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Lysophosphatidylcholine Attenuates Endothelium-dependent Relaxation Responses through Inhibition of ACh-induced Endothelial $[Ca^{2+}]_i$ Increase

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Lysophosphatidylcholine (LPC), which accumulates in atherosclerotic arteries, has been reported to inhibit endothelium-dependent relaxation (EDR) in many different species. However, the underlying mechanism of LPC-induced inhibition of EDR is still uncertain. In the present study, we measured simultaneously both isometric tension and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in rabbit carotid strips, and examined the effect of LPC on tension and $[Ca^{2+}]_i$. In carotid strips with intact-endothelium, high K^+ (70 mM) increased both tension and $[Ca^{2+}]_i$, and cumulative addition of acetylcholine (ACh) from 0.1 to 10μM induced dose dependent increase of $[Ca^{2+}]_i$ with concomitant relaxation. In the presence of L-NAME (0.1 mM), ACh increased $[Ca^{2+}]_i$ without affecting the amplitude of high K^+-induced tension. These ACh-induced change of $[Ca^{2+}]_i$ and tension was abolished by removal of endothelium or 10 nM 4-DAMP (muscarinic receptor antagonist) pretreatment. Pretreatment of LPC (10μM) inhibited ACh (10μM)-induced change of tension and $[Ca^{2+}]_i$ in endothelium-intact carotid artery. On the other hand, LPC had no effect on ACh-induced change of tension and $[Ca^{2+}]_i$ in endothelium denuded artery. In Ca^{2+}-free external solution, ACh transiently increased $[Ca^{2+}]_i$, and pretreatment of LPC significantly inhibited ACh-induced transient $[Ca^{2+}]_i$ change. Based on the above results, it may be concluded that LPC inhibits the ACh-induced $[Ca^{2+}]_i$ change through inhibition of Ca^{2+} mobilization in vascular endothelial cells, resulting in decreased production of NO and concomitant inhibition of endothelium-dependent vascular relaxation.

Key Words: Vascular endothelium, LPC, EDR, Endothelial $[Ca^{2+}]_i$

INTRODUCTION

Impairment of endothelium-dependent relaxation (EDR) is thought to play an important role in the development of abnormal vascular responses in atherosclerotic arteries (Flavahan, 1992), and oxidized low-density lipoprotein (LDL), which is accumulated in the vessel wall in atherosclerosis, plays an important role in impairment of EDR (Berliner et al, 1995). The inhibitory effect of oxidized LDL, which is not shared by native LDL, is mainly mediated by lysophosphatidylcholine (LPC) (Murohora et al, 1994). When LDL is oxidized, the production of LPC from the phosphatidylcholine is increased 3 to 5 folds (Parthasarathy et al, 1985), and exogenous application of LPC causes an endothelial dysfunction similar to that observed in atherosclerotic arteries (Miwa et al, 1994; Murohora et al, 1994; Huang et al, 2002). Although LPC-induced inhibition of EDR has been examined in many studies, details of its mechanism are not clear at present.

Vascular endothelium secrets a variety of bioactive agents including nitric oxide (NO), prostacyclin, and endothelium-dependent hyperpolarizing factors. These bioactive agents play an important role in controlling vascular tone (Gryglewski et al, 1988). A rise in endothelial cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to various chemical or physical stimuli is an essential step in the synthesis or release of these bioactive agents (Boulaner & Vanhoutte, 1997). There is a positive correlation between increase in endothelial $[Ca^{2+}]_i$ and release of NO in rat aortic endothelium, suggesting that Ca^{2+} is prerequisite for activation of nitric oxide synthase (Busse & Mulsch, 1990). In endothelial cells, the agonist-induced elevation of $[Ca^{2+}]_i$ was due to the release of Ca^{2+} from internal stores by 1,4,5-triphosphate (IP_3) or the influx of Ca^{2+} from external space (Adams et al, 1989). In fact, LPC has been reported to inhibit the agonist-induced increase in $[Ca^{2+}]_i$ in cultured endothelial cells (Inoue et al, 1992) or in endothelium-intact tissue preparations (Miwa et al, 1994; Huang et al, 2002). Thus, LPC-induced inhibition of EDR may be due to inhibition of IP_3-induced Ca^{2+} release or Ca^{2+} influx from external space. However, which one is responsible for the

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ABBREVIATIONS: ACh, addition of acetylcholine; EDR, endothelium-dependent relaxation; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; NO, nitric oxide; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration.

inhibition of endothelial $[Ca^{2+}]_i$ in intact vascular strips is still uncertain.

