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EFFECT OF CYTOCHROME C ON FORMATION OF LIPID PEROXIDES IN RAT BRAIN *IN VITRO*

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Antioxidant acitivity of exogenous cytochrome c was investigated *in vitro* using the whole brain homogenate, mitochondrial fraction and postmitochondrial supernatant containing microsomes prepared from rat brains. Increments in the amount of lipid peroxides were observed in each fraction when incubated at 30°C, while the addition of cytochrome c (200 mM) effectively suppressed the production of peroxides. This depressive effect of cytochrome c was more prominent in the supernatant than in the mitochondrial fraction. Although the peroxidation was enhanced markedly by the addition of NADPH (2 mM), particularly in the mitochondrial fraction, cytochrome c was able to prevent its acceleration. This inhibitory mechanism might be explained by the fact that cytochrome c deprived superoxide radicals of electrons generated in ischemic insult. The results of the present study suggest that exogenous cytochrome c has free radical scavenging or antioxidant activity, which might be responsible in part for its cerebral protective action during ischemia.

KEY WORDS: Cytochrome c, lipid peroxidation, free radical, radical scavenger, brain ischemia.

INTRODUCTION

Free radical reactions generating lipid peroxides have been considered to play a pivotal role in various pathophysiological responses, particularly in ischemic neuronal damage and postischemic reperfusion cell injury.[1] Once peroxidative reactions are initiated, they proceed as a chain-propagation in the biomembrane, leading to ultimate membrane disintegrity. Much interest has been focused on agents which can act as a radical scavenger or antioxidant, because of their presumable cerebral protective action against ischemic brain damage.

Cytochrome c, which is a water-soluble heme-protein situated on the cytoplasmic side of mitochondrial inner membranes and one of the important components of the electron-transport chain in mitochondria, is concerned in cellular respiration and production of indispensable bioenergy.[2] This substance is known to be clinically effective in a variety of cases of tissue anoxia, such as mycocardial damage, liver dysfunction or CO intoxication, and it is also effective in ameliorating cerebral hypoxia through the increase of oxygen-consumption efficiency.[3] However, little information is available concerning the precise molecular mechanism for brain protection by cytochrome c. Cytochrome c essentially has a potency to scavenge

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superoxide radicals. Hence, in this study, we attempted to assess the effect of exogenous cytochrome c on lipid peroxidation in rat brain *in vitro*.

MATERIALS AND METHODS

Chemicals

Cytochrome c, derived from horse myocardium, was kindly donated by Mochida Pharmaceutical Co. Ltd. (Tokyo). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Kohjin Co. Ltd (Tokyo). Other reagents used were of analytical grade and commercially available.

Brain Preparation

Male Sprague-Dawley albino rats, weighing about 200 g, had free access to food and water until the time of the experiment. After decapitation without anasthesia, the whole brain was immediately picked up, weighed and homogenized with 10 volumes of 0.25 M sucrose, 0.02 M Tris-HCl buffer (pH 7.4) in an ice-cold, loosely fited glass-teflon homogenizer. The crude homogenate was centrifuged at 1,100 × g for 5 min, and the resulting supernatant was employed for further experiments as "the homogenate". Part of the homogenate was subjected to further centrifugation at 20,000 × g for 20 min. The supernatant fluid was used as a sample of a post-mitochondrial supernatant fraction. The pellet was resuspended in aliquots of the same medium and was centrifuged again at 20,000 × g for 20 min. The sedimented mitochondria were resuspended in the same buffer to yield a protein concentration of approx. 10 mg/ml with a teflon homogenizer, and this material was used in the experiments.

Each sample of homogenate, mitochondrial and postmitochondrial fractions was divided into 4 groups as follows: 1) control, 2) addition of NADPH (2 mM), 3) addition of cytochrome c (200 mM), and 4) addition of both NADPH and cytochrome c. 0.5 ml of each sample was transferred into a test tube and incubated for 5, 10, 20 or 30 min at 30°C in a shaking water bath. The reaction was stopped by cooling in ice.

