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Arachidonate-induced Oxygen Radical Production and Cellular Damage in Ischemic-Reperfused Heart of Rat

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ABSTRACT

The present study was conducted to assess the possible contribution of arachidonic acid to generation of reactive oxygen metabolites and myocardial damage in ischemic-reperfused heart. Langendorff preparations of isolated rat heart were made ischemic by hypoperfusion (0.5 ml/min) for 45 min, and then followed by normal oxygenated reperfusion (7 ml/min). The generation of superoxide anion was estimated by measuring the SOD-inhibitable ferricytochrome C reduction. The myocardial cellular damage was observed by measuring LDH released into the coronary effluent. Oxygenated reperfusion following a period of ischemia produced superoxide anion, which was inhibited by both indomethacin (60 nmole/ml) and ibuprofen (30 μg/ml). Sodium arachidonate (10^{-7}-10^{-2} μg/ml) administered during the period of oxygenated reperfusion stimulated superoxide anion production dose-dependently. The rate of arachidonate-induced superoxide generation was markedly inhibited by indomethacin, a cyclooxygenase inhibitor; nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, and by eicosatetraynoic acid (ETYA), a substrate inhibitor of arachidonic acid metabolism. The release of LDH was increased by Na arachidonate and was inhibited by superoxide dismutase. The release of LDH induced by arachidonic acid was also inhibited by indomethacin, NDGA and ETYA. In conclusion, the presnt result suggests that arachidonic acid metabolism is involved in the production of reactive oxygen metabolite and plays a contributory role in the genesis of reperfusion injuy of myocardium.

Key Words: Arachidonic acid, Reactive oxygen metabolite, Ischemic-reperfusion injury

INTRODUCTION

Reactive oxygen free radicals (O_2^-; superoxide anion; H_2O_2, hydrogen peroxide; OH•, hydroxyl radical) may be involved in the genesis of reperfusion injury of ischemic hearts. It has been suggested that intracellular accumulation of reducing equivalents during ischemia leads to increased generation of oxygen free radicals when ischemic tissue is reoxygenated by reperfusion (Hess & Manson, 1984). This has been supported by the observations that scavengers of oxygen free radicals and antioxidants protect the hearts from the development of oxidative damages during reperfusion of ischemic hearts (Kim & Akera, 1987; Simpson et al., 1987; Van der Vusse & Reneman, 1985). Many investigators have reported that oxygen free radicals are produced from various sources, including xanthine oxidase, mitochondria and leukocyte in normal as well as in certain pathological conditions (Chambers et al., 1985; Forman & Boveris, 1982; Simpson & Lucchesi, 1987).

Arachidonic acid is a major polyunsaturated fatty acid consisting lipid bilayers of myocardial sarcolemmal membrane. The experimental con-

ditions with high oxidative stresses cause peroxidation of sarcolemmal lipids and resultant release of unsaturated fatty acid residues from the phospholipids (Freeman & Crapo, 1982; Kramer et al., 1984). In the ischemic-reperfused hearts, it has been reported that arachidonic acid is released (De Deckere et al., 1977), and the myocardial cellular damages are prevented by non-steroidal antiinflammatory drugs, which inhibit arachidonic acid metabolism (Gunn et al., 1985; Karmazyn, 1986). In in vitro studies, it has been also reported that arachidonic acid produces superoxide anion during its metabolism via both cyclooxygenase and lipooxygenase pathways (Kontos 1984, 1985; Kukreja et al., 1986).

In spite of the potential effects of arachidonic acid metabolism on cardiac function, few studies have been reported on the exact role of arachidonates in oxygen free radical-mediated myocardial changes. Therfore, the present study was performed to investigate the contributions of arachidonic acid, in particular as a possible source of oxygen free radicals, in the development of ischemic-reperfusion injury of heart. Effects of exogenously administered arachidonic acid and its metabolic inhibitors on oxygen radical production and myocardial damage were observed in ischemic-reperfusion model of isolated hearts of rats.

