Propionyl-L-carnitine induces eNOS activation and nitric oxide synthesis in endothelial cells via PI3 and Akt kinases

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A B S T R A C T

Propionyl-L-carnitine (PLC) is a natural short-chain derivative of L-carnitine (LC), a natural amino acid that plays an important role in fatty acid metabolism. Recent studies suggest that PLC has vascular protective effects. Because of the importance of endothelial nitric oxide synthase (eNOS) and its product, antiatherogenic molecule nitric oxide (NO), in vascular endothelial function, we sought to elucidate that if PLC would stimulate eNOS and its upstream activators Akt and phosphatidylinositol 3-kinase (PI3 Kinase) in cultured human aortic endothelial cells (HAEC). PLC caused eNOS phosphorylation at Ser-1177, and dominant negative Akt and a novel Akt-selective inhibitor MK-2206 inhibited both PLC-mediated phosphorylation and activation of the enzyme. PI3 kinase inhibition also blocked the phosphorylation and activation of eNOS by PLC. Studies with specific drug inhibitors PD173955 and PP2 showed that the non-receptor tyrosine kinase, src, is an upstream stimulator of the PI3 kinase-Akt pathway in this pathway. In addition, PLC significantly decreased intracellular ATP/ADP ratio and activate AMPK, subsequently leading to Src activation. Finally, we demonstrated that the effects of PLC to augment eNOS activity were associated with a net increase in NO release from endothelial cells.

1. Introduction

L-carnitine (LC) has been described as a natural cellular amino acid synthesized by the body, which is required for the conversion of fat to chemical energy. LC may function as a vehicle to transport subunits of enzyme. PI3 kinase inhibition also blocked the phosphorylation and activation of eNOS by PLC. Studies with specific drug inhibitors PD173955 and PP2 showed that the non-receptor tyrosine kinase, src, is an upstream stimulator of the PI3 kinase-Akt pathway in this pathway. In addition, PLC significantly decreased intracellular ATP/ADP ratio and activate AMPK, subsequently leading to Src activation. Finally, we demonstrated that the effects of PLC to augment eNOS activity were associated with a net increase in NO release from endothelial cells.

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1. Introduction

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2. Materials and methods

2.1. Materials

The following antibodies were purchased from Cell Signaling: rabbit polyclonal phospho-eNOS (Ser1177), rabbit polyclonal phospho-Akt (Ser473), rabbit polyclonal Akt. Rabbit polyclonal eNOS and mouse monoclonal actin antibodies were respectively obtained from Abcam and Chemicon. Monoclonal antibodies specific for src (m327) was obtained from Calbiochem. MM-2206 was purchased from Selleck.

Abbreviations: eNOS, endothelial nitric oxide synthase; DN-Akt, dominant-negative mutant of protein kinase B (Akt); HAEC, human aortic endothelial cells; LC, L-carnitine; NO, nitric oxide; PI3 Kinase, phosphatidylinositol 3-kinase; PLC, Propionyl-L-carnitine; SHR, spontaneously hypertensive rats.

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PD173955 and PD173955 were obtained from Symansis. PP2, Wortmannin, compound C and LY294002 were purchased from Calbiochem. The dominant-negative mutant of protein kinase B (Akt) (DN-Akt) was obtained from VectorBiolabs. Pyruvate kinase was purchased from Lee Biosolutions, Inc. All other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology. The specific AMPK-targeted SAMS peptide used in AMPK activity assays was from GenScript (Piscataway, NJ).

2.2. Cell culture

All experiments were done in human aortic endothelial cells (HAEC, purchased from Shanghai Bioleaf Biotech Co., Ltd) propagated in EGM-2 and used at passages 4 to 6. The transfected cells were used 48 h after transfection, and 70–80% transfection efficiency was typically achieved. cDNAs for DN-Akt was prepared as described previously [22].

2.3. Western blotting

Western blot analysis was performed according to standard procedures. Proteins from whole cell lysates of HAECs were separated on 4–12% Tris-glycine gels and transferred to nitrocellulose membranes (Invitrogen). Membranes were then probed with antibodies against eNOS, phospho-eNOS, Akt, phospho-Akt, and actin followed by incubation with appropriate horseradish peroxidase-associated secondary antibodies before signals were visualized with the enhanced chemiluminescence detection system (Amer sham Bioscience). Phospho-eNOS and phospho-Akt measurements were performed in HAECs that had been treated with the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin (50 nM) or LY-294002 (50 μM) for 2 h, the tyrosine kinase inhibitor PD173955 (5 μM) or PP2 (0.1 μM) for 30 min, the novel PKB-selective inhibitors MK-2206 (100 nM) for 2 h, NG-nitro-L-arginine methyl ester (L-NAME) (1 mM) for 30 min or transfected with DN-Akt. All cells, regardless of pretreatment, were stimulated with PLC (50 μM) for 6 h.

