Protective Role of Intracoronary Fatty Acid Binding Protein in Ischemic and Reperfused Myocardium

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In this study, fatty acid binding protein was used to protect an ischemic heart from reperfusion injury. Isolated rat heart was preperfused in the presence of 1.4 μM liposome-bound fatty acid binding protein for 15 minutes, followed by 30 minutes of ischemia and 30 minutes of reperfusion. Our results indicated better preservation of myocardial high-energy phosphate compounds (including ATP and creatine phosphate), reduced creatine kinase and lactate dehydrogenase release from the heart, and improved coronary flow in hearts treated with fatty acid binding protein compared with untreated controls. Fatty acid binding protein enhanced reacylation of arachidonic acid into phospholipids, thereby preserving membrane phospholipids and reducing free fatty acid contents during ischemia and reperfusion. In addition, fatty acid binding protein-bound long-chain free fatty acids and their thioesters as well as carnitine esters were increased in the cytosolic compartment of the heart. These results suggest that fatty acid binding protein may be used as a possible therapeutic agent to improve myocardial function during reperfusion of ischemic heart. (Circulation Research 1990;66:1535–1543)

The precise biochemical mechanisms for membrane injury during myocardial ischemia and reperfusion remain obscure despite many investigations. However, a growing body of evidence shows that among many factors, accumulation of membrane-derived fatty acids and their esters may play a significant role in the pathogenesis of ischemic and reperfusion injury. Several factors may be responsible for the accumulation of fatty acids and their esters during ischemia. First, β-oxidation of fatty acids is significantly inhibited during ischemia, which leads to the accumulation of free fatty acids (FFAs), fatty acyl coenzyme A (CoA) esters, and long-chain fatty acyl carnitines. Another possibility is the breakdown of membrane phospholipids during ischemia and reperfusion, resulting in accumulation of nonesterified fatty acids and lysophospholipids. These accumulated lipids can cause severe membrane injury and may represent one of the causes for ischemic and reperfusion injury.

Recent studies indicate the presence of an intracellular fatty acid binding protein (FABP) in the heart that can bind to FFAs and their thioesters with high affinity. These FABPs, which represent about 4% of the soluble protein in the heart, were found to be lost during ischemia and reperfusion. Therefore, we hypothesized that if additional FABPs could be added to the heart, they might provide adequate protection to the ischemic and reperfused heart.

In this study, we perfused isolated rat heart with highly purified FABP before the ischemic insult. Reperfusion of heart treated with FABP demonstrated significant improvement in preserving high-energy phosphate compounds and maintaining coronary flow, and showed less tissue injury compared with control hearts.

Materials and Methods
Isolation and Purification of Fatty Acid Binding Protein

Rat heart cytosolic FABP was prepared according to the procedure described by Ockner et al with slight modification. In brief, the cytosol (100,000g supernatant containing 5 mg protein/ml) was dialyzed against 10 mM Tris HCl (pH 7.5) (buffer A), and the dialyze was loaded onto a Sephadex G-75 (100×1 cm) column (Pharmacia, Piscataway, New
Delipidation of Purified Fatty Acid Binding Protein and Assay of Fatty Acid Binding

Purified FABP was subjected to delipidation using Lipidex 1000 (Packard Instrument Co., Downers Grove, Illinois), as described by Glatz et al. In brief, 2 mg FABP was loaded onto a column of Lipidex (0.5 x 5 cm) equilibrated with 100 mM Tris HCl buffer (pH 7.4) and 1 mM 1,4-dithiothreitol (DTT) at 37°C. The delipidated FABP emerged in the void volume. Oleate binding was determined essentially according to the procedure described by Offner et al. The reaction mixture contained increasing amounts of [1-14C]oleic acid (Na+ salt) (10,000 cpm), 2 nmol FABP, 1.5 mM DTT, and 100 mM Tris HCl (pH 7.4) in a total volume of 0.35 ml. Binding was allowed for 5 minutes at 37°C, after which the test tubes were cooled in ice. The unbound fatty acid was removed by mixing with 200 μl ice-cold Lipidex 1000 and buffer suspension (1:1, vol/vol) for 10 minutes at 0°C. The radioactivity remaining in the supernatant was determined to calculate nanomoles oleate bound/nanomoles FABP.