MATERIALS AND METHODS

Isolated heart preparations and perfusion

Hearts were quickly removed from heparinized (100 IU, i.p.) Sprague-Dawley rats of either sex weighing about 200 g. Isolated hearts were perfused through the aorta in the retrograde manner (Langendorff preparation) with oxygenated Krebs-Henseleit (K-H) solution (in mM: NaCl 118, $NaHCO_3$ 27.2, KCl 4.8, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1, $CaCl_2$ 1.25, glucose 11.1, pH 7.4 with 95% O_2+5% CO_2) under a constant pressure of 100 cm H_2O or at a constant flow of 7 ml/min. After equilibration for 15 min, perfusion was switched to ischemic K-H solution (saturated with 95% N_2+5% CO_2, equimolar mannitol instead of glucose)at a reduced flow rate of 0.5 ml/min for 45 min. At the end of ischemic period, perfusion was returned to oxygenated K-H solution and normal flow rate to induce reperfused state of ischemic heart.

Arachidonic acid (10^{-7}~10^{-2} μg/ml) ws administered during the reperfusion period by peristaltic pump connected to the aortic cannula. Archidonic acid stock solution (10 mg/ml) was made by dissolving Na arachidoniate (Sigma Chem. Co., U.S.A.) in sodium carbonate (3 mg/ml) solution and soted at −70°C. Just before use, the stock solution was diluted to desired concentrations. Various inhibitors of arachidonic acid metabolism were used to assess the effects of arachidonic acid in the ischemic-reperfused hearts. Cyclooxygenase inhibitors (indomethancin 60 nmoles/ml, ibuprofen 30 μg/ml), a lipooxygenase inhibitor (nordihydroguaiaretic acid, NDGA, 0.1 μmole/ml) and a substrate inhibitor (eicosatetraynoic acid, ETYA, 1 μg/ml) were adiministered via aortic cannula from 25 min before returning to the normal oxygenated flow and throughout the reperfusion period.

Lactic dehydrogenase (LDH) release

LDH released into the coronary perfusate was measured as an index of myocardial cellular damage. Coronary perfusate was collected at the indicated time intervals during first 5 min of reperfusion period. LDH activity was assayed by UV-spectrophotometric method (Bergmeyer & Bernt, 1974). A 0.5 ml aliquot of sample was added into a cuvette containing 2.5 ml of reaction mixture consisted of 48 mM phosphate buffer (pH 7.5), 0.6 mM pyruvate and 0.18 mM NADH. The rate of change of absorbance was measured with UV-spectrophotometer (Hewlett-Packard, 8452A) at 340 nm and 25°C.

Superoxide anion production

Reduction of exogenusly administered ferricytochrome C was used to measure superoxide anion production (Salin & McCord, 1974). Starting with reperfusion, ferricytochrome C solution (100 μM) either containing SOD (300 U/ml) and inhibitors of arachidonic acid metabolism or not was infused through the aortic cannula at a rate of 0.5 ml/min. The coronary perfusate was collected at an interval of 30 sec. Immediately after determination of volume, optical density was measured at 418 nm with UV-spectrophotomer.

Superoxide anion production was estimated from SOD-inhibitable portion of ferricytochorme C reduction. The extent of ferricytochrome C reduction during reperfusion was calculated by using the difference of molar extinction coefficient ($\triangle E_{418}=7.0\times10^4$/M/Cm) between reduced ferrocytochrome C and oxidized ferricytochrome C. The total amount of cytochrome C in the perfusate was estimated after full reduction of ferricytochrome C by addition of a few crystals of sodium dithionite.

Chemicals

Sodium arachidonate, superoxide dismutase (SOD), ferricytochrome C, nordihydroguaiaretic acid (NAGA), indomethacin, ibuprofen were purchased from Sigma Chemical Co. (St. Louis, U.S.A), and eicosatetraynoic acid (ETYA) was a gift from Hoffman-LaRoche (Nutley, U.S.A.). All other reagents were of analytical grade.

