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KEYWORDS: myocardial necrosis, lipid peroxides, ?-tocopherol

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CHANGES OF LIPID PEROXIDES AND α-TOCOPHEROL IN RATS WITH EXPERIMENTALLY INDUCED MYOCARDIAL NECROSIS

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Abstract. Myocardial necrosis was produced in rats by injection of isoproterenol (80 mg per kg body weight). Lipid peroxides were measured by the thiobarbituric acid reaction. α-Tocopherol was assayed by fluorometric analysis. Immediately after isoproterenol injections, serum lipid peroxides increased and serum α-tocopherol decreased, then gradually returned to the pre-injection levels. Lipid peroxides increased more rapidly in the heart and liver than in serum. α-Tocopherol decreased in the heart and liver, then gradually returned to the pre-injection levels. These results indicate that increase in serum lipid peroxides reflects production of peroxides in myocardial tissue and in liver. The decrease in α-tocopherol may be due to consumption as anti-oxidants in the vascular system and organs.

Key words : myocardial necrosis, lipid peroxides, α-tocopherol.

Under certain experimental conditions, "peroxides" form which can cause extensive destruction of lipids. Peroxidation of lipids in tissue preparation by active oxygen or free radicals leads to toxic effects that include structural changes in plasma membranes (1, 2) and damage to polypeptide chains of proteins (3). Although little is known about the role of "peroxide" formation *in vivo*, it is assumed that lipid peroxides are important in the pathogenesis of aging (4, 5), atherosclerosis (6), oxygen toxicity in the lung (7, 8), retinopathy of prematurity (9), drug toxicity (10-16) and liver disease (17).

Antioxidants such as superoxide dismutase (SOD), ascorbic acid, SH-radical and α-tocopherol prevent peroxidation *in vivo* (7, 18). α-Tocopherol was discovered by Evans *et al.* originally as an anti-sterility hormone (19) and is believed to be one of the strongest antioxidants *in vivo*. Einarson and Ringsted suggested that the "seroid" seen in chronic vitamin E deficient rats had similar characteristics to the lipofuscin seen in aging tissue (20). Since then, much attention has been given to the role of α-tocopherol in the pathogenesis of certain diseases (21). Horning *et al.* showed that α-tocopherol decreased in patients with apoplexy (22) and this report stimulated workers to study the relationship between α-tocopherol and vascular accidents. Kibata *et al.* reported that serum

α-tocopherol concentration was low in the acute phase of apoplexy and myocardial infarction in contrast to the high values of lipid peroxides in these diseases (23-25).

In the present study, experiments were undertaken to measure the serum levels of α-tocopherol and lipid peroxide in rats with experimentally induced myocardial necrosis (MN rat). The results obtained from the present animal model system are compared with clinical data.

MATERIALS AND METHODS

Male Wister rats weighing 200 to 300 gm were from a commercial source and 4 rats were housed in each metal cage. The rats were given commercial diets and drinking water *ad libitum.*

All chemicals were of reagent grade and were obtained from the following sources: 2-thiobarbituric acid and 1. 1. 3. 3-tetraethoxypropane were from Wako Pure Chemical Industries, Osaka, Japan; phosphotungstic acid was from Nakarai Chemical, Kyoto, Japan; dl-α-tocopherol was a gift from the Esai Pharmaceutical Co., Tokyo, Japan; and dl-isoproterenol hydrochloride was from the Sigma Chemical Co., St. Louis, U.S.A.

Induction of myocardial necrosis in rats. Myocardial necrosis was induced in rats by the method of Rona *et al.* (26, 27). Rats were injected with dl-isoproterenol hydrochloride in distilled water at a dose of 80 mg/kg body weight. This was given into the dorsal subcutaneous space and the injection was repeated 24 h later. The rats were anesthetized with ether, and blood was collected from the inferior vena cava 24 h after the last injection. Control rats were injected with distilled water and treated similarly thereafter. All rats were killed by venesection.

Time course of myocardial damage. Myocardial necrosis was induced in 60 rats. Five rats were killed at each of the following times: before injection, at 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 5 days, 7 days and 14 days after the first injection. Each rat was perfused with large doses of 1.15 % KCl solution through the portal vein after venous collection, then the heart and liver were isolated. These tissues were homogenized in KCl solution at 4 ℃. The hearts were sectioned and stained with hematoxylin and eosin and with Azan.

