A Study on Dilazep: II. Dilazep Attenuates Lysophosphatidylcholine-Induced Mechanical and Metabolic Derangements in the Isolated, Working Rat Heart

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ABSTRACT—The effects of dilazep, d-propranolol and lidocaine on the mechanical and metabolic changes induced by lysophosphatidylcholine (LPC) were studied in isolated, perfused working rat heart. After a stabilization period, the heart was perfused for 5 min with LPC (10 μM) alone, LPC plus dilazep (5, 10 or 20 μM), LPC plus d-propranolol (30 or 50 μM) or LPC plus lidocaine (30 or 100 μM) and then perfused with normal Krebs-Henseleit bicarbonate (KHB) buffer for a further 20 min. Perfusion with LPC for 5 min followed by KHB for 20 min irreversibly decreased cardiac mechanical function, decreased the tissue levels of adenosine triphosphate and creatine phosphate significantly, and increased the tissue levels of lactate and free fatty acids including arachidonic acid. Dilazep or d-propranolol significantly attenuated the mechanical and metabolic changes induced by LPC, but lidocaine did not. These results indicate that the exogenous LPC causes ischemia-like changes, suggesting that LPC is one of the important factors in producing ischemia-reperfusion derangements in terms of mechanical and metabolic functions, and that both dilazep and d-propranolol can prevent the LPC-induced myocardial damage.

Keywords: Dilazep, Lysophosphatidylcholine, Working heart, Adenosine triphosphate, Free fatty acid

In the preceding report (1), we concluded that the anti-ischemic action of dilazep is probably due to its energy-sparing effect. Other factors, however, cannot be excluded from the mechanism of the anti-ischemic action of dilazep. One of the candidates believed responsible for ischemia-induced changes is lysophosphoglycerides, because during ischemia lysophosphoglycerides increase in the heart and in the effluents from myocardium (2–4). In addition, lysophosphoglycerides produce cardiac arrhythmias (2, 5, 6), suggesting that lysophosphoglycerides contribute to cardiac arrhythmias that occur during ischemia and post-ischemic reperfusion (simply expressed as reperfusion in the present study). If lysophosphoglycerides are responsible for arrhythmias during ischemia and reperfusion, there is no reason to discard the view that they are also responsible for other ischemic changes including mechanical dysfunction and metabolic derangements. In support of this view, lysophosphatidylcholine (LPC) has been shown to produce myocardial cell injury in addition to arrhythmias (7).

The present study therefore was designed to examine for the first time whether exogenous LPC could cause ischemia-like mechanical and metabolic changes in the isolated working rat heart and whether dilazep attenuates the LPC-induced changes. LPC was used as one of the lysophosphoglycerides because it accumulates in the ischemic heart (2). To exclude extracardiac factors, we employed an isolated heart preparation. As reference drugs, d-propranolol and lidocaine were employed because these drugs, like dilazep, attenuate ischemia-reperfusion-induced mechanical and metabolic changes of the heart (8, 9).

MATERIALS AND METHODS

Heart perfusion

Male Sprague-Dawley rats (280–340 g; Sankyo Labo Service Corporation, Sapporo) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After thoracotomy, the hearts were quickly removed and perfused according to the Langendorff method at a constant pressure

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of 90 cmH\textsubscript{2}O for 10 min (stabilization period), and then they were perfused according to the working heart method at a left atrial filling pressure of 12.5 cmH\textsubscript{2}O and an afterload pressure of 90 cmH\textsubscript{2}O for 35 min (8, 10). The solution for perfusion was a modified Krebs-Henseleit bicarbonate (KHB) buffer (119.4 mM NaCl, 4.7 mM KCl, 2.9 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 25.0 mM NaHCO\textsubscript{3}, 11.0 mM glucose and 0.5 mM EDTA-2Na) equilibrated with a gas mixture of 95\% O\textsubscript{2} + 5\% CO\textsubscript{2} and maintained at 37 °C. Aortic pressure and heart rate were monitored with a pressure transducer placed in the aortic cannula. Cardiac mechanical function was defined as RPP (peak aortic pressure multiplied by heart rate). Coronary flow (defined as the flow coming from the cannula inserted into the pulmonary artery) was measured with a graded glass cylinder.

