[Chem. Pharm. Bull. 36(4)1514—1518(1988)]

# Influence of Coenzyme $Q_{10}$ on Doxorubicin Uptake and Metabolism by Mouse Myocardial Cells in Culture

KYOKO TAKAHASHI,*[,1)] TADANORI MAYUMI, and TAKEO KISHI

*Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Nishi-ku, Kobe 673, Japan*

(Received August 27, 1987)

In order to test the hypothesis that coenzyme $Q_{10}$ ($CoQ_{10}$) might prevent myocardial cell uptake of doxorubicin (DX), $^3$H-DX uptake in cultured myocardial cells was studied. When cultured myocardial cells were incubated with 3.5 $\mu$M DX for 24—72 h or 140 $\mu$M DX for 1—4 h, 120 $\mu$M $CoQ_{10}$ added in advance protected their beating from DX toxicity. However, $CoQ_{10}$ had no effect on $^3$H-DX uptake by the cells under the above conditions (3.5 $\mu$M DX for 24—72 h or 140 $\mu$M DX for 1—4 h). Most of the radioactivity in the cells was recovered as DX itself in both the medium with and without $CoQ_{10}$. Furthermore, the effects of a CoQ homolog (coenzyme $Q_9$ ($CoQ_9$)) or an analog (plastoquinone ($PQ_9$)) on the beat inhibition by DX were compared using this system. It was shown that $CoQ_9$ prevents, though only partially, the inhibition of the myocardial cell beating by 3.5 $\mu$M DX. $PQ_9$ was without effect. It is suggested that the protective effect of $CoQ_{10}$ against DX cardiotoxicity was not caused by inhibition of DX uptake or by alteration of DX metabolism in the cells.

**Keywords**——doxorubicin; coenzyme $Q_{10}$; coenzyme $Q_9$; plastoquinone; beating; cellular uptake; metabolism; cardiac cell culture; cardiotoxicity

## Introduction

Doxorubicin (DX), an anthracycline antibiotic, is one of the most effective chemotherapeutic agents for solid tumors.[2)] However, DX is known to produce cardiomyopathy which limits its prolonged use.[3)]

Cultured myocardial cells are separated anatomically and functionally from nerves, connective tissue and blood vessels. They can therefore be used to study the direct effects of ions and other cardioactive agents on myocardial cells.[4)] We suggested previously that the beating state of myocardial cells is a sensitive and reliable parameter in this culture system for studies on the cardiotoxicity of DX.[5)]

There are many reports which discuss the prophylactic effect of coenzyme $Q_{10}$ ($CoQ_{10}$) on DX cardiotoxicity.[6,7)] We also observed that $CoQ_{10}$ protected cultured mouse myocardial cells against the inhibition of beating by DX.[8)] Since DX may induce cardiotoxicity by the inhibition of $CoQ_{10}$-dependent enzymes,[9)] and since $CoQ_{10}$ is a redox carrier in the cellular respiratory chain,[10)] this redox role may be important in the mechanism of protection against DX cardiotoxicity by $CoQ_{10}$ treatment.[6,7)] However, the mechanism has not been clarified. On the other hand, the anti-tumor action of DX is not affected by $CoQ_{10}$,[11)] so $CoQ_{10}$ might not affect DX uptake by the cells.

Hence, we must examine whether $CoQ_{10}$ works by affecting both uptake and metabolism of DX by the cells before we can discuss the bioenergetic role of $CoQ_{10}$ in relation to DX toxicity. Thus, the purpose of this study was to determine the influence of $CoQ_{10}$ on DX uptake and metabolism by mouse myocardial cells. Furthermore, we investigated whether CoQ analogs would prevent the inhibition by DX.

## Experimental

**Materials**——$^3$H(G)-Labelled DX (198.2 $\mu$ Ci/mg) and unlabelled DX were kindly donated by Kyowa Hakko Co., Chiyoda-ku, Tokyo, Japan. $CoQ_{10}$, coenzyme $Q_9$ ($CoQ_9$), plastoquinone ($PQ_9$) and their solvent (HCO-60) were supplied by courtesy of Eizai Co., Bunkyo-ku, Tokyo, Japan. The other agents were of analytical reagent grade.