2.4. eNOS activity assay

eNOS activity was detected by measuring the conversion of L-[3H]arginine to L-[3H]citrulline at 37 °C for 30 min with the eNOS assay kit (Calbiochem–Nova Biochem) as described [28]. Unlabeled L-arginine was added to L-[3H]arginine (specific activity, 60 Ci/mmol) at a ratio of 3:1. Samples incubated in the presence of the competitive NOS inhibitor, L-NAME (1 mM), were used to determine nonspecific activity. Nonspecific activity accounted for 20–35% of total activity.

2.5. Akt kinase assay

As previously described [17], anti-Akt antibody was incubated in 96-well protein G-coated plates overnight. Lysates were then added and incubated overnight as well. Subsequent exposure to [γ-32P]ATP and Aktide substrate initiated an in vitro reaction that was subsequently terminated after 30 min by addition of phosphoric acid. Reaction mixtures were then transferred to a phosphocellulose filter plate and filter bound [γ-32P]-substrate was quantified using a scintillation counter. Count per minute readings were normalized to lysate concentrations.

2.6. Measurement of NO production

NO quantification was performed using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; Cayman Chemical, Ann Arbor, MI) as described previously [10]. Briefly, HAECs were seeded in 96 well-plates (3 x 10⁴ cells/well), cultured for 24 h and treated with 50 μM PLC for 6 h in the presence or absence of I-LAME, PD173955, LY294002, MK-2206 or compound C. The cells were then serum starved for 1 h in phenol red-free EB3 supplemented with K-arginine (100 μM) and uric acid (UA, 100 μM). Cells were then loaded with DAF-2DA (5 μM final concentration) for 10 min at 37 °C. After being loaded with DAF-2 DA, cells were washed three times with EB3 at 37 °C and kept in the dark. The fluorescence intensity was measured with a multilabel plate reader Wallac 1420 Victor2 (PerkinElmer) using 485 nm as excitation and 535 nm as emission wavelengths. The fluorescence intensities were corrected by subtracting the non-specific fluorescence in wells without addition of DAF-2 DA and in wells without cells.

2.7. Src kinase assays

Src kinase assays were performed as described previously [21] with minor modifications. Briefly, total cellular lysates were harvested in modified RIPA buffer [1% sodium deoxycholate, 1% NP40, 0.1% SDS, 150 mm NaCl, and 10 mm sodium phosphate (pH 7.2)] supplemented with 10 μm aprotinin, 10 μm leupeptin, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Total cellular lysates (350 μg) were incubated with antibodies specific for the src family kinase and immunoprecipitated using protein G-Sepharose. Immunoprecipitates were washed twice in cold lysis buffer and once in kinase buffer and added to an in vitro kinase reaction consisting of 50 mm PIPES (pH 7.0), 10 mM MnCl₂, 10 mM DTT, 10 μM ATP, 2 μg of acid-denatured enolase, and 5 μCi of [γ-32P]ATP. Reactions were allowed to proceed at 30 °C for 5 min and then stopped immediately by boiling in sample buffer, products separated on a 10% SDS-PAGE gel, transferred to membrane, and exposed to film.

2.8. Assay of AMP kinase activity

After treatment, cells were immediately washed with 2 ml of ice-cold phosphate-buffered saline buffer and scraped with a rubber spatula in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). EC lysates were incubated with SAMS peptide and (γ-32P)ATP, and the catalytic activity of AMPK was determined by the incorporation of 32P into SAMS peptide as described previously [31].

2.9. Determination of adenine nucleotides

HAEC were cultured in 6-well plates, treated with PLC for 6 h, washed with PBS, and scraped in 0.3 ml of PBS. ATP and ADP were then measured in quadruplicate by a luminometric method as described elsewhere [25].

2.10. Statistical analysis

All data are given as the mean ± SD. Data were analyzed by using paired and unpaired Student’s t test and one-way ANOVA. A p value of <0.05 was taken as significant difference between data sets.

