Calcium-independent phospholipase A_2 mediates CREB phosphorylation in double-stranded RNA-stimulated endothelial cells

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Abstract **One of the products of a calcium-independent phospholipase A_2 (iPLA$_2$) attack of plasmenylcholine, lysoplasmenylcholine, has previously been shown to activate cAMP-dependent protein kinase (PKA). Because endothelial cells respond to some agonists in part by the activation of iPLA$_2$, the present study was designed to determine whether double-stranded RNA (dsRNA), the primary activator of the antiviral response in endothelial cells, elicits cAMP response element binding protein (CREB) phosphorylation through a mechanism mediated by iPLA$_2$. dsRNA stimulated CREB phosphorylation in bovine pulmonary artery endothelial cells that was inhibited by the iPLA$_2$ inhibitor, bromoenol lactone, and the PKA inhibitor, H-89. Additionally, the product of iPLA$_2$ hydrolysis of plasmenylcholine and lysoplasmenylcholine elicited CREB phosphorylation in bovine pulmonary endothelial cells. Taken together, the present studies suggest that dsRNA as well as other agonists of endothelial cells elicit signaling mechanisms that include in part CREB phosphorylation mediated by iPLA$_2$.**—Martinson, B. D., C. J. Albert, J. A. Corbett, R. B. Wysolmerski, and D. A. Ford. **Calcium-independent phospholipase A_2 mediates CREB phosphorylation in double-stranded RNA-stimulated endothelial cells. *J. Lipid Res.* 2003.** 44: **1686–1691.**

Supplementary key words endothelium • cAMP response element binding protein • cAMP-dependent

Lysophospholipid production in most mammalian cells is regulated by the coordinated activities of the phospholipase A_2 family and lysolipid acyl CoA transferases. Calcium-independent phospholipase A_2 (iPLA$_2$) has previously been shown to be the predominant phospholipase A_2 in mammalian myocardium and is activated during brief episodes of ischemia (1–3). Because purified cAMP-dependent protein kinase (PKA) is activated by both lysophosphatidylcholine and lysoplasmenylcholine through a cAMP-independent mechanism, a putative role of iPLA$_2$ in the ischemic heart was predicted as a mediator resulting in PKA activation (4). Indeed, recent studies have demonstrated that iPLA$_2$ likely mediates cAMP response element binding protein (CREB) phosphorylation and subsequent c-*fos* expression in response to brief ischemia through the activation of PKA by lysoplasmenylcholine or lysophosphatidylcholine (5).

Double-stranded RNA (dsRNA) accumulates at various stages of viral replication and plays a role in the activation of the antiviral response in virally infected cells (6). We have recently shown that dsRNA elicits iPLA$_2$-mediated CREB phosphorylation in RAW 264.7 cells (7). Because one of the primary host target cells of viral infection are the vascular endothelial cells, the role of iPLA$_2$ as a mediator of dsRNA stimulation of endothelial cell signaling was determined. The present studies demonstrate that dsRNA elicits CREB phosphorylation in endothelial cells that is sensitive to inhibition by the iPLA$_2$ inhibitor, bromoenol lactone (BEL). Additionally, the mechanism of this signaling appears to be mediated by lysoplasmenylcholine or lysophosphatidylcholine because they independently elicit CREB phosphorylation. Furthermore, dsRNA-stimulated CREB phosphorylation likely is mediated through iPLA$_2$-derived lyso choline glycerophospholipids stimulating PKA because this signaling pathway was susceptible to inhibition by the PKA inhibitor, H-89.

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Abbreviations: BEL, bromoenol lactone; CMC, critical micellar concentration; dsRNA, double-stranded RNA; iPLA$_2$, calcium-independent phospholipase A_2; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; pCREB, phospho cAMP response element binding protein; PMA, phorbol myristate acetate; poly IC, polyinosinic-polycytidylic acid.