**Experimental protocol and heart groups**

After 10 min of working heart perfusion, the perfusion solution was changed for 5 min to the KHB buffer containing LPC alone, LPC plus dilazep (5, 10 or 20 \mu M), LPC plus d-propranolol (30 or 50 \mu M), LPC plus lidocaine (30 or 100 \mu M) or LPC plus bovine serum albumin (BSA) (10 \mu M). The concentration of LPC used in the present study was 10 \mu M. After treatment with LPC or LPC plus drugs for 5 min, the hearts were perfused with KHB buffer for a further 20 min. In some experiments, the hearts were perfused with LPC alone for 5 or 25 min.

The hearts were divided into 12 groups: control (n=10), LPC 5 min (n=6), LPC 25 min (n=6), LPC (LPC 5 min + KHB 20 min) (n=12), LPC plus dilazep (5 \mu M) (n=8), LPC plus dilazep (10 \mu M) (n=12), LPC plus dilazep (20 \mu M) (n=10), LPC plus d-propranolol (30 \mu M) (n=8), LPC plus d-propranolol (50 \mu M) (n=5), LPC plus lidocaine (30 \mu M) (n=4), LPC plus lidocaine (100 \mu M) (n=4) and LPC plus BSA (10 \mu M) (n=5). In the control group, the hearts were perfused with KHB by the working heart perfusion method for 35 min without LPC or any drug. The hearts were freeze-clamped at the end of KHB perfusion. In the LPC 5 min and LPC 25 min groups, the hearts were perfused with LPC alone for 5 min and 25 min, respectively. These hearts were freeze-clamped at the end of LPC perfusion. In the LPC (LPC 5 min + KHB 20 min) group, hearts were perfused with LPC for 5 min and then perfused with KHB for 20 min. In LPC plus drug or LPC plus BSA groups, hearts were perfused with LPC plus drug or LPC plus BSA, respectively, for 5 min and then perfused with KHB for 20 min. In these groups, the hearts were freeze-clamped at the end of the 20 min perfusion with KHB. Freeze-clamping of the heart was performed by the use of a Wollenberger's clamp chilled in liquid nitrogen (−173 °C). The frozen myocardial samples were stored at −173 °C until the bio-chemical analysis was performed.

**Assay of the tissue high-energy phosphates and lactate**

A part of the frozen cardiac tissue sample (about 0.8 – 1 g) was pulverized in a mortar and pestle cooled to the temperature of liquid nitrogen. High-energy phosphates (adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine monophosphate, AMP; and creatine phosphate, CrP) and lactate were extracted from the pulverized tissue sample with perchloric acid and then neutralized with KOH. These products of energy metabolism were assayed by standard enzymatic methods (11–13) using a spectrophotometer (Gilford system 2600; Gilford Instrument Laboratories, Inc., Oberlin, OH, USA). Energy charge potential (ECP) was calculated according to the following formula:

\[ ECP = \frac{(ATP + 0.5 \text{ ADP})}{(ATP + \text{ ADP} + \text{ AMP})} \]

**Assay of the tissue FFA**

The levels of tissue free fatty acids (FFA) were measured according to the method described in our previous study (10). Briefly, the frozen cardiac tissue (about 150 mg) was pulverized in a mortar cooled with liquid nitrogen, and tissue FFA were extracted from the pulverized tissue with chloroform/methanol (2:1) containing 0.05\% butylated hydroxytoluene (as an anti-oxidant), and FFA in the extract were then converted to their fluorescent derivatives with 9-anthryldiazomethane in methanol. After incubation at room temperature for 1 hr, the fluorescent derivatives of FFA were filtered with a Millipore\textsuperscript{TM} filter (FH 0.5 \mu m; Nihon Millipore Kogyo K.K., Yonezawa) and injected into a reverse-phase high-performance liquid chromatography system with a Zorbax-ODS column (0.46 x 25 cm; DuPont, Philadelphia, PA, USA). Methanol/distilled water (100:80) was used as the mobile phase. The level of individual FFA was determined by comparing the peak height of the FFA with that of a known amount of heptadecanoic acid (an internal standard).