Preparation of Liposomes Containing Fatty Acid Binding Protein

FABP was incorporated into liposomes by sonication of a mixture of phosphatidylcholine (1 mg/ml) and FABP (1 mg/ml) in an ice-cold bath for 10 minutes. The liposome-bound FABP was separated from free FABP and bovine serum albumin by using G-50 gel filtration.

Perfusion of Heart With Fatty Acid Binding Protein

Isolated and perfused rat heart was prepared according to the Langendorff technique, as described previously. Hearts were perfused with Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.4) equilibrated with a gas mixture of 95% O2 and 5% CO2 in a nonrecirculating mode for 15 minutes, then by a recirculating mode. Liposome-bound FABP (1.4 μM) was then added into the perfusion circuit, and perfusion continued for 15 minutes. Control experiments were performed by perfusing the hearts for 15 minutes with liposome only. Ischemia was then induced for 30 minutes by stopping coronary flow, and reperfusion was reinstated with a fresh recirculatory KHB buffer (50 ml) to which tracer amounts of [1-14C]arachidonic acid (1 μCi) had been added. Reperfusion continued for 30 minutes, and perfusate samples were withdrawn at 5-, 10-, 20-, and 30-minute intervals. Coronary flow was also measured by collecting a known volume of perfusate over a time period. At the end of reperfusion, the heart was freeze-clamped at liquid nitrogen temperature for biochemical assay.

Assay for ATP, Creatine Phosphate, Lactic Acid Dehydrogenase, and Creatine Kinase

Lactic acid dehydrogenase (LDH) and creatine kinase (CK) were assayed in the coronary perfusate, whereas ATP and creatine phosphate (CP) were estimated in the heart. ATP and CP were assayed using high-performance liquid chromatography, as described elsewhere.

Measurement of Myocardial Contractility

A polyethylene catheter was inserted into the left ventricle through the apex. This, in turn, was connected to a pressure transducer (Statham P23, Gould, Oxnard, California) to measure the left ventricular pressure and to record its first derivative (dP/dt). The tracings were recorded by a Honeywell recorder (Pleasantville, New York).

Assay for Lipid Peroxidation

The extent of lipid peroxidation was measured in the perfusate buffer by assaying malondialdehyde, as described previously. Briefly, 1 ml perfusate was treated with 1 ml ice-cold 30% HClO4 and 1 ml 0.75% theobarbituric acid dissolved in 0.50% sodium acetate. The samples were boiled for 20 minutes and air-centrifuged to remove the pellet. The color of the supernatant was read as 535 nm. The concentration of malondialdehyde was measured using a molar extinction coefficient of 156 mM⁻¹ cm⁻¹.

Arachidonic Acid Uptake by the Heart and Its Incorporation Into Membrane Phospholipid and Tissue Fatty Acid Pool

A known amount of [14C]arachidonic acid was added to the perfusion circuit and before ischemia, as described. Perfusates were withdrawn at regular intervals and counted for radioactivity. A portion of the ventricular biopsies was also counted for radioactivity at the end of the experiment. Lipids were extracted from another portion of the biopsies and...
separated into individual phospholipids and neutral lipids using thin-layer chromatography, as described by Das et al. The spots corresponding to authentic standards were scraped off and counted for radioactivity using a scintillation counter (Packard).

**Isolation and Estimation of Cytosolic Fatty Acid Binding Protein–Bound Free Fatty Acids, Long-Chain Acyl Coenzyme A, and Long-Chain Acyl Carnitines**

Ventricular biopsies were homogenized in 0.25 M sucrose containing 10 mM sodium phosphate buffer (pH 7.2) (5 ml/g), using a Polytron homogenizer (Brinkmann Instruments, Westbury, New York). The homogenate was subjected to subcellular fractionation, and 100,000g supernatant (cytosol) was used for assaying protein-bound fatty acid content.