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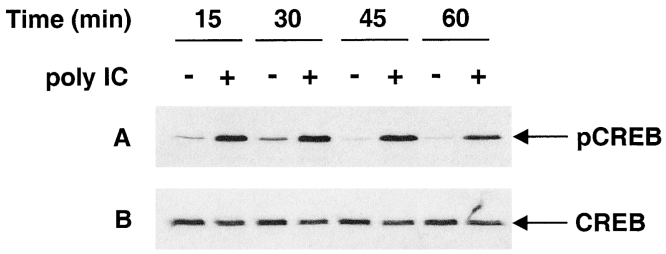

Fig. 1. Polyinosinic-polycytidylic acid (Poly IC)-stimulated cAMP response element binding protein (CREB) phosphorylation in bovine pulmonary artery endothelial cells. Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in serum-free media subjected to treatments either without or with 250 μg/ml poly IC for indicated time intervals, as described in Materials and Methods. Lysates were then subjected to SDS-PAGE and Western blot analysis using either anti-phospho CREB (pCREB) or CREB as the primary antibodies. The blots illustrated are representative of over four independent experiments.

MATERIALS AND METHODS

Endothelial cell culture

Bovine pulmonary artery endothelial cells were cultured to confluency in MEM supplemented with 10% heat-inactivated fetal calf serum and washed in MEM in the presence or absence of serum (as indicated) immediately prior to experiments utilizing 35 mm collagen-coated culture dishes. The cells were incubated with indicated concentrations of either polyinosinic-polycytidylic acid (poly IC), lipopolysaccharide (LPS), phorbol myristate acetate (PMA), or sonicated lysoplasmenylcholine added in 25 μl MEM as indicated for selected time intervals at 37°C. Incubations were terminated by removing the media from the dishes, scraping the cells in SDS sample buffer containing DTT, 400 U/ml DNase I, 200 μM Na_3VO_4, and 50 mM NaF (phosphatase inhibitors).

Western blotting

Samples from cell culture experiments were boiled for 3 min and sequentially subjected to SDS-PAGE utilizing 15% polyacrylamide gels followed by the transfer of proteins to polyvinylidene difluoride (PVDF)-plus membranes for Western blot analysis. Anti-CREB and anti-phospho CREB (pCREB) antibodies were utilized as primary antibodies (1:1,333 dilution, respectively) along with the horseradish peroxidase-conjugated secondary antibody (1:7,000 dilution). Immunoreactive bands were then visualized by chemiluminescence detected on X-ray film utilizing the enhanced chemiluminescence (ECL) system after 1 h exposure. Multiple exposures of film to blots were developed. Exposures that had linear levels of grain development were used for quantitation of band intensity utilizing NIH Image software following scanning and conversion of autoradiographic data to TIFF file formats using a Macintosh 5500/225 computer and a Linocolor-Hell Jade scanner. Quantitative analysis of autoradiographic data was performed using the public domain NIH Image program.

Preparation of lysoplasmenylcholine

The lysoplasmenylcholine molecular species, 1-*O*-hexadec-1′-enyl-GPC, was prepared from bovine heart lecithin and purified as previously described (8). Briefly, bovine heart lecithin was subjected to base methanolysis and the reaction product, lysoplasmenylcholine, was purified by preparative straight-phase HPLC. Subsequently, the lysoplasmenylcholine molecular species, 1-*O*-hexadec-1′-enyl-GPC, was purified by semipreparative reversed-phase HPLC. Synthetically prepared lysoplasmenylcholine was determined to be greater than 95% pure by thin-layer chromatography, straight phase HPLC, reversed-phase HPLC, and capillary gas chromatography of the aliphatic constituents. Lysoplasmenylcholine was subjected to acid methanolysis in the presence of arachidic acid (20:0 fatty acid) as an internal standard and quantified by capillary gas chromatography by comparisons of the integrated peak area of the dimethyl acetal of palmitaldehyde (derived from the *sn*-1 aliphatic chain of lysoplasmenylcho-