In the present study, we investigated the mechanism of LPC-induced inhibition of EDR in the rabbit carotid arteries. For this, we used a method that allows simultaneous measurements of endothelial $[Ca^{2+}]_i$ and vascular tone, and then measured effect of LPC on ACh-induced endothelial $[Ca^{2+}]_i$ and tension response in rabbit carotid arteries. Our results suggest that LPC suppressed ACh-induced endothelial $[Ca^{2+}]_i$ elevation via inhibition of Ca^{2+} release from internal stores in rabbit carotid strips.

METHODS

Tissue preparations

New Zealand male white rabbits (1.5～2 kg) were anesthetized with pentobarbital sodium (30 mg/kg body weight IV), and the right and left carotid arteries were isolated and cleaned of surrounding tissues. Carotid arteries were cut into helical strips of 1.5- to 2-mm width and 8 to 10-mm length and placed in physiological salt solution (PSS) of the following composition (in mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, EDTA 0.01, glucose 5.5. Ethylenediaminetetraacetic acid (EDTA) at 0.01 mM was also added to chelate the contaminated heavy metal ions. High K^+ solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with 95% O_2 and 5% CO_2 at 37°C to maintain pH at 7.4.

Simultaneous measurements of isometric tension and $[Ca^{2+}]_i$ in isolated carotid strips

$[Ca^{2+}]_i$ was measured according to the method described by Sato et al. (1990) using the fluorescent Ca^{2+} indicator fura-2. Muscle strips were exposed to the acethoxymethyl ester of fura-2 (fura-2/AM, 10μM) in the presence of 0.02% cremophor EL for 5～6 hr at room temperature (22～24°C). The muscle strip loaded with fura-2 was held horizontally in a temperature-controlled organ bath filled with normal PSS, and the endothelial side of strip was faced on the aperture of UV light. The carotid strip was illuminated alternately at a cycle of 48 Hz with two excitation wavelengths of 340 nm and 380 nm, and the fluorescence emitted from strip was collected into a photomultiplier tube through a 500 nm filter. The amount of emitted fluorescence induced by 340 nm excitation wave (F_{340}) and that induced by 380 nm excitation wave (F_{380}) was measured successively, and the ratio of theses two fluorescence values (F_{340}/F_{380}) was calculated. The ratio was used as an indicator of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). To measure the $[Ca^{2+}]_i$ change in endothelial cells, endothelial surface of the strip was faced to the bottom of the chamber, and emitted fluorescence was detected in the presence or absence of endothelium. Experiments were performed with a bio-fluorimeter (CAF-110, Japan Spectroscopic, Tokyo, Japan). The resting $[Ca^{2+}]_i$ in normal PSS and the sustained $[Ca^{2+}]_i$ in the presence of 70 mM KCl were taken as 0% and 100%, respectively.

Statistics

The results of the experiments are expressed as mean± S.E.M. Paired Student's *t*-test was used for statistical analysis of the results, and the number of preparations taken from separate animals was indicated by n. P values of less than 0.05 were considered significant.