Measurement of Lipid Peroxides

The levels of lipid peroxides or malondialdehyde (MDA) precursors were measured by thiobarbituric acid (TBA) reaction according to the modification of the procedure of Kogure *et al.*[4] To each sample, 0.5 ml of 7% (W/V) sodium dodecyl sulfate, 2 ml of 0.1 N hydrochloric acid and 0.3 ml of 10% (W/V) phosphotungstic acid were added and mixed in this order. Then, 1 ml of 0.5% TBA was added, and the reaction mixture was immediately vortexed. The contents were then heated at 95°C for 45 min in boiling water and then immediately cooled in ice. Five ml of n-butanol was then added to the system and mixed for 15 sec. The n-butanol layer was withdrawn after centrifugation at 1,100 × g for 10 min, and fluorescence intensity was measured at 565 nm (Ext. 535 nm) by spectrofluoremetry (Hitachi MPE-3). The amount of TBA reactants was calculated in comparison with a standard MDA solution prepared by hydrolysis of 1,1,3,3-tetraethoxy propane, which yields one mole MDA per mole under these

conditions. Total protein was assayed by the method of Lowry *et al.*[5] using bovine serum albumin as a standard protein.

RESULTS

The level of TBA-reactive substances was found to increase linearly with progress of incubation in the control homogenate as shown in Figure 1. The level increased 1.5 fold after 30-min incubation over the level in the homogenate without incubation (expressed as 0 min). However, the formation of MDA was depressed significantly by the addition of cytochrome c. On the other hand, the addition of NADPH enhanced generation of lipid peroxides when compared to the control homogenate (Figure 2). The increment ratios of NADPH-induced MDA production during 5 and 30 min of incubation were 1.4 and 1.6 fold, respectively. Cytochrome c exerted a significant depressive effect on MDA generation during incubation of the brain homogenate even in the presence of NADPH.

The elevation in the amount of TBA-reactive materials was not obvious in the mitochondrial fraction compared to the homogenate, with a 1.2 fold increase at 30 min (Figure 3). The basal value was in good agreement with that obtained by

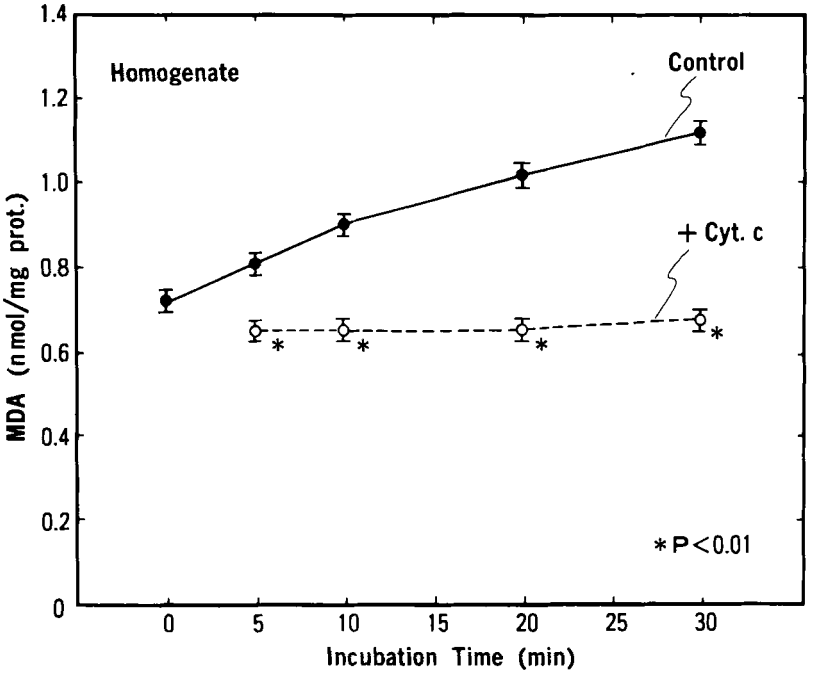

FIGURE 1 Time courses of malondialdehyde (MDA) formation and effect of cytochrome c in rat brain homogenate. The homogenates were incubated with or without addition of cytochrome c (200 mM) at 30°C under room conditions, and the reaction was terminated by cooling in ice. The amount of MDA formed was measured by the thiobarbituric acid method. Each value was expressed as means of triplicate determinations ± S.D.. The asterisk indicates statistically significant differences from the control values, $p < 0.01$.

