Decreases in ANP Secretion by Lysophosphatidylcholine Through Protein Kinase C

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Abstract—Lysophosphatidylcholine (LPC) is an endogenous phospholipid released from the cell membrane during ischemia, and it has potent, local effects on cardiac tissues. LPC has been implicated in arrhythmogenesis during ischemia by increasing intracellular Ca\(^{2+}\). However, it is not known whether LPC influences atrial release of atrial natriuretic peptide (ANP). The aim of this study was to investigate the effect of LPC on ANP secretion from isolated, perfused, beating rat atria. LPC (10 and 30 \(\mu\)mol/L) caused decreases in ANP secretion in a dose-dependent manner, with slight increases in intra-atrial pressure and extracellular fluid (ECF) translocation. Therefore, the ANP secretion in terms of ECF translocation was markedly decreased by LPC. The order of the suppressive effect of ANP release was stearoyl-LPC>myristoyl-LPC>lauroyl-LPC. Staurosporine and wortmannin significantly attenuated suppression of the ANP release and an increase in intra-atrial pressure by LPC. High extracellular Mg\(^{2+}\) also attenuated the LPC-induced suppression of ANP release. However, other protein kinase C inhibitors such as chelerythrine, GF 109203X, and tamoxifen citrate did not affect LPC-induced suppression of ANP release. In single atrial myocytes, LPC caused increases in intracellular Ca\(^{2+}\) in a dose-dependent manner. The order of an increase in intracellular Ca\(^{2+}\) by LPC was stearoyl-LPC>myristoyl-LPC>lauroyl-LPC. An increase in intracellular Ca\(^{2+}\) by LPC was attenuated by staurosporine. These results suggest that LPC-induced suppression of ANP release through protein kinase C/Ca\(^{2+}\) and phosphoinositol-3-kinase might in part play an important role in the development of hypertension. (Hypertension. 2003; 41:1317–1323.)

Key Words: atrial natriuretic factor ■ phospholipases ■ extracellular space ■ calcium ■ protein kinases ■ heart

Lysophosphatidylcholine (LPC) is naturally formed by phospholipase A\(_1\)-induced hydrolysis of a main membrane phospholipid, phosphatidylcholine, in all eukaryotic and many prokaryotic cells.\(^1\) LPC is produced intracellularly during normal phospholipid turnover and accumulates during myocardial ischemia.\(^2-4\) A marked increase in LPC levels observed within 10 minutes of myocardial ischemia\(^9\) produces potent, reversible, and localized effects in the heart.\(^3,4\) LPC causes membrane depolarization as a result of decreases in potassium conductance\(^3,10\) and modulates the cardiac Na\(^{+}\)/current through protein kinase C (PKC)-dependent phosphorylation.\(^11\) Therefore, LPC has been implicated in arrhythmogenesis during ischemia.\(^9,12,13\) Increasing intracellular calcium ([Ca\(^{2+}\)]) induced by LPC\(^14-17\) also plays an important role in the generation of arrhythmias, even though the mechanisms responsible for this effect are still controversial. It has been reported that cardiac accumulation of [Ca\(^{2+}\)], by LPC is due to inhibition of Na,K-ATPase\(^16\) and is attenuated by inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger.\(^17\)

On the other hand, the cardiac atrium is now well known as an endocrine organ that secretes atrial natriuretic peptide (ANP), participating in the regulation of body fluid and blood pressure.\(^18\) ANP is released mainly from cardiac muscle cells in response to various physiological and pathological conditions to induce atrial stretch.\(^19-22\) However, the exact nature of stretch-secretion coupling in atrial cardiomyocytes remains to be elucidated. Atrial cardiomyocytes are involved in both mechanical and endocrine functions of the heart, which are mainly mediated by intracellular Ca\(^{2+}\).\(^23-27\) Ca\(^{2+}\) may be 1 of the most important factors affecting ANP secretion, even though controversy still persists.\(^25,26\) When these results are considered, there is a possible role for endogenously generated LPC in ANP secretion. However, there are few reports about the relation between LPC and ANP secretion. Therefore, the aim of the present study was to define the effect of LPC on ANP secretion and to characterize its mechanism.