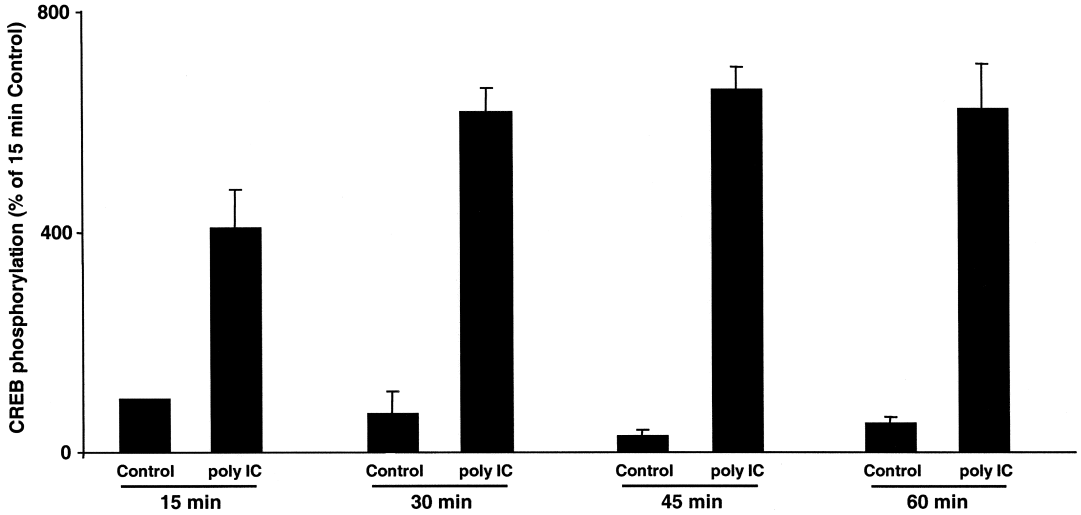

Fig. 2. Quantification of the temporal course of poly IC-stimulated CREB phosphorylation in bovine pulmonary artery endothelial cells. Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in serum-free media subjected to treatments either with or without 250 μg/ml poly IC for indicated time intervals and were subjected to SDS-PAGE. Following SDS-PAGE, Western blot analyses with densitometric quantification were performed using either anti-pCREB or CREB as the primary antibodies, as described in Materials and Methods. Values represent the mean ± SEM of four independent determinations and are normalized to the amount of CREB detected in each sample and to the results measured for the samples prepared from the 15 min control.

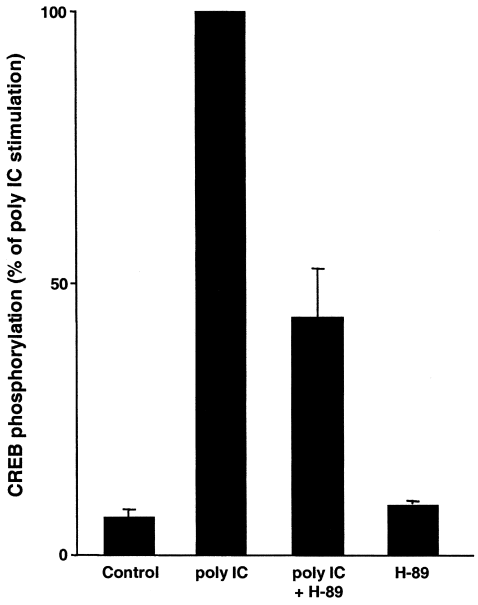

Fig. 3. Inhibition of poly IC-stimulated CREB phosphorylation in bovine pulmonary artery endothelial cells by H-89. Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in serum-free media subjected to treatments either with or without (Control) 250 μg/ml poly IC for 30 min in the presence or absence of 10 μM H-89. Following SDS-PAGE, Western blot analyses with densitometric quantification were performed using either anti-pCREB or CREB as the primary antibodies, as described in Materials and Methods. Values represent the mean ± SEM of four independent determinations and are normalized to the amount of CREB detected in each sample and compared with the results measured for the samples stimulated with poly IC with no other additions.

line) to that of the methyl ester of arachidonic acid (derived from the internal standard).

Materials

PVDF-plus membranes were purchased from Micron Separations. Anti-CREB and anti-pCREB (Ser133) antibodies were purchased from Cell Signaling. X-OMAT AR X-ray film was purchased from Kodak. The ECL system was purchased from Amersham. Poly IC and LPS (*Escherichia coli* serotype 0111:B4) were purchased from Sigma. PMA was from Calbiochem. HPLC-grade solvents were purchased from Fisher Scientific Co. BEL was purchased from Cayman Chemical. All other reagents, chemicals, or enzymes were purchased from Aldrich, Sigma, or Fisher.

