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Research Paper

ATP-Loaded Liposomes Effectively Protect the Myocardium in Rabbits with an Acute Experimental Myocardial Infarction

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Purpose. We assessed whether the infusion of ATP-loaded liposomes (ATP-L) can limit the fraction of the irreversibly damaged myocardium in rabbits with an experimental myocardial infarction.
Methods. ATP-L, empty liposomes (EL), or Krebs–Henseleit (KH) buffer were administered by intracoronary infusion, followed by 30 min of occlusion and 3 h of reperfusion. Unisperse Blue dye was used to demarcate the net size of the occlusion-induced ischemic zone (area at risk) and nitroblue tetrazolium staining was used to detect the final fraction of the irreversibly damaged myocardium within the total area at risk.
Results. The total size of the area at risk in all experimental animals was approx. 20% wt. of the left ventricle. The final irreversible damage in ATP-L-treated animals was only ca. 30% of the total area at risk as compared with ca. 60% in the group treated with EL ($p < 0.009$) and ca. 70% in the KH buffer-treated group ($p < 0.003$).
Conclusions. ATP-L effectively protected the ischemic heart muscle in rabbits with an experimental myocardial infarction as evidenced by a significantly decreased fraction of the irreversibly damaged heart within the total area at risk. ATP-L may provide an effective exogenous source of the ATP *in vivo* to protect ischemically damaged cells.

KEY WORDS: ATP; experimental myocardial infarction; heart muscle; ischemia; liposomes; mechanical functions; rabbits; reperfusion.

INTRODUCTION

In myocardial ischemia, a portion of the myocardium is deprived of nutrients and oxygen, and the level of ATP to drive the contractile process drops by 80% (1), which can only be maintained by the breakdown of the myocardial glycogen to produce small amounts of ATP via glycolysis. However, in the continued absence of an oxygen supply, this temporary metabolic pathway also becomes depleted, and ATP-dependent ion pumps in the outer membranes of myocytes cease to function, the ion balance of the cells is lost, and myocytes swell and burst, releasing their contents into the circulation. Cell death can proceed by apoptosis or oncosis depending on both the duration of the ischemic stress and the availability of intracellular ATP (2). Thus, the key factor responsible for the decrease in the ATP supply/demand ratio during the myocardial ischemia is the relative lack of an ATP supply. Theoretically, the timely application of the exogenous ATP might help in the restoration of its normal cellular level in myocytes thereby protecting the myocardium from the ischemia–reperfusion damage. However, the native ATP has a very short half-life in the blood being hydrolyzed into adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine by extracellular ectonucleotidases (3). We have recently shown that encapsulating ATP into liposomes can protect it from the hydrolysis in the presence of ATPase (4).

Liposomes are widely used as nanosized drug delivery vehicles for active and passive targeting (5). Accumulation of these lipid based vesicles in regions of experimental myocardial infarction has been demonstrated (6–8), which proceeds via the enhanced permeability and retention (EPR) effect (9,10). Liposomes may also "plug" and "seal" the damaged myocyte membranes and protect cells against ischemic and reperfusion injury *in vitro* (11). Several methods for loading ATP into liposomes have been described (12–15). The highest degree of ATP incorporation into liposomes was achieved by the freezing–thawing method (16).

Earlier, we hypothesized that the supply of the exogenous ATP loaded into liposomes may protect ischemic myocardium by delivering ATP to hypoxically compromised cells thus reducing the fraction of the irreversibly damaged

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ABBREVIATIONS: ATP, adenosine-5′-triphosphate; ATPase, adenosine-5′-triphosphatase; ATP-L, liposomes loaded with ATP; DOTAP, 1,2-dioleoyl-3-trimethyl-ammonium-propane; EL, empty liposomes; EPR, enhanced permeability and retention; KH, Krebs–Henseleit; NBT, nitroblue tetrazolium; PEG-PE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; USB, unispearse blue dye.

heart within the total area at risk, and demonstrated such protection in an isolated rat heat model (4). This protection was associated with the accumulation of liposomes and increase in ATP level in the infarcted tissues. An anti-ischemic action of ATP-loaded liposomes in different tissues was also demonstrated in several other studies (14,15,17).