RESULTS

Effect of ACh on $[Ca^{2+}]_i$ and tension in high K^+-stimulated carotid strips

Typical recordings of the response to ACh in carotid arterial strips with endothelium are shown in Fig. 1, Application of high K^+ (70 mM K^+) evoked the contraction with simultaneous increase of $[Ca^{2+}]_i$ in endothelium-intact carotid strips. ACh, administered after high K^+-stimulated responses, reached to steady state, and elicited a concentration-dependent additional increase in $[Ca^{2+}]_i$, which was accompanied by a simultaneous decrease in muscle tension (Fig. 1A). ACh (10μM) caused a decrease of muscle tension by 53.0±10.0% with concomitant increase in $[Ca^{2+}]_i$ by 78.4± 10.1% from the steady state values of high K^+-induced responses. In contrast to endothelium-intact strips, ACh had no significant effect on steady-state values of high K^+-induced tension and $[Ca^{2+}]_i$ in endothelium-denuded carotid strips (Fig. 1B). To test whether the ACh-induced changes in $[Ca^{2+}]_i$ represent the endothelial $[Ca^{2+}]_i$ transient, we measured the relationship between ACh-induced $[Ca^{2+}]_i$ transients and the magnitude of relaxations (Fig. 1C), and found a positive correlation between them (correlation coefficient=0.97, $p<0.01$). We also measured the effects of muscarinic receptor antagonist and nitric oxide synthase (NOS) inhibitor on ACh-induced tension and $[Ca^{2+}]_i$ transient in endothelium-intact carotid strips (Fig. 2). Pretreatment with 10 nM 4-DAMP, a muscarinic receptor antagonist, had no effect on high K^+-induced $[Ca^{2+}]_i$ increase and tension development. However, it completely abolished the ACh-induced increase in $[Ca^{2+}]_i$ (% change of $[Ca^{2+}]_i$ in control vs. 4-DAMP treated group; 53.3±7.8% vs. 0%, n=5) and relaxation responses (% change of relaxation in control vs. 4-DAMP treated group; 48.1±6.7% vs. 0%, n=5). Pretreatment with 0.1 mM L-NAME, a NOS inhibitor, significantly inhibited the ACh-induced relaxation (48.1± 6.7% vs. 3.4±2.3% in control vs. L-NAME treated group, n=5), while it had no significant effect on the ACh-induced $[Ca^{2+}]_i$ increase (53.3±7.8% vs. 48.4±6.3% in control vs. L-NAME treated group, n=5).

Effect of lysophosphatidylcholine on ACh-induced additional $[Ca^{2+}]_i$ increase and tension

As shown in Fig. 3, LPC (10μM) had no significant effect on high K^+-induced $[Ca^{2+}]_i$ and tension development in endothelium-intact carotid strips. However, ACh-induced elevations of $[Ca^{2+}]_i$ and relaxation responses were significantly attenuated by pretreatment with LPC (10μM). In control experiment, ACh caused a relaxation (48.1±6.7% of high K^+-stimulated level) and elevation of $[Ca^{2+}]_i$ (53.3± 7.8% of high K^+-stimulated level, n=8). In the presence of 10μM LPC, the magnitude of the ACh-induced relaxation was decreased to 5.2±4.2% of the high K^+-stimulated level, and the ACh-induced $[Ca^{2+}]_i$ transient was also decreased to 5.5±3.6% of the high K^+-stimulated level. This inhibitory effect of LPC on ACh-induced tension and $[Ca^{2+}]_i$ responses was reversible, because the ACh-induced $[Ca^{2+}]_i$ elevations and relaxation responses gradually returned to