RESULTS

Archidonate-induced superoxide anion production

Reduction of exogenous ferricytochrome C was increased upon reperfusion of previously ischemic rat hearts. The peak reduction (8.2 nmol/30 sec/g wet wt) was observed at 2 min after starting reperfusion. SOD (300 U/ml) added to the coronary flow inhibited the reduction of ferricytochrome C to the value of 5.2 nmol/30 sec/g wet wt. Indomethacin and ibuprofen, the non-steroidal antiinflammatory drugs which inhibit cyclooxygenase, prevented the reduction of ferricytochrome C as almost same extent as SOD (Fig. 1). When arachidonate was administered during reperfusion, ferricytochrome C reduction was increased in a dose-dependent manner. With 10^{-3} μg/ml of arachiodonate the peak reduction was 22.5 nmol/30 sec/g wet wt at 1 min after the reperfusion. This arachidonate induced ferricytochrome C reduction was significantly suppressed by simultaneous administration of SOD to the value of 13.5 nmole/30 sec/g wet wt (Table 1, Fig. 2).

Effects of inhibitors of arachidonate metabolism on superoxide anion production

Indomethacin, an inhibitor of cyclooxygenase decreased the arachidonate-induced reduction of ferricytochrome C to the same degree as SOD. Similarly, the ferricytochrome C reduction was reduced by concomittant administration of NDGA, an inhibitor of lipooxygenase pathway of arachidonate metabolism (Table 1, Fig. 3). ETYA, which inhibits both cyclooxygenase and lipooyxgenase pathways by competing with natural substrates, also suppressed the reduction of ferricytochrome C somewhat larger extent than SOD (Table 1, Fig. 4).

Arachidonate-induced myocardial damage

Activity of LDH was measured in the coronary

Table 1. Effects of superoxide dismutase and inhibitors of arachidonate metabolism on Na arachidonate-induced superoxide anion generation in hypoxic-reperfused heart of rat

Treatment	No. of exp.	Max. ferricytochrome C reduction[1] (n moles/30 sec./g wet wt.)
Control	7	8.22±0.96
Control+SOD	7	5.19±0.84*
Na Arachidonate, 10^{-3} μg/ml	10	22.46±2.24
〃 +SOD[2]	11	13.46±1.98**
〃 +Indo[3]	8	14.52±1.47**
〃 +NDGA[4]	6	11.27±1.86**
〃 +ETYA[5]	7	10.99±1.30**

1: Mean±SEM, 2: Superoxide dismutase, 300 U/ml, 3: Indomethacin, 60 nmole/ml
4: Nordihydroguaiaretic acid, 0.1 μmole/ml, 5: Eicosateraynoic acid, 1 μg/ml, *: $p<0.01$ vs Control
**: $p<0.01$ vs Na arachidonate