RESULTS

dsRNA stimulation of endothelial CREB phosphorylation

Because previous studies have shown that lysoplasmenylcholine and other choline lysoglycerophospholipids can activate PKA (4, 5), experiments were performed to demonstrate that this mechanism is active in intact cells. Bovine pulmonary artery endothelial cells were subjected to poly IC treatment and the phosphorylation state of the PKA target, CREB, was determined. **Figure 1A** shows that CREB is phosphorylated within 15 min of poly IC stimulation of endothelial cells, and the CREB phosphorylation state remains high over a 1 h period of stimulation. Figure 1B shows that CREB levels are nearly constant in each sample tested. Similar data from multiple Western blot analyses were quantified by densitometry, and these data are summarized in **Fig. 2**. Significant CREB phosphorylation occurs in response to poly IC treatments of endothelial cells at all time intervals examined in this study (e.g., 15 min, 30 min, 45 min, and 60 min).

Inhibition of dsRNA stimulation of CREB phosphorylation by $iPLA_2$ and PKA inhibitors

The mechanism of poly IC-stimulated CREB phosphorylation was explored using specific inhibitors of PKA, H-89, and $iPLA_2$, BEL. **Figure 3** demonstrates that the phosphorylation of CREB in response to poly IC is likely mediated in part by PKA because H-89 is inhibitory to the phosphorylation mediated by poly IC (Fig. 3). Additionally, H-89 had no effect on basal phosphorylation of CREB (Fig.

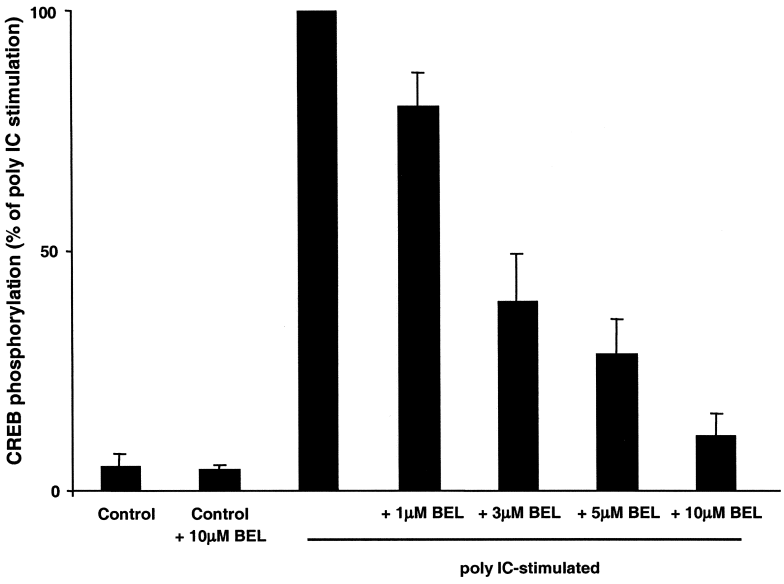

Fig. 4. Inhibition of poly IC-stimulated CREB phosphorylation in bovine pulmonary artery endothelial cells by bromoenol lactone (BEL). Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in serum-free media subjected to treatments either with or without (Control) 250 μg/ml poly IC for 30 min in the presence or absence of indicated concentrations of BEL. Following SDS-PAGE, Western blot analyses with densitometric quantification were performed using either anti-pCREB or CREB as the primary antibodies, as described in Materials and Methods. Values represent the mean ± SEM of four independent determinations and are normalized to the amount of CREB detected in each sample and compared with the results measured for the samples stimulated with poly IC with no other additions.