In this work, we present the *in vivo* data showing a significant cardioprotective effect of ATP-loaded liposomes in rabbits subjected to a 30-min coronary artery occlusion. These results can be considered as the next significant step towards the protection of the ischemic myocardium against damage resulting from an inadequate ATP supply.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), cholesterol (Ch), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG-PE), and 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). ATP was purchased from Sigma (St. Louis, MO, USA). All other chemicals and buffer components were of analytical grade.

Preparation of Liposomes

To prepare ATP-L by the freezing–thawing method as described in (16,18), a chloroform solution of egg phosphatidylcholine (0.26 mmol), cholesterol (0.12 mmol), PEG-PE (1.8 μmol), and DOTAP (10.8 μmol) was evaporated, and the film formed was hydrated with 5 mL of 400 mmol ATP in the Krebs–Henseleit (KH) buffer. The dispersion was frozen at −80°C for 30 min followed by the thawing at 45°C for 5 min. This cycle was repeated five times. The liposomes were extruded five times through each of 800, 600, 400, and 200 nm pore size polycarbonate membranes (Whatman) to produce samples with a narrow size distribution. Nonencapsulated ATP was separated by dialysis against the KH buffer at 4°C overnight. The liposomal formulations were diluted to form a preparation containing 45 mg of lipids and 12 mg of ATP per milliliter (16), which were additionally supplemented with 1.7 mM $CaCl_2$ prior to infusion into the rabbit heart. Control EL were prepared without the addition of ATP.

Characterization of Liposomes

The size and size distribution of EL and ATP-L were determined by the dynamic light scattering technique using a Coulter N4 MD Submicron Particle Analyzer (Beckman-Coulter). Zeta potentials of the liposome preparations were measured at 25°C in water (0.08–0.015 mg lipid/mL) by using a Zeta Plus z-potential analyzer (Brookhaven Instruments, Brookhaven, NY, USA).

Experimental Myocardial Infarction in Rabbits

The procedure was previously described elsewhere (19). Briefly, 2.5–3.5 kg New Zealand White rabbits (Millbrook Breeding Labs) were anesthetized subcutaneously with 80 mg/kg of ketamine and 8 mg/kg of xylazine, intubated via a tracheostomy, and ventilated with room air at a tidal volume of 18–22 mL at 46–50 strokes/min using a Harvard rodent positive-pressure ventilator (Harvard Apparatus Co.). Anesthesia was maintained with a dilute pentobarbital Na infusion via a marginal ear vein at 20–25 mg/h. The heart was exposed through a parasternal thoracotomy. The pericardium was dissected to insert a flexible plastic catheter into the left atrium for rapid infusions into the heart. An anterior branch of the left coronary artery was isolated with a 3–0 suture for control of flow with an occlusive snare. ECG was continuously monitored with a PageWriter M1700A electrocardiograph (Hewlett-Packard). Approximately 3 mL of a test solution of ATP-L (a total of 135 mg of lipid and 36 mg of ATP), EL (a total of 135 mg of lipid, no ATP), or KH buffer, pH 7.4, was infused through the coronary arteries by a brief (5–10 s) clamping of the aorta during the infusion with trapping of infusate in the area occluded by tightening the snare as the infusion ended. After 30 min, the snare was released and reperfusion established for 3 h. The coronary artery was then reoccluded, and 3 mL of a 1:5 diluted Unisperse Blue (USB; Ciba) was infused via the atrial catheter to demarcate the net size of the occlusion-induced ischemic zone (USB stains the normoxic tissues, whereas the ischemic zone termed "the area at risk" remains unstained). The anesthetized animal was immediately sacrificed by exsanguination and the heart was removed. The excised left ventricle (LV) was sliced transversely between apex and base into five to six approximately equal thickness slices, which were digitally photographed from both sides to register the site of the occlusion-induced area at risk by the absence of blue staining. Slices were then incubated for 20 min in a 40–45°C 0.05% PBS-buffered nitroblue tetrazolium (NBT) solution for the detection of the infarcted portion of the area at risk by delineation of noninfarcted areas as the stained zones containing intact myocyte dehydrogenases, whereas irreversibly damaged necrotic tissues within the net ischemic zone remain unstained. This technique relies on the ability of dehydrogenase enzymes and cofactors in the tissue to react with tetrazolium salts to form a formazan pigment. The slices were rephotographed and finally weighed. The area at risk and the fraction of the irreversibly damaged myocardium were determined from planimetry of both sides of all slices using Adobe Photoshop 7.0. The total LV weight at risk and the weight infarcted were calculated in two independent runs and averaged. The infarction size was expressed as the percent of the total weight at risk.