Mesurement of serum lipid peroxides (thiobarbituric acid reactive substance, TBARS). TBARS was measured by the 2-thiobarbituric acid reaction, which measures malondialdehyde evolved as a breakdown product of peroxidized lipids, according to the method of Yagi *et al.* (28, 29). Serum (0.02 ml), 4.0 ml of 1/12 N sulfuric acid, and 0.5 ml of 10 % phosphotungstic acid solution were mixed in a glass-stoppered brown centrifuge tube which was then left at room temperature for 5 min. The mixture was centrifuged at 3000 rpm for 10 min, and the supernatant decanted. Two ml of 1/12 N sulfuric acid and 0.3 ml of 10 % phosphotungstic acid solution were overlaid on the sediment, followed by thorough mixing. After re-centrifugation at 3000 rpm for 10 min, the sediment was mixed with 4.0 ml of distilled water. As a standard solution, 0.1 ml of 1. 1. 3. 3-tetraethoxypropane (5 nmol/ml) and 4.0 ml of distilled water were mixed. All mixtures were processed similarly thereafter. One ml each of 1 : 1 volume mixture of 0.67 % thiobarbituric acid and acetic acid (TBA test solution) was added to the mixture, which was then allowed to react at 95 ℃ for 60 min in a glass-stoppered brown centrifuge tube. The mixture was cooled in tap water for 5 min, then 5.0 ml of n-butanol was added. The tube was agitated vigorously by a mixer and the extracted mixture was centrifuged at 3000 rpm for 10 min. A portion of the n-butanol phase was removed for

fluorometric measurement at 515 nm excitation and 553 nm emission using a RF-510 fluorospectrophotometer (Shimazu Manuf. Co. Ltd., Tokyo, Japan).

Tissue TBARS analysis. Tissue TBARS analysis was done according to the method of Masugi *et al.* (30) with slight modification. Tissue homogenate (0.5 ml) was placed in a glass-stoppered brown centrifuge tube and gently mixed with 0.2 ml of 7 % sodium dodecylsulfate (SDS) solution. After adding 2.0 ml of 0.1 N hydrochloric acid, the mixture was shaken gently to dissolve the homogenate and 0.25 ml of 10 % phosphotungstic acid solution was added. Following addition of 1.0 ml of TBA test solution, the mixture was heated at 95 ℃ for 45 min. After cooling in tap water for about 5 min, 3.5 ml of n-butanol was added and the mixture was shaken vigorously. The mixture was centrifuged at 3000 rpm for 10 min and about 3 ml of n-butanol phase was removed for spectrophotometry at a wave length of 532 nm (Shimazu UV-100-01 Spectrophotometer). Tetraethoxypropane (5 nmole/ml) was used as the standard.

Measurement of serum α-tocopherol. Serum α-tocopherol was assayed by a fluorometric method (31). Serum (0.2 ml), 1.0 ml of distilled water and 1.0 ml of absolute ethanol were mixed in a glass-stoppered brown centrifuge tube, 5.0 ml of n-hexane added, and vigorously mixed on a mixer. The mixture was centrifuged at 3000 rpm for 10 min and a portion of the hexane phase was removed for fluorometric measurement at 286 nm excitation and 330 nm emission. Standards were prepared by dissolving α-tocopherol in absolute ethanol to make a final α-tocopherol concentration of 2 ng/ml.

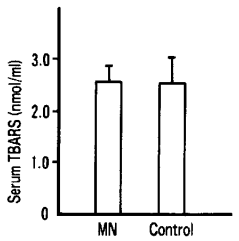
Tissue α-tocopherol analysis. α-Tocopherol analysis in tissues was done by the method of Taylor *et al.* (32). The usual saponification mixture contained 1.5 ml of homogenate, 0.5 ml of 25 % ascorbic acid and 1.0 ml of absolute ethanol. The mixture was preincubated at 70 ℃ for 5 min in a glass-stoppered centrifuge tube. Following addition of 1.0 ml of 10 N KOH, the mixture was saponified for 30 min at 70 ℃. After cooling in tap water, 4.0 ml of hexane was added and the saponified mixture shaken vigorously on a mixer. The mixture was centrifuged at 3000 rpm for 10 min and about 3 ml of the hexane phase removed for fluorometric measurement. The analytical conditions and external standard were the same as for measurement of serum α-tocopherol.

