Augmentation of Eicosanoids in Ischemic Heart Muscle in Dogs: its Role in the Deterioration of the Ischemic Lesion

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The dissolution of infarcted myocardium occurs after the infiltration of leukocytes. In the search for a mechanism of the leukocyte infiltration, we measured the production of lipoygenase metabolites of arachidonic acid in the canine myocardium after ligation of the circumflex branch of the left coronary artery. At least 2 lipoygenase products, namely 5- and 12-hydroxyeicosatetraenoic acids (HETEs), were augmented in myocardium subjected to ischemia lasting more than 6 hours, with levels of the latter being raised much more than the former.

Augmentation of the HETEs in ischemic myocardium appeared to occur prior to any significant infiltration of leukocytes. More than 12 hours after coronary ligation, the infiltration of leukocytes became prominent and an increase in 12-HETE was observed. Calcium content in the infarcted myocardium appeared to be increased several hours before the increase in 12-HETE.

These data suggest that the initial increment in 12-HETE may result from it being a product of infarcted myocardium, where Ca²⁺ is accumulated in the cell, and that the increased HETEs work as a leukocyte chemoattractant in infarcted myocardium. This hypothesis is supported by the independent experiment which showed that cultured cardiomyocytes produced lipoygenase metabolites of arachidonic acid, including 12-HETEs etc., which exhibited neutrophil-chemoattractant activity when they were challenged by calcium ionophore and/or arachidonic acid. Azelastine-HCl, a lipoygenase inhibitor, attenuated not only the above production of HETEs from the cardiomyocytes, but also production of HETEs and infiltration of neutrophils in ischemic myocardium, resulting in attenuation of the fibrous scar of infarcted myocardium.

It is well known that infarcted heart muscles develop so-called coagulation necrosis followed by infiltration of leukocytes, and the resulting myocardial dissolution is finally replaced by a fibrotic scar. However, it is not yet understood exactly how these histological alterations are induced in the lesion. Clarification is necessary in order to establish new therapeutic methods for salvaging the infarcted lesion. For this reason, we have undertaken biochemical and histological investigations using ischemic myocardia produced by ligating the coronary artery of dogs²,³

From our results, it is evident that infiltration of neutrophils into the infarcted lesion is found

Key words:
- 5-hydroxyeicosatetraenoic acid (5-HETE)
- 12-hydroxyeicosatetraenoic acid (12-HETE)
- Infarcted myocardium
- Lipoygenase metabolites
- Lipoygenase inhibitor (Azelastine)
- Cultured cardiomyocyte
- Neutrophil chemoattractant activity

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in association with an increase in eicosanoids (lipoxygenase metabolites of arachidonic acid) more than 8 hours after coronary ligation, and that the dissolution of the infarcted myocardia, with preceding infiltration of neutrophils, is found more than 12 hours after ligation.

Thus, it appears that the infiltration of neutrophils is essential for the dissolution of infarcted myocardia and the inhibition of eicosanoids production in the ischemic lesion may lead to attenuation of this leukocyte infiltration, preventing the further degradation of the infarcted myocardia.

In this paper, we describe our findings in support of the above hypothesis, together with findings that the substantial increase in myocardial Ca" in the very early stage of ischemia may play a role as a trigger for the induction of pathologic alterations in the ischemic myocardia.

MATERIALS AND METHODS

1) Animal preparation

Mongrel dogs weighing 8.0 to 10.0 kg were anesthetized with sodium diethybarbiturate (30 mg/kg i.v.), ventilated with room air at positive pressure and subjected to ligation of the circumflex of the left coronary artery at seg. 11 with either nylon-thread for permanent ischemia or a rubber string for reperfusion experiments. The myocardial infarction was established by elevation of the ST-segment on the ECG. The operation wound was closed. In the permanent ischemia-experiment, the myocardial ischemia was maintained for 1, 3, 6, 9, 12, and 24 hours, and 4 weeks.

In the reperfusion experiment, the myocardial ischemia was maintained for either 3 or 8 hours, followed by reperfusion for 16 or 21 hours, or for 4 weeks.