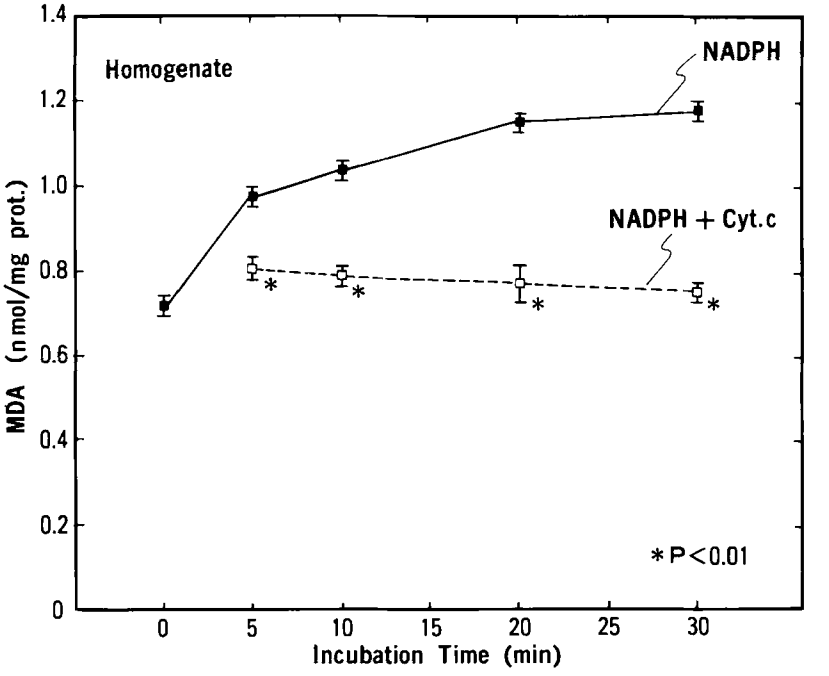

FIGURE 2 Facilitation of MDA production by the addition of NADPH (2 mM) and inhibitory effect of cytochrome c in rat brain homogenate. See legend to Figure 1 for details.

Majewska *et al.*[6] However, a striking rise in MDA formation was observed in the mitochondrial fraction in the presence of NADPH (Figure 4). The level was elevated 2.4 fold at 5 min and 2.6 fold at 30 min, but this increase was attenuated significantly by the added cytochrome c.

In the postmitochondrial supernatant, which contains microsomes, the amount of lipid peroxides was smaller that that of the mitochondrial fraction, merely showing a 1.3 fold increase at 30 min (Figure 5). Addition of cytochrome c diminished the lipid peroxidation in the supernatant. NADPH enhanced the lipid peroxidation in the supernatant fluid, and cytochrome c exerted a suppressive effect against MDA formation (Figure 6).

DISCUSSION

In recent years, it has been well documented that cerebral ischemia causes a remarkable accumulation of free fatty acids released from membrane phospholipids in the brain.[7,8] Among free fatty acids, polyunsaturated fatty acids, especially arachidonic acid, are known to produce a variety of bioactive mediators such as prostaglandins, thromboxanes or leukotrienes, which are closely coupled to the pathophysiology of brain ischemia.[9] In addition, polyunsaturated fatty acids are inclined to be converted into their peroxy radicals, and then into hydro- and endoperoxides.[10] Moreover, polyunsaturated fatty acids are most susceptible to free radical damage. Demopoulos *et al.*[11] first advocated the free radical hypothesis during brain ischemia. According to