Tissue protein analysis. Protein analysis in tissues was done by the Biuret method (33). Homogenate (1.0 ml) and 4.0 ml of Biuret solution were mixed and reacted at room temperature for 30 min. If the mixture was not clear, kerosene ether was added and mixed to render the mixture clear. After centrifugation at 3000 rpm for 5 min, the Biuret phase was removed for spectrophotometry at a wave length of 540 nm. The protein concentration was determined from a standard protein curve obtained using albumin solutions (1 mg/ml to 10 mg/ml).

Serum cholesterol, GOT and GPT concentrations were assayed by enzyme methods (34, 35).

RESULTS

The serum TBARS, α-tocopherol and cholesterol concentrations in rats with myocardial necrosis (MN) are shown in Fig. 1, 2, 3. The α-tocopherol-cholesterol ratio is shown in Fig. 4. TBARS in MN rats was 2.56 ± 0.36 nmol/ml and 2.54 ± 0.50 nmol/ml in control rats. This difference was not statistically significant (Fig. 1). Serum α-tocopherol concentration in MN rats was 0.36 ± 0.06 mg/dl and 0.48 ± 0.08 mg/dl in control rats. This difference was statistically



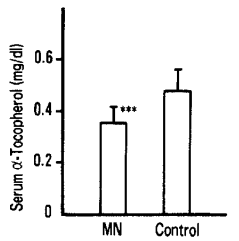
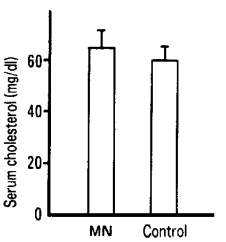


Fig. 1. *(left)* Serum TBARS of rats with myocardial necrosis (MN) and control rats (mean+SE). Number of MN : 10, number of control rats : 8.

Fig. 2. *(right)* Serum α-tocopherol of MN and control rats (mean+SE). Number of MN : 10, number of control rats : 8. *** indicates $p<0.01$



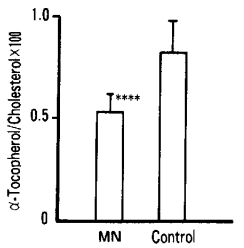


Fig. 3. *(left)* Serum cholesterol of MN and control rats (mean+SE). Number of MN : 10, number of control rats : 8.

Fig. 4. *(right)* Serum α-tocopherol-cholesterol ratio of MN and control rats (mean+SE). Number of MN : 10, number of control rats : 8. **** indicates $p<0.001$

significant ($p<0.01$) (Fig. 2). Serum cholesterol concentration in MN rats was 65.1 ± 6.9 mg/dl and 60.1 ± 5.8 mg/dl in control rats. This difference did not reach a statistically significant level (Fig. 3). The serum α-tocopherol-cholesterol ratio in MN rats was 0.54 ± 0.09, and 0.83 ± 0.15 in control rats. The ratio was significantly lower in the MN rats ($p<0.001$) (Fig. 4).