To ascertain the effect of a lipoxygenase-inhibitor on the ischemic myocardia, azelastine-HCl [AZ : (±)-(4-chlorobenzyl)-2-(hexahydromethyl-1H-azepin-4-yl)-2H-phthalazine-HCl]—a lipoxygenase inhibitor used as a therapeutic agent for bronchial asthma—was administered to some experimental dogs that were reperfused after coronary ligation for 3 hours. We have already established that myocardium subjected to ischemia for more than 3 hours is replaced by fibrous tissue even when coronary reperfusion is maintained for 4 weeks, as found in persistent ischemia.

In the AZ group, the drug was injected 4 times—just before and after coronary ligation, just after onset of reperfusion and 8 hours after reperfusion—in a dose of 1 mg/kg body weight in 10 mg/ml of saline.

In the control group, saline alone was administered in the same manner as in the AZ group. Some dogs in each group were sacrificed at 24 hours after the initial coronary ligation and their left ventricular myocardia were treated as described later to measure 12-HETE, myeloperoxidase (MPO) and creatine phosphokinase (CPK) in myocardia subjected to ischemia-reperfusion and in non-ischemic myocardia. Other dogs were sacrificed at 4 weeks after the initial coronary ligation. The hearts were removed and treated as described later to estimate histologically the fibrous scar area.

The experimental animals were kept in their respective cages until they were sacrificed accord-
ing to the experimental schedule.

2) Examination of the myocardium

Since we previously established that infarcted myocardium was completely replaced by fibrotic scar tissue at 3 weeks after the coronary ligation, the extent of the scar in myocardium subjected to ischemia with or without reperfusion was histologically estimated 4 weeks after coronary ligation. The heart was sliced transversely into 5 sections of equal width, from the apex to the base, and each slice was stained with hematoxylin-eosin and Azan-Mallory. The myocardium was graded according to the ratio (%) of the scar area to the entire area of left ventricle in each slice (Fig. 1-A).

The left ventricular myocardium obtained within 24 hours after coronary ligation was serially divided into pieces 0.5 cm long and 1.0 cm wide, starting from the ischemic center to the non-infarcted area on the endocardial surface, as shown in Fig. 1-B, and was examined biochemically and histologically. In the present experiments, the ischemic center was always located at the top of the posterior papillary muscle. Areas 0-1.5 cm, 2.0-2.5 cm and 4.0-4.5 cm from the ischemic center were assigned as the infarcted, marginal, and non-infarcted areas, respectively, regardless of the duration of ischemia.

a) Biochemical study

Ca$^{2+}$, neutrophil-specific MPO enzyme activity, and monohydroxy fatty acids of lipoxigenase metabolites of arachidonic acid were measured in the serial myocardial pieces.

For measurement of myocardial calcium, the specimen (1 g wet weight) was reduced to ash by placing it in a stream of O$_2$ under an ultra violet ray lamp and the ash was dissolved in HCl to measure Ca$^{2+}$ by the atomic absorption method.

Myeloperoxidase was measured in the serial myocardial pieces by an adaptation of the method of Bradley et al$^4$ according to the procedure of Bednar et al$^5$. The results were expressed as units (u) of MPO/0.1 g tissue (wet weight), where 1 unit of MPO activity is defined as that degrading 1 $\mu$mol of peroxide/min at 25°C.

For measurement of HETEs in the myocardial pieces, the myocardial specimen was first homogenized in 50 mM phosphate buffer (pH 7.0) with prostaglandin B$_2$ (PG.B$_2$: 100 ng in 0.2 ml of methanol) added as an internal standard. The eicosanoids were extracted with ethylacetate by shaking, followed by centrifugation. The supernatant was concentrated to less than 50 $\mu$l through evaporation under a stream of nitrogen.
and dissolved in 100 μl of pure methanol. An aliquot was analyzed for HETEs and PG.B₂ by liquid chromatography (RP-HPLC) using a column packed with Nucleosil C₁₈ (4.6 x 150 mm). The solvent system consisted of methanol-water-acetic acid (65:35:0.1, v/v). Detection was carried out with an ultraviolet (UV) detector operating at 235 nm for HETEs and at 280 nm for PG.B₂. The amounts of HETEs were calculated from the peak area ratio to PG.B₂. Protein was determined by the biuret method with bovine serum albumin as standard. Respective peaks corresponding to 12-, 5-HETE of eicosanoids were purified by liquid chromatography and identified by a gas chromatography mass spectrometer (GC-MS).