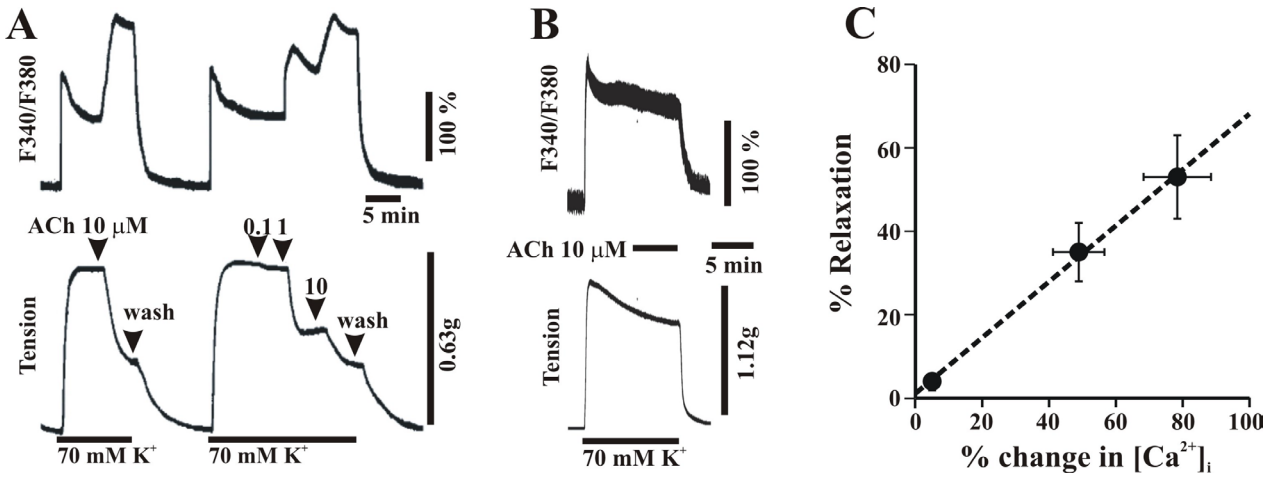

Fig. 1. ACh-induced changes of $[Ca^{2+}]_i$ and tension in endothelium- intact or denuded rabbit carotid strips. Typical recordings of $[Ca^{2+}]_i$ (indicated by F_{340}/F_{380}; upper trace) and tension (lower trace) in response to ACh in endothelium-intact (A) or endothelium-denuded (B) carotid arterial strips. Muscle strips were pre-contracted with high K^+ (70 mM) and relaxed by cumulative addition of ACh. Changes in $[Ca^{2+}]_i$ were expressed by measuring the fura-2 fluorescence ratio (F_{340}/F_{380}). The steady-state response to high K^+, obtained before the application of ACh, was taken as 100% and the resting $[Ca^{2+}]_i$ level as 0%. The magnitude of ACh-induced relaxation was expressed as a percent of high K^+-induced contraction. (C) Relationship between ACh-induced increases in $[Ca^{2+}]_i$ (abscissa) and relaxation (ordinate) in endothelium-intact carotid strips. Each point represents the mean of 6 to 8 experiments. S.E.M. is shown by vertical and horizontal bar. The regression line was drawn by least-square method (correlation coefficient=0.97, $p < 0.01$).

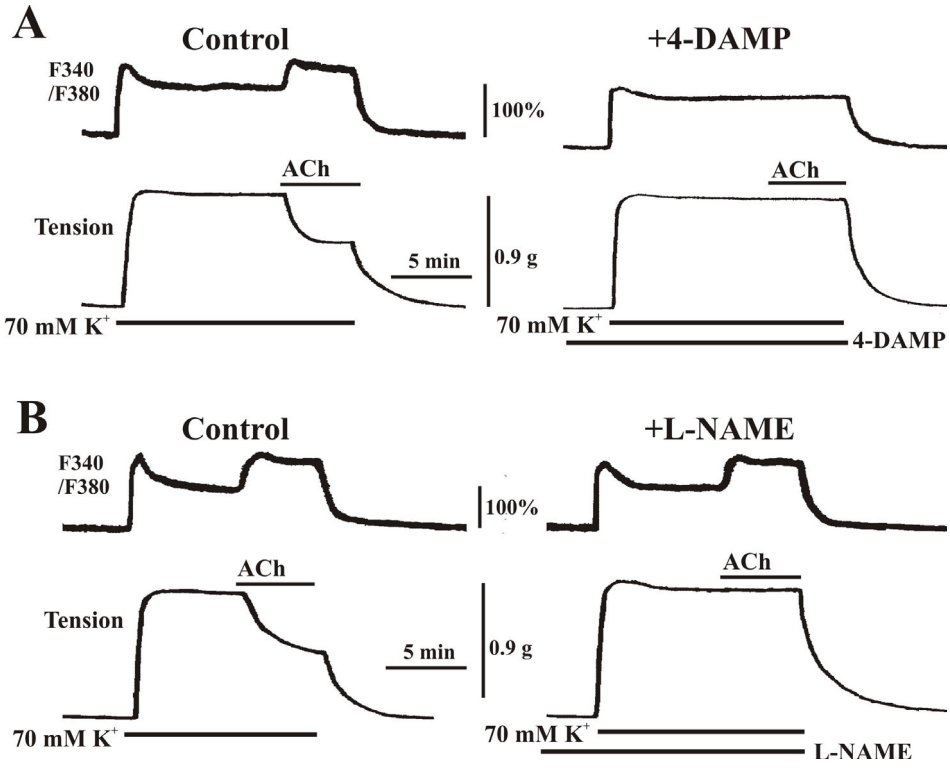

Fig. 2. Effect of 4-DAMP and L-NAME on ACh-induced changes of $[Ca^{2+}]_i$ and tension. Typical recordings of the ACh-induced changes of $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) in the presence of 10 nM 4-DAMP (A) or 0.1 mM L-NAME (B).