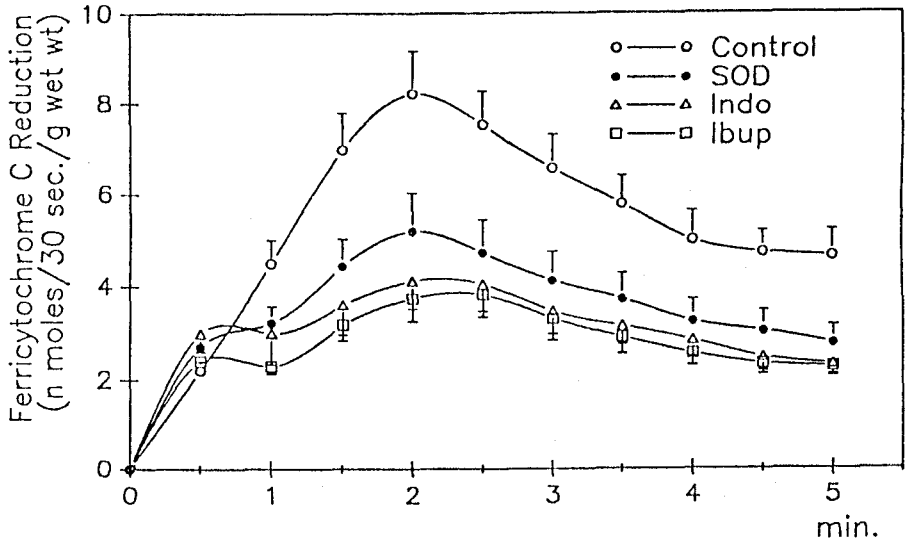

Fig. 1. Ferricytochrome C reduction upon oxygenated reperfusion of hypoxic myocardium.
Langendorff preparations of isolated rat heart were made hypoxic by hypoperfusion (0.5 ml) for 45 min, and then followed by oxygenated reperfusion (7 ml/min). Starting with the reperfusion, ferrictytochrome C (100 uM) was infused to the heart by way of aortic cannula at a rate of 0.5 ml/min. Superoxide dismutase (SOD, 300 U/ml), indomethacin (Indo, 60 nmole/ml) and ibuprofen (Ibup, 30 ug/ml) were administered from 25 min before reperfusion. Results are Mean±SEM of 6~8 experiments.

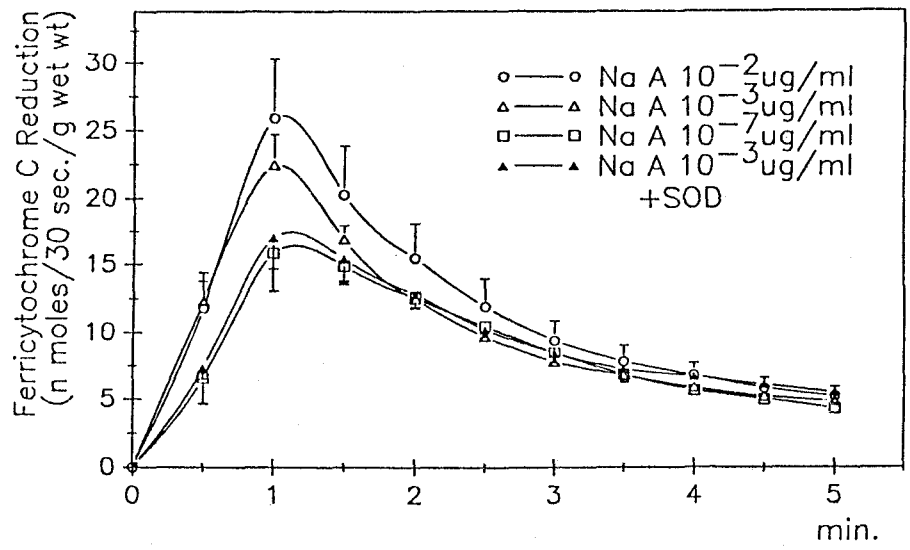

Fig. 2. Arachidonic acid-induced ferricytochrome C reduction in hypoxic-reperfused heart of rat.
Na arachidonate (NaA, 10^{-7}~10^{-2} ug/ml) was administered with start of oxygenated reperfusion following 45 min of hypoperfusion. Others are same as in Fig. 1. Results are Mean±SEM of 7~10 experiments.

