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FREE RADICAL SCAVENGING BY MYOCARDIAL FATTY ACID BINDING PROTEIN

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Recent investigations have indicated the presence of a fatty acid binding protein (FABP) in mammalian heart. This protein binds free fatty acids and their esters with high affinity, however, its physiological role remains unknown. Since FABP constitutes a significant amount of cystolic protein, it is likely that it would be a target for free radical attack. To test this hypothesis, FABP was examined for scavenging against free radicals such as the superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and hypochlorite radical ($OCl^\cdot$) which may be present in an ischemic reperfused heart. Our results suggest that FABP scavenges O_2^-, $OH^\cdot$ and $OCl^\cdot$ as indicated by the FABP inhibition of O_2^--dependent reduction of cytochrome c, $OH^\cdot$-dependent hydroxybenzoic acid formation and $OCl^\cdot$-mediated chemiluminescence response. FABP was found to be a more potent scavenger of these free radicals compared to bovine serum albumin. Furthermore, FABP was more effective in scavenging $OH^\cdot$ than O_2^-, and inhibited $OH^\cdot$ mediated lipid peroxidation process. These results indicate that FABP can scavenge free radicals which may be present in an ischemic/reperfused heart and, thus, may play a significant physiological role in the heart during ischemia and reperfusion.

KEY WORDS: Free radicals, oxygen, heart, fatty-acid binding protein.

INTRODUCTION

The reperfusion of ischemic myocardium is associated with the generation of free radicals[1,2] which presumably attack the polyunsaturated fatty acids (PUFA) of membrane phospholipids causing their breakdown.[3,4] These processes result in the accumulation of free fatty acids and their thioesters,[5,6] which are known to cause arrhythmia and severe myocardial dysfunction by virtue of their detergent actions on the membrane and their enzyme inhibitory properties.[7] Recently a specific fatty acid binding protein (FABP) present in several tissues including heart has been identified.[8,9] This FABP is capable of binding fatty acids and their esters with high affinity,[10] and can therefore protect the myocardium from fatty acid mediated injury. FABP has also been implicated in several other biochemical functions including the transport of fatty acids to mitochondria which subsequently enter the β-oxidation pathway.[11]

Oxygen derived free radicals such as the superoxide anion (O_2^-) and hydroxyl radical ($OH^\cdot$) can modulate myocardial membrane by attacking membrane lipids and proteins.[12–14] Since, FABP constitutes about 4% of the cystosolic proteins,[15] it could also be a target of free radical attack and as a consequence, it might be able to directly scavenge these radicals. Free radical scavenging by FABP may be important because of the recent findings that reperfusion of ischemic myocardium is also associated with the loss of cystolic FABP.[10] The results of our study indicate that FABP can scavenge

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O_2^-, $OH^\cdot$, and hypochlorite radicals ($OCl^\cdot$) plus hypochlorous acid (HOCl) and suggest that FABP can function as a scavneger of free radicals during ischemia and reperfusion of myocardium.

MATERIALS

Xanthine, xanthine oxidase, fatty acid free bovine serum albumin, and NaOCl were received from Sigma Chemical Company, St. Louis, MO., U.S.A.

METHODS

Preparation of rat heart cytosolic FABP

Rat heart cytosolic FABP was essentially prepared according to the procedure described by Ockner *et al.*[11] The cytosolic fraction (100,000 g supernatant containing 10.5 mg protein/ml) was dialyzed against 10 mM Tris-HCl (pH 7.5) (buffer A) and the dialysate was loaded onto a Sephadex G-75 (100 × 1 cm) column, previously equilibrated buffer A. The second peak eluting in the low molecular weight region 10–25,000 daltons was loaded onto a DEAE-cellulose (10 × 2 cm) column previously equilibrated with 10 mM Tris-HCl, pH 8.5 (buffer B). After adsorption, the column was washed with 2 bed volumes of buffer B and linear gradient of 0–0.4 M KCl (2 bed volumes) in buffer B. The fractions eluting in 0.1 M KCl were pooled and concentrated by ultrafiltration using a YM-5 membrane. The concentrate was subjected to a second Spehadex G-75 gel filtration and the fractions capable of binding oleate and eluting after myoglobin were pooled (18,000 daltons). The purity of the preparation was determined by 15% polyacrylamide gel electrophoresis under denaturing and reducing conditions.