Methods

Preparation of Perfused, Beating Rat Atria

Isolated, perfused, beating atria from male Sprague-Dawley rats were prepared by using a previously described method,\(^28,29\) with minor modifications. In brief, the right atrium was dissected from the heart after the animal was killed, and sinoatrial nodal tissue was removed. A cannula containing 3 small catheters sealed within it was inserted into the atrium and secured by ligatures. The cannulated atrium was transferred to an organ chamber, immediately perfused with oxygenated HEPES buffer solution at 36.5°C, and paced at 1.3
Hz (duration 0.3 ms, voltage 40 V), as described previously. The composition of the HEPES buffer solution was as follows: NaCl 118 mmol/L, KCl 4.7 mmol/L, CaCl2 2.5 mmol/L, MgSO4 1.2 mmol/L, NaHCO3 25 mmol/L, HEPES 10 mmol/L, glucose 10 mmol/L, and bovine serum albumin 0.1%. The pericardial buffer solution, which contained [3H]inulin to measure the translocation of extracellular fluid (ECF), was also oxygenated by placing silicone tubing coils inside the organ chamber. The atrium was perfused for 100 minutes to stabilize the secretion of ANP and to maintain a steady-state [3H]inulin level in the extracellular space. The perfusate was collected at 2-minute intervals at 4°C.

Experimental Protocols
Experiments were performed with 3 groups. Group 1 was the time-control group (n=7). In this group, HEPES buffer was perfused throughout the experiment. Group 2 included the LPC-perfused groups. In this experimental group, LPC (type V from bovine brain, 10 or 30 μmol/L; n=5 or 8; Sigma) was introduced into the atrial lumen after a 10-minute control collection period, and perfusate was collected for 60 minutes. Three types of LPCs (each 30 μmol/L)—stearoyl-LPC (n=7), lauroyl-LPC (n=8), and myristoyl-LPC (n=6)—were also used.

Group 3 included the modifier-pretreated groups. To modify the LPC effect on ANP secretion, inhibitors for PKC (staurosporine [1 μmol/L, n=7], chelerythrine chloride [1 to 3 μmol/L, n=10], GF 109203X [10 μmol/L, n=6], or tamoxifen citrate [1 μmol/L, n=5]) or phosphoinositol-3-kinase (PI3 kinase) (wortmannin [0.3 μmol/L, n=6]) were administered as a pretreatment at 40 minutes after start of the perfusion. Then, LPC was simultaneously infused after a 10-minute control collection period. LPC was also perfused under high-extracellular-Mg2+ conditions (2.5 mmol/L, n=5).

Radioimmunoassay of ANP
The concentration of immunoreactive ANP in the perfusate was measured by using a specific radioimmunoassay, as described previously.22,27

Measurement of Intracellular Ca2+ Concentration in Single Atrial Myocytes
Single rat atrial myocytes were isolated by using a previously described technique,28,29 and changes in [Ca2+]i were measured by using a fluorescence digital imaging microscopic system (Attofluor, Atto Instruments). After loading with 2 μmol/L fura 2-AM (Molecular Probes) for 20 minutes at room temperature, atrial myocytes were attached to a perfusion chamber coated with matrix gel, stimulated at 1 Hz, and perfused with HEPES buffer containing Ca2+ at a concentration of 1 mmol/L at a rate of 0.7 mL/min for 5 minutes. LPC (10, 30, or 100 μmol/L; n=8 to 10) was perfused for 5 minutes. Three types of LPCs (n=8 to 10)—stearoyl-LPC, lauroyl-LPC, and myristoyl-LPC—were also used. To modify the effect of LPC, staurosporine (0.3 μmol/L) was used as a pretreatment for 5 minutes and LPC was simultaneously infused. Cells were imaged with an intensified charge-coupled device camera and analyzed with the Attofluor image processing software.

Statistical Analysis
The results are given as the mean±SEM. The Student unpaired t test was used (Figure 1). The statistical significance of the differences was assessed with a 2-way ANOVA for repeated measures (Figure 2). The Student unpaired t test was also used (Figure 3). A 2-way ANOVA for repeated measures was also used (Figures 4 and 5). The critical level of significance was set at P<0.05.