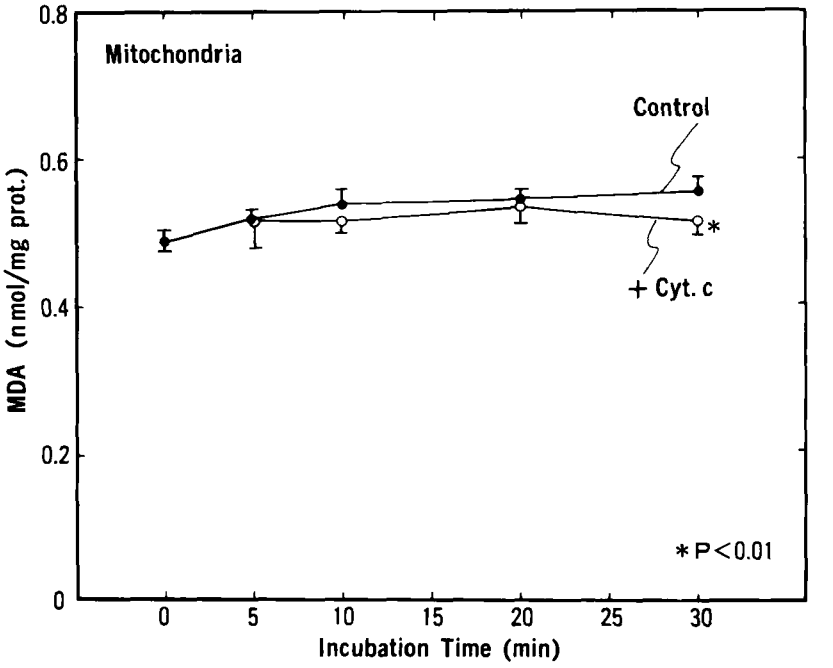

FIGURE 3 Development of MDA in mitochondrial fraction by incubation and effect of cytochrome c. See legend to Figure 1 for details.

their deliberations, the lack of oxygen, an acceptor for electrons from the mitochondrial respiratory chain, leads to dislocation of free radicals which in turn attack polyunsaturated fatty acids, not only liberated from, but also bound to neighbouring membrane phospholipids, resulting in a loss of structural and functional integrity of the biomembranes. Since the proposal of the free radical theory, an inreasing number of investigators have dealt with this subject regarding ischemic brain damage.[1] In consequence, it is now accepted that the generation of free radicals with subsequent lipid peroxidation may be involved intimately in the development of ischemic membrane disintegrity.

Although the configuration of free radical formation has well been known to be enhanced during the reperfusion period after ischemia, controversy still arises as to whether this process also occurs during the intraischemic period or not. Kogure *et al.*[4] have examined the development of MDA in minced brains under aerobic and anaerobic incubations, and have found that 100% oxygen facilitates MDA formation, while in contrast, 100% nitrogen blocks it. Thus, they concluded that lipid radical intermediates and associated chain peroxidation process were potentiated by ischemia and occurred during tissue reoxygenation. There are several reports describing how the generation of free radicals could be detected during ischemic period using electron spin resonance spectroscopy[12] or chemiluminescence[13] analysis. In the present study, this subject was not followed because the incubation was carried out under normal room temperature conditions.

On the other hand, those agents which possess radical scavenging or antioxidant activities, such as α-tocopherol,[14] vinpocetin[15] or flunarizine,[16] are capable of protect-

