Altered Lipoxygenase Metabolites and Leukocyte Involvement in an Acute Occlusion-Reperfusion Model of Canine Myocardial Infarction

TSUNEHIKO KUZUYA, M.D., SHIRO HOSHIDA, M.D., MASASHI NISHIDA, M.D.
YONJUN KIM, M.D., TAKENOBU KAMADA, M.D.
AND MICHHIKO TADA, M.D.*

We compared amounts of lipoxygenase products with the extent of leukocyte infiltration in the ischemic myocardium with an occlusion-reperfusion model of open-chest dog. Changes in peripheral leukocyte count and leukocyte function estimated by neutrophil aggregation induced by calcium ionophore A23187 were also examined. The ischemic tissue (120 ± 40 ng/g, mean ± SEM) showed a marked increase in 12-hydroxyeicosatetraenoic acid (HETE) production compared with the normal tissue (13 ± 1 ng/g, p < 0.01). The production of 5-HETE in the ischemic tissue was also augmented as well. When we examined the correlation between production of either 12-HETE or 5-HETE and leukocyte infiltration in the ischemic tissue, the former was augmented markedly in proportion to the extent of the latter. Leukocyte count in peripheral circulation was gradually increased after reperfusion. Similarly, neutrophil aggregation was significantly augmented during reperfusion. These results indicate that production of lipoxygenase metabolites associated with leukocyte infiltration in the reperfused ischemic tissue was increased during the course of myocardial infarction, which was accompanied by activation of leukocyte in peripheral circulation. Further studies should be done to clarify the importance of lipoxygenase metabolites in the evolution of reperfusion-induced myocardial injury.

It has been well recognized that an imbalanced synthesis of thromboxane A₂ (TXA₂) and prostacyclin between platelets and vascular wall plays an important role in the disintegration of coronary circulation, leading to the aggravation of myocardial ischemia.¹ ² We previously reported that the infarct size could be effectively limited by the administration of a TXA₂ synthetase inhibitor during the hyperacute phase of myocardial infarction.³ As yet, however, the mechanism by which such platelet activation occurs in the ischemic myocardium remains to be seen. Recent experimental evidences suggest that leukotrienes and hydroperoxyeicosatetraenoic acids (HPETEs), lipoxygenase products from leukocytes, cause microcirculatory disorders by their direct actions⁴ ⁵ or by modulating cyclooxygenase pathways in platelets and vascular endothelium.⁶ ⁷ The issue of leukocyte activation is also important in understanding the pathophysiology of inflammatory reaction during ischemic myocardial cell damage. In fact, interventions to deplete circulating neutrophils were

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Division of Cardiology, First Department of Medicine and *Department of Pathophysiology, Osaka University School of Medicine, Osaka, Japan
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Mailing address: Tsunehiko Kuzuya, M.D., Division of Cardiology, First Department of Medicine, Osaka University School of Medicine, 1-1-50 Fukushima, Fukushima-ku, Osaka 553, Japan

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found to suppress the expansion of myocardial necrosis. In this study, we evaluated myocardial lipoxygenase activity and its relation to leukocyte infiltration in the ischemic tissue with an acute occlusion-reperfusion model of open-chest dog.

**Measurement of lipoxygenase metabolites by high pressure liquid chromatography**

To determine the amounts of lipoxygenase products, reverse phase high pressure liquid chromatography (HPLC) was performed using a C_{18} column (YMC Model A-302). A variable wave-length ultraviolet (UV) visible spectrophotometer (UVIDEC-100-III, Japan Spectroscopic Co.) was used for detection. The pump was from Hitachi (model 655 A-11). Solvent consisted of acetonitrile: distilled water: methanol: acetic acid (30 : 25 : 20 : 0.1). Lipoxygenase products were eluted at a rate of 1.0 ml/min with monitoring for UV absorbance at 270 nm from 0 to 12 min for the detection of leukotriene B_{4} (LTB_{4}) and at 237 nm from 13 to 40 min for mono hydroxylicosatetraenoic acids (HETEs) (Fig. 1, panel A). Peak height was used to quantify these lipoxygenase products. Peak height observed after repeated injections of a single amount (2–200 ng) of each authentic eicosanoid was highly reproducible, and there was a linear relationship between the amounts of each eicosanoid injected and the observed peak height (Fig. 1, panel B).