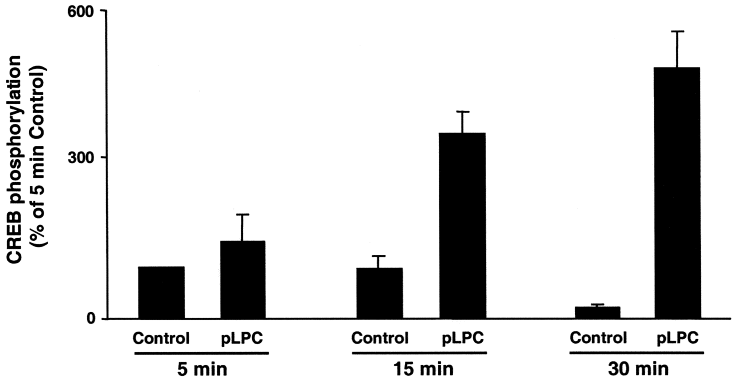

Fig. 5. Quantification of the temporal course of lysoplasmenylcholine-stimulated CREB phosphorylation in bovine pulmonary artery endothelial cells. Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in serum-free media subjected to treatments either with or without 5 μM lysoplasmenylcholine for indicated time intervals and were subjected to SDS-PAGE. Following SDS-PAGE, Western blot analyses with densitometric quantification were performed using either anti-pCREB or CREB as the primary antibodies, as described in Materials and Methods. Values represent the mean ± SEM of four independent determinations and are normalized to the amount of CREB detected in each sample and compared with the results measured for the samples prepared from the 5 min control.

3). **Figure 4** shows that poly IC-stimulated CREB phosphorylation was partially inhibited by the iPLA$_2$ inhibitor, BEL, at concentrations as low as 1 μM and was nearly completely inhibited by BEL at 10 μM . Furthermore, the apparent IC$_{50}$ for BEL inhibition of endothelial cell CREB phosphorylation elicited by poly IC was <3 μM (Fig. 4).

Endothelial cell CREB phosphorylation in response to lysoplasmenylcholine

Because previous studies have linked iPLA$_2$ to CREB phosphorylation through activation of PKA by lysoplasmenylcholine, further studies were performed to demonstrate that CREB phosphorylation could be directly modulated by treating endothelial cells with lysoplasmenylcholine. Within 5 min of treatment of endothelial cells with lysoplasmenylcholine (5 μM), CREB phosphorylation was increased about 75% over control CREB phosphorylation (**Fig. 5**). Following 15 min and 30 min of lysoplasmenylcholine treatment, CREB phosphorylation in endothelial cells continued to increase. **Figure 6** shows the effects of different concentrations of lysoplasmenylcholine on endothelial cell CREB phosphorylation. CREB phosphorylation increased in response to increasing amounts of lysoplasmenylcholine between 1 μM and 20 μM (Fig. 6). It should be appreciated that under these conditions, endothelial cells remained attached to tissue culture plates and appeared healthy. Furthermore, media lactate dehydrogenase (LDH) levels did not increase in the presence of lysoplasmenylcholine concentrations below 20 μM in comparison to that from control-treated cells (data not shown). Concentrations above 20 μM (such as 100 μM) were above the critical micellar concentration (CMC) for lysoplasmenylcholine and accordingly were not used in these studies. In fact, treating endothelial cells with 200 μM lysoplasmenylcholine resulted in a >175-fold increase in media LDH levels compared with control-treated endothelial cells. Taken together, lysoplasmenylcholine elicits an increased CREB phosphorylation state in a time- and concentration-dependent manner in bovine pulmonary artery endothelial cells. Although the previous demonstration that lysoplasmenylcholine directly activates PKA (4) leads to the conclusion that the enhanced CREB phosphorylation state in endothelial cells is mediated by lysoplasmenylcholine activation of PKA, it is possible that lysoplasmenylcholine may modulate the CREB phosphorylation state by inhibiting protein phosphatases.

iPLA$_2$-mediated endothelial CREB phosphorylation in response to LPS and PMA stimulation

Further experiments were performed to determine if other agonists of endothelial cells elicited CREB phosphorylation through mechanisms mediated by iPLA$_2$. **Figure 7** demonstrates that both LPS and PMA stimulate CREB phosphorylation in bovine pulmonary artery endothelial cells. Furthermore, CREB phosphorylation to both agonists is inhibited by the specific iPLA$_2$ inhibitor, BEL, at both time points studied (Fig. 7). Further analysis of the sensitivity of LPS-stimulated CREB phosphorylation to BEL inhibition demonstrated that LPS-stimulated CREB