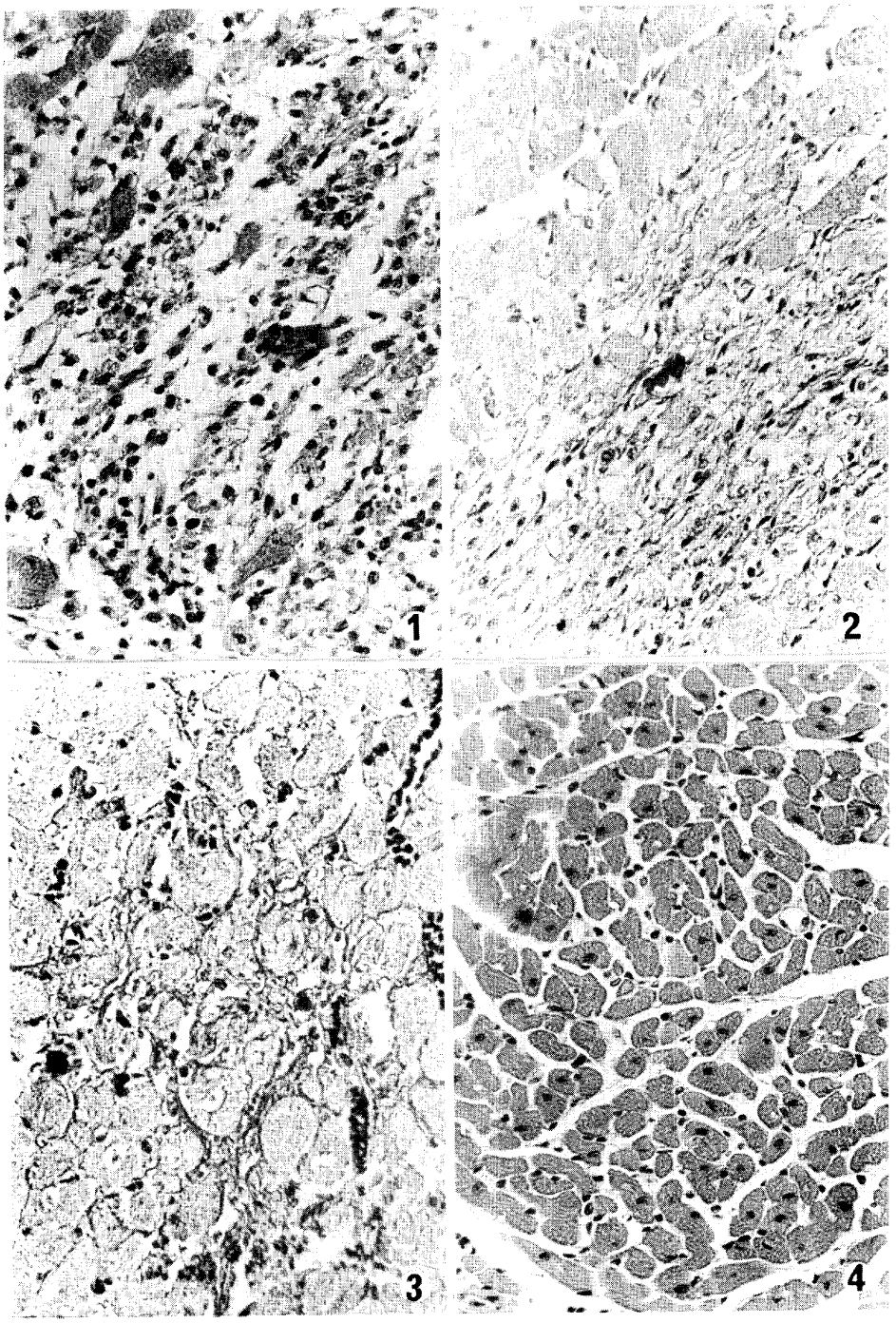
The time course of histological changes in myocardial tissue after induction of myocardial necrosis is shown in photos 1-4. Forty-eight hours after the first injection of isoproterenol, diffuse myocardial necrosis associated with cellular infiltration was seen (photo 1). This first appeared 12 h after the first injection and reached maximum intensity 48 h after the first injection. Necrotic tissues gradually regenerated thereafter. Decreased cell infiltration and fibrosis were seen in the specimen taken 7 days after the first injection (photo 2, 3). Myocar-

Photo. 1. Myocardium 48 h after the first injection of isoproterenol. Diffuse myocardial necrosis associated with cellular infiltration is seen. This first appeared 12 h after the first injection and reached maximum intensity by 48 h. (Hematoxylin-Eosin stain, ×400)

Photo. 2. Myocardium 7 days after the first injection of isoproterenol. Decreased cell infiltration and fibrosis are seen. (Hematoxylin-Eosin stain, ×400)

Photo. 3. Myocardium 7 days after the first injection of isoproterenol. (Azan stain, ×400)

Photo. 4. Myocardium of a control rat. (Hematoxylin-Eosin stain, ×400)


1
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dial tissues of control rats injected with distilled water are shown in photo 4.

