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ORIGINAL ARTICLE

Antioxidant role of endogenous coenzyme Q against the ischemia and reperfusion-induced lipid peroxidation in fetal rat brain

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Background. Ischemia and subsequent reperfusion induce lipid peroxidation in the cerebrum of the fetal rat. The present study evaluated the antioxidant activity of endogenous coenzyme Q in protecting against the lipid peroxidation induced in the fetal rat brain by ischemia/reperfusion.
Methods. We used wistar rats at day 19 of pregnancy. Fetal ischemia was induced by bilateral occlusion of the utero-ovarian artery for 20 minutes. For reperfusion, the occlusion was released and the circulation was restored for 30 minutes. Control rats underwent sham operation. We determined the levels of thiobarbituric acid-reactive substances, the concentrations of coenzyme Q9, coenzyme Q10, and the mitochondrial respiratory control index in fetal brains.
Results. Occlusion for 20 minutes significantly reduced the respiratory control index ($p<0.01$), but did not alter the levels of thiobarbituric acid-reactive substances, coenzyme Q9 or coenzyme Q10. Subsequent reperfusion, however, significantly increased the level of thiobarbituric acid-reactive substances (from 6.53 ± 1.54 to 11.46 ± 3.31 nM/mg of protein, $p<0.01$) and significantly decreased the level of coenzyme Q9 (from 291.73 ± 108.94 to 162.44 ± 56.83 pM/mg of protein, $p<0.05$) and that of coenzyme Q10 (from 153.10 ± 75.24 to 79.84 ± 30.40 pM/mg of protein, $p<0.05$). The respiratory control index was still significantly lower following reperfusion than in controls ($p<0.01$). Significant negative correlations were observed between the level of thiobarbituric acid-reactive substances and the concentrations of either coenzyme Q9 ($r=-0.68$, $p<0.001$) or coenzyme Q10 ($r=-0.70$, $p<0.001$).
Conclusion. Endogenous coenzyme Q may protect the fetal rat brain against the lipid peroxidation induced by ischemia/reperfusion.

Key words: antioxidant; coenzyme Q; fetal rat brain; ischemia/reperfusion; lipid peroxidation

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The encephalopathy induced by hypoxia or ischemia in the prenatal period is a major cause of neurological disabilities in children. Oxygen-free radicals might be important in the pathogenesis of such hypoxic/ischemic brain damage (1, 2). Ischemia and subsequent reperfusion produce reactive oxygen-free radicals and induce lipid peroxidation (3–5). Since the cell membranes consist primarily of lipids, lipid peroxidation may cause cellular dysfunction and, even, cell death.

Brain tissue is highly sensitive to damage induced by oxygen-free radicals because of its high oxygen utilization; its high concentrations of polyunsaturated fatty acids (PUFAs) and of transition metals, such as iron; and its low levels of cytosolic antioxidants (6). We previously demonstrated that

Abbreviations:
CoQ: coenzyme Q; TBARS: thiobarbituric acid-reactive substances; RCI: respiratory control index; ATP: adenosine triphosphate; ADP: adenosine diphosphate; PUFAs: polyunsaturated fatty acids; ANOVA: analysis of variance.

fetal ischemia and subsequent reperfusion induced the oxidation of fetal cerebral lipids and DNA (7), thereby possibly contributing to an impairment of the fetal central nervous system.

The activities of such antioxidants as catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase are underdeveloped in the fetal brain early in gestation (8). Although the rate of lipid peroxidation was higher in term brain, the levels of peroxidation products were greater in preterm brain (1). The levels of vitamin C, a water soluble antioxidant, are maintained higher in umbilical cord plasma even in cases where the maternal vitamin nutrition is poor (9). However, the levels of vitamin E, a major lipid soluble antioxidant, are substantially lower in placental and umbilical cord plasma than that in maternal plasma (10). These observations suggest that the fetal brain may be particularly susceptible to the oxidative damage induced by oxygen-free radicals.

Coenzyme Q (CoQ) is a lipid-soluble benzoquinone that is localized in the mitochondrial respiratory chain as well as in other internal membranes. CoQ is directly involved in energy transduction and aerobic adenosine triphosphate (ATP) production because it transports electrons in the respiratory chain, as well as couples the respiratory chain to oxidative phosphorylation (11). Besides its role in electron transfer reactions, CoQ is a powerful antioxidant that can protect the cell structures against oxidative damage during the reperfusion following ischemia (12, 13). Thus, CoQ, which can be regenerated, can modulate the use and production of energy as well as scavenge oxygen-free radicals. The antioxidant activity of CoQ in the fetal brain, however, has not yet been evaluated.

Our objective was to evaluate the antioxidant role of endogenous CoQ in protecting the fetal rat brain against the lipid peroxidation induced by ischemia/reperfusion.