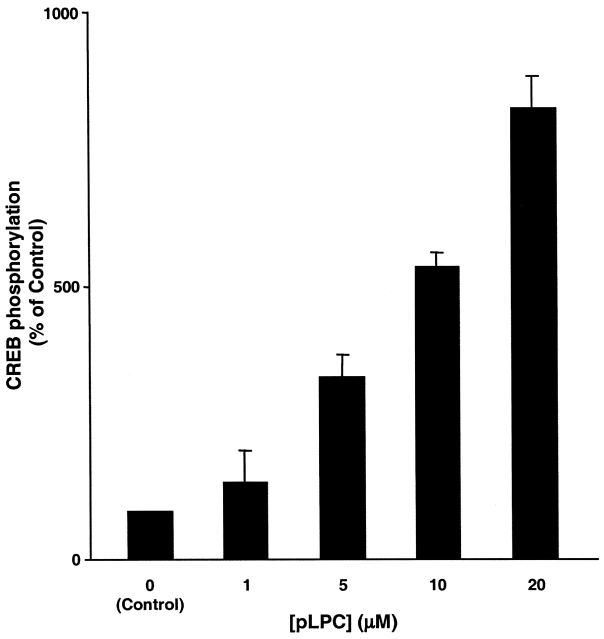

Fig. 6. Lysoplasmenylcholine stimulation of CREB phosphorylation in bovine pulmonary artery endothelial cells. Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in serum-free media subjected to treatments with selected concentrations of lysoplasmenylcholine for 15 min. Following SDS-PAGE, Western blot analyses with densitometric quantification were performed using either anti-pCREB or CREB as the primary antibodies, as described in Materials and Methods. Values represent the mean ± SEM of four independent determinations and are normalized to the amount of CREB detected in each sample and compared with the results measured for the samples prepared from the control samples.

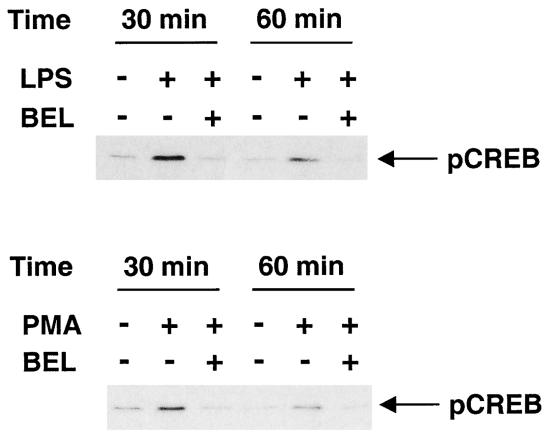

Fig. 7. Lipopolysaccharide (LPS)- and phorbol myristate acetate (PMA)-stimulated CREB phosphorylation in bovine pulmonary artery endothelial cells is calcium-independent phospholipase A_2 mediated. Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in media containing 10% heat-inactivated serum subjected to treatments with 10 μg/ml LPS or 500 nM PMA for 30 min and were subjected to SDS-PAGE. In indicated conditions, 30 μM BEL was included. Following SDS-PAGE, Western blot analyses were performed using either anti-pCREB or CREB as the primary antibodies, as described in Materials and Methods.

phosphorylation was substantially inhibited by the $iPLA_2$ inhibitor, BEL, at concentrations as low as 1 μM, and was nearly completely inhibited by BEL at 10 μM (**Fig. 8**). The apparent IC_{50} for BEL inhibition of endothelial cell CREB phosphorylation elicited by LPS was ~3 μM (Fig. 8).

DISCUSSION

The present studies demonstrate that $iPLA_2$ mediates CREB phosphorylation in bovine pulmonary artery endothelial cells in response to the dsRNA poly IC through a mechanism that may include lyso choline glycerophospholipid activation of PKA. These conclusions are supported by the observations that *1*) poly IC elicits CREB phosphorylation in bovine pulmonary artery endothelial cells and this phosphorylation is inhibited by the specific $iPLA_2$ inhibitor, BEL; *2*) the product of $iPLA_2$-mediated hydrolysis of plasmenylcholine, lysoplasmenylcholine, at concentrations below its CMC stimulates CREB phosphorylation; and *3*) poly IC-stimulated CREB phosphorylation is inhibited by the specific PKA inhibitor, H-89. Further studies also revealed that both PMA- and LPS-stimulated CREB phosphorylation in endothelial cells is likely mediated through a similar biochemical mechanism because BEL inhibited CREB phosphorylation in response to both stimuli.