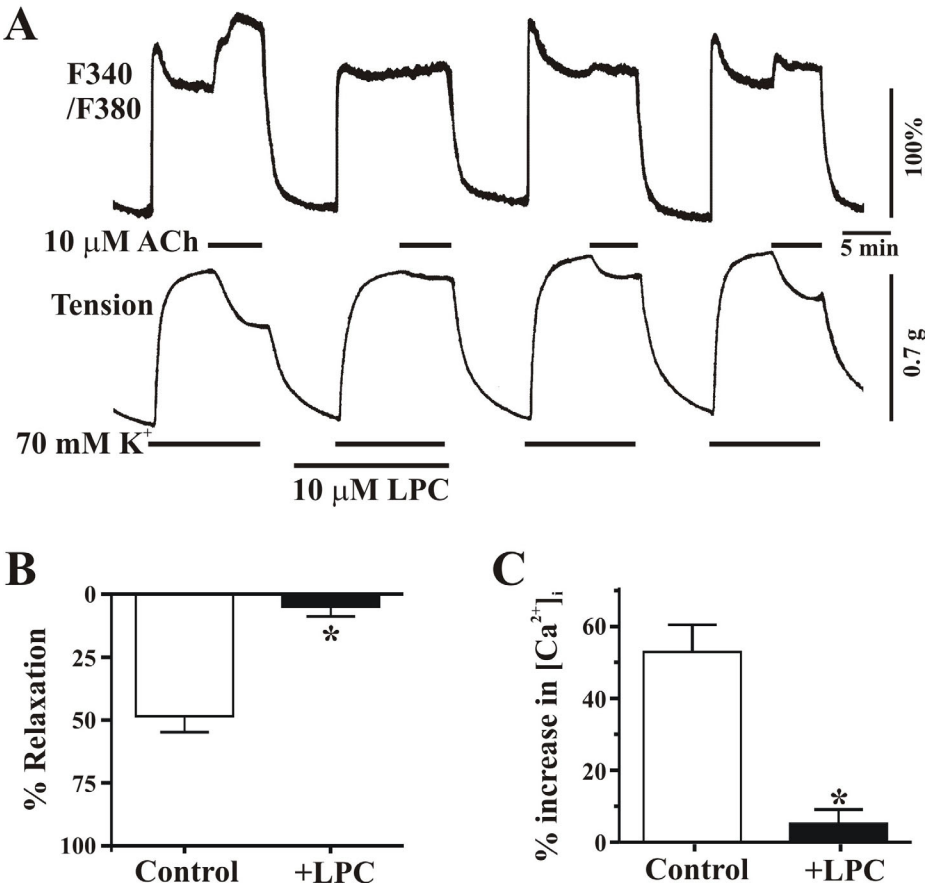

Fig. 3. Effect of LPC on ACh-induced changes of $[Ca^{2+}]_i$ and tension in carotid arterial strips. (A) Typical recordings of simultaneous measurement of ACh-induced changes of $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) in the presence of 10μM LPC. (B) Mean changes of LPC-induced inhibition of ACh-induced relaxation (B) and increase in $[Ca^{2+}]_i$ (C). Results are expressed as mean±S.E. (n=4). *Significantly different from ACh-induced response with $p < 0.05$.