Materials and methods

Experimental protocol

Wistar rats were maintained in accordance with the Kochi Medical School Guidelines for the Care and Use of Laboratory Animals. All research protocols were performed according to the guidelines of the Animal Research Committee of the Kochi Medical School.

Pregnant rats underwent a laparotomy on day 19 of pregnancy following the induction of anesthesia produced by administering an injection of ketamine (40 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Fetal ischemia was induced by occluding the utero-ovarian artery bilaterally for 20 minutes, using forceps covered with soft polyvinyl tubing ($n=7$). Reperfusion was achieved by declamping the utero-ovarian artery and restoring the circulation for 30 minutes ($n=8$). The complete interruption and restoration of the blood flow was confirmed directly under the operating microscope. Control rats ($n=8$) underwent a similar sham operation. At the end of the experimental period, the fetal brain tissue retrieved from each pregnant rat was pooled and homogenized in a medium consisting of 0.24 M mannitol, 0.06 M sucrose, 50 μM EDTA, and 5 mM Tris-HCl, at pH 7.2 (7).

Mitochondrial respiratory activity

The fetal brain tissue homogenate was centrifuged at 490×g for 5 minutes, and the supernatant was collected and centrifuged at 7800×g for 10 minutes. The resultant pellet was suspended in a medium consisting of 0.25 M sucrose and 1 mM Tris-HCl, at pH 7.2 and used as the brain mitochondrial fraction. All procedures were carried out at 0–4°C. The protein concentration of the mitochondrial fraction was measured using the technique of Lowry et al. (14).

Oxygen consumption was measured polarographically at 25°C using 1.0–2.5 mg of protein from the fresh mitochondrial in 2.0 mL of incubation medium consisting of 100 mM KCl, 0.05 mM EDTA, 10 mM Tris-HCl, and 0.1 M sucrose, at pH 7.4 using a Clark-type electrode. Mitochondrial respiration was initiated by adding 150 μM adenosine 5 diphosphate (ADP) with 5 mM succinate as the respiratory substrate. The oxygen consumption measured in the presence of added ADP was defined as state 3 respiration, while that measured following the consumption of ADP was defined as state 4 respiration. The respiratory control index (RCI) was calculated as the ratio of state 3 to state 4 respiration and used as a marker of mitochondrial respiratory activity (15, 16).

Determination of CoQ concentration

The fetal brain tissue homogenate was saponified by adding 3 vol. 1% pyrogallol dissolved in methanol and 0.2 vol. 50% potassium hydroxide and heated at 100°C for 10 minutes. After the mixture was cooled on ice, CoQ was extracted by adding 1 vol. water and 5 vol. n-hexane. The n-hexane layer was evaporated under a nitrogen stream at 30°C and then dissolved in dioxane. The amounts of CoQ9 and CoQ10 were analyzed by high performance liquid chromatography (IRICA, Kyoto, Ja-

Table I. Mitochondrial RCI and levels of TBARS, CoQ9 and CoQ10 in fetal rat brain in the presence or absence of ischemia/reperfusion

Treatment					
Occlusion (minutes)	Reperfusion (minutes)	RCI	TBARS (nM/mg of protein)	CoQ9 (pM/mg of protein)	CoQ10 (pM/mg of protein)
0	0	3.24±1.40	6.53±2.00	291.73±108.94	153.10±75.24
20	0	2.29±0.17**	7.99±1.81	216.47±81.82	103.42±19.62
20	30	2.22±0.29**	11.46±3.31***	162.44±56.83*	79.84±30.40*

Data represent the mean±s.d.
RCI=Respiratory control index; TBARS=Thiobarbituric acid reactive substances; CoQ=Coenzyme Q.
* $p<0.05$, ** $p<0.01$, *** $p<0.005$ versus sham-operated animals.

pan), using ultraviolet absorbance detection at 275 nm.

Determination of TBARS concentration

Brain tissue homogenate aliquots containing 1.5 mg of protein as determined by the method of Lowry et al. were mixed with 100 μL 8.1% sodium dodecyl sulfate. Next, 1.5 mL 20% acetic acid (pH 3.5) and 1.5 mL of a 0.8% thiobarbituric acid solution were added to the mixture, and the volume brought to 4.0 mL with distilled water. The mixture was shaken thoroughly and heated in an oil bath at 95°C for 60 minutes. After the mixture was cooled in tap water, 1.0 mL of distilled water and 5.0 mL of butyl alcohol and pyridine (15:1, v/v) were added, and the samples were shaken gently for 5 minutes. After centrifugation at 1,500×g for 10 minutes, the butyl alcohol-pyridine phase containing the thiobarbituric acid-reactive substances (TBARS) was separated and its absorbance measured at 532 nm. Results were expressed as mol equivalent malondialdehyde/mg of protein, using malondialdehyde from tetramethoxypropane as a standard and double-distilled water as a control (17).