**Drugs**

Dilazep was supplied by Kowa Company (Tokyo). d-Propranolol hydrochloride was supplied by ICI Pharma (Osaka). LPC, biochemicals, reagents and enzymes were purchased from Sigma Chemical Company (St. Louis, MO, USA).

**Statistical analyses**

All data are expressed as means±S.E.M. The significance of differences between means was analyzed by means of the analysis of variance, followed by Duncan's multiple range test for unpaired observations and by Student's \( t \)-test for paired observations. \( P < 0.05 \) was considered significant.
RESULTS

Cardiac mechanical function

Changes in RPP during 35 min of working heart perfusion in the presence or absence of LPC alone or LPC plus drugs are illustrated in Figs. 1 and 2. Values of RPP of the hearts before treatment with LPC alone or LPC plus drugs were similar in all the groups. In the control hearts, RPP increased when Langendorff perfusion was switched to working heart perfusion, and the RPP remained nearly constant until the end of working heart perfusion. LPC was given to the heart for 5 min from 10 min after the working heart perfusion. LPC decreased the heart rate to 0/min and it also decreased aortic pressure to 0 mmHg; therefore, RPP became 0 mmHg/min after 5 min of LPC treatment. Data on coronary flow before the beginning of LPC perfusion (10 min in Figs. 1 and 2) and those at the end of working heart perfusion (35 min in Figs. 1 and 2) are shown in Table 1.

To determine whether dilazep, d-propranolol or lidocaine could counteract the harmful effects of LPC on the myocardium, a series of hearts were perfused with a combination of LPC and one of these drugs. When dilazep (10 or 20 μM) (Fig. 1) or d-propranolol (30 or 50 μM) (Fig. 2) were present with LPC for 5 min, RPP significantly recovered after 20 min of KHB perfusion. When lidocaine (30 or 100 μM) was present with LPC, however, RPP did not recover (Fig. 2). When BSA was present with LPC, RPP did not decrease but remained unchanged from the control level (Fig. 1), suggesting that only LPC that is not bound to protein produces the cardiac effect. Table 1 indicates that dilazep (10 and 20 μM) and d-propranolol (30 μM) significantly increased the coronary flow compared to LPC (LPC 5 min + KHB

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Table 1. Effects of dilazep, d-propranolol and lidocaine on the changes of coronary flow (ml/min) after 10 min of working heart perfusion (before LPC addition) and at the end of 35 min working heart perfusion.

|                   | n  | Before LPC  | After 35 min of working heart perfusion |
|-------------------|----|-------------|----------------------------------------|
| Control           | 10 | 18.11±0.57  | 11.69±0.89                              |
| LPC 25 min        | 6  | 17.98±0.74  | 2.67±0.21*                              |
| LPC (LPC 5 min + KHB 20 min) | 12 | 17.49±0.83  | 3.86±0.32*                              |
| LPC + 5 μM Dilazep| 8  | 17.73±0.55  | 4.37±0.32*                              |
| LPC + 10 μM Dilazep| 12 | 18.17±0.77  | 6.73±0.75*                              |
| LPC + 20 μM Dilazep| 10 | 17.24±0.31  | 5.73±0.24*                              |
| LPC + 30 μM d-Propranolol | 8  | 18.75±0.51  | 7.43±0.66*                              |
| LPC + 50 μM d-Propranolol | 5  | 17.45±0.29  | 5.03±0.32*                              |
| LPC + 30 μM Lidocaine | 4  | 17.28±0.68  | 3.73±0.21*                              |
| LPC + 100 μM Lidocaine | 4  | 18.73±0.35  | 3.78±0.20*                              |
| LPC + BSA         | 5  | 17.50±0.55  | 11.34±0.85                              |