Results
Effect of LPC on ANP Secretion
Figure 1 shows the effect of LPC on ANP secretion, ECF translocation, and pulse pressure in beating rat atria. After 100 minutes of stabilization, the perfusate was collected 5 times every 2 minutes to serve as a control period. Then, LPC...
was infused at a concentration of 10 or 30 μmol/L. ANP secretion was gradually decreased (Figure 1A), and ECF translocation was gradually increased (Figure 1B). Therefore, the ANP secretion in terms of ECF translocation (interstitial ANP concentration) was markedly suppressed in a dose-dependent manner (Figure 1C). The pulse pressure was maintained constantly during the early period of LPC infusion and then slightly but not significantly decreased (Figure 1D).

There are different types of LPC. To compare the potency of the suppressive effect of LPC on ANP secretion, stearoyl-LPC, myristoyl-LPC, or lauroyl-LPC was also perfused. We obtained the relative percent change from the mean of 5 control values and last 5 experimental values exposed to LPC, compared with mean of 5 control values from Figure 1. S-LPC caused an increase in pulse pressure, which increased ECF translocation, compared with the LPC-infused group. Myristoyl-LPC and lauroyl-LPC did not cause any significant changes in pulse pressure and ECF translocation. Therefore, the order of potency of suppressive effect on ANP release was stearoyl-LPC > myristoyl-LPC = lauroyl-LPC (Figure 2C). Time indicates time control. Values are mean ± SEM. *P ≤ 0.05, **P ≤ 0.01 vs the group infused with LPC.

Figure 2. Comparison of effects of LPC (n = 8), stearoyl-LPC (S-LPC, n = 7), myristoyl-LPC (M-LPC, n = 8), and lauroyl-LPC (L-LPC, n = 6) on ANP secretion (A), ECF translocation (B), and pulse pressure (D). Values were expressed as percent changes of last 5 experimental values exposed to LPC, compared with the LPC-infused group. Myristoyl-LPC and lauroyl-LPC did not cause any significant changes in pulse pressure and ECF translocation. Therefore, the order of potency of suppressive effect on ANP release was stearoyl-LPC > myristoyl-LPC = lauroyl-LPC (C). Time indicates time control. Values are mean ± SEM. *P ≤ 0.05, **P ≤ 0.01 vs the group infused with LPC.

Modification of the Suppressive Effect of LPC
To modify the effect of LPC on ANP secretion, several PKC inhibitors, such as staurosporine, chelerythrine, GF 109203X, or tamoxifen citrate, and a PI3 kinase inhibitor, such as wortmannin, were used. As shown in Figure 3, suppression of ANP secretion (Figure 3A) and an increase in ECF translocation (Figure 3B) by LPC was markedly attenuated by staurosporine. Values are mean ± SEM. *P ≤ 0.05, **P ≤ 0.01 vs the LPC-infused group.

Figure 3. Modification of suppressive effect of ANP secretion by LPC in the presence of 1 μmol/L staurosporine, a PKC inhibitor (n = 7). Staurosporine was infused 40 minutes after the start of perfusion, and LPC was simultaneously infused after a 10-minute control period. Staurosporine attenuated a decrease in ANP secretion (A) and an increase in ECF translocation (B) by LPC. Therefore, the suppression of ANP secretion in terms of ECF translocation (interstitial ANP concentration, C) by LPC was markedly attenuated by staurosporine. Values are mean ± SEM. *P ≤ 0.05, **P ≤ 0.01 vs the LPC-infused group.

from Figure 1. A decrease in ANP secretion by LPC was similar to that by stearoyl-LPC but was more prominent than that by myristoyl-LPC or lauroyl-LPC (Figure 2A). Stearoyl-LPC caused an increase in ECF translocation, compared with the LPC-infused group (Figure 2B). Therefore, the order of the suppressive effect of ANP release was stearoyl-LPC > myristoyl-LPC = lauroyl-LPC (Figure 2C). However, myristoyl-LPC and lauroyl-LPC did not cause any significant differences in pulse pressure and ECF translocation, compared with the LPC-infused group (Figure 2D).
with staurosporine. Therefore, the LPC-induced suppression of ANP secretion in terms of ECF translocation was markedly attenuated by staurosporine (Figure 3C). The LPC-induced increase in pulse pressure was attenuated by staurosporine (Figure 3D).