Statistical analysis

Data are expressed as the mean±standard deviation. One-way analysis of variance (ANOVA) was used to compare the mitochondrial RCI, and the TBARS, CoQ9 and CoQ10 levels among the 3 groups of animals (sham-operated control animals; animals subjected to ischemia for 20 minutes; animals subjected to ischemia for 20 minutes followed by reperfusion for 30 minutes). If the ANOVA showed a significant difference, Scheffe's multiple comparison procedure was applied to determine which values differed. Regression lines

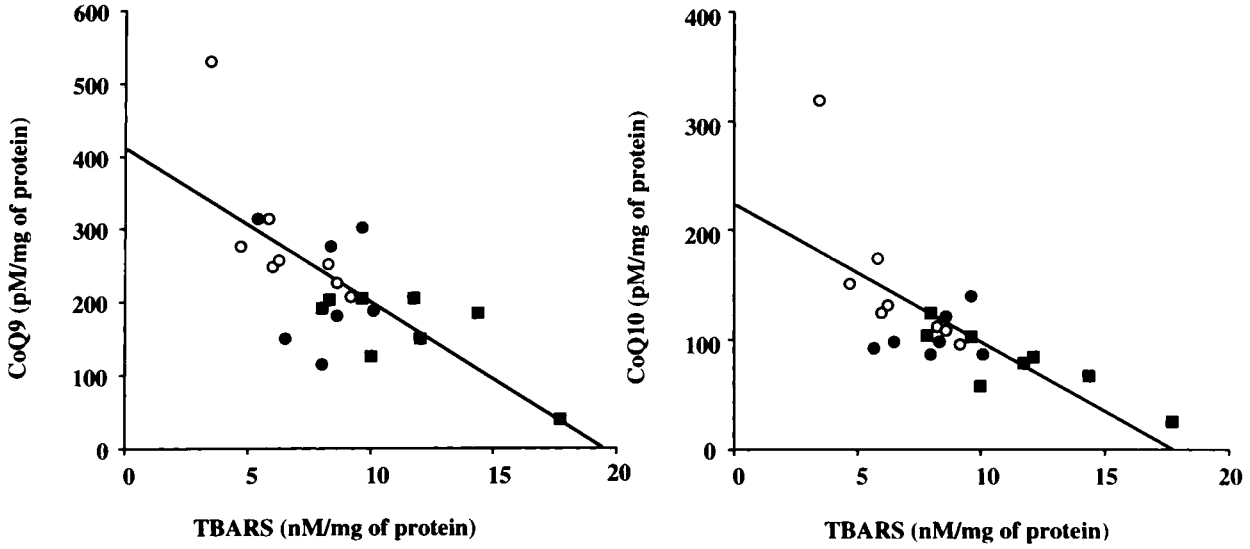

Fig. 1. Relationship between the levels of thiobarbituric acid-reactive substances (TBARS) and the concentrations of coenzyme Q9 (CoQ9) and coenzyme Q10 (CoQ10) in the fetal rat brain. Open circles indicate sham-operated control animals. Closed circles indicate animals subjected to ischemia induced by a 20-minute occlusion of the utero-ovarian artery. Closed squares indicate animals subjected to ischemia followed by reperfusion for 30 minutes.

were determined using the least squares method. A level of $p<0.05$ was accepted as statistically significant.

Results

We observed significant differences in the values for the mitochondrial RCI and in the levels of TBARS, CoQ9, and CoQ10 among the sham-operated (control) animals, those subjected to ischemia, and those subjected to ischemia plus reperfusion. Ischemia induced by a 20-minute occlusion of the utero-ovarian artery significantly decreased the RCI ($p<0.01$), but did not significantly alter the levels of TBARS or of CoQ9 and CoQ10. Subsequent reperfusion for 30 minutes, however, significantly increased the TBARS level ($p<0.005$) and significantly reduced the levels of CoQ9 ($p<0.05$) and CoQ10 ($p<0.05$). The mitochondrial RCI remained significantly lower after reperfusion compared with the control animals ($p<0.01$) (Table I). TBARS levels exhibited a significant negative correlation with those of CoQ9 ($y=-21.09x+408.01$, $r=-0.68$, $p<0.001$) and of CoQ10 ($y=-12.60+222.53$, $r=-0.70$, $p<0.001$) (Fig. 1).

Discussion

The present study evaluated the ability of the endogenous antioxidant CoQ to protect the fetal rat brain against damage by the lipid peroxidation induced by ischemia and reperfusion. Ischemia and subsequent reperfusion have been shown to produce oxygen-free radicals in the brain (3, 4), that may oxidize membrane lipids, proteins and DNA, thereby leading to cell damage. Lipid peroxidation is initiated by the oxygen-free radical attack on PUFAs, substances that are easily to be oxidized. This reaction results in the formation of lipid radicals, which eventually generate peroxyl radicals that are sufficiently reactive to propagate the lipid peroxidation. During the final steps of lipid peroxidation, non-radical products such as alkanes and carbonyl compounds are formed that can be measured based on their reactivity with TBA.