b) Histological and electron microscopic studies
The myocardia obtained within 24 hours after coronary ligation were also examined histologically to check myofibrillar degradation and infiltration of leukocytes. Electron microscopic studies were undertaken in myocardia obtained within 2 hours after coronary ligation.

c) Study using cultured mouse fetal cardiomyocytes
Cultured mouse fetal cardiomyocytes were obtained according to the method of Goshima and Wakabayashi. There was little contamination of other cells such as fibroblasts. These cells (10⁷ per flask), cultured for 2 days in Eagle Minimum Essential Medium buffered with HaHCO₃, were used in the experiments. The cells were further incubated for 15 min at 37°C in the presence of either: 1) 5 μM calcium ionophore (A-23187); 2) 30 μM arachidonic acids; 3) a combination of 1 and 2; 4) 3 plus azelastine HCl (lipoxygenase-inhibitor, 2.4 x 10⁻⁴ M) or 5) methanol only, as a control. After ethylacetate and PG.B₂ (100 ng) were added to each flask, the flasks were shaking vigorously to extract the lipoxygenase metabolites of arachidonic acid. The mixture was centrifuged to obtain the supernate. The organic layer was removed up and evaporated to measure the lipoxygenase metabolites of arachidonic acid in RP-HPLC as described above.

The neutrophil-chemoattractant activity of the above obtained extracts or 12-HETE was estimated by the Boyden's method using neutrophils from human blood. The neutrophils were suspended in "Hanks solution" in a Boyden's chamber and incubated for 4 hours at 37°C.

RESULTS
(1) Production of HETEs and increase of calcium in myocardium subjected to ischemia
As shown in Fig. 2, RP-HPLC of ethylacetate extracts from myocardia subjected to ischemia for 24 hours showed 3 noticeable large peaks in the absorption curve at 235 nm, which were different from those in the case of non-infarcted myocardium. The large peak at 18.25 min and the other samloer peak at 20.00 min were determined to be 12- and 5-HETE, respectively, in comparison with the elution time of authentic samples as well as by gas chromatography mass spectrometry analysis. The content of 12-HETE and 5-HETE in the infarcted myocardium was estimated to be 38.05 ng/mg protein and 10.8 ng/mg protein, respectively. In non-

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infarcted myocardium, the content of 12-HETE and 5-HETE was estimated to be 0.62 ng/mg protein and a trace (nondetectable amount), respectively. Thus, the behavior of eicosanoids in ischemic myocardium was estimated through a trend of 12-HETE. The finding that 12-HETE was augmented in ischemic myocardium was further confirmed by analysis of myocardial pieces from the ischemic center to the non-infarcted area (Fig. 1-B) of the heart subjected to coronary ligation for 24 hours.

As shown in Fig. 3, 12-HETE was high in the ischemic myocardium and then decreased as the myocardial specimen approached the non-infarcted area. Similar results were obtained in the myocardial specimen in which the coronary artery had been ligated for 8 hours and then reperfused for 16 hours. In this case, the highest level of 12-HETE (40.2 ng/mg protein) was found in the specimen 1.0–1.5 cm from the ischemic center. In the non-infarcted myocardia the 12-HETE level ranged between 0.62 and 1.92 ng/mg protein.