Delipidation of Purified FABP and Assay of Fatty Acid Binding

The purified FABP was subjected to delipidation using Lipidex 1000 as described by Glatz *et al.*[12] A 2 mg sample of FABP was loaded on a column of Lipidex (0.5 × 5 cm) equilibrated with 100 mM Tris-HCl buffer, pH 7.4 containing 1 mM dithiothreitol at 37°C. The delipidated FABP emerged in the void volume. The oleate binding capacity was determined essentially according to the procedure described by Offner *et al.*[15] The reaction mixture contained increasing amounts of [1-^{14}C] oleic acid (Na^+ salt) (10,000 c.p.m.), 2 nmol FABP, 1.5 mM dithiotreitol and 100 mM Tris-HCl, pH 7.4, in a total volume of 0.35 ml. The binding reaction was allowed to take place for 5 min at 37°C, after which the reaction tubes were cooled on ice. The unbound fatty acid was removed by mixing with 200 μl of ice-cold Lipidex 1000 and buffer suspension (1:1 v/v) for 10 min at 4°C. The radioactivity remaining in the supernatant was determined to calculate nmol oleate bound/nmol FABP after subtracting for non-specific binding.[8]

Generation and Measurement of Free Radicals

The $O2^-$ was generated by the reaction of xanthine oxidase with xanthine.[1] The reaction mixture contained 100 μM xanthine and 10 μM EDTA in a total volume of

1 ml. To generate $OH^{\cdot}$, 100 μM EDTA were added to the O_2^{-} generating system.[16] The reaction mixture to generate OCl^{-} contained 100 μl of luminol 10 mM Hank's buffer (pH 10) and 500 μM NaOCl in a total volume of 1 ml.

The generation of O_2^{-} was measured by following the reduction of cytochrome c spectrophotometrically at 550 nm[1]. The generation of $OH^{\cdot}$ was measured by trapping the radical with sodium salicylate and analyzing hydroxylated products 2,3-and 2,5-dihydroxybenzoic acids[17] on HPLC. 100 μl of concentrated HCl and 2 nmol of sodium fluoride were added to terminate the reaction. The resultant mixture was degassed and filtered through Rainin Nylon-66 membrane filter (0.45 μM size). 5 μl of sample was injected into an Altex Ultrasphere 3 μODS column (75 × 4.6 mm) inserted in a Waters Associates HPLC unit consisting of Model 510 pump and Model 460 electrochemical detector (Waters, Milford, MA). The hydroxylated products of salicylic acids were eluted with the buffer (degassed and filtered) containing 0.03 μM sodium citrate (pH 4.5) at a flow rate of 0.8 ml/min. The detector potential was maintained at 0.6V using a Ag/AgCl reference electrode. The time required for each analysis was about 10 min.

The generated OCl^{-} was measured by monitoring the chemiluminescence response with a luminometer, model 1250 (LBK Biotech Inc., Gaithersburg, MD).[1]

Measurement of Free Radical Scavenging Action of Albumin, FABP and Dialyzed Cytosol