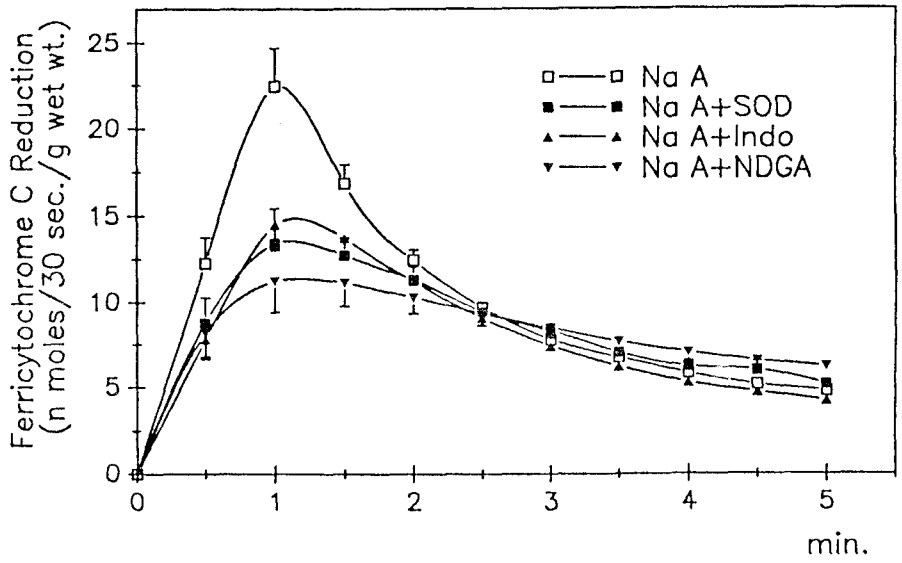

Fig. 3. Effects of cyclooxygenase and lipooxygenase inhibitors on arachidonate-induced ferricytochrome C reduction in hypoxic-reperfused heart of rat. Na arachidonate (NaA, 10^{-3} ug/ml) was administered with start of oxygenated reperfusion. Indomethacin (Indo, 60 nmole/ml) and nordihydroguaiaretic acid (NDGA, 0.1 umole/ml) were administered from 25 min before and throughout the reperfusion period. Others are same as in Fig. 1. Results are Mean±SEM opf 7~11 experiments.

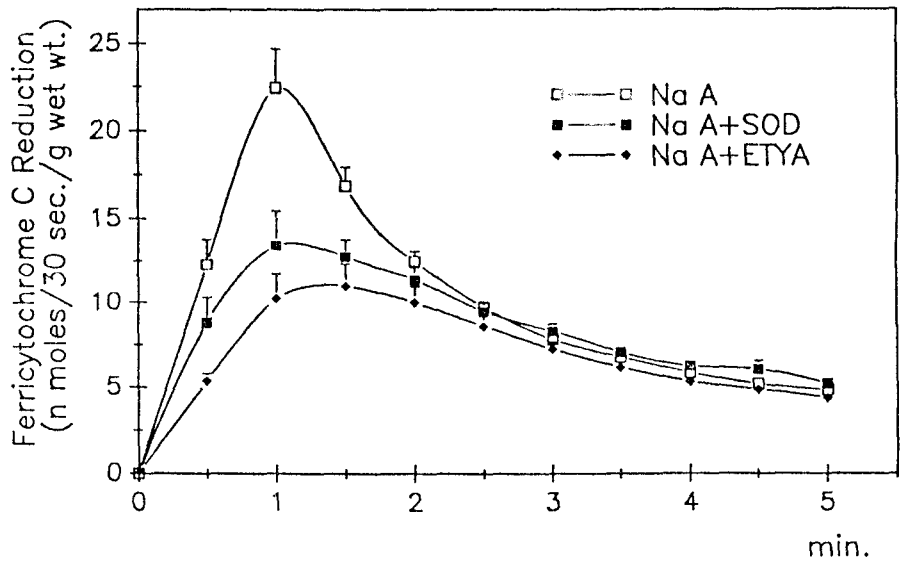

Fig. 4. Effect of substrate inhibitor of arachidonic acid metabolism on arachidonate-induced ferricytochrome C reduction.

Eicosatetraynoic acid (ETYA, 1 ug/ml) was administered during a period (45 min) of hypoperfusion. Others are same as in Fig. 1. Results are Mean±SEM of 7 experiments.

Table 2. Effects of superoxide dismutase and inhibitors of arachidonate metabolism on Na arachidonate-induced LDH release in hypoxic-reperfused heart of rat

Treatment	No. of exp.	LDH(units/5 min./g wet wt.)[1]
Control	7	150.4±14.4
Na Arachidonate, 10^{-3} μg/ml	7	189.2±12.6*
〃 +SOD[2]	6	36.5± 9.1**
〃 +Indo[3]	6	123.0±21.6**
〃 +NDGA[4]	7	101.0±13.2**
〃 +ETYA[5]	6	96.4± 9.1**