As shown in Fig. 4, it was found that 12-HETE was increased in myocardium subjected to ischemia lasting more than 6 hours, and the increase was accelerated as ischemia was prolonged for more than 12 hours. That is, the 12-HETE level was 1.97 ± 0.83, 8.71 ± 4.60, 11.50 ± 5.63, and 38.68 ± 12.69 ng/mg protein in infarcted myocardium subjected to ischemia for 6, 9, 12, and 24 hours, respectively. Following myocardial ischemia lasting less than 6 hours, the 12-HETE level was less than 0.5 ng/mg protein, which is similar to that in normal myocardium. In the non-infarcted area the 12-HETE level was 0.56 ± 0.44, 0.59 ± 0.34, 0.30 ± 0.13 ng/mg protein at 6, 9, and 12 hours after coronary ligation, respectively. In coronary ligation for 24 hours, the 12-HETE level in non-infarcted myocardium was 1.85 ± 0.55 ng/mg protein, which is slightly higher than that in normal myocardium. In the marginal area, 12-HETE increased significantly under coronary ligation lasting more than 9 hours and the high level was maintained thereafter without any increase until 24 hours.
These results might indicate that the increase of 12-HETE in ischemic myocardium was brought about by leukocytes infiltrating the ischemic area. However, little infiltration of leukocytes was found in myocardia subjected to ischemia for 3–9 hours. Therefore, it was suggested that the ischemic myocardial cells might produce lipoxygenase metabolites from arachidonic acid, as activated leukocytes do.

On the other hand, as shown in Fig. 5, myocardial Ca\(^{2+}\) content increased even in ischemia lasting for only a few hours, increasing considerably thereafter as the duration of ischemia was prolonged. That is, in the infarcted area Ca\(^{2+}\) content was 51.5 ± 6.7, 67.0 ± 11.8, 86.1 ± 10.5, 93.0 ± 6.2 and 336.9 ± 25.1 μg/g tissue wet weight at 3, 6, 9, 12, and 24 hours after coronary ligation, respectively. In the non-infarcted area, Ca\(^{2+}\) content was 29.3 ± 4.1, 25.7 ± 6.8, 26.9 ± 6.3, 22.2 ± 3.6 and 43.5 ± 3.3 μg/g tissue wet weight at 3, 6, 9, 12, and 24 hours after coronary ligation, respectively. In the marginal area, too, Ca\(^{2+}\) content increased significantly in coronary ligation lasting more than 3 hours.

(2) Infiltration of leukocytes in ischemic myocardium after coronary ligation

In histological examination of ischemic myo-
cardia obtained at various intervals after coronary ligation, infiltration of leukocytes was not conspicuous in the infarcted area until 9 hours after coronary ligation.

Little myocardial degradation was seen following ischemia for less than 12 hours (Fig. 6).

The extent of infiltration of leukocytes into the infarcted area was also estimated biochemically by measuring MPO activity in myocardia obtained at various intervals after coronary ligation. This was done because the MPO activity regarded as the marker enzyme specific for neutrophils correlated exactly with the number of leukocytes (≤10⁶/ml). As shown in Fig. 7, the enzyme activity in the myocardium subjected to ischemia lasting less than 9 hours was very low, being similar to that in non-infarcted myocardium in the experimental animal. However, in the myocardium subjected to ischemia lasting longer than 9 hours, there was a remarkably high level of enzyme activity, correlating with the increase in 12-HETE in infarcted myocardia shown in Fig. 4.

(3) Cardiomyocytes produce eicosanoids possessing neutrophil chemoattractant activity.

The above finding led us to examine whether cardiomyocytes did or did not produce lipoxygenase products with a neutrophil chemoattractant activity from arachidonic acid. Cultured mouse fetal cardiomyocytes were stimulated by Ca²⁺-ionophore with or without arachidonic acid. Analysis of the incubation medium by RPV-

![Graph](image)

**Fig. 7.** Myeloperoxidase activity in myocardium in relation to the duration after coronary occlusion. 
- : infarcted area, ----- : marginal area, --- : non-infarcted area. Experimental cases: 10 except n = 7 in ischemia for 12 and 24 hours.