$iPLA_2$ has previously been shown to be activated in endothelial cells in response to thrombin (9, 10). The mechanism of $iPLA_2$ activation and translocation to membranes is not understood. Although $iPLA_2$ contains consensus phosphorylation sites for several serine PKAs, active phosphorylated $iPLA_2$ has not been demonstrated (11). One common mechanism through which both poly IC and LPS activate $iPLA_2$, resulting in CREB phosphorylation, is through the commonality of their receptor, TLR-3 (12). Additionally, Steer et al. have recently demonstrated that PMA stimulates $iPLA_2$ activity through activation of protein kinase C (PKC)-ε (13). Taken together, it appears that both LPS and poly IC stimulate $iPLA_2$ through the TLR-3 receptor, and evidence suggests that PKC might be involved in activation. Furthermore, the present results suggest that $iPLA_2$ is a common signaling pathway for poly IC, LPS, and PMA that converge to activate PKA.

Viral infection of endothelial cells of the vascular wall represents a key mechanism believed to mediate vascular wall disease, including atherosclerosis. dsRNA accumulates at various stages of viral infection and is considered to be a key stimulus to the antiviral response of infected cells (6). We have previously demonstrated in RAW 264.7 cells that $iPLA_2$ activation in response to poly IC elicits an

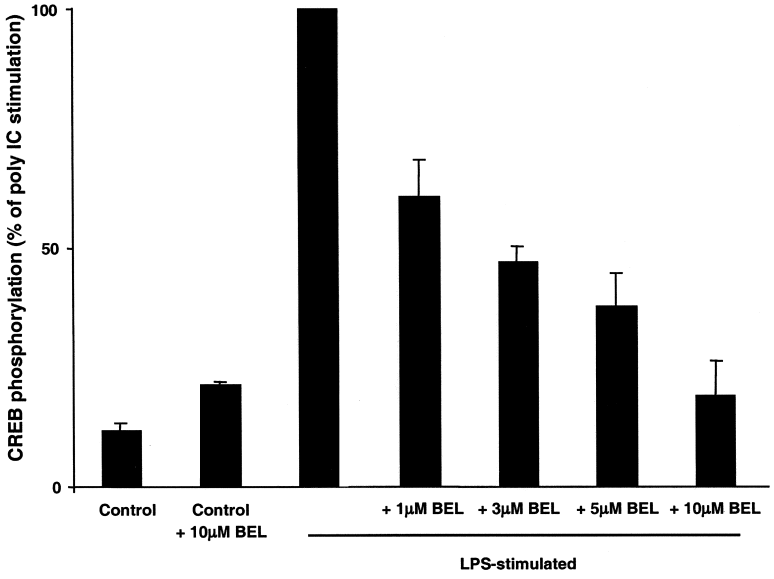

Fig. 8. Inhibition of LPS-stimulated CREB phosphorylation in bovine pulmonary artery endothelial cells by BEL. Cell lysates were prepared in SDS-sample buffer from bovine pulmonary artery endothelial cells in serum-free media subjected to treatments either with or without (Control) 10 μg/ml LPS for 30 min in the presence or absence of indicated concentrations of BEL. Following SDS-PAGE, Western blot analyses with densitometric quantification were performed using either anti-pCREB or CREB as the primary antibodies, as described in Materials and Methods. Values represent the mean ± SEM of four independent determinations and are normalized to the amount of CREB detected in each sample and compared with the results measured for the samples stimulated with LPS with no other additions.

antiviral response by signaling inducible nitric oxide synthase (iNOS) production (7). In comparison to RAW 264.7 cells, poly IC did not stimulate iNOS production in endothelial cells (data not shown). This illustrates the disparate mechanisms that host cells use to respond to viral infection. CREB phosphorylation is critical for the activation of the CRE element that elicits the transcription of new proteins. It remains to be determined which new proteins are stimulated in response to poly IC in the endothelial cells and whether they either contribute to the antiviral response leading to protection from vascular wall disease or, alternatively, represent an initiating step toward vascular wall disease elicited by viral infection.

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