Values represent mean±S.E.M. (ml/min). n = number of experiments. Groups are the same as those described in Figs. 1 and 3. *P<0.05 vs control group, **P<0.05 vs LPC (LPC 5 min + KHB 20 min) group.
20 min) group at the end of the 35-min working heart perfusion, although d-propranolol at 50 μM increased the RPP significantly without any significant increase in coronary flow.

**High-energy phosphates**

In the control group, the ATP value was 14±0.2 μmol/g dry wt. The levels of ATP in the LPC 25 min and LPC (LPC 5 min + KHB 20 min) groups were significantly lower than that in the control group (Fig. 3). In the LPC 5 min group, the ATP level was similar to that in the control group. The levels of ATP in the LPC+5 μM Dila, LPC+10 μM Dila, LPC+20 μM Dila, LPC+30 μM d-Pro, LPC+50 μM d-Pro and LPC+BSA groups were significantly higher than that in the LPC (LPC 5 min + KHB 20 min) group, whereas the levels of ATP in the LPC+30 μM Lido and LPC+100 μM Lido groups were not significantly higher than that in the LPC (LPC 5 min + KHB 20 min) group.

Changes in the levels of CrP (Fig. 4) and ECP (Fig. 5) were similar to those of ATP. These results indicate that the depletion of high-energy phosphate induced by LPC were attenuated by dilazep or d-propranolol but not by lidocaine. It should be noted that there is no difference between the LPC 5 min and control groups in the ATP and ECP levels, although the CrP level in the LPC 5 min group was lower than that in the control group.

**Lactate**

The tissue level of lactate in the control group was 4.55±0.68 μmol/g dry wt (Fig. 6). The level of lactate, a marker of ischemia in tissues, increased significantly in LPC 5 min, LPC 25 min and LPC (LPC 5 min + KHB 20 min) groups compared to the control group. In the LPC+5 μM Dila, LPC+10 μM Dila, LPC+20 μM Dila, LPC+60 μM d-Pro, LPC+50 μM d-Pro, LPC+100 μM Lido and LPC+BSA groups, the tissue levels of lactate were significantly lower than that in the LPC (LPC 5 min + KHB 20 min) group, whereas the level of lactate in the LPC+30 μM Lido group was not significantly different from that in the LPC (LPC 5 min + KHB 20 μM) group.

**Arachidonic acid and other FFA**

The levels of arachidonic acid are shown in Fig. 7. In the control group, the value of arachidonic acid was 18.39±1.34 nmol/g dry wt. The levels of arachidonic acid in the LPC 25 min and LPC (LPC 5 min + KHB 20 min) groups were significantly higher than that in the control group. In the LPC 5 min group, the arachidonic acid level was almost the same as that in the control group. The levels of arachidonic acid in the LPC+5 μM Dila, LPC+10 μM Dila, LPC+20 μM Dila, LPC+30 μM d-Pro, LPC+50 μM d-Pro and LPC+BSA groups were significantly lower than that in the LPC (LPC 5 min + KHB 20 min) group. The effect of LPC on the tissue levels of linoleic acid (Fig. 8), palmitoleic acid (Fig. 9) and stearic acid (Fig. 10) was essentially the same as that on the levels of arachidonic acid.