To measure scavenging action of the albumin, FABP and dialyzed cytosol on O_2^{-}, varying amounts of these proteins were included in the O_2^{-} generating system which also contained 100 μM cytochrome c. The reduction of cytochrome c was followed spectrophotometrically at 550 nm at an interval of 20 seconds. The amount of cytochrome c reduced was calculated using a molar extinction coefficient at 26 mM^{-1} cm^{-1}. To measure the scavenging action of $OH^{\cdot}$, various amounts of protein were added to the generating system. The $OH^{\cdot}$ generating system also contained 2 mM salicylate to trap the generated $OH^{\cdot}$ radical. The generation was allowed to proceed for 15 min at 25°C after which the reaction was terminated and the products were analyzed. The $OH^{\cdot}$ scavenging action of FABP was also examined by following the $OH^{\cdot}$-catalyzed oxidation of deoxyribose, as described in Halliwell *et al.*[18] The reaction mixture contained a total volume of 1 ml, increasing amounts of FABP or albumin, 28 mM deoxyribose, 20 mM Tris-HCl buffer pH 7.4, containing 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2 and 100 μM ascorbate. The reaction mixture was incubated at 37°C for 1 hr, after which malondialdehyde formation from deoxyribose oxidation was measured using the thiobarbituric acid reaction.[18,19]

To measure the scavenging of OCl^{-}, the reaction mixture contained luminol buffer, (pH 10) and various amounts of protein. The reaction was started with the addition of 500 μM NaOCl. The chemiluminiescense response was immediately measured.

RESULTS

The FABP purified from rat heart by the method of Ockner *et al.*[1] was found to be homogenous as judged by SDS-PAGE (Figure 1). The apparent molecular weight was calculated to be 14,000 daltons which is similar to that reported for rat heart FABP.[8] Before examining the effects of FABP on the free radical generating systems, it is

FIGURE 1 SDS-PAGE of Purified FABP. From left to right: A DEAE fraction: B, second G-75 fraction; C, first G-75 fraction: D, cytosolic proteins, E, standard proteins: bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); trypsin inhibitor (22,000); and myoglobin (17,000); trypsinogen (24,000) and lysozyme (14,000).

necessary to demonstrate that the isolated rat heart FABP could bind stoichiometric amounts of fatty acid. In this study, when the oleate concentration was increased, increasing amounts of the fatty acid were bound and saturation was reached at a concentration of 2 nmol of oleate bound/nmol of FABP (Figure 2).

The effect of increasing concentration of FABP, albumin and dialyzed cytosol on the scavenging of O_2^- generating by the action of xanthine oxidase on xanthine is given in Figure 3. An increase in the FABP concentration resulted in the decrease of O_2^- dependent reduction of cytochrome c, and at 40 μM FABP, a maximum of 30% inhibition was observed. Although, the inhibition of O_2^- dependent cytochrome c

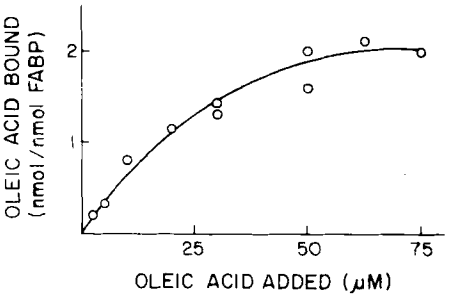

FIGURE 2 Binding of radiolabeled oleic acid to rat heart FABP. Varying amounts of oleic acid were incubated with 2 nmol of FABP for 5 min at 37°C, after equilibration protein bound and unbound oleic acid were separated by the use of Lipidex at 0°C as described in "Experimental Procedures."