**Lipoxygenase metabolites in reperfused ischemic myocardium**

We quantified the amounts of lipoxygenase products in reperfused ischemic myocardium. Adult mongrel dogs were anesthetized with pentobarbital sodium (30 mg/kg, i.v.), intubated with a cuffed endotracheal tube and artificially ventilated with room air. The heart was exposed by a thoracotomy at the 5th intercostal space. The left anterior descending coronary artery was dissected free at the level of the first major diagonal branch and a silk ligature passed around it. The coronary artery was occluded for 90 min followed by 5 hours of reperfusion. Then, 30 ml of whole blood was collected into a tube containing heparin (10 units/ml in final concentration). The coronary artery was re-occluded and then cannulated distal to the snare. The blood previously withdrawn was infused into the
TABLE 1  PRODUCTION OF MONO HETEs
IN THE NORMAL AND ISCHEMIC MYOCARDIUM

<table>
<thead>
<tr>
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<th>12-HETE (ng/g ± SEM)</th>
<th>5-HETE</th>
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<tbody>
<tr>
<td>Normal</td>
<td>non-stimulated 13 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>Ischemic</td>
<td>non-stimulated 120 ± 40*</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td>stimulated 268 ± 60**</td>
<td>50 ± 7</td>
</tr>
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*aIschemic myocardium was cocultured with
A23187 (20 μM) and arachidonate (100 μM),
HETE = hydroxyeicosatetraenoic acid; ND = not detected.
*p < 0.05, **p < 0.01 vs Normal.

Cannulated artery under mean blood pressure. Simultaneously, 20 ml of Evans blue dye (Sigma Chemical Co.) were injected into the femoral vein so that the coronary vascular bed perfused with the pre-occluded coronary artery (area at risk) could be demarcated clearly. The dog was killed and its heart was removed.

Myocardial tissue from the normal area of the posterior ventricular wall and from the ischemic area (area at risk) were finely chopped on ice in saline and were spun down. The supernatants were removed and the tissue was incubated in Dulbecco's phosphate buffered saline (pH 7.4) at 37°C. After 15 min incubation, the reaction was stopped by adding ethanol and the incubation medium was spun down. The supernatant was collected and diluted with distilled water to bring the final concentration of ethanol to 15%, and then was acidified to pH 3 by adding hydrochloride (1N). Lipoxygenase metabolites were extracted and partially purified from the acidified supernatants by chromatography on Sep-Pak C18 cartridges (Waters Associates), according to the modified method described by Powell10. Briefly, the acidified supernatant was applied on the cartridge, which was eluted sequentially with 20 ml of 15% ethanol, 20 ml of distilled water, 20 ml of petroleum ether, and 10 ml of ethyl acetate. The ethyl acetate fractions were evaporated and replaced with 250 μl of methanol/distilled water (3 : 2). Reverse phase HPLC was performed to examine lipoxygenase metabolites as described above.

Figure 2 represents typical traces of analysis for lipoxygenase products in the normal and ischemic myocardium by HPLC. The ischemic tissue showed marked production of 12-HETE as well as other metabolites like 15-HETE or 5-HETE compared with the normal tissue. Under these conditions, LTB4 production was not detectable. Amounts of 5-HETE and 12-HETE produced in the normal and ischemic myocardium are summarized in Table I. The ischemic myocardium showed a marked increase in 12-HETE production (120 ± 40 ng/g) compared with the normal myocardium (13 ± 1 ng/g, p < 0.05). The production of 5-HETE was augmented as well as in the ischemic myocardium. These increases in HETE production were found to be augmented when the myocardial tissue was coincubated with calcium ionophore A23187 and...
arachidonate (Table I).

It has been noted that infiltration of leukocytes, mainly neutrophils, into the ischemic myocardium is enhanced by reperfusing the obstructed coronary artery. Since such infiltrated leukocytes might cause deleterious effects on the jeopardized myocardium by producing vasoactive lipoxygenase metabolites, we examined the correlation between production of 12-HETE or 5-HETE and extent of leukocyte infiltration in the ischemic myocardium. Sampling for histologic analysis was done by taking transmural slices of myocardium from the ischemic regions. Tissue samples were fixed in phosphate-buffered formalin (10%). The sections of tissue blocks were stained with hematoxylin and eosin. Leukocyte infiltration was examined by scoring on a semiquantitative basis, where + = cells few, ++ = moderate, +++ = many. The production of 12-HETE or 5-HETE was markedly augmented in proportion to the extent of leukocyte infiltration (Fig. 3), suggesting that the former is probably the function of the latter.