In the present study, a 20-minute occlusion of the utero-ovarian artery did not change the TBARS concentrations in the fetal cerebrum, but reduced the mitochondrial RCI. When mitochondrial respiration begins, the electrons derived from intermediates of the Krebs cycle and other metabolic pathways flow through the mitochondrial respiratory chain, and ATP is produced via oxidative phosphorylation. Since the RCI represents mitochondrial respiratory activity (15, 16), a reduction in RCI indicates mitochondrial dysfunction and an impairment of ATP production. These findings suggest that an ischemic insult can damage the mitochondria in the fetal cerebrum by causing hypoxic, not oxidative, stress. Subsequent reperfusion, however, led to an increase in TBARS levels, consistent with findings of our previous study (7). An ischemic insult induces the release of PUFAs from the phospholipid in the cell membrane in the brain. PUFAs, in turn, are particularly sensitive to peroxidation during reoxygenation (18). Increased PUFAs concentrations have been demonstrated in the fetal brain (8). Consequently, the ischemia induced by the occlusion may result in the accumulation of PUFAs in the fetal brain. During the subsequent reperfusion, oxygen-free radicals may attack the PUFAs, leading to lipid peroxidation. The RCI, however, remained inhibited despite reperfusion and the associated reoxygenation. This observation indicates that the lipid peroxidation that is induced by ischemia and reperfusion may be accompanied by an oxidative impairment of the mitochondria in the fetal brain.

Lipid peroxidation can be prevented by administering antioxidants such as vitamin E, beta-carotene and CoQ. CoQ is a strong endogenous antioxidant that inhibits lipid peroxidation (12, 13). For example, CoQ preserves and potentiates the normal cellular defenses against the oxidative stress induced by adriamycin in rat hepatocytes (19). CoQ also protects low-density lipoprotein, the major cholesterol-carrying lipoprotein in plasma, that has been implicated in the formation of the atherosclerotic lesion against lipid peroxidation (20, 21). The beneficial effects of CoQ also have been demonstrated in cardiovascular disorders, hypertension, cerebrovascular disorders, muscular dystrophy, neurogenic atrophies, and periodontal diseases (22).

Although, in the present study, the levels of CoQ9 and CoQ10 did not change in response to the occlusion of the utero-ovarian artery, subsequent reperfusion led to a decrease in those levels. Ischemia and reperfusion have been shown to induce lipid peroxidation and a reduction in CoQ concentrations in the rat heart (23), liver (24), and brain (25). Lipid peroxidation is a free radical reaction that progresses via a cycle chain reaction. The ischemia/reperfusion-induced increase in TBARS levels and reduction in endogenous CoQ9 and CoQ10 concentrations suggest that CoQ is consumed as an antioxidant during lipid peroxidation in the fetal rat brain. This conclusion is supported by the finding that TBARS levels were negatively correlated with the concentrations of endogenous CoQ9 and CoQ10. Thus, both CoQ9 and CoQ10 may be important antioxidants in protecting the fetal brain against ischemia/reper-

fusion-induced oxidative stress. Because CoQ is an essential cofactor in the mitochondrial respiratory chain that is coupled to oxidative phosphorylation, reduced CoQ levels resulting from lipid peroxidation could decrease the rate of electron transfer, thereby contributing to mitochondrial dysfunction.

Because CoQ is widely distributed throughout the cells, its antioxidant action is not limited to the mitochondria, but affects all the cell membranes (13). It quenches hydroxyl radicals (26) as well as peroxyl radicals (27, 28). Furthermore, CoQ recycles and regenerates vitamin E, an important antioxidant that is derived from the tocopherol radicals induced by oxygen-free radicals (29). The fetal brain may be particularly susceptible to oxidative damage, because its defense against the damage induced by oxygen-free radicals reportedly is underdeveloped (8). CoQ may scavenge oxygen-free radicals and protect against oxidative stress more efficiently than other intracellular antioxidants in the fetal rat brain.

Clinically, intrapartum asphyxia is considered to be a possible cause of fetal cerebral damage in only about 10% of the cases (30, 31), with most of the causal events occurring prenatally (32, 33). A recent study demonstrated that oxygen-free radicals and released neurotransmitters such as glutamate promote necrosis of the fetal brain (34). The occurrence of free radical-induced oxidative cerebral damage during the fetal period may have a key role in the pathogenesis of such damage. Further studies are required to investigate the roles of other endogenous oxygen-free radical scavengers during lipid peroxidation in the fetal rat brain.

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