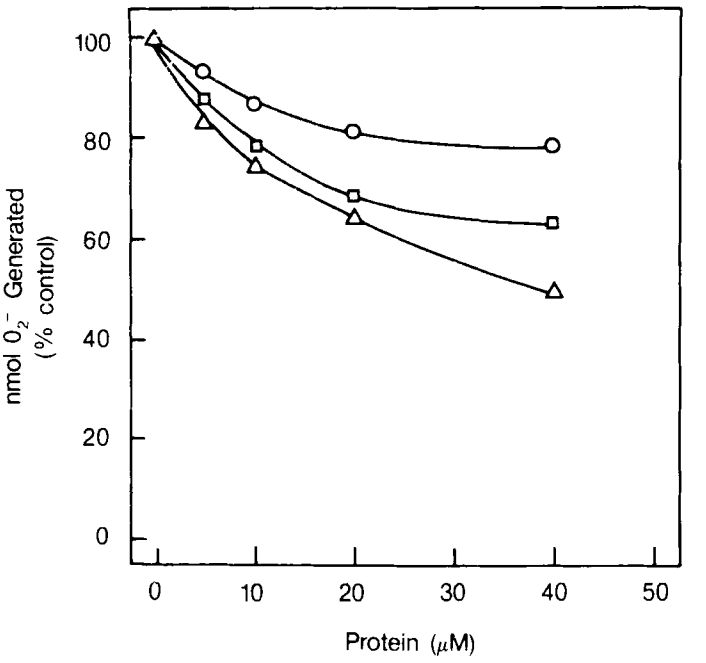

FIGURE 3 Effect of varying concentration of FABP, albumin and dialyzed cytosol on the O_2^- generation. The reaction mixture contained in 10 μM Tris-HCl (pH 7.4), 100 μM hypoxanthine, 100 μM cytochrome c, 10 μM EDTA and indicated amounts of albumin (○), FABP (△) and dialyzed cytosol (□) in a final volume of 1 ml. The reaction was started by the addition of 8 mU of xanthine oxidase and the cytochrome C reduction was followed spectrophotometrically at 550 nm.

reduction also increased with increasing albumin concentration, the maximum inhibition obtained at 40 μM BSA appears to be only 20%. The dialyzed cytosolic proteins also scavenged O_2^- and assuming an average molecular weight of 50,000 daltons for cytosolic proteins, 50% inhibition of O_2^--dependent cytochrome c reduction was observed at 40 μM protein concentration.

The $OH^{\cdot}$ was generated by the action of Fe^{3+} and EDTA on the O_2^- generating system and its generation was monitored by trapping it with salicylic acid and analyzing the hydroxylaxted products by HPLC. Figure 4 shows that a zero xanthine concentration, two response peaks eluting at 3.5 min and 3.8 min were observed. However, at 40 μM and 100 μM concentration of xanthine, in addition to the increase in the response of the 3.5 min and 3.8 min peaks, a third peak emerging at 7.5 min also increased. The authentic standards of 2,3-and 2,5 dihydroxybenzoic acids (data not shown) were also found to elute at 7.5 min indicated that indeed the hydroxylated products of salicylic acids (2,3-and 2,5-dihydroxybenzoic) were being measured. The results included in Figure 5 show that concentration of FABP, albumin, or cytosol inhibited the formation of hydroxylated products of salicylic acid. The inhibition on the formation of hydroxylated products of salicylic acid. The inhibition on the formation of hydroxylated products progressively increased with increasing concentrations of FABP and at 25 μM more than 80% inhibition was observed. Further-

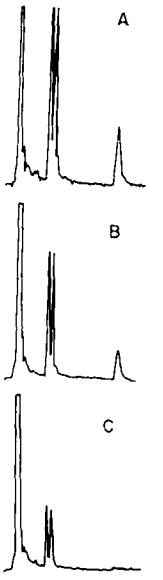

FIGURE 4 HPLC identification of the hydroxylated products of salicylic acid. The incubation mixture contained in a total volume of 1.8 ml; 10 mM Tris-HCl (pH 7.4), 2 mM salicylic acid, 8 mU of xanthine oxidase, 100 μM EDTA, and (A) 0 μM, (B) 40 μM, and (C) 100 μM xanthine.

more, the extent of inhibition of $OH^{\cdot}$ generation by increasing the cytosolic protein appears to be similar to that produced by FABP. Thus at 25 μM cytosolic protein concentration (assuming an average molecular weight of 50,000 daltons), 80% of the hydroxylated salicylic acid formation was inhibited. However, as observed with O_2^- and also with $OH^{\cdot}$, albumin appears to be a relatively poor scavenger compared to FABP to cytosolic proteins. Thus, with 25 μM albumin only 38% inhibition was observed, while at 50 μM 55% of the $OH^{\cdot}$ generation was inhibited.