**Mouse Embryo Cardiac Cell Culture**——The method of myocardial cell culture was described elsewhere.[5] In brief, hearts from 14- to 16-day-old mouse embryos (ICR strain) were minced and digested with 5 ml of 0.125% trypsin–0.025% collagenase solution at 37 °C for 15—20 min. The dispersed cardiac cells were filtered off then collected by centrifugation at $200 \times g$ for 10 min. Cardiac cells ($2—4 \times 10^5$ per dish) were seeded into Petri dishes (35 mm i.d.) each containing 1 ml of Ham's F-12 medium supplemented with 10% newborn calf serum. The cells were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$-95% air. Two different cell types, myocardial cells and fibroblast-like cells, could be easily distinguished under a phase-contrast microscope. In our experiments, the ratio of myocardial cells to fibroblast-like cells was 4 : 1. $CoQ_{10}$, $CoQ_9$, $PQ_9$ or its solvent (HCO-60) were mixed with culture medium, at the time of seeding the cardiac cells. The applications of DX were started after a 24 h preincubation, at which period 90% of cultured myocardial cells showed rhythmical spontaneous beating.

**Measurement of Beating**——The beating status of cultured myocardial cells was monitored with an inverted phase-contrast microscope at a magnification of 150 to 400 in a chamber controlled-at 37 °C. In each culture dish, about twenty cells or clusters, which were beating regularly at the mean beating rate of 106 beats/min, were selected for each experiment, and their beating rates, shapes and locations in the dish were recorded before application of the test agents. Beating of each cell or cluster was usually counted for 60 s at the time indicated. Each experiment was repeated at least three times by using separate cell cultures, and the results were averaged.

**Determination of Uptake and Metabolism of DX**——$^3$H(G)-DX instead of unlabelled DX was dissolved in culture medium (Ham's F-12 medium supplemented with 10% newborn calf serum) and added to the culture. At the time indicated, the cultured cells were rinsed 3 times with 0.5 ml of phosphate-buffered saline (PBS), and then dissolved in 0.5 ml of 0.1 M sodium hydroxide solution for 30 min. Two-thirds of the solution was transferred to a scintillation vial and mixed with 12 ml of ACS-II. The radioactivity was measured with a Packard Tri-Carb 460 liquid scintillation counter. The remaining part of the solution was used for the measurement of protein content by the method of Lowry *et al.*[12] For analysis of DX metabolities, labelled compounds were extracted with methanol from the cells disrupted by freezing, and divided into fractions of DX, doxorubicinone and the remaining eluate by high-performance liquid chromatography (HPLC) as described by Haneke *et al.*[13] The radioactivities were determined with a scintillation counter. The HPLC column was Finepak $C_{18-5}$ (5 $\mu$m particles, 250 × 4.6 mm i.d.) and the flow rate was 0.8 ml/min.

## Results

The effect of $CoQ_{10}$ on subsequent DX-induced beat inhibition is shown in Table I. The doses of 3.5 and 140 $\mu$M DX were tested, since they correspond to the doses causing chronic and acute toxicities, respectively.[5]

At the low dose of 3.5 $\mu$M DX, 120 $\mu$M $CoQ_{10}$ added in advance did not appreciably protect beating of the cells at 24 h, but did significantly protect it at 48 and 72 h. $CoQ_{10}$ increased the beating rate (BR) from $32 \pm 4$% of the control (none) to $46 \pm 4$% at 48 h (Table I). Further, at 72 h $CoQ_{10}$ increased the beating cell number (BCN) from 5% of the control (none) to 18% and BR from $4 \pm 2$% of the control (none) to $17 \pm 4$%. At the high dose of 140 $\mu$M DX, $CoQ_{10}$ also significantly protected BCN from DX toxicity. $CoQ_{10}$ did not protect the cells from DX toxicity at concentrations of less than 96 $\mu$M (results not shown).

Then, we investigated whether $CoQ_{10}$ affected $^3$H-DX uptake by the cells. Although addition of $CoQ_{10}$ in advance reduced DX uptake slightly in comparison to the control (none), no significant difference was observed. Namely, $CoQ_{10}$ did not affect $^3$H-DX uptake by the cells under the conditions used (3.5 $\mu$M DX for 24—72 h or 140 $\mu$M DX for 1—4 h). Most of the radioactivity in the cells was confirmed by HPLC analysis to be due to DX itself, and no significant difference was observed in the ratio of intact DX to total uptake between the control (none) and $CoQ_{10}$-treated group (Table I), suggesting no change of DX metabolism.