1: Mean±SEM, 2: Superoxide dismutase, 300 U/ml, 3: Indomethacin, 60 nmole/ml
4: Nordihydroguaiaretic acid, 0.1 μmole/ml, 5: Eicosateraynoic acid, 1 μg/ml, *: $p<0.01$ vs Controi
**: $p<0.01$ vs Na arachidonate

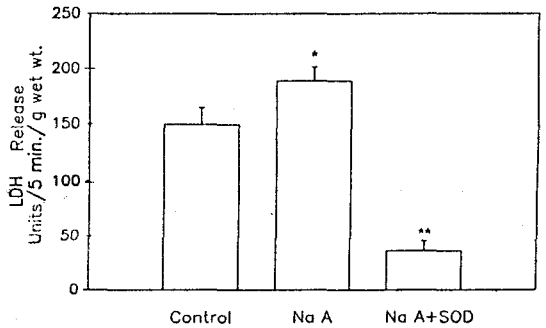

Fig. 5. Effect of arachidonic acid and superoxide dismutase on LDH release in hypoxic-reperfused heart of rat.

Activity of lactic dehydrogenase released into the coronary effluent upon oxygenated reperfusion was measured as described in the text. The methods of preparation of hypoxic-reperfused heart and administration of Na arachidonate(NaA, 10^{-3} ug/ml) and superoxide dismutase (SOD, 300 U/ml) were same as in Fig. 1 & 2. *: $p<0.05$ vs Control **: $p<0.01$ vs NaA.

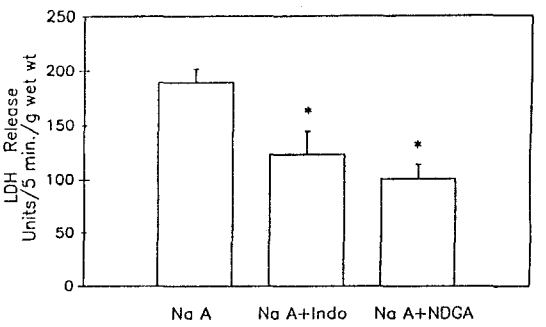

Fig. 6. Effects of cyclooxygenase and lipooxygenase inhibitors on arachidonate induced LDH release in hypoxic-reperfused heart of rat.

Methods of administrations of Na arachidonate(NaA), indomethacin(Indo), nordihydroguarietic acid (NDGA) and others are same as in Fig. 3 & 5. Results are Mean±SEM of 7 experiments.
*:$p<0.01$ vs NaA.

perfusate as an index of myocardial cellular injury. LDH released during first 5 min of reperfusion was 150 U/g wet wt. When arachidonate (10^{-3} μg/ml) was administered, LDH activity was increased to 189 U/5 min/g wet wt and it was significantly decreased with SOD treatment (36.5 U /5 min/g wet wt) (Table 2, Fig. 5). Simultaneous additions of the arachidonate inhibitors significantly prevented the LDH release induced by exogenously administered Na-arachidonate to the same degree as SOD (Table 2, Fig. 6 & 7).

DISCUSSION

The involvement of reactive oxygen species in oxidative damages during reperfusion of ischemic heart has been observed in vitro as well as in vivo studies, in which oxygen radical scavengers and antioxidants protect the hearts from reperfusion injury (Hess & Manson, 1984; Kim & Akera, 1987; Simpson et al., 1987; Van der Vusse

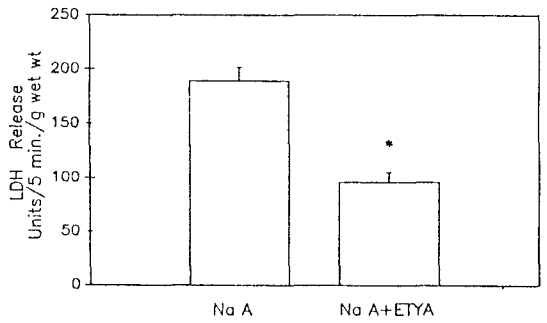

Fig. 7. Effect of substrate inhibition of arachidonic acid metabolism on arachidonate-induced LDH release in hypoxic-reperfused heart of rat.