### TABLE 1 AMOUNTS OF 12-HYDROXYEICOSATETRAENOIC ACID (12-HETE) PRODUCED FROM CULTURED MOUSE CARDIOMYOCYTES INCUBATED IN THE PRESENCE OF Ca²⁺-IONOPHORE AND/OR ARACHIDONIC ACID WITH OR WITHOUT AZELASTINE (AZ), A LIPOOXYGENASE-INHIBITOR, AND THEIR EFFECTS ON THE NEUTROPHIL CHEMOATRACTANT ACTIVITY BY THE BOYDEN'S METHOD

<table>
<thead>
<tr>
<th>Added agents</th>
<th>12-HETE (ng/10⁷ cells) mean ± S.D.</th>
<th>Migrated* Leukocytes counts/field mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Control (Methanol only)</td>
<td>n = 4</td>
<td>4.15 ± 1.50</td>
</tr>
<tr>
<td>(B) A-23187</td>
<td>n = 4</td>
<td>10.52 ± 2.10</td>
</tr>
<tr>
<td>(C) Arachidonic acid</td>
<td>n = 4</td>
<td>28.56 ± 8.15</td>
</tr>
<tr>
<td>(D) A-23187 + Arachidonic acid</td>
<td>n = 4</td>
<td>30.50 ± 8.20</td>
</tr>
<tr>
<td>(E) (D) + AZ (2.4 × 10⁻⁴ M)</td>
<td>n = 4</td>
<td>7.20 ± 2.0</td>
</tr>
</tbody>
</table>

*(Boyden's method)*

*Japanese Circulation Journal Vol. 52, July 1988*
HPLC revealed that the cardiomyocytes challenged by the above agents produced some lipoxygenase metabolites of arachidonic acid including 12-, 5-HETE and leukotriene B4-like substances. Among these a significant increase in 12-HETE was detected (Table I). This production of 12-HETE was inhibited by the administration of the lipoxygenase inhibitor, AZ.

Using Boyden’s chamber, it was demonstrated that migration of leukocytes was accelerated according to the amount of 12-HETE released from fetal cardiomyocytes challenged with the above agents (Table I and Fig. 8), illustrating the chemoattractant properties of 12-HETE. Furthermore, it was evident that migration of leukocytes was inhibited in the sample treated with AZ.

In another experiment using Boyden’s chamber, it was also confirmed that authentic 12-HETE possessed chemoattractant activity for leukocytes at the levels of 10–20 µg/ml.

(4) Lipoxygenase inhibitor inhibits production of 12-HETE in ischemic myocardium and reduces the fibrous area in myocardium subjected to 4 weeks’ reperfusion after ischemia for 3 hours.

We then investigated whether or not administration of the lipoxygenase inhibitor, AZ, to the experimental dogs inhibited degradation of infarcted myocardium through suppression of 12-HETE production in myocardium subjected to ischemia.

As Fig. 9 shows, the augmentation of both 12-HETE and MPO, and the decrease in CPK, in myocardium (without hemorrhage) subjected to reperfusion for 21 hours after ischemia for 3 hours was significantly inhibited in dogs treated with AZ. However, this therapeutic effect of AZ was not found in reperfused myocardium that had suffered hemorrhage.

In the myocardium reperfused for 4 weeks after ischemia for 3 hours, the ratio of fibrous area to the total myocardial area in 5 horizontal sections of left-ventricle was significantly smaller in the AZ group (8.17 ± 3.0%, n = 7) than in the control group (16.0 ± 5.5%, n = 7).

**DISCUSSION**

In this paper, it was shown that the lipoxygenase metabolites of arachidonic acid, including 12- and 5-HETE with neutrophil chemoattractant activity, increased in myocardium subjected to ischemia lasting more than 9 hours. It was also shown that administration of a lipoxygenase inhibitor resulted in a reduction of scar-size in the infarcted myocardium through attenuation of eicosanoid production.