Table II shows the effects of a CoQ homolog and an analog, $CoQ_9$ and $PQ_9$, respectively. When cultures were incubated with 3.5 $\mu$M DX, $CoQ_9$ protected the beat inhibition

TABLE I. Effects of Coenzyme $Q_{10}$ (120 $\mu$M) on Doxorubicin Uptake and Metabolism by Cardiac Cells and Inhibition of Beating

| DX ($\mu$M) | Incubation time (h) | Beating cell number[a] (%) | | Beating rate (%) | | Total untake of $^3$H-DX[e] pmol DX eq/$\mu$g protein ($n$) | | DX found[e] % of total uptake ($n$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | None | $CoQ_{10}$ | None | $CoQ_{10}$ | None | $CoQ_{10}$ | None | $CoQ_{10}$ |
| 3.5 | 24 | 99/134 (74) | 100/134 (75) | 70±6 | 74±5 | 3.67±0.68 (12) | 3.58±0.22 (15) | 90±3 (5) | 90±2 (5) |
| | 48 | 51/134 (38) | 60/134 (49) | 32±4 | 46±4[b] | 4.91±0.64 (16) | 4.40±0.30 (20) | 90±2 (4) | 86±2 (4) |
| | 72 | 6/134 (5) | 24/134[d] (18) | 4±2 | 17±4[c] | 3.36±0.58 (11) | 3.05±0.19 (17) | 82 (2) | 87 (2) |
| 140 | 1 | 56/108 (52) | 82/108[d] (76) | 35±4 | 38±3 | 18.6±1.48 (8) | 17.6±0.80 (8) | — | — |
| | 4 | 26/108 (24) | 55/108[d] (51) | 15±3 | 20±3 | 32.1±1.62 (8) | 32.4±2.25 (8) | — | — |

Beating cell number and beating rate are given as the ratio of beating cells at the indicated time to that at 0 h. *a*) Figures in parentheses show the percentage of beating cell number. Beating rate is expressed as the averaged beating rates (mean±S.E.) of individual cells. In the case of the control medium (none), only the solvent for $CoQ_{10}$ solution was added. $CoQ_{10}$ (120 $\mu$M) and its solvent were added to the medium at the seeding time. Values with superscripts *b*), *c*) and *d*) are significantly different from the corresponding control with $p<0.05$, $p<0.01$ and $p<0.001$, respectively, by Student's $t$-test or the $X^2$-test. *e*) The data are expressed as mean±S.E. and figures in parentheses are the number of experiments. The total uptake of $^3$H-DX was estimated by measuring radioactivity incorporated in the cells. DX found in the cells was determined by HPLC as described in the text.

TABLE II. Protective Effects of Coenzyme $Q_9$ and Plastoquinone on Inhibition of Beating by 3.5 $\mu$M Doxorubucin

| Incubation time (h) | Beating cell number (%) | | | Beating rate (%) | | |
|---|---|---|---|---|---|---|
| | None | $CoQ_9$ | $PQ_9$ | None | $CoQ_9$ | $PQ_9$ |
| 24 | 41/53 (77) | 41/54 (76) | 37/60 (62) | 63±6 | 69±8 | 43±8[a] |
| 48 | 17/53 (32) | 23/54 (43) | 17/60 (28) | 32±7 | 40±7 | 22±6 |
| 72 | 2/53 (4) | 14/54 (26)[b] | 1/60 (2)[c] | 2±2 | 28±7[d] | 4±2[e] |

Beating cell number and beating rate are given as the ratio of beating cells at the indicated time to that at 0 h. Figures in parentheses show the percentage of beating cell number. Beating rate is expressed as the averaged beating rates (mean±S.E.) of individual cells. In the case of the control medium (none), the solvent for $CoQ_9$ and $PQ_9$ solution was added. $CoQ_9$ (120 $\mu$M), $PQ_9$ (120 $\mu$M) and their solvent were added to the medium at the seeding time. Values with superscripts *b*) and *d*) are significantly different from the corresponding control (values of none) with $p<0.05$ and $p<0.001$ by the $X^2$-test or Student's $t$-test, respectively. Values with superscripts *a*), *c*) and *e*) are significantly different from the corresponding $CoQ_9$ with $p<0.005$, $p<0.001$ and $p<0.001$ by the $X^2$-test or Student's $t$-test, respectively.

significantly at 72 h. $CoQ_9$ increased BCN and BR from 4% to 26% and from 2% to 28%, respectively. No significant difference was observed between the effects of $CoQ_9$ and $CoQ_{10}$ (Tables I and II). On the other hand, $PQ_9$ did not protect the beating from DX toxicity at all. Further, significant differences were observed between the effects of $CoQ_9$ and $PQ_9$ on the inhibition of beating by 3.5 $\mu$M DX for 24 and 72 h (Table II).