Figure 4 shows a comparison of the blocking effect of PKC inhibitors on the suppressive effect of LPC. Staurosporine, wortmannin, and high extracellular Mg\(^{2+}\) (2.5 mmol/L) caused an attenuation of decreased ANP secretion (Figure 4A) without affecting the LPC-induced increase in pulse pressure. GF 109203X, a potent specific PKC inhibitor, staurosporine, and wortmannin attenuated the increased ECF translocation by LPC (Figure 4B). Therefore, LPC-induced suppression of ANP secretion in terms of ECF translocation was significantly attenuated by staurosporine, wortmannin, and high extracellular Mg\(^{2+}\) (Figure 4C). Staurosporine and GF 109203X attenuated the increased pulse pressure by LPC (Figure 4D). However, chelerythrine or tamoxifen citrate did not affect LPC-induced changes in pulse pressure, ECF translocation, and ANP secretion.

Effects of LPC on [Ca\(^{2+}\)] in Single Atrial Myocytes

Changes in [Ca\(^{2+}\)] by LPC were measured in single, beating atrial myocytes. Basal [Ca\(^{2+}\)] in atrial myocytes was 139.2 ± 8.2 nmol/L (n = 15). As shown in Figure 5, LPC and stearoyl-LPC caused increases in [Ca\(^{2+}\)] in a dose-dependent manner. An increase in [Ca\(^{2+}\)] by stearoyl-LPC was greater than that by LPC (1.12 ± 0.03- vs 1.05 ± 0.03-fold at 30 \(\mu\)mol/L; 1.25 ± 0.05- vs 1.14 ± 0.09-fold at 100 \(\mu\)mol/L; all P < 0.05). These effects were attenuated by pretreatment with staurosporine (0.3 \(\mu\)mol/L). Staurosporine itself caused a slight increase in [Ca\(^{2+}\)]. However, lauroyl-LPC and myristoyl-LPC did not cause any significant changes in [Ca\(^{2+}\)].

Discussion

This is the first study to evaluate the effect of LPC on ANP secretion from beating rat atria. The major finding of the present study is that LPC is a potent inhibitor of atrial ANP release through PI3 kinase and the PKC/Ca\(^{2+}\) pathway.

LPC produced during normal phospholipid turnover increases from 69.1 to 178.0 \(\mu\)mol/L in the coronary sinus in patients with pacing-induced ischemia and is increased by 2-fold in effluent from cat myocardium after 10 minutes of ischemia. LPC is known to cause many local cardiac effects, as well as other effects. LPC causes membrane depolarization and modulates the cardiac Na\(^+\) current. Therefore, LPC has been implicated in arrhythmogenesis during ischemia. Anti-LPC antibodies might contribute a novel factor in the development of hypertension and athero-
sclerosis. To investigate the role of elevated LPC in the regulation of blood pressure, the present study was performed to define the effect of LPC on ANP secretion from rat atria. LPC at a dose of 30 μmol/L caused ~60% suppression of ANP release with an increase in atrial pressure. The dose used in this study is lower than that observed during ischemia.8,9 The LPC-induced suppression of ANP was also observed in stearoyl-LPC, lauroyl-LPC, and myristoyl-LPC infusion groups, even though the potency might depend on the species of the fatty acid residue of the lipid. With the observation of decreased antibodies to LPC in LPC-injected SHR,14,15 we suggest that LPC might participate in the induction of hypertension by directly decreasing ANP secretion from the atria.