Cytosol (0.85–1.1 ml) was incubated with an equal volume of 1:1 Lipidex 1000 phosphate buffer suspension for 20 minutes to adsorb free fatty acyl derivatives. After centrifugation, the supernatant containing FABP-bound fatty acids and esters was divided into two parts for lipid estimations.

One portion of the supernatant was adjusted to pH 12.4 and incubated for 2 hours at 70°C to hydrolyze long-chain acyl carnitines and long-chain acyl CoAs. After hydrolytic treatment, total cytosolic lipids were extracted. Heptadecanoic acid was used as the internal standard.

FABP-bound FFAs were estimated by extraction of lipids from the other portion of cytosol that was not subjected to hydrolytic treatment.

The extracted fatty acids were methylated and separated by gas chromatography (Hewlett-Packard, Palo Alto, California). Quantification of acyl groups was done by comparing the areas with the heptadecanoyl methyl ester areas. Long-chain acyl CoAs and long-chain acyl carnitines were estimated as the differences of the fatty acid levels in the hydrolyzed sample (total cytosolic lipids) and the fatty acid levels of the nonhydrolyzed sample.

Long-chain acyl CoA, long-chain acyl carnitine, and FFA were also measured in the whole heart using our previously described methods.

**Statistical Analysis**

Comparison between two groups was performed by two-sample t tests. For further comparison between three or more groups, analysis of variance followed by Scheffe's test was done. Data were expressed as mean±SEM and were considered statistically significant only if p<0.05.

**Results**

**Perfusion of Heart With Fatty Acid Binding Protein**

FABP purified from rat heart by the method of Ockner et al 14 was found to be homogeneous as judged by SDS-PAGE (Figure 1). The apparent molecular weight was calculated to be 14 kDa, which was similar to that reported by others for heart FABP. Before using this FABP as therapeutic intervention to bind FFAs released from heart, we wanted to demonstrate that purified FABP could bind stoichiometric amounts of FFA. In this study, we used oleate because this was one of the fatty acids found to increase significantly during ischemia. As shown in Figure 2, when oleate concentration was
increased, increasing amounts of oleate were bound. The saturation was reached at a concentration of 2 nmol oleate bound/nmol FABP.

**Effect of Fatty Acid Binding Protein on Coronary Flow**

Both treated and untreated groups showed a decrease in coronary flow during the first 15 minutes of perfusion after mounting the heart in the Langendorff apparatus; however, these changes were not statistically significant. These coronary flows stabilized from an average of 12–14 ml/min/g heart weight to about 10 ml/min/g heart weight within 15 minutes, and remained unchanged even after 30 minutes of perfusion (data not shown). These changes were consistently reflected in each rat and may be attributed to the stabilization process. If FABP was added after this stabilization, no further drop of coronary flow was noticed. The flow remained unchanged in control heart not subjected to ischemia and reperfusion for up to 30 minutes after the addition of FABP (10.5 ± 1.2 ml/min/g), suggesting that FABP did not cause any inotropic effect.

In the present study, FABP-treated heart showed significant improvement in coronary flow compared with the untreated group. For example, the coronary flow after 5 minutes of reperfusion was only 10.5 ml/min/g in control heart, compared with 11.7 ml/min/g in the experimental group (Figure 3). After 30 minutes of reperfusion, the coronary flow in the FABP-treated heart was 14 ml/min/g, which was significantly higher compared with the corresponding control group (8.5 ml/min/g). This indicated better myocardial performance for the FABP-treated hearts.