Methods of administrations of Eicosatetraynoic acid(ETYA), Na arachidonate, and others are same as in Fig. 4 & 5. Results are Mean±SEM of 7 experiments. *: $p<0.01$ vs NaA.

& Reneman, 1985). Although there are many discussions on the pathophysiological significances, many intra- and extracellular mechanisms have been suggested as the sources of oxygen free radical production in ischemic-reperfused hearts. Steps of electron transport chain of mitochondria (Forman & Boveris, 1982), infiltrated leukocytes in the ischemic region (Simpson & Lucchesi, 1987), coronary endothelial xanthin oxidase system (Chambers et al., 1983; Lim & Kim, 1988) and catecholamines released from sympathetic nerve terminals in the ischemic lesion (Singal et al., 1983) are among the candidates. The present study suggests also that arachidonic acid is another possible canidate for the production of oxygen free radicals in the ischemic-reperfused heart of rat.

Arachidonic acid and its metabolic products have been reported to be increased in the ischemic-reperfused hearts (Berger et al., 1976; Chien et al., 1985; Gunn et al., 1985; Karmazyn, 1986), and also in the hearts treated with the exogenous oxygen radical-generating system (Basu & Karmazyn, 1987). When arachidonate metabolism is inhibited by the non-steroidal antiinflammatory drugs in the ischemic hearts, recovery of cardiac functions on reperfusion is much better than that seen in the absence of the drugs (Karmazyn et al., 1981; Karmazyn 1986). These observations may indicate that endogenous arachidonate metabolism mediates, at least in part, ischemia and reperfusion-associated myocardial dysfunction.

Several investigators have reported that the functional and morphological abnormalities of cerebral blood vessels in acute hypertension are prevented by pretreatments with the inhibitors of arachidonate metabolism or with the oxygen free radical scavengers (Kontos et al., 1981; Wei et al., 1981). And the cerebrovascular abnormalities seen in acute hypertension were reproduced either by topical application of arachidonate (Kontos et al., 1984) or by exogenous oxygen radical preoducing xanthine oxidase system (Wei et al., 1985). This is thought to be indicative that oxygen free radicals are generated in the course of acute hypertension in association with increased arachidonate metabolism, and that these oxygen radicals are responsible for the vascular injuries. Similarly, in the present study with isolated hearts, exogenous administration of arachidonic acid produced myocardial admage which was prevented by treatments with inhibitors of arachidonate metabolism as well as with an oxygen radical scarvenger, SOD. Furthermore, the administration of arachidonate showed to increase the generation of superoxide anion, which was significantly reduced by metabolic inhibitors of arachidonic acid. It is considered from these results that, also in the myocardial tissue, arachidonic acid may play a contributory role in the genesis of cellular damage in association with production of oxygen free radicals.

However, the detailed mechanism of oxygen radical production during arachidonate metabolism in the myocardial cell has not been firmly established yet. In in vitro studies with microsomal fractions from ram seminal vesicles or purified enzymes, many investigators (Eagan et al., 1981; Kalyanaraman et al., 1982; Kukreja et al., 1986) observed that prostaglandin synthetase produced superoxide radical. Prostaglandin synthetase is a hemoprotein which has two activities; that is responsible for the oxidation of arachidonate to the hydroperoxide PGG_2 (cyclooxygenase activity) and for the peroxidation of the 15-hydroperoxy group of PGG_2 to a 15-hydroxy group, thus producing PGH_2 (hydroperoxidase activity). The prostaglandin hydroper-

oxidase, like other peroxidases, is capable of oxidizing a large number of reducing cosubstrates, including the reduced pyridine nucleotides (NADH, NADPH). These oxidations frequently follow chain reactions involving the formation of free radicals (below) (Eagan et al., 1979, 1981; Kukreja et al., 1986).

$$P + P\text{-}OOH + NAD(P)H \rightarrow P' + P\text{-}OH + \cdot NAD(P)$$
$$\cdot NAD(P) + O_2 \rightarrow NAD(P)^+ + O_2^{-}\cdot$$