Time course of changes in serum TBARS and α-tocopherol concentrations. Serum TBARS concentration rose to 3.00±0.11 nmol/ml ($p<0.01$) by 12 h after the first injection then slightly decreased by 24 h, and increased to 3.08 ± 0.51 nmol/ml by 36 h after the first injection (Fig. 5). The concentration gradually decreased

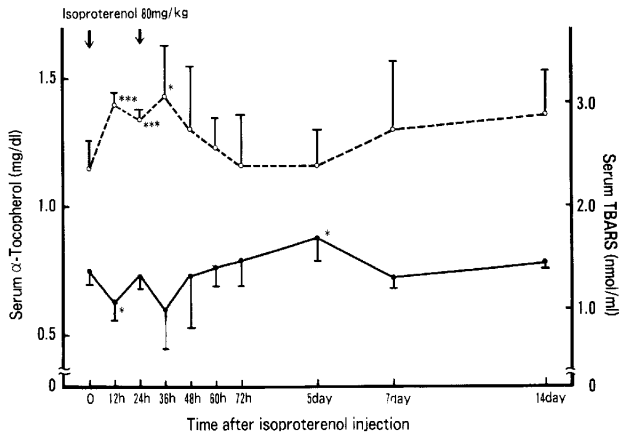

Fig. 5. Changes in serum α-tocopherol and TBARS after isoproterenol injection (mean±SE). Each group contained 4 to 5 rats. α-tocopherol (●), TBARS (○)
* indicates $p<0.05$, *** indicates $p<0.01$.

to 2.40 ± 0.35 nmol/ml by 5 days, rose slightly by 7 days and thereafter failed to vary significantly. At 14 days, the value was 2.89 ± 0.43 nmol/ml, which was slightly higher than the value before the first injection.

Serum α-tocopherol concentration decreased to 0.63 ± 0.07 mg/dl by 12 h after the first injection and then increased to 0.73 ± 0.05 mg/dl by 24 h and decreased to 0.60 ± 0.15 mg/dl by 36 h after the first injection. The concentration gradually rose, then decreased slightly by 7 days. The concentration was 0.78 ± 0.02 mg/dl at 14 days. The changes in serum TBARS and α-tocopherol with time showed a reciprocal pattern after the injection.

Time course of changes in TBARS and α-tocopheral in the hearts of rats injection with isoproterenol. TBARS in rat myocardial tissue was 0.70 ± 0.08 nmol/mg prot. and increased rapidly to 1.20 ± 0.42 nmol/mg prot. by 12 h after the injections ($p<0.05$) (Fig. 6). There was moderate increase in TBARS after the second injection. At 14 days, TBARS was 0.94 ± 0.12 nmol/mg prot. This value was higher than TBARS before injection ($p<0.02$).

In contrast, α-tocopherol in rat myocardial tissue decreased rapidly from 0.158 ± 0.015 μg/mg prot. to 0.118 ± 0.023 μg/mg prot. by 12 h after the injections ($p<0.01$). The concentration recovered to 0.151 ± 0.019 μg/mg prot. but decreased to 0.135 ± 0.012 μg/mg prot. by the second injection. It increased gradually up to 0.158 ± 0.032 μg/mg prot. by 60 h but had fallen by 72 h. By

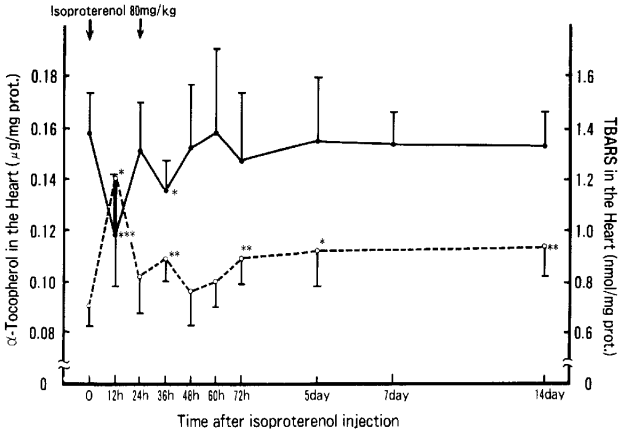

Fig. 6. Changes in α-tocopherol and TBARS in the heart after isoproterenol injection (mean ± SE). Each group contained 4 to 5 rats. α-tocopherol (●), TBARS (○)
* indicates $p<0.05$, ** indicates $p<0.02$, *** indicates $p<0.01$.