**Effects of Fatty Acid Binding Protein on the Release of Creatine Kinase and Lactic Acid Dehydrogenase**

The release of CK and LDH is considered to be a reliable indicator for cellular injury and tissue necrosis. Release of CK (Figure 4) and LDH (Figure 5) from the heart followed a similar pattern and increased progressively during the entire period of reperfusion. However, increases in CK and LDH release during reperfusion were less in the FABP group compared with the nontreated group. At the end of 30 minutes of reperfusion, release of CK and LDH was significantly less (p < 0.05) in heart of the FABP group compared with corresponding controls.
Effects of Fatty Acid Binding Protein on High-Energy Phosphate Compounds in the Heart

After 30 minutes of ischemia and 30 minutes of reperfusion, the FABP-treated ischemic hearts showed significant preservation of ATP content. The ATP content of FABP-treated heart was two times higher as compared with control heart (Figure 6) after 30 minutes of ischemia and after 30 minutes of reperfusion. There was no difference in preischemic control values of ATP among treated and untreated groups. CP values were reduced significantly in both groups compared with preischemic control; however, FABP-treated heart showed better preservation of CP compared with the untreated control group, both during ischemia and during reperfusion.

Effects of Fatty Acid Binding Protein on Myocardial Functions

The changes in left ventricular contractility after 30 minutes of ischemia and 30 minutes of reperfusion are shown in Table 1. The contractile parameters did not change after the addition of FABP. In both groups, left ventricular diastolic pressure and left ventricular dP/dt were significantly decreased after ischemia; however, these values were significantly higher in the FABP-treated group. After 60 minutes of reperfusion, the treated group showed significant improvement in myocardial contractility compared with the control group.

Effects of Fatty Acid Binding Protein on Lipid Peroxidation

Formation of malondialdehyde was increased by only 20–25% in both groups during early reperfusion (Figure 7). In the nontreated group, however, it increased to 35% after 30 minutes of reperfusion, whereas in the FABP-treated group, no further malondialdehyde formation was noticed.

Effects of Fatty Acid Binding Protein on Arachidonic Acid Uptake by the Heart

When a tracer amount of radioactive arachidonic acid was added into the perfusion circuit and its
disappearance was monitored by counting radioactivities from perfusate withdrawn at regular intervals, FABP was found to enhance arachidonic acid uptake by isolated heart (Figure 8). Although the enhanced arachidonic acid uptake was noticed during the reperfusion phase, significant differences were observed after 30 minutes of reperfusion in the presence of FABP.

Effects of Fatty Acid Binding Protein on [14C]Arachidonic Acid Incorporation Into Membrane Phospholipids and Tissue Fatty Acids

As shown in Table 2, FABP stimulated the incorporation of isotopic arachidonic acid into membrane phospholipids and reduced its incorporation into tissue FFA. For example, incorporation of [1-14C]arachidonic acid into myocardial phospholipid was twofold higher in the FABP-treated group as compared with the control group. Increased incorporation of [1-14C]arachidonic acid into membrane phospholipids was also accompanied by decreased radiolabels in the FFA fraction of myocardium as compared with the control group.

Effects of Fatty Acid Binding Protein on Free Fatty Acids and Their Long-Chain Thioesters

FABP was found to decrease significantly the contents of FFAs and their long-chain thioesters in the reperfused heart (Table 3). Thus, after 30 minutes of reperfusion following 30 minutes of ischemia, FABP reduced total FFAs by 20% and their long-chain acyl CoAs by 25%. The drop in long-chain acyl carnitines, however, was not statistically significant.

Effects of Fatty Acid Binding Protein on Fatty Acid Composition of Protein-Bound Long-Chain Fatty Acids and Their Acyl Coenzyme As and Carnitine Esters

Because FABP is known to bind FFAs and their esters, we separated the FABP-bound fatty acids and their esters from the FFAs in the cytosolic fraction by Lipidex chromatography. Analysis of the fatty acid profiles of cytosolic protein-bound FFAs revealed that FABP bound all the long-chain fatty acids (Table 4). In particular, 18:1, 18:2, 20:4, and total FFAs showed significantly higher binding with FABP compared with the control group. In addition, FABP also bound with long-chain fatty acyl CoAs and long-chain fatty acyl carnitines (Table 5). The fatty acid profiles of those long-chain fatty acyl esters demonstrated that FABP significantly enhanced binding in the 18:1, 18:2, and total FFAs compared with corresponding controls.