**DISCUSSION**

Before the experiments with drugs, we examined the effect of LPC on the cardiac metabolism in three kinds of experiments: experiments with LPC 5 min, experiments with LPC 25 min and experiments with LPC 5 min followed by KBH for 20 min. We found in the isolated working rat heart for the first time that LPC 5 min did not change the ATP and FFA levels, while LPC 25 min decreased the ATP level and increased the FFA levels markedly, and that LPC 5 min followed by KBH for 20 min decreased the ATP level and increased the FFA levels as did the LPC 25 min, although the degrees of change were smaller. The concentration of LPC was chosen as 10 μM,
Fig. 4. The level of creatine phosphate (CrP) in the heart. Groups and symbols are the same as those in Fig. 3.

Fig. 5. The level of energy charge potential (ECP) in the heart. Groups and symbols are the same as those in Fig. 3.

Fig. 6. The level of lactate in the heart. Groups and symbols are the same as those in Fig. 3.

Fig. 7. The level of arachidonic acid in the heart. Groups and symbols are the same as those in Fig. 3.
because in the preliminary experiment, we found that this was the minimum concentration that produces irreversible mechanical dysfunction at the end of KHB perfusion for 20 min following LPC perfusion for 5 min. It is of interest to note that after 5 min of LPC perfusion, the level of ATP did not change, although the level of CrP decreased and the level of lactate increased, indicating that 5 min LPC perfusion does not inflict severe damage to the heart. However, after 20 min of the KHB perfusion following 5 min LPC perfusion, metabolic changes became very severe, suggesting that LPC perfusion for 5 min predisposes the heart to metabolic damage. From the results obtained above, we employed the experiments with LPC 5 min followed by KHB for 20 min as a control for experiments with drugs. Interestingly, dilazep and d-propranolol significantly attenuated both mechanical and metabolic alterations induced by LPC (Figs. 1 and 2, Table 1). These results demonstrate for the first time that dilazep and d-propranolol counteract the effect of exogenous LPC that produces cardiac damage in isolated working rat hearts. There was an increase in coronary flow in experiments with dilazep or d-propranolol (Table 1), except for 50 μM d-propranolol. This increase in coronary flow can be one of the reasons why dilazep and d-propranolol attenuated the LPC-induced mechanical dysfunction and metabolic changes. Nevertheless, there is no coronary vasodilating effect of dilazep or d-propranolol (1), and therefore one cannot determine whether the drug-induced attenuation of the cardiac dysfunction and metabolic changes induced by LPC is a cause or a result of the increase in coronary flow.

Why does LPC produce ischemia-like changes in the heart? Because the pattern of metabolic changes induced
by LPC is very similar to those induced by ischemia and reperfusion, it is possible to assume that LPC affects oxidative production of ATP in the cardiac mitochondria. This view is partially supported by the fact that LPC uncouples oxidative phosphorylation in the rat liver mitochondria (14). Another possible mechanism of LPC-induced derangements is Ca\(^{2+}\)-overload that leads to damage of the heart cells. It has been shown that LPC produces Ca\(^{2+}\)-overload in rabbit cardiac myocytes (15) and potentiates Ca\(^{2+}\) accumulation in rat cardiac myocytes (16). Although the exact mechanism by which LPC induces intracellular Ca\(^{2+}\)-overload remains speculative, several mechanisms have been proposed: 1) LPC has a direct effect to increase the sarcolemmal permeability to Ca\(^{2+}\) (17), 2) LPC increases the level of myocardial cyclic AMP (18), which may cause Ca\(^{2+}\)-influx via slow Ca\(^{2+}\) channel (19), 3) LPC activates protein kinase C directly (20, 21) or indirectly (22) leading to Ca\(^{2+}\)-overload, 4) LPC inhibits myocardial Na\(^{-}\)-K\(^{+}\) ATPase and therefore may increase the intracellular Na\(^{-}\) concentration with consequent acceleration of Na\(^{+}/Ca\(^{2+}\) exchange (23, 24) leading to Ca\(^{2+}\)-overload, 5) LPC produces long-lasting bursts of Na\(^{+}\) channel openings (25) leading to Ca\(^{2+}\)-overload. If there is Ca\(^{2+}\)-overload, mitochondrial damage would also occur. Ver Donk et al. (15) found that on exposure to LPC mitochondria showed complete deterioration of ultrastructure.