The $OH^{\cdot}$ has been shown to oxidase deoxyribose yielding malondialdehyde which appear to be similar to the process of lipid peroxidation.[19] Therefore, we examined the effects of FABP and albumin on the $OH^{\cdot}$ catalyzed formation of malondialdehyde from deoxyribose. The results shown in Figure 6 demonstrate that both FABP and albumin inhibited the formation of malondialdehyde. As in the salicylate assay for $OH^{\cdot}$ generation, FABP was again found to be more effective than albumin in inhibiting deoxyribose oxidation. Thus, at 40 μM, FABP and albumin inhibited malondialdehyde formation by 50% and 30% respectively.

The chemiluminescense response of the $OCl^{\cdot}$ generated from NaOCl was also inhibited by FABP, albumin and dialyzed cytosol Figure 7. It is interesting to note that the concentration of these proteins required to scavenge $OCl^{\cdot}$ was much lower compared to those required to scavenge either $OH^{\cdot}$ and O_2^-. Thus at 10 μM of FABP, albumin and cytosolic proteins, 60%, 45% and 65%, of chemiluminiescense responses were inhibited respectively. Again, the inhibition by all these proteins increased with increase in protein concentration.

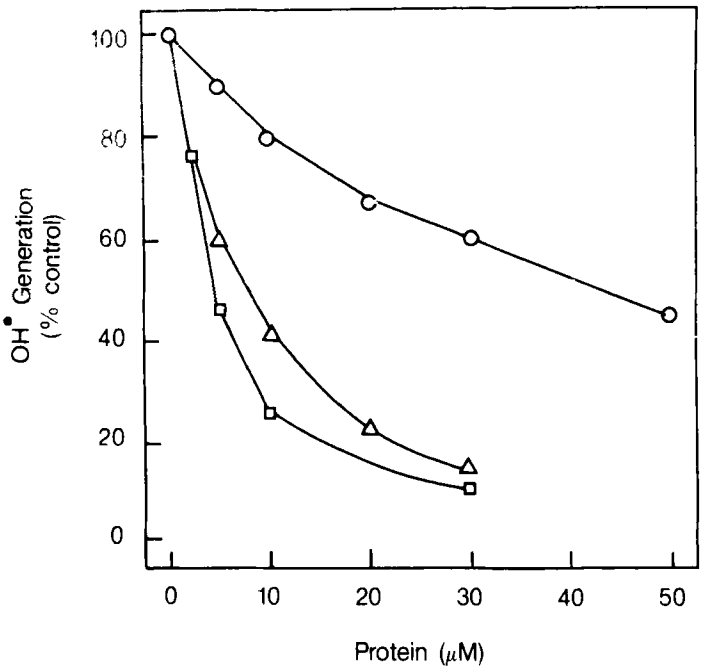

FIGURE 5 Effect of varying concentrations of FABP, albumin and dialyzed cytosol on the OH· formation. The reaction mixture contained 10 mM, Tris-HCl (pH 7.4), 100 μM hypoxanthine, 100 μM $FeCl_3$, 100 μM, $FeCl_3$, 100 μM EDTA, 2 mM slicylic acid and indicated amounts of albumin (○), FABP (△) and dialyzed cytosol (□) in a final volume of 1 ml. The reaction was started by the addition of 8 mU of xanthine oxidase and incubated 10 min at 25°C. The termination of reaction and HPLC analysis of hydroxylated products of salicylic acids are carried out as described under methods.

DISCUSSION

Fatty acid binding proteins have been identified in the cytosol of several tissues including heart.[8-12] Since these proteins have high affinity for fatty acids and their esters, several investigators attempted to examine the physiological roles of this protein. Myocardial FABP has been implicated in the intracellular transport of fatty acids between subcellular organalles,[10] and in the attenuation of acyl CoA mediated denaturation of membrane bound and cytosolic proteins.[10,11] Recent work of Haq *et al.*[20] has shown that palimitoyl CoA bound to FABP may serve as a better substrate for glycerophosphate acyl transferase during phospholipid biosynthesis.[20]