Discussion

Among the many factors responsible for myocardial cellular injury during ischemia and reperfusion,
accumulation of nonesterified fatty acids (including arachidonic acid and lysophospholipids) represents a major concern.\(^1\)\(^-\)\(^7\) Accumulation of arachidonic acid has also been observed after a relatively brief period of ischemia followed by reperfusion.\(^6\)\(^,\)\(^7\) It is generally believed that arachidonic acid and lysophospholipids are derived from the breakdown of membrane phospholipids primarily because of the activation of the deacylation pathway and a defective reacylation system.\(^6\)\(^,\)\(^7\) Recent studies from our laboratory have also indicated that these lipids may be derived from other metabolic pathways, including the signal transducing systems.\(^1\)\(^7\) In addition, inhibition of \(\beta\)-oxidation during ischemic insult also results in accumulation of long-chain fatty acids and their acyl CoA thioesters.\(^4\)\(^,\)\(^5\)

The detrimental effects of these membrane-derived lipids in the pathogenesis of ischemic and reperfusion injury have been reviewed.\(^1\)\(^-\)\(^2\) FFAs and lysophospholipids can cause myocardial arrhythmias by their detergentlike action.\(^1\)\(^-\)\(^2\) They can also inhibit a number of key enzymes, including Na\(^+\),Ca\(^++\)-ATPase\(^2\)\(^2\) and adenosine nucleotide translocase.\(^2\)\(^3\) Accumulation of nonesterified fatty acids in concert with loss of membrane phospholipids can also alter the fluidity of the membrane lipid bilayer.\(^2\)\(^4\) Prolonged ischemia can ultimately lead to physical disruption of the myocardial membrane and loss of its ability to function as a protective barrier, resulting in massive calcium influx during reperfusion, which leads to cell death.\(^1\)\(^,\)\(^2\)

Once the accumulation of lipids occurs, mammalian heart becomes defenseless. Unless outside interventions are immediately administered, the lipids may cause serious myocardial injury. Until recently, it was generally believed that there had been no naturally occurring myocardial defense system against lipid abnormality. Recent studies, however, recognize that such a system may exist in the heart. These are nonenzyme proteins (referred to as FABP), which exist in appreciable quantities in mammalian hearts (4–8% of soluble proteins). A very limited number of studies have been performed on myocardial FABP, and its precise nature and physiological functions within the cell remain largely speculative. It has been suggested that FABP may temporarily store or sequester FFAs and fatty acyl CoA esters, thereby modulating or buffering their detrimental effects.\(^9\)\(^-\)\(^10\) In addition, FABP may help to shuttle the poorly soluble long-chain fatty acids and fatty acyl CoAs from the site of entry or synthesis to the site of esterification or oxidation, thus behaving as an intracellular counterpart of serum albumin.\(^2\)\(^5\) Either of these properties of FABPs makes them ideal protective agents during myocardial ischemia. Recent studies demonstrate a significant loss of FABP from heart during ischemia and reperfusion.\(^1\)\(^3\) Therefore, we hypothesized that if additional FABP can be supplied

**TABLE 2. Incorporation of Isotopic Arachidonic Acid Into Membrane Phospholipids and Fatty Acids During Perfusion of Isolated Rat Heart**

<table>
<thead>
<tr>
<th>Incorporation of [(1^4)C]arachidonic acid into myocardial phospholipids (% [(1^4)C]arachidonic acid)</th>
<th>Radioactivity of [(1^4)C]arachidonic acid in myocardial FFA (% [(1^4)C]arachidonic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 2.04±0.30</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>FABP 4.54±0.59*</td>
<td>0.07±0.01*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. FFA, free fatty acid; FABP, fatty acid binding protein.

\(*p<0.05\) compared with control.