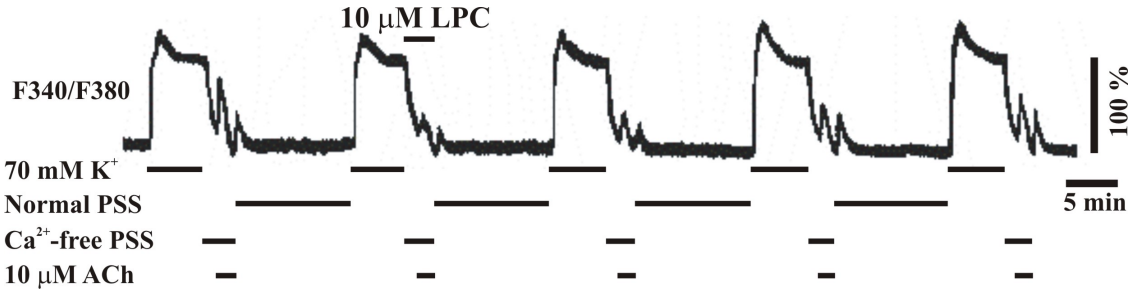

Fig. 4. Effect of LPC on ACh-induced changes of $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. Typical recordings of ACh-induced changes of $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. Muscle strips were stimulated by 70 mM K^+ followed by a wash with Ca^{2+}-free PSS containing 2 mM EGTA. One minute after washing with Ca^{2+}-free PSS, ACh (10μM) was added to the bath and measured the fluorescence change in the presence and absence of LPC (10μM). Effect of LPC on Ca^{2+} transient was evaluated by comparing the peak amplitude of Ca^{2+} transients. 100% represents the 70 mM K^+-stimulated $[Ca^{2+}]_i$ before the addition of Ca^{2+}-free PSS and the resting $[Ca^{2+}]_i$ level as 0%.

control level after washout of LPC (Fig. 3A).

To elucidate the mechanism of LPC-induced inhibition of ACh-evoked rise in $[Ca^{2+}]_i$, we measured the effect of LPC on $[Ca^{2+}]_i$ transient in the absence of external Ca^{2+}, in which agonist-induced $[Ca^{2+}]_i$ transients was solely due to Ca^{2+} release from internal stores. As shown in Fig. 4, removal of external Ca^{2+} decreased the high K^+-induced tension and $[Ca^{2+}]_i$ to the resting level. Application of ACh after removal of external Ca^{2+} transiently increased $[Ca^{2+}]_i$ (68.7±10.3% of high K^+-stimulated level, n=6). Pretrea-

tment with LPC significantly inhibited these ACh-induced transient change in $[Ca^{2+}]_i$ (43.3±7.3% of high K^+-stimulated level, n=6).

DISCUSSION

In this study, we simultaneously measured both endothelial $[Ca^{2+}]_i$ and vascular tension, in order to elucidate the mechanism of LPC-induced suppression of endothelium-dependent relaxation (EDR) at the tissue level. We first examined whether ACh-induced change in Fura-2 signal was due to changes in endothelial $[Ca^{2+}]_i$ or not, since in vascular strips which contain both endothelium and smooth muscle cells, the Fura-2 signals measured might be due to the changes in $[Ca^{2+}]_i$ in endothelium or smooth muscle cells. As seen in Fig. 1A & B, ACh-induced changes in Fura-2 signal were observed only in endothelium-intact strips, but not in endothelium-denuded strips. Moreover, there was a positive correlation between the ACh-induced increase in $[Ca^{2+}]_i$ and the magnitude of relaxation in endothelium-intact strips (Fig. 1C). These findings suggest that, in our experimental conditions, ACh-induced change of Fura-2 signals was almost entirely originated from the vascular endothelium, and it represents endothelial $[Ca^{2+}]_i$ change. It is possible that ACh-induced increase in endothelial $[Ca^{2+}]_i$ might have been underestimated, because ACh decreases $[Ca^{2+}]_i$ in smooth muscle cells by releasing EDRF from endothelium (Rapoport et al, 1983; Grygleswki et al, 1988; Karaki et al, 1988). However, the contribution of ACh-induced reduction of $[Ca^{2+}]_i$ in smooth muscle to the ACh-induced changes in $[Ca^{2+}]_i$ could be negligible, because there was a close relationship between the ACh-induced increase in $[Ca^{2+}]_i$ and the magnitude of relaxation ($p < 0.01$, n=6).