Peripheral leukocyte function during the course

of myocardial infarction

It has been reported that during the early phase of myocardial infarction leukocyte count in peripheral circulation is increased, indicating that augmented production of chemo-attractive agents such as complements and LTBA may take place under these conditions, resulting in activation of peripheral leukocytes. In an acute occlusion-reperfusion model of canine myocardial infarction, we investigated changes in peripheral leukocyte count and leukocyte function estimated by neutrophil aggregation induced by calcium ionophore A23187 (Fig. 4). Neutrophils were isolated by Percoll gradient centrifugation. Heparinized venous blood was collected and centrifuged at 180g for 10 min. After the removal of platelet rich plasma, residual portion of blood was recentrifuged at 1600g for 5 min to obtain the buffy coat. The 2 ml buffy coat was layered over 3 ml of 55% isotonic Percoll solution and centrifuged at 1600g for 20 min. The neutrophil rich band on the erythrocyte layer

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was taken and contaminated erythrocytes were lysed with 0.15 M NH₄Cl. Obtained leukocytes were resuspended in saline and centrifuged at 180g for 5 min. The supernatants were removed and Tris buffer (50 mM, pH 7.5) was added to adjust the final leukocyte count to approximately 1 x 10⁷/ml. Leukocytes prepared in this manner contained >92% neutrophils. Neutrophil aggregation was monitored in a model RAM Multi-channel Aggregometer (Rikadenki) with stirring at 1,100 rpm and was performed with 0 and 100% light transmission preset with the neutrophil-rich suspension (1 x 10⁷/ml) and its diluted suspension (5 x 10⁶/ml), respectively. Neutrophil aggregation was induced by calcium ionophore A23187 diluted in Tris buffer. Leukocyte count in peripheral circulation was gradually increased after reperfusion (Fig. 4A). Similarly, neutrophil aggregation was markedly augmented during reperfusion (Fig. 4B). The addition of 10⁻⁸ to 10⁻⁵ M A23187 to neutrophil suspensions resulted in dose-dependent aggregation. The results indicate that activation of leukocyte in peripheral circulation has occurred during the course of myocardial infarction, thus suggesting its relation to leukocyte-induced myocardial cell damage.

Role of altered arachidonate metabolism in myocardial ischemia

Leukocytes produce a line of arachidonate metabolites through lipoxygenase enzymes, causing the expansion of myocardial ischemia through inflammatory processes. Leukocytes are known to accumulate in ischemic myocardial tissue, and this facilitates tissue lysis and resorption. More importantly, leukocytes have been shown to play a detrimental role, in that they not only participate in repair process, but augment myocardial tissue damage by releasing a variety of mediators including lipoxygenase metabolites in the early phase of myocardial infarction. LT₄, a compound formed via a 5-lipoxygenase enzyme, is chemotactic for leukocyte and promotes leukocyte adhesion to the vascular endothelium and acts as a calcium ionophore at high concentrations to increase intracellular calcium and to promote the release of lysosomal enzymes. LTC₄ and LTD₄ may also be formed, which induce coronary vasoconstriction and impairs contractility. Lipoxigenase pathways are therefore particularly important in the understanding of mechanism by which myocardial cells undergo reperfusion-induced myocardial injury. In our study with an occlusion-reperfusion model, we found a marked increase in the production of 5-HETE and 12-HETE associated with augmented leukocyte infiltration in ischemic myocardial tissue, which was accompanied by peripheral leukocyte activation. Though LT₄ production was not prominent in the ischemic tissue, it is possible that this eicosanoid could play a role in evolving myocardial infarction because of its potent biological actions.

Recent studies also demonstrated that platelets were selectively trapped into ischemic myocardial tissue and suppression of platelet accumulation by a cyclooxygenase inhibitor could cause a significant reduction of infarct size. Activated platelets may release vasoactive substances such as TXA₂ in the coronary circulation and cause platelet aggregation and vasoconstriction, resulting in microcirculatory disorders. Since platelet accumulation was found to be significantly suppressed by leukocyte depletion in experimental myocardial infarction, it may be secondary to leukocyte activation by releasing vasoactive substances. Alternatively, it could take place without preceding leukocyte activation in vascular disorders. In view of these findings, it is important to clarify the interrelation between arachidonate metabolites derived from these blood corpuscles. The extent to which arachidonate metabolism is associated with reperfusion-induced myocardial cell damage remains to be examined. Further studies should provide more insights into the roles of cyclooxygenase and lipoxygenase pathways in the evolution of ischemic myocardial injury.

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