Mullane et al. recently reported that in myocardium subjected to reperfusion for up to 5
hours after 1 hour of ischemia:

1) Infarcted myocardium produced 10 times 12-HETE from arachidonic acid than the adjacent "normal" area of the ventricle; 2) the production of 12-HETE in ischemic myocardium was attributed to the invading leukocytes; and 3) agents inhibiting either lipoxygenase or both lipoxygenase and cyclooxygenase activity in the metabolism of arachidonic acid attenuated leukocyte infiltration into infarcted myocardium. Thus, in myocardium subjected to permanent ischemia, it should be established whether 12-HETE is produced by ischemic myocardial cells prior to infiltration of leukocytes into the infarcted lesion, which differs from the reperfused myocardium. This question follows the observation that infiltration of leukocytes is found to occur much earlier and more markedly in reperfused myocardium than in the myocardium subjected to permanent ischemia.12

It has been stated that in myocardial infarction without reperfusion, infiltration of leukocytes into the infarcted area, except the marginal area, occurs more than 12 hours after the onset of ischemia. This finding was also confirmed by the present study (Figs. 6 and 7). Therefore, the metabolic activity in ischemic myocardium within 12 hours of the onset of ischemia is important for invasion of leukocytes into the infarcted lesion. In research along these lines several workers reported that myocardial damage by ischemia was induced by leukocytes invading the ischemic area; this invasion was due to the leukotactic effect of a complement cascade activated by binding of the first component of complement to a substance from mitochondria in the presence of Ca2+.13,14

On the other hand, the finding that depletion of ATP in cardiomyocytes brings about significant accumulation of arachidonic acid released from phospholipid in the cell membrane has recently been shown by Chien et al.15–19 Depletion of ATP in the cell has been considered to bring about an abnormal increase in intracellular Ca2+ by its release from sarcoplasmic reticulum and/or subsarcolemmal cisternae. A rise in calcium in ischemic myocardium was confirmed in this paper. This is considered to be due to an increase in net Ca2+ influx through phospholipid degradation in the cell membrane of ischemic cells.18,19

The increase of calcium in myocardium is considered to induce various metabolic alterations in the cells; that is, activation of Ca2+-activated neural protease20 deterioration of mitochondrial function, binding of complement to mitochondria, and activation of some phospholipase etc.21 Therefore we examined the lipoxygenase metabolites of arachidonic acid in ischemic myocardium; these were assumed to be released from phospholipids in the cell membrane by Ca2+-activated phospholipase A2 or by some
other mechanism. From the results of our study, it was found that 12- and 5-HETE increased more in the infarcted area than in the adjacent non-infarcted area. In this investigation, 12-HETE was more dominant than 5-HETE. An explanation of this will require more intensive analysis of arachidonic acid metabolism.

In our study, the increase of 12-HETE in ischemic myocardium appeared subsequent to an increase in calcium prior to the infiltration of leukocytes into the ischemic lesion. Thus, it is conceivable that, in the ischemic lesion, not only leukocytes and/or platelets but also ischemic cardiomyocytes produce lipoxygenase metabolites from arachidonic acid.

In fact, cultured cardiomyocytes produced some lipoxygenase metabolites of arachidonic acid in the presence of Ca\(^{2+}\)-ionophore and/or arachidonic acid, and their production was inhibited in the presence of the lipoxygenase inhibitor AZ. Furthermore, we found that normal canine adult myocardial tissue produced a significant amount of the lipoxygenase metabolites of arachidonic acid—mainly 12-HETE—when incubated with Ca\(^{2+}\)-ionophore and/or arachidonic acid.

In another experiment, we confirmed that both 12- and 5-HETE showed dose-dependent and significant neutrophil chemoattractant activity in the Boyden's chamber although their activity was less than that of leukotriene B\(_4\). Therefore, it is conceivable that 12- and 5-HETE produced in ischemic myocardium may act as a neutrophil chemoattractant, promoting infiltration of neutrophils into the infarcted lesion. It also seems likely that these neutrophils promote cellular injury in ischemic myocardium by production of both myeloperoxidase and protease. This hypothesis is supported by the finding that administration of lipoxygenase inhibitor to the experimental dogs significantly suppressed augmentation of 12- and 5-HETE as well as MPO in the infarcted myocardium, resulting in a reduction in the development of fibrous scar tissue.

Acknowledgement

Authors wish to thank Dr. T. Shimizu (Tokyo Univ. Med. School) for his sincere help in identification of 12-HETE and valuable discussions.

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