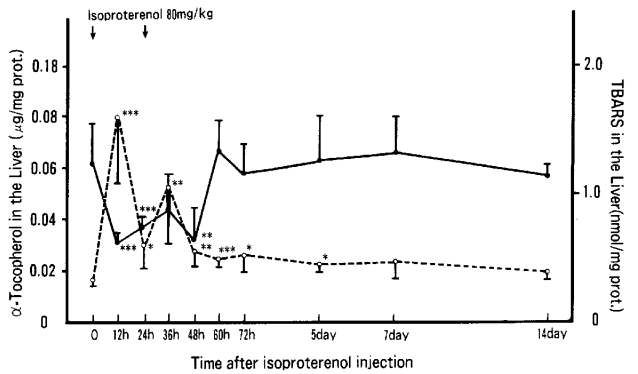

Fig. 7. Changes in α-tocopherol and TBARS in the liver after isoproterenol injection (mean ± SE). Each group contained 4 to 5 rats. α-tocopherol (●), TBARS (○)
* indicates $p<0.05$, ** indicates $p<0.02$, *** indicates $p<0.01$.

14 days, α-tocopherol concentration recovered to 0.153 ± 0.013 μg/mg prot.

Time course of changes in TBARS and α-tocopherol in rat liver tissues after isoproterenol injections. TBARS in rat liver tissue before the injection was 0.33 ± 0.05 nmol/mg prot. and had increased rapidly to 1.59 ± 0.54 nmol/mg prot. by 12 h after the first injection ($p<0.01$) (Fig. 7). It then decreased to 0.60 ± 0.19 nmol/mg prot., increased again up to 1.06 ± 0.47 nmol/mg prot. by the second njection ($p<0.02$), then decreased gradually to 0.38 ± 0.06 nmol/mg prot. at 14 days.

α-Tocopherol in rat liver tissue before injection was 0.062 ± 0.015 μg/mg prot. and decreased rapidly to 0.031 ± 0.003 μg/mg prot ($p<0.01$). Thereafter, it increased gradually but decreased again to 0.032 ± 0.012 μg/mg prot. by 24 h

after the second injection ($p < 0.02$). Next, it increased rapidly up to 0.067 ± 0.011 μg/mg prot. and reached a plateau. α-Tocopherol concentration at 14 days was 0.057 ± 0.004 μg/mg prot. This was slightly lower than the value before injection.

Time course of changes in serum GOT and GPT concentrations after the isoproterenol injections. GOT increased moderately after the injection (Fig. 8). Changes in GPT concentrations were minimal.

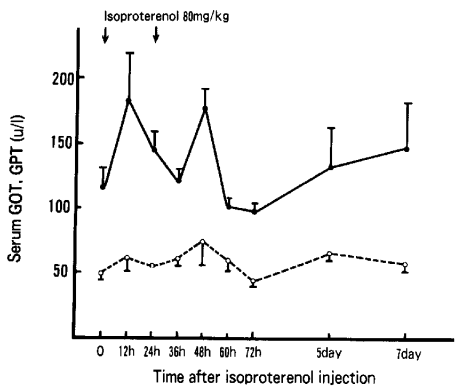

Fig. 8. Changes in serum GOT, GPT after isoproterenol injection (mean ± SE). Each group contained 2 to 5 rats.
GOT (●), GPT (○)

DISCUSSION

Rona *et al.* produced myocardial necrosis by injecting rats with isoproterenol (26, 27). This agent is known to stimulate the heart, to increase the oxygen requirement of heart muscle, and, at the same time, to cause dilatation of coronary vessels. Therefore, the basis of the myocardial necrosis is apparently not vascular occlusion or spasm, but myocardial hypoxia resulting from an oxygen supply that is inadequate to support the increased myocardial work. This experimentally induced MN closely resembles human myocardial infarction in various pathological aspects. In the present study, variations in lipid peroxides and α-tocopherol were measured using MN rats as the experimental model.