Ethics

Protocols for this study were approved by the IACUC, Northeastern University (Boston, MA, USA), and conform to the guidelines specified in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985).

Statistical Data Analysis

Data analysis was carried out with the software package Microcal Origin, Version 6. Results were expressed as mean ± standard error (n = 4–8 independent samples). Statistically significant differences were determined by using

the analysis of variance (ANOVA) followed by Student's *t* test. A *p* value of less than 0.05 was considered significant.

RESULTS

Liposome Size, Size Distribution, and Zeta Potential

The freezing–thawing/extrusion method resulted in vesicles with an average size of ca. 200 nm, a relatively high ATP encapsulation efficiency (ca. 0.40 μmol of ATP per μmol of total lipids), and narrow size distribution for both EL and ATP-L (Fig. 1A, B). Zeta-potential values were almost identical for EL and ATP-L (13.27 ± 0.59 and 12.91 ± 0.41 mV, respectively; with the difference being nonsignificant) indicating that ATP was encapsulated into liposomes rather than associated with the liposomal membrane. In our earlier experiments, we showed that the release of ATP from liposomes prepared by the freezing–thawing/extrusion method is approximately 12% of the total liposomal ATP after 24 h long incubation in the KH buffer at 37°C (20).

Infarct Size in Rabbits with Experimental Myocardial Infarction

The temporary occlusion of a branch of the left circumflex artery in rabbits resulted in myocardial infarction as confirmed by the comparison of the electrocardiograms (ECG) of postocclusion animals with preocclusion ECG patterns (see typical ECGs in Fig. 2). The elevation of the ST segment in animals with the occluded artery (Fig. 2B) indicated the development of an infarction.

The average LV weight in all animals was 3.57 ± 0.11 g (n = 19). The mean size of the "area at risk" (the net hypoxic area developed as a result of occlusion) in all infarcted animals was 20.4 ± 2.3% of the total LV (n = 19) as shown by the USB staining (Fig. 3A, C, E).

The effect of the ATP-L and control (EL, KH buffer) treatments on the size of the final infarction was estimated by measuring the fraction of the area at risk (USB staining data), which by the end of the experiment underwent irreversible damage according to the NBT staining. In control animals receiving either the EL or KH buffer, the final size of the infarction (irreversibly damaged tissue) was 59.5 ± 7.2 and 68.6 ± 3.9% of the total area at risk, respectively (Fig. 4). The 60–70% infarction of the area at risk after 30 min of ischemia

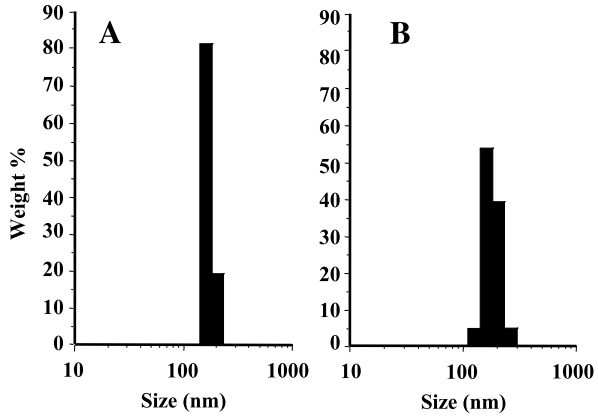

Fig. 1. The size and size distribution of EL (A) and ATP-L (B).