Agonist-induced changes in endothelial $[Ca^{2+}]_i$ is an essential steps in the production of bioactive agents, such as nitric oxide (NO). Chelation of endothelial Ca^{2+} inhibits the production of NO and abolishes the EDR in vascular strips (huang et al, 2001). In the present study, we showed that blocking the ACh-induced $[Ca^{2+}]_i$ elevation by 4-DAMP completely abolished the ACh-induced EDR responses. And pre-treatment of the strips with L-NAME completely eliminated the ACh-induced relaxation response with little effect on endothelial $[Ca^{2+}]_i$ elevation (Fig. 2). These findings suggest that elevation of endothelial $[Ca^{2+}]_i$ is an essential step for the production of endothelial bioactive agents, and NO is the major bioactive agents released from endothelium in our experimental conditions.

Impairment of EDR is a characteristic feature of atherosclerotic artery, and it is well documented that LPC is involved in the pathogenesis of atherosclerosis through inhibition of EDR (Flavahan, 1992; Murohora et al, 1994; Berliner et al, 1995). Similarly, we also observed that LPC inhibited ACh-induced EDR in carotid arteries. There is a possibility that LPC-induced reduction in EDR might be due to lysis of endothelial cells by its cytotoxic effect (Weltzien, 1979). However, the inhibitory effect of LPC was gradually abolished by washing the strip several times with normal PSS solution (Fig. 3A). Therefore, the LPC-induced reduction in EDR was not due to lysis of endothelial cells, but rather due to reversible alteration of endothelial function. However, it is still possible that LPC-induced overproduction of superoxide anion might perturb the efficacy of NO by formation of peroxynitrite, contributing to the LPC-induced inhibition of EDR (Ohara et al, 1994).

In endothelial cells, the agonist-induced elevation of $[Ca^{2+}]_i$ was due to the Ca^{2+} released from internal stores by 1,4,5-triphosphate (IP_3) or the influx of Ca^{2+} from external space. The release of Ca^{2+} from internal stores represents the transient component in agonist-induced increase in $[Ca^{2+}]_i$, whereas the influx of Ca^{2+} from external stores through capacitive Ca^{2+} entry pathway represents the sustained component (Putney, 1986; Adams et al, 1989). In the present study, LPC (10μM) significantly blocked the ACh-induced elevation of endothelial $[Ca^{2+}]_i$ in the absence of external Ca^{2+} (Fig. 4), suggesting the possibility that LPC inhibits the release of Ca^{2+} from internal stores via disturbing the G-protein coupled Ca^{2+} signaling in endothelial cells (Hirata et al, 1992; Flavahan, 1993).

Transmembrane Ca^{2+} influx from external space depends on the membrane potential of endothelial cells. Agonist-induced hyperpolarization through activation of Ca^{2+}-activated K^+ channels augments the driving force for the Ca^{2+} influx through capacitive Ca^{2+} entry pathway (Mehrke et al, 1991; Nilus, 1991). Therefore, depolarization with any causes can attenuate the amount of Ca^{2+} influx from external space. Terasawa et al. (2002) reported that LPC depolarized the coronary smooth muscle cells through activation of nonselective cation channels. Thus, LPC may depolarize membrane potential of endothelial cells, leading to the inhibition of transmembrane Ca^{2+} influx. In the present study, we measured the effect of LPC after 70 mM K^+-induced contraction reached steady-state. In this high external K^+ solution, it is expected that the membrane potential of endothelial cells shows strong depolarization, and that the possibility of further depolarization by LPC will be negligible. However, we did not directly evaluate this possibility in the present study. Whether or not LPC inhibits Ca^{2+} influx through depolarization needs to be investigated further in isolated endothelial cells.

In summary, LPC inhibits the ACh-induced EDR through perturbation of ACh-induced $[Ca^{2+}]_i$ regulation in rabbit carotid strips. LPC-induced inhibition of endothelial $[Ca^{2+}]_i$ transient might be due to inhibition of Ca^{2+} release from internal stores in endothelial cells. Present results are expected to provide us one possible explanation for LPC-induced impairment of endothelium-dependent vascular relaxation.

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