The FABP from bovine and rat heart contains significant amounts of SH-containing amino acids such as cystine, methionine, as well as aromatic acids like phenylalanine, tyrosine and histidine.[11] Since FABP constitutes about 4–5% of total cytosolic proteins which also contain free radical susceptible amino acids, it is reasonably to speculate that FABP may be a target of free radical attack. Since the formation of free radicals has been indicated in the ischemic reperfused heart, it may be presumed that these free radicals can attack the oxidizable amino acids present in FABP. This process should reduce the concentration of free radicals that can reach the mitochon-

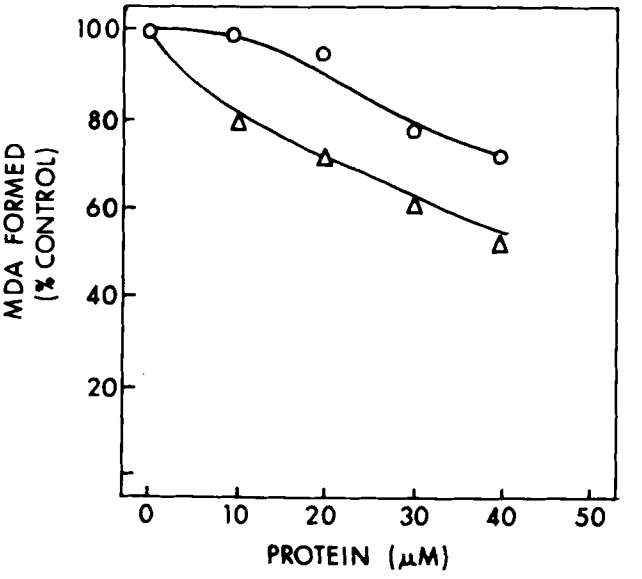

FIGURE 6 Effect of varying concentrations of FABP and albumin on the $OH^{\cdot}$ dependent formation of malondialdehyde from deoxyribose. The reaction mixture contained 20 mM Tris-HCl (pH 7.4), 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2, 100 μM ascorbate and indicated amounts of FABP (△) and albumin (○), in a final volume of 1 ml. The incubation was carried out at 37°C for 1 hour, following which malondialdehyde formation was measured.

drial and sarcoplasmic membrane fractions of myocardium. The present results indicate that FABP inhibited the O_2^- dependent cytochrome c reduction, $OH^{\cdot}$ -dependent dihydroxybenzoic acid formation and the chemiluminiescense response of $OCl^{\cdot}$, suggesting a role for FABP as an intracellular free radical scavenger.

The free radical scavenging effect of purified FABP has been compared with the extracellular fatty acid binding protein, albumin and dialyzed cytosol to ascertain that the scavenging action of free radical by FABP is not an enzyme mediated process such as that of superoxide dismutase and catalase. Between FABP and albumin, FABP appears to be more efficient in scavenging O_2^-, $OH^{\cdot}$ and $OCl^{\cdot}$ FABP scavenges 20%, 70% and 50% of O_2^-, $OH^{\cdot}$, and $OCl^{\cdot}$, respectively at 10 μM concentration. These results also indicate that FABP is more effective in scavenging $OH^{\cdot}$ and $OCl^{\cdot}$ than O_2^-. Recent studies by Starke and Farber[21] and Hoffmann *et al.*[22] indicate that among O_2^-, $OH^{\cdot}$ is the most detrimental to cells. Such an interpretation is based on the observation that O_2^- and H_2O_2, in the presence of Fe^{3+} chelator, desferoxamine did not cause any cellular damage, while the omission of desferoxamine resulted in significant cellular injury. Consistent with these studies are our recent findings that $OH^{\cdot}$ but not O_2^- caused the degradation as well as lipid peroxidation of membranes phospholipids.[23] Furthermore, although H_2O_2 caused the degradation and lipid peroxidation of membrane phospholipids, the concentration required was at least 1,000 fold higher than the physiological concentration.[23] These results together with the present finding that FABP is more effective in scavenging $OH^{\cdot}$ than O_2^- suggest that FABP may have a physiological role as a free radical scavenger for most deleterious $OH^{\cdot}$. The inhibition of malondialdehyde formation from the $OH^{\cdot}$ cat-