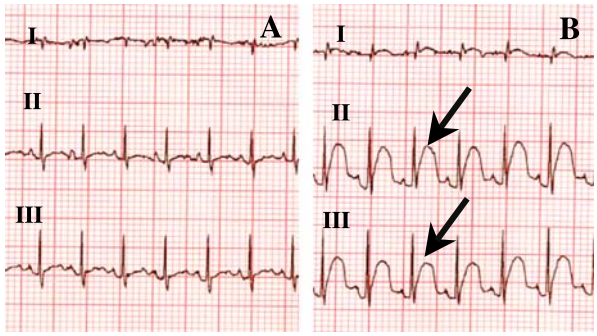

Fig. 2. Typical ECGs of the noninfarcted rabbit (A), and ECG with the elevated ST segment after coronary occlusion in the rabbit following an acute experimental myocardial infarction (B).

is in good agreement with previous observations in a similar model (21). At the same time, the administration of ATP-L resulted in a substantial decrease in the size of the irreversibly damaged heart within the total area at risk as depicted in Fig. 4. The intracoronary administration of the ATP-L (36 mg of ATP total) effectively protected the ischemic heart muscle in rabbits with experimental myocardial infarction limiting the irreversible tissue damage to only 29.4 ± 6.5% of the total area at risk ($p < 0.003$ when compared with controls). Thus, in KH buffer- and EL-treated animals, the infarcted area seems not much smaller than the total area at risk (see the typical data in Fig. 3, red areas in A and B, C and D), whereas in the ATP-L-treated animal the developed infarct constituted a significantly smaller fraction of the total area at risk (see the typical data in Fig. 3, red areas in E and F) because of the protective action of ATP-L. To keep the number of experimental animals reasonably low, the KH buffer and EL were used as controls for the ATP-L treatment, because using the free ATP as an additional control does not make any sense due to its instant degradation *in vivo* (3,22).

We have also performed an analysis of covariance for the group effect on the weight of the tissue that developed necrosis and the weight of the total area at risk. Figure 5 shows the relationship between the absolute weight of the final necrosis and the weight of the ischemic zone. Because there was no significant difference between the final infarct size in EL- and KH buffer-treated animals (Fig. 4), these two control groups were combined for the comparison with the ATP-L group. There was good linear correlation between the weight of infarcted tissue and the weight of the total area at risk for the combined controls ($r^2 = 0.91$; $Y = -0.13 + 0.82X$), although the data for the ATP-L-treated group were more variable ($r^2 = 0.41$; $Y = -0.09 + 0.41X$) (Fig. 5). However, results of this analysis still clearly show that treatment with ATP-L significantly decreased the size of the irreversibly damaged heart tissue within the total area at risk by ca. 55% (over the weight range of the LV at risk varying from ca. 0.4 to ca. 1.4 g) compared to the combined controls (Fig. 5).

DISCUSSION

Our results clearly demonstrate a pronounced protective effect of the ATP-L (a total intracoronary administered dose of 36 mg ATP in these experiments) on the survival of the