## Discussion

Basically the role of CoQ in living organisms has been established as an essential co-factor of the electron transport chain coupled to oxidative phosphorylation in the inner membrane of mitochondria.[14] In addition to its role in bioenergetics, $CoQ_{10}$ appears to have other functions, such as an antioxidant effect.[15]

We observed previously that $CoQ_{10}$ protected cultured mouse myocardial cells from the inhibition of beating by DX; the protective effect of $CoQ_{10}$ was stronger than that of α-tocopherol, which has a much stronger antioxidative effect than $CoQ_{10}$.[8] Hosono *et al.*[16] indicated that $^{14}C$-labelled $CoQ_{10}$ was located in mitochondria and in the cell membrane of cultured mouse myocardial cells. If the protective effect of $CoQ_{10}$ against DX cardiotoxicity involved preventing cell uptake of DX at the cell membrane, $CoQ_{10}$ might reduce the antitumor effect of DX. However, our results show that $CoQ_{10}$ did not affect uptake or metabolism of DX by the cells. Yamanaka *et al.*[11] indicated that $CoQ_{10}$ increased the antitumor effect of DX against L1210 leukemia cells. On the basis of our results, that $CoQ_{10}$ affects neither uptake nor metabolism of DX by the cells, their finding may be reasonable.

Thus, the protective effect of $CoQ_{10}$ seemed to be based on biochemical actions other than reduction of lipid peroxidation and inhibition of DX uptake. Moreover, it was demonstrated that CoQ also protected the cardiac muscle from ischemic injury by maintaining the oxidative phosphorylating and adenosine triphosphate (ATP) generating capacity of mitochondria.[17] Therefore, we were interested in whether or not there is any interrelation between the effect of $CoQ_{10}$ on DX toxicity and the bioenergetic role of $CoQ_{10}$.

In animals only CoQ occurs naturally, and $PQ_9$ is neither found nor absorbed. $CoQ_9$ and $CoQ_{10}$ are predominant and minor forms in mouse heart, respectively. $PQ_9$ is primarily concentrated in the green parts of plants[18] and is specific for the plant photosynthesis system.[19] $CoQ_9$ as well as $CoQ_{10}$ protected beating myocardial cells, though only partially, from the inhibition by DX, whereas $PQ_9$ did not. The differences between the effectiveness of $CoQ_9$, $CoQ_{10}$ and $PQ_9$ for the prevention of the beat inhibition by DX were significant. When beef heart mitochondria are extracted with acetone and pentane, their succinate oxidase and nicotinamide adenine dinucleotide (NADH) oxidase activities are lost.[20] $CoQ_9$ and $CoQ_{10}$ are specifically required to restore the activities of such CoQ-enzymes after acetone or pentane treatment of mitochondria, whereas $PQ_9$ is coenzymically inactive in both system.[20] It is not clear why $CoQ_9$ and $CoQ_{10}$ are effective and why $PQ_9$ is not in preventing DX toxicity. However, it is interesting that $CoQ_9$ and $CoQ_{10}$, occurring in mouse heart and having respiratory activity, were effective.

The myocardium is the most energy-consuming tissue, and the energy for the myocardial function is generated and supplied mainly through the mitochondrial respiratory chain, which is abundant in the heart tissue. DX is known to produce structural and functional alterations in heart mitochondria.[21] Recently, the relation between mitochondrial injury and cardiomyopathy was emphasized by the observations that DX produced a reduction in the cardiac store of ATP in the isolated perfused rat heart[22] and that DX associated strongly with cardiolipin, an phospholipid specific to the mitochondrial inner membrane.[23] We have indicated previously that DX depressed cellular respiration[24] and ATP content in the cardiac cells.[5] Further, it was recognized that DX inhibited beating and cellular respiration in mouse cardiac cells, and the activities of CoQ-enzymes in beef heart mitochondria, at about the same concentrations.[7,24] We have also shown that $CoQ_{10}$ reduced the inhibitory effect of DX on succinate oxidase and NADH oxidase systems.[7] Ohhara *et al.*[22] suggested that $CoQ_{10}$ protected the cardiac muscles from the decrease of ATP level by DX in isolated perfused rat hearts.