The concentrations of lipid peroxides in myocardial tissue taken from rats 24 h after the second injection of isoproterenol did not differ from that of the controls, but α-tocopherol in similar experimental conditions decreased significantly compared to that of controls. Since α-tocopherol is a lipid soluble vitamin, it is possible that decrease in α-tocopherol concentration is correlated to decrease in serum lipid concentration (36). However, serum cholesterol concentration did not decrease significantly during the period studied. Moreover, the α-tocopherol-cholesterol ratio was significantly lower when compared to that of controls, suggesting absolute reduction of α-tocopherol.

In the acute phase of human myocardial infarction, serum α-tocopherol levels were low and values of lipid peroxides were high (24). In the present

experiment, serum lipid peroxides did not increase after induction of myocardial necrosis. This difference seemed to be due to the time gap between the peak of serum lipid peroxide concentration and the time of blood collection, so the time course of changes in serum lipid peroxides and α-tocopherol was studied. Prompt increase in serum lipid peroxides and decrease in serum α-tocopherol after isoproterenol injection followed gradual recovery to pre-injection levels. This reflects well the time course of changes in human myocardial infarction (24).

It seemed reasonable to suppose that this increase in serum lipid peroxides concentration during the acute phase of myocardial necrosis reflects increased production of lipid peroxides in damaged tissue. Tomita *et al.* showed, using stroke prone spontaneously hypertensive rats (SHRSP), that the serum lipid peroxide concentration increased in the acute phase of apoplexy and that lipid peroxides also markedly increased in the brain and liver (37, 38). Yoshioka *et al.* reported that lipid peroxides in the liver of patients who died of apoplexy was higher than that of patients who died of gastric cancer (39). Furthermore, lipid peroxides in the heart of MN rats was higher than in controls (40). In the present study, the increase in lipid peroxides in tissue seemed to be higher in the liver than in the heart. The protein content of the liver is much higher than that of the heart, since the liver is the biggest organ in the rat body. Therefore, production of lipid peroxides in the liver may play an important role in increasing serum lipid peroxide concentration. In the present experiment, a large dose of isoproterenol was used to induce myocardial necrosis so the possibility of direct impairment of liver by the drug can not be ruled out. However, the elevation of serum GPT after the injection was mild compared to the elevation of serum GOT, suggesting that the degree of liver damage due to the drug was mild or negligible. Therefore, the increase in liver lipid peroxides may be due to impairment of oxygen metabolism following acute circulation failure resulting from myocardial necrosis.

In contrast to lipid peroxides, α-tocopherol decreased In the heart and liver after injection. The α-tocopherol concentration, together with the decrease in lipid peroxides, returned gradually to pre-injection levels. However, the rate of decreases of the α-tocopherol concentration was higher in the liver than in the heart. This might be due to a greater increase of lipid peroxides in the liver than in the heart. Rapid increase in the concentration of lipid peroxides in sera and tissues may cause consumption of α-tocopherol as an antioxidant, resulting in the decrease in the concentration of α-tocopherol.

Therefore, the degree of tissue damage was in reverse proportion to the concentration of α-tocopherol in the tissue. It was reported that glutathion peroxidase and SOD decreased after apoplexy attacks in SHRSP (38). Glutathion peroxidase and SOD activity in SHRSP that died within several days of a cerebral hemorrhage were low compared to that in rats that survived more than

8 days. The non-surviving rats seemed to be susceptible to damage by lipid peroxides. When SHRSP were fed continuously with α-tocopherol, the incidence of apoplexy did not change but the life span increased (41). The administration of α-tocopherol during the acute phase of apoplexy inhibited the increase in lipid peroxide concentration (25).

These results suggest a protective effect of α-tocopherol. Moreover, increase in lipid peroxides in lung damage due to oxygen was successfully inhibited by the administration of antioxidants (7). The cardiotoxicity of adriamycin, which might be due to increase in lipid peroxides, was reduced by administration of α-tocopherol (15, 16). On the basis of the present results and recently available data, a trial administration of large doses of antioxidants such as α-tocopherol to patients with fresh vascular accidents is warranted in an attempt to prevent tissue damage due to rapid increases in lipid peroxides. Administration of large doses of α-tocopherol to patients with apoplexy or myocardial infarction in the acute phase is needed to assess the clinical effectiveness of α-tocopherol.

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