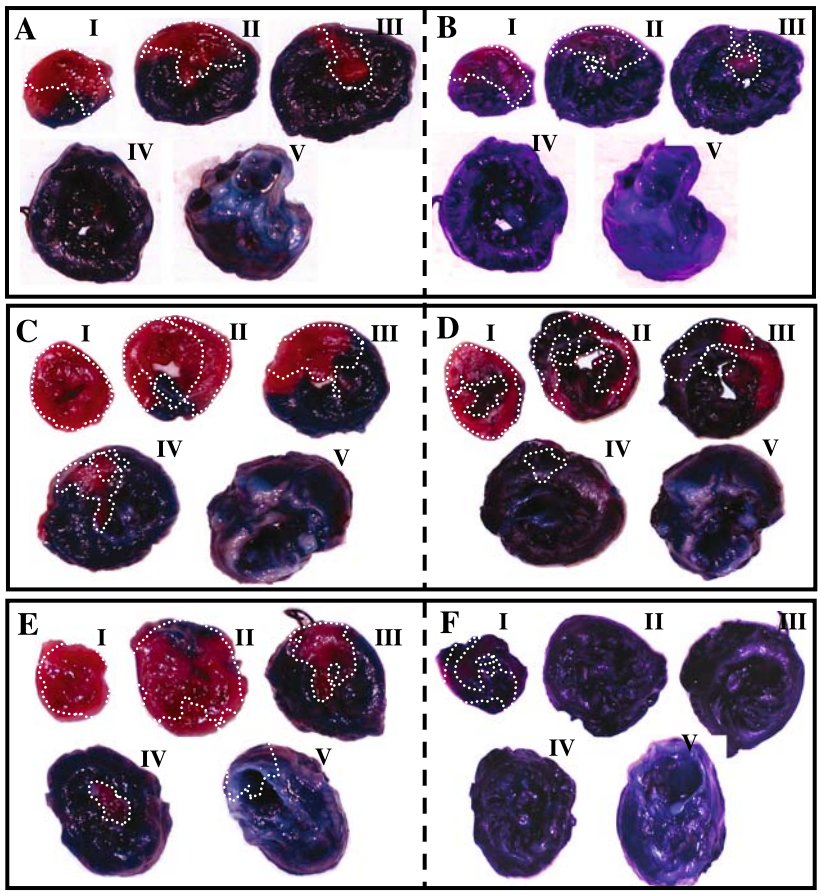

Fig. 3. USB- and NTB-stained sections of infarcted myocardium. Cardioprotective effect of ATP-L after 30 min of coronary occlusion and following 3 h of reperfusion in rabbits with an acute experimental myocardial infarction. (A, B) Control KH buffer-treated animal; (C, D) EL-treated animal; (E, F) ATP-L-treated animal. (A, C, and E) Area at risk (USB-unstained red tissue) developed as a result of occlusion; (B, D, and F) infarcted area at the end of occlusion/reperfusion experiment (NBT-unstained tissue); heart slices I to V represent base to apex.

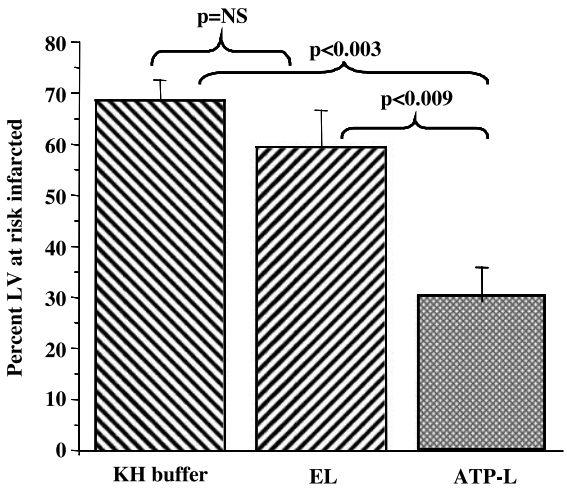

Fig. 4. Summary graph showing the fraction of infarcted area as a percentage of the total area at risk in the KH buffer-treated control group, EL-, and ATP-L-treated group; (mean ± SE), n = 4–10.