The important finding in the present study is that $CoQ_{10}$, but not $PQ_9$, protected the beating of the cells from DX toxicity in spite of affecting neither uptake nor metabolism of DX by the cells. Although the mode of action of $CoQ_{10}$ was not determined in our experiments, it is possible that $CoQ_{10}$ acts as an essential co-factor in the mitochondrial electron transport chain.

**References and Notes**

1) Present address: *Third Department of Internal Medicine, Osaka University Medical School, Fukushima-ku, Osaka*

*553, Japan.*
2) R. H. Blum and S. K. Carter, *Ann. Intern. Med.*, **80**, 249 (1974).
3) L. Lenaz and J. A. Page, *Cancer Treat. Rev.*, **3**, 111 (1976).
4) K. Goshima, *J. Mol. Cell. Cardiol.*, **8**, 217 (1976).
5) K. Takahashi, Y. Fujita, T. Mayumi, T. Hama, and T. Kishi, *Chem. Pharm. Bull.*, **35**, 326 (1987).
6) C. Bertazzoli, L. Sala, and M. G. Tosana, *Int. Res. Commun. Sys. Med. Sci.*, **3**, 367 (1975).
7) T. Kishi, T. Watanabe, and K. Folkers, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 4653 (1976).
8) K. Takahashi, T. Mayumi, and T. Kishi, *Chem. Pharm. Bull.*, **35**, 4626 (1987).
9) Y. Iwamoto, I. L. Hansen, T. H. Porter, and K. Folkers, *Biochem. Biophys. Res. Commun.*, **58**, 633 (1974).
10) P. Michell, *Ann. Rev. Biochem.*, **46**, 996 (1977).
11) N. Yamanaka, T. Kato, K. Nishida, T. Fujikawa, M. Fukushima, and K. Ota, "Biomedical and Clinical Aspects of Coenzyme Q," Vol. 2, ed. by Y. Yamamura, K. Folkers, and Y. Ito, Elsevier, North-Holland Biomedical Press, Amsterdam, 1980, pp. 213—222.
12) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
13) A. C. Haneke, J. Crawfold, and A. Aszalos, *J. Pharm. Sci.*, **70**, 1112 (1981).
14) J. W. Depierre and L. Ernster, *Ann. Rev. Biochem.*, **46**, 201 (1977).
15) T. Ozawa, "Coenzyme Q," ed. by G. Lenaz, A Wiley Interscience Publication, Chichester, 1985, pp. 441—456.
16) K. Hosono, H. Ishida, M. Okuda, N. Suzuki, and T. Nakamura, "Biomedical and Clinical Aspects of Coenzyme Q," Vol. 3, ed. by K. Folkers and Y. Yamamura, Elsevier, North-Holland Biomedical Press, Amsterdam, 1981, pp. 269—278.
17) W. G. Nayler, "Biomedical and Clinical Aspects of Coenzyme Q," Vol. 2, ed, by Y. Yamamura, K. Folkers, and Y. Ito, Elsevier, North-Holland Biomedical Press, Amsterdam, 1980, pp. 409—425.
18) T. Ramasaruma, "Coenzyme Q," ed. by G. Lenaz, A. Wiley Interscience Publication, Chichester, 1985, pp. 67—81.
19) A. Trebst, "Coenzyme Q," ed. by G. Lenaz, A Wiley Interscience Publication, Chichester, 1985, pp. 257—284.
20) G. Lenaz, G. D. Daves, and K. Folkers, *Arch. Biochem. Biophys.*, **123**, 539 (1968).
21) M. E. Ferreero, E. Ferrero, G. Gaja, and A. Bernelli-Zazzera, *Biochem. Pharmacol.*, **25**, 125 (1975).
22) H. Ohhara, H. Kanaide and M. Nakamura, *J. Mol. Cell. Cardiol.*, **13**, 741 (1981).
23) E. Goormaghtigh, G. Pollakis, and J. M. Ruysschaert, *Biochem. Pharmacol.*, **32**, 889 (1983).
24) K. Takahashi, N. Matsumoto, T. Okamoto, and T. Kishi, *Yakugaku Zasshi*, **10**, 727 (1987).