P': Prostaglandin hydroperoxidase
P-OOH: Prostaglandin hydroperoxide, PG G_2
P-OH: Prostaglandin hydroxide, PG H_2
· NAD (P): NAD (P) radical
$O_2^{-}\cdot$: Superoxide radical

In connection with the ischemic-reperfusion injury of myocardial tissue, the findings that prostaglandin hydroperoxidase is capable of oxidizing NADH and NADPH in a series of reactions which involve the formation of the oxygen radicals are of particular interest. Since pyridine nucleotides are known to be existed largely in reduced state in the ischemic myocardial cells (Jennings et al., 1981), these reduced pyridine nucleotides may promote the production of oxygen radicals during arachidonate metabolism in the ischemic-reperfused hearts.

In the present study, inhibitors of lipooxygenase, NDGA and ETYA also prevented both myocardial damage and superoxide anion production induced by exogenously administered arachidonic acid. These results may indicate that arachidonate metabolism via lipooxygenase pathway is also responsible for the prodcution of oxygen radical-mediated myocardial damage. Similarly in the cerebral tissue, Christman et al (1984) reported that topical application of 15-HPETE (15-hydroperoxyeicosatetraenoic acid), a lipooxygenase product of arachidonate metabolism produced cerebrovascular abnormalities, which were prevented by oxygen radical scavengers, SOD and catalase. It should be noted that 15-HPETE is as good substrate of the prostaglandin hydroperoxidase as the natural substrate PG G_2 (Eagan et al., 1979), and that superoxide radicals are also generated during the action of lipooxygenase in the presence of NADH and NADPH (Kukreja et al., 1986).

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= 국문초록 =

허혈-재관류 적출심장에서 Arachidonic Acid에 의한 산소라디칼 생성 및 심근손상

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허혈심근의 재관류시 arachidonic acid가 반응성 산소대사물의 발생원으로서 심근 손상에 미치는 영향을 검토하였다. Langendorff 관류장치를 이용하여 흰쥐 적출심장을 0.5 ml/min의 저용량으로 관류 (45분)한 후 정상관류 (7 ml/min)로 복귀 시키므로써 실험적인 허혈-재관류 심장을 만들었다. 재관류시 Na arachidonate (10^{-7}~10^{-2} μg/ml)를 투여한 후 superoxide anion 생성을 관찰하고, 심근 손상의 지표로 lactic dehydrogenase(LDH)유리를 측정 하였으며 이들에 대한 각종 arachidonic acid 대사 억제 약물의 영향을 비교 검토하였다. Superoxide anion 생성은 SOD-억제성 ferricytochrome C 환원 반응을 이용하였다.

연구성적은 다음과 같다.

1) 저용량 관류후 재관류시 ferricytochrome C환원은 superoxide dismutase (SOD, 300 U/ml) 및 indomethacin (60 nmole/ml), ibuprofen (30 μg/ml)에 의하여 억제되었다.

2) Na arachidonate는 용량의존적으로 ferricytochrome C 환원을 증가 시켰으며 반응성 산소대사물 제거효소인 superoxide dismutase (SOD, 300 U/ml)에 의하여 현저히 억제되었다.

3) Na arachidonate (10^{-3} μg/ml)에 의한 superoxide anion 생성은 cyclooxygenase 억제약물인 indomethacin (60 nmol/ml), lipooxygenase 억제약물인 nordihydroguaiaretic acid (NDGA, 0.1 μmole/ml), arachidonic acid의 substrate inhibitor인 eicosatetraynoic acid (ETYA, 1 μg/ml)에 의하여 현저히 억제되었다.

4) Na arachidonate는 LDH 유리를 증가시켰으며 SOD에 의하여 유의하게 억제 되었다.

5) Na archidonate에 의한 LDH 유리증가는 indomethacin, NDGA, ETYA에 의하여 유의하게 억제되었다.

이상의 결과로 흰쥐의 허혈-재관류심근에서 arachidonic acid는 그 대사 과정에서 반응성 산소대사물을 발생하고 이는 심근세포손상에 부분적으로 기여할 수 있을 것으로 여겨졌다.