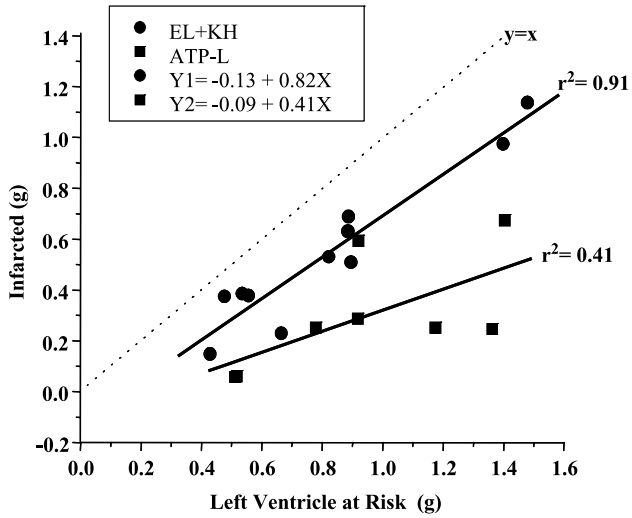

Fig. 5. Scatter plot showing the relationship between the weight of ischemic myocardium at risk and necrotic zone of the combined controls and ATP-L-treated groups.

myocardium during an experimental acute myocardial infarction *in vivo* resulting in an approx. 2-fold decrease in the size of the irreversibly damaged area of the heart (revealed by the NBT staining) within the total size of the area at risk (revealed by the USB staining), when compared to the animals receiving control treatments with KH buffer or EL (Fig. 4).

It is known that the most effective approach for decreasing the myocardial necrosis is the quick and complete coronary artery reperfusion. Further attenuation of myocardial necrosis can be achieved if timely reperfusion is supplemented with other interventions, such as an augmentation of the collateral flow, slowing metabolic degradation of ATP, reducing reperfusion injury (21), or the supply of the exogenous ATP using, for example, a delivery system such as liposomes that protects ATP from degradation by ATPases (4). The administration of ATP with a liposomal delivery system clearly protected the ischemic heart after 30 min of coronary occlusion followed by 3 h of reperfusion.

From these data [well supported by our previous experimental data from the isolated rat heart model (4)], we conclude that the infusion of ATP-L eventually resulted in an elevated level of ATP in cardiomyocytes under the conditions of the coronary occlusion in this rabbit myocardial infarction model. Thirty minutes of occlusion may have provided a sufficient time for ATP-L to accumulate in the ischemic zone via the earlier reported EPR effect (4,8,10), and "unload" at least a part of the liposomal ATP into the ischemic cells in concentrations sufficient to protect them from the irreversible damage. This possibility agrees well with earlier observations that ATP-L are rapidly taken up by isolated rat hearts during a Langendorff perfusion (23) and also protect the rat liver during 30 min of hypovolemic shock (15).

The mechanism of the protective action of ATP-L on the ischemic myocardium may involve the combination of the earlier demonstrated nonspecific "plugging and sealing" of the damaged cell membranes (11) and the delivery of the ATP into the ischemic myocytes at the same time (4). Earlier reported results support our hypothesis on the intracellular delivery of an ATP by ATP-L: positive ionotropic effect of ATP-L infusion in isolated rat hearts; an extensive association of liposomes that were double-labeled with hydrophilic and lipophilic fluorescent dyes in the ischemic areas of the rat heart; and an increase by almost 50% in the level of ATP found in ATP-L-treated rat hearts (4).

Liposomes have been shown to cross the capillary endothelium and deliver ATP intracellularly by passing through the endothelial tight junction and by increased endothelial endocytosis (24,25). Liposomes and micelles have also been reported to accumulate in the ischemic myocardium via the EPR effect when passing through the damaged blood vessels (4,8–10). It was also reported that liposomes could cross the continuous wall of myocardial capillaries in the isolated heart through endocytosis (26).

In conclusion, ATP-L effectively protected the myocardium from the ischemia/reperfusion damage by diminishing the size of the irreversibly damaged zone in rabbits with an acute experimental myocardial infarction. ATP liposomes may provide an effective exogenous source of the ATP *in vivo* and a tool for cardiomyocyte protection in ischemically damaged hearts suffering from an inadequate ATP supply during an ischemic attack, although the exact application setup may be quite different from our proof-of-principle experiment.

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