**TABLE 3. Effects of Fatty Acid Binding Protein on Content of Free Fatty Acid, Long-Chain Acyl Coenzyme A, and Long-Chain Acyl Carnitine in Rat Heart**

<table>
<thead>
<tr>
<th>Content</th>
<th>Control</th>
<th>FABP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total FFA (nmol/g)</td>
<td>220±18</td>
<td>180±11*</td>
</tr>
<tr>
<td>Long-chain acyl coenzyme A (nmol/g)</td>
<td>410±28</td>
<td>317±15*</td>
</tr>
<tr>
<td>Long-chain acyl carnitine (nmol/g)</td>
<td>425±31</td>
<td>388±22</td>
</tr>
</tbody>
</table>

Values are mean±SEM. FABP, fatty acid binding protein; FFA, free fatty acid.

\(*p<0.05\) compared with control.
to the heart, it might provide adequate protection to ischemic and reperfused myocardium.

FABP isolated and prepared from rat heart was found to be of high purity by SDS-PAGE. Purified FABP was found to bind 2 nmol fatty acid/nmol protein. Even though FABP used in this study was of relatively low molecular weight (14 kDa), we still used liposome-bound FABP in our study to ascertain the entrance of FABP inside the cell. FABP was found to provide adequate protection to the ischemic reperfused heart as judged by the preservation of high-energy phosphate compounds, increased coronary flow, and reduced release of CK and LDH, presumptive markers for cellular injury. However, most of the beneficial effects were found after 30 minutes of reperfusion, so we performed some additional experiments to examine whether these beneficial effects persisted beyond 30 minutes of reperfusion. Our results indicated higher coronary flow and lower LDH, and CK release by FABP persisted even after 60 minutes of reperfusion (results not shown). In this study, we used FABP before the ischemic insult. Our attempt to use FABP at the onset of reperfusion was not successful owing to its failure to provide any myocardial protection.

FABP by itself did not appear to have any effect on the heart. When an isolated heart was perfused for up to 30 minutes in the presence of FABP without any intervention, no apparent changes in coronary flow and myocardial contractility were observed. In each experiment, however, a drop in coronary flow was observed after mounting the heart in the perfusion apparatus, irrespective of FABP treatment. This drop in coronary flow was consistent for each experiment during its first 15 minutes, which might be attributed to stabilization.

Our results clearly demonstrated that the internally added FABP entered the cell and bound with FFAs and their esters. Because the majority of FABP is located in the cytosolic compartment, we isolated the FABP-bound FFAs and their esters in the heart cytosol to study the binding of FABP. In addition, FABP reduced arachidonic acid incorporation in the FFA pool, with corresponding increases in incorporation into the membrane phospholipids. This suggests that turnover of arachidonic acid and its reacetylation into phospholipids were enhanced by FABP. Because ischemia is known to induce a defective reacylation of arachidonic acid, it is likely that FABP, by stimulating the reacylation step, may help preserve the membrane phospholipids. Indeed, the contents of total FFAs and their esters were significantly lower in the FABP as compared with control, suggesting that FABP inhibited the breakdown of membrane phospholipids. Arachidonic acid, once formed, is degraded by the cyclooxygenase pathway into lipid hydroperoxide, endoperoxides, and, finally, malondialdehydes. Therefore, we measured the malondialdehyde content release from the heart, which primarily reflects the peroxidative products of phospholipids and lipids. Decreased malondialdehyde formation by FABP treatment additionally supported phospholipid degradation and FFA accumulation. Comparison of total myocardial FFAs and their triester contents with those in protein-bound fractions indicates that FABP protects ischemic heart by 1) preserving membrane phospholipids and enhancing arachidonic acid uptake by the heart by stimulating the reacylation step and 2) binding with FFAs and their esters, which may be produced during ischemic insult.

The protection of ischemic heart by FABP may be considered to be an interesting facet of the pathobiology of ischemic and reperfusion injury. Accumulation of FFAs and their long-chain esters and lysophospholipids in concert with loss of FABP during ischemia strongly suggests a role of FABP in myocardial ischemic and reperfusion injury. Our results support this hypothesis and clearly demonstrate a protective role of FABP in ischemic heart.

Acknowledgments

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