Since phospholipase A\(_2\) (PLA\(_2\)) is a Ca\(^{2+}\) dependent enzyme, LPC-induced Ca\(^{2+}\)-overload leads to an increase in the PLA\(_2\) activity (26). In fact, LPC at low concentration can directly increase the PLA\(_2\) activity (27), leading to release of lysophosphoglycerides and FFA. Furthermore, the low tissue level of ATP produced by either ischemia or LPC may also result in increased FFA accumulation (28–30). According to Sargent et al. (7), accumulation of LPC observed in isolated rat hearts after myocardial ischemia may not be solely responsible for the observed contractile dysfunction and LDH release. The results of the present study, however, show that the degree of increase of arachidonic acid after reperfusion following ischemia is similar to that induced by 5 min LPC (10 \(\mu\)M) perfusion followed by 20 min KHB perfusion (1). This suggests that LPC is one of the causes of ischemia-reperfusion damage to the heart, because accumulation of arachidonic acid may reflect accumulation of lysophosphoglycerides released from membrane phospholipids.

Why does dilazep or d-propranolol but not lidocaine attenuate the LPC-induced changes in the heart? According to Neufeld et al. (31), lidocaine is effective in the protection of the isolated rat heart from LPC-induced arrhythmias. There is a question as to why lidocaine does not attenuate the LPC-induced cardiac damage, whereas it attenuates the ischemia-induced (9) and H\(_2\)O\(_2\)-induced cardiac damage (32). Since lidocaine reduces the H\(_2\)O\(_2\)-induced accumulation of malondialdehyde in the heart (32), the mechanism of the protective effect of lidocaine is probably due to inhibition of lipid peroxidation or a free radical scavenging effect. It is clear from the results of the present study that the mechanism of the effect of lidocaine is not the anti-LPC effect. The mechanism of action of dilazep and d-propranolol, however, should be related to the anti-LPC effect.

There are some reports showing that dilazep produces a Ca\(^{2+}\) antagonizing effect in the guinea pig taenia coli (33) and in dog coronary artery (34). Dilazep also inhibits the histamine-stimulated cytosolic Ca\(^{2+}\) increase in cultured endothelial cells (35). If dilazep has the Ca\(^{2+}\) channel blocking action, it would decrease the intracellular concentration of Ca\(^{2+}\) effectively. The results of our recent study (K. Yazawa et al., unpublished observation) has revealed that dilazep inhibits the voltage-dependent Ca\(^{2+}\) channel in the guinea pig ventricular myocyte. Therefore, it is possible that dilazep protects the heart by preventing the increase of intracellular Ca\(^{2+}\) induced by LPC, leading to preservation of mitochondrial function that spares ATP. Sparing of ATP then leads to inhibition of accumulation of FFA (29). Furthermore, dilazep has a PLA\(_2\) inhibitory effect (36). Therefore, it is likely that dilazep can inhibit LPC-induced accumulation of FFA and hence attenuate LPC production. There is no information, however, as to the effect of d-propranolol on the Ca\(^{2+}\) channel. In regard to the effect of dilazep, there is a possibility that the drug protects against the LPC-induced cardiac damage via inhibiting adenosine uptake into cardiac cells and hence potentiating the effect of adenosine (37). In fact, stimulation of adenosine A\(_3\) receptor causes an inhibitory effect on ischemia-induced infarct size (38). The above assumption however is hypothetical, because there is no report suggesting that adenosine protects against the LPC-induced cardiac dysfunction and that d-propranolol inhibits adenosine uptake into cardiac cells.

In conclusion, although the exact mechanism still remains under study, our present results clearly indicate that LPC is an important factor in producing ischemic damage, and both dilazep and d-propranolol attenuate mechanical and metabolic changes induced by exogenous LPC.

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