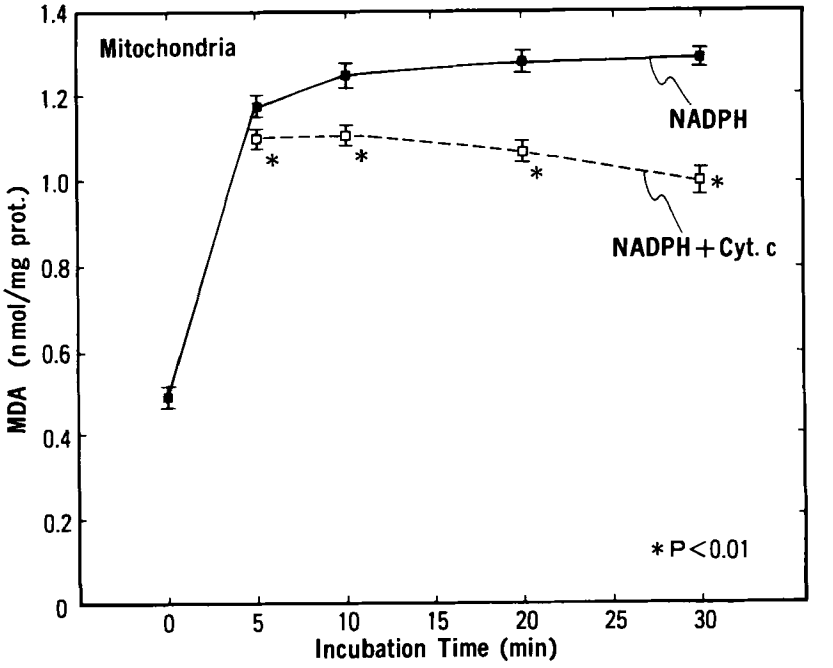

FIGURE 4 Inhibitory effect of cytochrome c on NADPH-induced lipid peroxidation in mitochondrial fraction. See legend to Figure 1 for details.

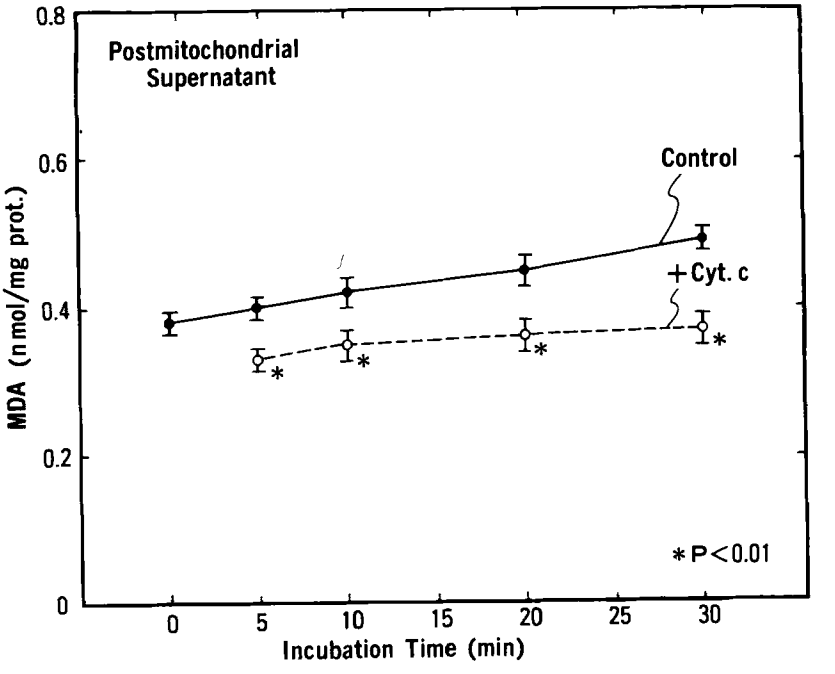

FIGURE 5 Development of MDA in postmitochondrial supernatant, which contains microsomes, by incubation and inhibitory effect of cytochrome c. See legend to Figure 1 for details.

