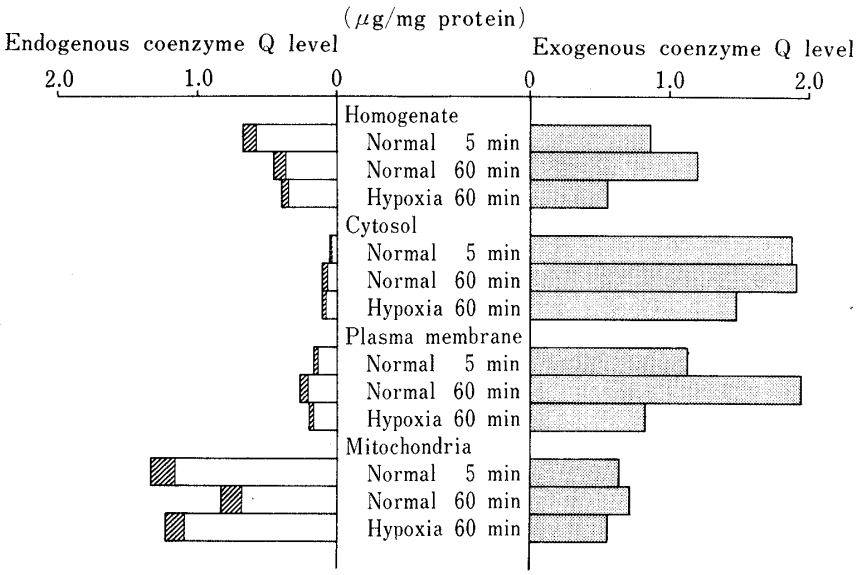

Fig. 7. Profile of Endogenous CoQ_9, CoQ_{10} and Exogenous CoQ_{10} Concentrations in Subfractions of Cultured Myocardial Cells after Incubation with CoQ_{10}-d_5 (200 μg/ml)

□, endogenous CoQ_9; ▨, endogenous CoQ_{10}; ▩, exogenous CoQ_{10}-d_5. The values represent the means of 3 experiments.

Intracellular distribution profiles of the CoQ did not change under either condition. CoQ_{10}-d_5 was found in the mitochondria, plasma membranes, and cytosol fraction under all conditions, but mainly in the latter two fractions.

Discussion

Cultured myocardial cells are a very useful experimental model because of the simplicity of the cell system.[22,23] The myocardial cells under normal culture conditions pulsate at a regular rate and irregular beating is frequent. In the present investigation, the irregular beating frequency of single myocardial cells under hypoxic conditions increased to about 6 times the control level. The K^+ concentration in the culture medium was also increased, though the ATP level of the myocardial cells was not changed. These results indicated that under these hypoxic conditions the cultured myocardial cells suffered cellular damage before the occurrence of mitochondrial dysfunction. This cellular damage may be the first step of ischemic cellular damage. The cellular damage decreased after the addition of CoQ_{10} to the culture medium after only a few minutes of exposure to the compound. It was found that CoQ_{10} limited the release of K^+ from the myocardial cells and decreased hypoxic cellular damage.

CoQ_{10}, in normal cultures of single myocardial cells, increased the beating rate and decreased the irregular beating frequency. Under the hypoxic conditions, CoQ_{10} did not change the beating rate but dramatically decreased the irregular beating frequency. Thus, CoQ_{10} appears to change the regular beating rate and decrease the irregular beating frequency in a manner that is dependent on the pO_2 level in the medium. During the early stage of cellular damage, the ATP level did not change, whereas CoQ_{10} showed its effect immediately after addition to the medium through an alteration in the function to the myocardial cells.

There are many reports that cardiac cAMP level and the chronotropic response are correlated.[24-26] In this study, we found that the cardiac cAMP level was well correlated to the beating rate, and exogeneous CoQ_{10}-d_5 changed the cardiac cAMP level depending on the

level of pO_2 in the culture medium. Although the mechanisms of action of exogenous CoQ_{10} are not clear, these results appear to show that exogenous CoQ_{10} protected the myocardial cells against hypoxic damge and controlled the cardiac cAMP level.

Deuterium labeled CoQ_{10} was used to determine the amounts of endogenous and exogenous CoQ at the same times and with the same samples. The distributions of endogenous CoQ_9, CoQ_{10}, and exogenous CoQ_{10}-d_5 were determined in the subcellular fractions of myocardial cells. After incubation with CoQ_{10}-d_5, the myocardial cells were washed with saline solution. The used CoQ_{10}-d_5 solution formed micelles, and we assumed that the CoQ_{10}-d_5 in the medium was washed out by the saline solution. Endogenous CoQ_9 and CoQ_{10} were found mainly in the fractions containing mitochondria and lysosomes under all conditions, whereas exogenous CoQ_{10}-d_5 was found in the mitochondrial, plasma membrane and cytosol fractions (especially in the plasma membrane and cytosol fraction). Moreover, exogenous CoQ_{10}-d_5 did not affect the distribution or content of endogenous CoQ. However, it is interesting that exogenous CoQ_{10} was largely incorporated into the plasma membrane and cytosol at the time of appearance of the effects of CoQ_{10}. It is not clear whether exogenous CoQ_{10}-d_5 in these fractions acted directly on cellular functions or whether the presence of CoQ_{10}-d_5 indicates the occurrence of transport of CoQ_{10}-d_5 from the medium to the mitochondria.

Recently, Higgins *et al.* suggested that cellular energy states may determine the resistance of the cell membrane to attack by phospholipase.[27] Furthermore, Katz and Messineo reported that the lipid-membrane interactions and membrane functions were modified in ischemic damage.[28] Two possibilities may be considered for the mechanism of the protective effects of CoQ. The first is that CoQ is incorporated into the mitochondria[9,29] and maintains the mitochondrial function in the ischemic state by altering the respiratory chain.[30,31] The second is that CoQ may act directly on the cell membrane and protect it against the action of hydrolytic enzymes such as phospholipase.[32] Our results suggest that, in protecting against cellular damage, CoQ_{10} not only works by improving ATP synthesis in the electron transport chain[33] but also acts upon the plasma membrane.

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