In vitro and in vivo studies have demonstrated that low concentrations of LPC activate PKC, and higher concentrations of LPC inhibit PKC activity in some cell types.11,35 In the case of vascular smooth muscle cells, LPC generates O2− through PKC activation, and the activity might be mediated by Ca2+ influx. However, LPC-mediated O2− generation in human neutrophils occurs through PI3 kinase activation but not through PKC. In ventricular myocytes, LPC modulates the Na+ current by PKC-dependent and tyrosine kinase–dependent phosphorylation.10 However, a controversy still exists, because LPC and PKC isoforms are not tissue-specific. Therefore, to determine whether LPC might suppress atrial release of ANP through PKC, several inhibitors for PKC or PI3 kinase were used. LPC-induced suppression of ANP secretion was attenuated by staurosporine and wortmannin but not by chelerythrine, GF 109203X, or tamoxifen citrate. These results suggest that PKC and PI3 kinase might be partly involved in the suppressive effect of LPC on ANP secretion. However, we do not know the reason for the different responses to PKC inhibitors in the modification of LPC effects at present. Interestingly, GF-109203X inhibited the positive inotropic effect of LPC, similar to staurosporine, but did not attenuate the LPC-induced suppression of ANP release. Staurosporine inhibited both LPC effects, but chelerythrine and tamoxifen citrate did not. Atrial hemodynamics is closely related to ANP secretion. However, our results showing dissociation of these 2 factors by our use of PKC inhibitors suggest that other factors as well as PKC might also participate in the regulation of ANP secretion.

It has also been reported that high extracellular Mg2+ (2 and 20 mM) might antagonize the actions of LPC in ventricular myocytes by inhibiting cellular Ca2+ overload, because Mg2+ is a critical factor in the myocardial ion pump. However, the antiarrhythmic effects of Mg2+ during ischemia might be mediated by inhibition of LPC-induced Ca2+ overload. In this study, LPC-induced suppression of ANP secretion was attenuated in a high-extracellular-Mg2+ (2.5 mM) buffer solution. These effects might be related to inhibition of LPC-induced Ca2+ overload by high Mg2+.

LPC is known to alter cellular Ca2+ homeostasis. LPC causes an accumulation of [Ca2+]i in a dose-dependent manner in ventricular myocytes,14–17 predisposing the heart to arrhythmia and mediating ischemic injury by generation of free radicals and activation of proteases. LPC also causes Ca2+ efflux from isolated rat ventricular myocytes through the Na+/Ca2+ exchanger. In the present study, we demonstrated that LPC also caused increases in [Ca2+]i in single atrial myocyte in a dose-dependent manner, which was attenuated by staurosporine. The order of LPC-induced increase in [Ca2+]i, was stearoyl-LPC > LPC > lauroyl-LPC = myristoyl-LPC. An increase in [Ca2+]i, by LPC was dependent on the fatty-acid side chain of LPC, and these results are consistent with another report observed in murine aortic endothelial cells. An increase in [Ca2+]i, by LPC might be an inhibitory effect on ANP secretion in our model. Lauroyl-LPC and myristoyl-LPC did not increase [Ca2+]i, in atrial myocytes. Low doses of LPC also did not increase [Ca2+]i, but 100 μmol/L LPC increased it by only 14%. However, these types of LPC showed the suppressive effect of ANP secretion and a positive inotropic effect in isolated beating atria. Thus, it is possible that [Ca2+]i, or the sensitivity to [Ca2+]i, in atrial tissue might be increased by LPC and other types. The potency of the suppressive effect on ANP secretion by different types of LPC also appears to be related to the potency of increase in [Ca2+]i. Therefore, the suppressive effect on ANP secretion by LPC might be partly related to [Ca2+]i.

In summary, LPC caused suppression of ANP release in a dose-dependent manner, with slight increases in intra-atrial pressure and ECF translocation. The order of the suppressive effect on ANP release and increased [Ca2+]i was stearoyl-LPC > LPC > myristoyl-LPC > lauroyl-LPC. Staurosporine and wortmannin attenuated suppression of the ANP release and an increase in intra-atrial pressure by LPC. High extracellular Mg2+ also attenuated the LPC-induced suppression of ANP release. These results suggest that LPC-induced suppression of ANP release might be through PKC/Ca2+ and PI3 kinase. Therefore, these LPC effects might in part play an important role in the development of hypertension. To clarify in greater detail the physiological role of LPC on the regulation of blood pressure, further studies are need in hypertensive animals.

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References


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