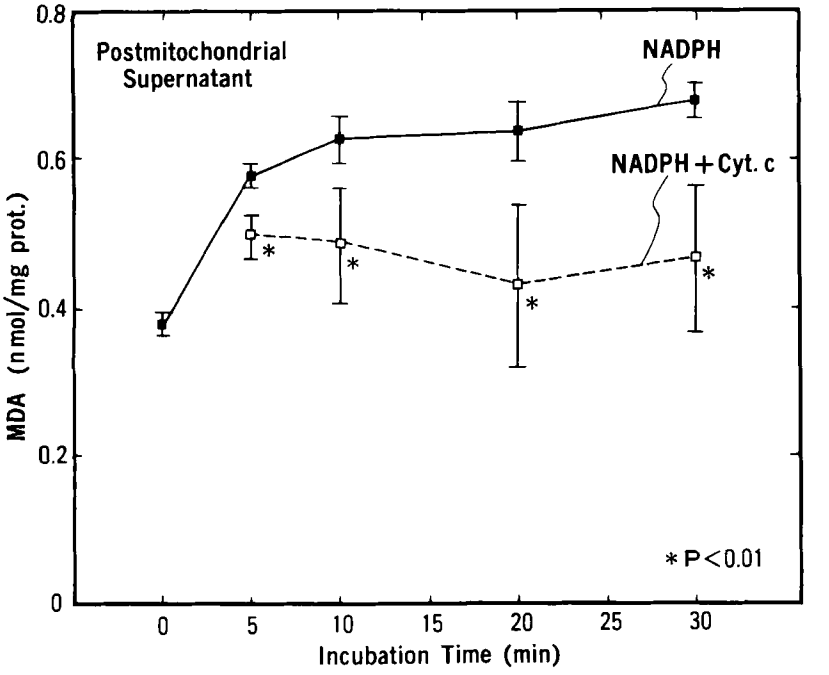

FIGURE 6 Inhibition of NADPH-induced lipid peroxidation by cytochrome c in postmitochondrial supernatant. See legend to Figure 1 for details.

ing the brain from ischemic insults. Barbiturate is also presumed to be a free radical scavenger,[6,11,17] although the evidence is still under dispute.[16] We have previously reported that barbiturate inhibits the activity of phospholipase C, and postulated that this inhibition might in part explain the mechanism for the brain protective action of barbiturate during ischemia.[18] In our present investigation, cytochrome c exhibited antioxidant activity, in particular against NADPH-induced lipid peroxidation. The

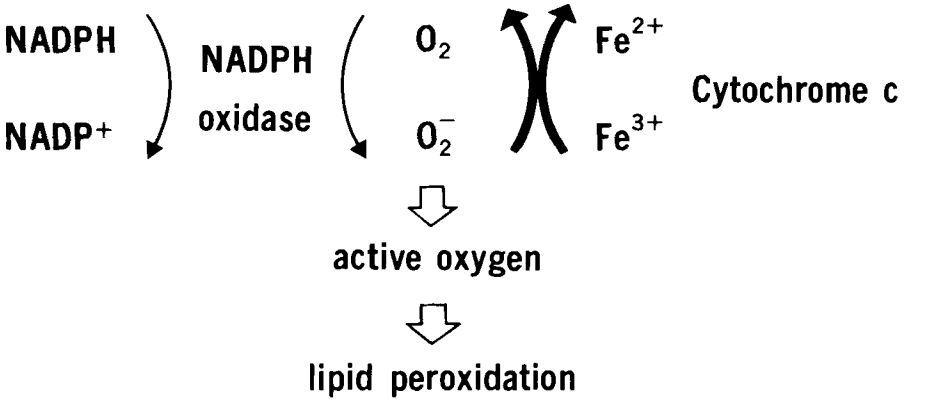

FIGURE 7 Proposed mechanism of cytochrome c action as superoxide scavenger, resulting in decreased formation of lipid peroxides.

inhibition mechanism of lipid peroxidation is not entirely clear, but can in part be interpreted by the fact that superoxide produced by NADPH reduces the ferric ions (Fe^{3+}) contained in cytochrome c to ferrous ions (Fe^{2+}) (Figure 7). This reduction of cytochrome c is used as the basis of the assay for superoxide dismutase activity.[19] Furthermore, transient formation of superoxide radicals was reported in the brain swelling induced by polyunsaturated fatty acid.[20]

Mitochondrial membranes have profuse polyunsaturated fatty acids and are consequently fragile against free radical damage, resulting in the production of lipid peroxides as we observed with NADPH loading. Inherently, cytochrome c is the endogenous substance localized preferentially in mitochondria, so that endogenous cytochrome c may serve substantially to defend mitochondrial membranes against free radical attack. It is, therefore, conceivable that the influence of exogenous cytochrome c predominantly appears in microsomal membranes possessing no cytochrome c.

Since cytochrome c is a high molecular compound, with a molecular weight of 12,400, a serious problem remains regarding whether this material can penetrate the cell membrane or not. However, several studies have demonstrated that exogenous cytochrome c does permeate across the plasma membrane under pathological conditions such as anoxia or intoxication of tissue, in which the permeability of the plasma membrane might be increased.[3,21]

Conclusively, our results strongly suggest that exogenous cytochrome c prevents lipid peroxidation during ischemia due to a radical scavenging action mainly in the microsomes, and this effect may be related to the biochemical process for cerebral protection of cytochrome c. Yet disclosure of the exact radical formation and scavenging action has to await further studies, including the detection of free radicals by other methods.

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