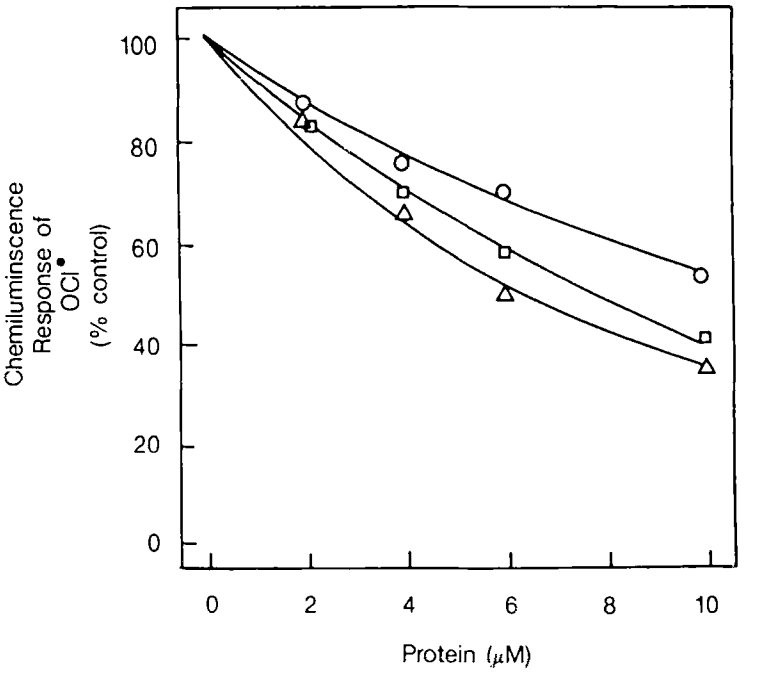

FIGURE 7 Effect of varying concentration of FABP, albumin and dialyzed cytosol on the chemiluminescense response of OCl^-. The reaction mixture contained 200 μl luminol buffer, pH 10, 500 μM NaOCl and indicated amounts of BSA (○), FABP (△) and dialyzed cytosol (□) in a final volume of 1 ml. The reaction was started by the addition of 500 mM NaOCl.

alyzed degradation of 2-deoxyribose has been suggested to be a valuable method for the determination of rate constant for reaction of most biological molecules with hydroxyl radicals.[18] Therefore, inhibition of malondialdehyde formation in $OH^\cdot$ catalyzed oxidation of deoxyribose by FABP and albumin suggest that FABP in myocardial cells can also reduce the lipid peroxidation of membrane phospholipids. The rate constants for reaction of FABP and BSA with $OH^\cdot$ as determined by the method of Halliwell *et al.* were 15.5×10^{10} and 6.1×10^{10} M^{-1} S^{-1}, respectively.

It is surprising to observe that albumin, having 5 times higher molecular weight than FABP, was less effective than FABP in scavenging of $OH^\cdot$, OCl^- and O_2^-. These differences are probably arising from the differences in the primary and secondary structures of these proteins.[13,14,25] Thus, rat heart FABP contain less of free radical susceptible amino acids such as Methionine (1.76 mole%) and tyrosine (1.68 mole%) as compared to 4.77 mole% and phenylalanine (4.77 mole%). On the other hand albumin contain 35 mole% cysteine residues as compared to zero in FABP. In addition, free radicals may be generated intracellularly where albumin being an extracellular protein may not be available in their immediate vicinity to scavenge them. FABP being an intracellular protein can easily perform the scavenging function.

Since FABP level has been shown to decrease during ischemia and reperfusion, it is reasonable to speculate that cellular injury may be arising from the decrease in FABP-linked free radical scavenging activity. In normal cells, the free radical scaveng-

ing activity of FABP may spare the attack of free radicals on membrane phospholipids, thus, preserving membrane integrity.

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