3. Results

3.1. PLC increases eNOS phosphorylation and activity in a concentration-and time-dependent fashion

In the first series of experiments, we determined whether PLC activates eNOS in human endothelial cells. The activity of eNOS is regulated by phosphorylation at multiple sites. The two most thoroughly studied sites are the activation site Ser1177 and the inhibitory site Thr495 [4]. As shown in Fig. 1A, PLC upregulated endothelial eNOS phosphorylation at Ser1177 in a concentration-dependent manner with the highest concentration of PLC used (100 μM) evoking a response
that was 2.5-fold higher than that in the control group \((p < 0.05)\). PLC also elevated eNOS phosphorylation in a time-dependent manner (Fig. 1B), with the maximum response seen at 6 h postinitiation of incubation in endothelial cells. PLC treatment did not change Thr495 phosphorylation (data not shown). Next, we performed western blot analysis to detect inducible nitric oxide synthase (iNOS) protein expression. As shown in Fig. 1C (upper panel), 6 h incubation of PLC did not change iNOS protein expression. To further substantiate the observed effects of PLC on eNOS activation, we directly evaluated eNOS activity by measuring \([3H]\)-arginine to \([3H]\)-citrulline conversion in HAECs following incubation with PLC. PLC exposure evoked a significant increase in eNOS activity, with 50 \(\mu\)M PLC increasing eNOS activity by 350\% over the control value \((p < 0.05)\) (Fig. 1C). Given that the activity assay used may not distinguish eNOS from iNOS, HAECs were treated with 20 \(\mu\)M N-iminoethyl-L-lysine (L-NIL), a specific iNOS inhibitor. As indicated in Fig. 1C, iNOS inhibition did not block PLC activation of eNOS activity, suggesting that the influence of iNOS can be excluded.

3.2. PLC stimulates eNOS phosphorylation at Ser1177 via Akt activation

Several stimuli are known to activate eNOS through Akt-mediated phosphorylation of the enzyme. Hence we evaluated the involvement of Akt in the phosphorylation and stimulation of eNOS by PLC. As shown in Fig. 2A and B, Akt phosphorylation at Ser473 was increased by PLC with a concentration- or time-dependent manner. Akt kinase assay also confirmed PLC-induced Akt activation (Fig. 2C).

To further determine if Akt is responsible for the phosphorylation of eNOS by PLC, either sham plasmid (EV) or a dominant negative Akt mutant (DN-Akt) was expressed in HAECs. The transfected cells were incubated with PLC (50 \(\mu\)M) for 6 h and the phosphorylation of eNOS at Ser-1179 and Akt at Ser-473 were assessed. As shown in Fig. 2D, PLC caused phosphorylation of eNOS and Akt in cells expressing EV, but no eNOS phosphorylation was observed in cells expressing dominant negative Akt (DN-Akt). In addition, inhibition of Akt with MK-2206, a novel allosteric inhibitor of Akt, blocked PLC-induced p-eNOS activation, suggesting that PLC stimulates Akt, which in turn phosphorylates eNOS.

3.3. PI3 kinase is responsible for Akt activation and eNOS activation by PLC

Akt is activated by PI3-kinase through recruitment to the plasma membrane where Akt becomes phosphorylated at Ser473 and Ser308. Since PI3 kinase is a putative upstream activator of Akt, we next examined the effect of PI3 kinase inhibitors on both Akt and eNOS phosphorylation and activation. HAECs were pre-incubated with the PI3 kinase inhibitor wortmannin (50 \(\mu\)M) or LY294002 (50 \(\mu\)M) for 30 min before PLC stimulation (50 \(\mu\)M), and the phosphorylation of eNOS at Ser-1177 and Akt at Ser-473 was assessed. In parallel experiments, eNOS activity was measured. As shown in Fig. 3, Wortmannin and LY294002 inhibited both eNOS activation and Akt phosphorylation, as well as eNOS activity. Taken together, these results suggest that PLC phosphorylates and stimulates eNOS via PI3 kinase-mediated Akt activation.

3.4. PLC stimulates eNOS phosphorylation at Ser1177 via a tyrosine kinase Src

Tyrosine kinases of the Src family are involved in different signal transduction pathways in cells. PI3 kinase is associated with Src proteins in transformed fibroblasts or activated B- and T-cells [3,11]. Direct interaction between the p85 subunit of PI3 Kinase and SH3 domains of different Src proteins has been demonstrated in several works [18,26]. To determine the effects of the src kinase family as an activator of PI3-kinase by PLC, we evaluated the effect of PD173955 and PP2, the specific src kinase inhibitors, on PLC-induced eNOS phosphorylation and activity. As shown in Fig. 4A and B, PD173955 and PP2 suppressed PLC-induced increase in eNOS phosphorylation and activity. To further substantiate the role of Src in PLC activation of eNOS, the activity of the src-related kinase was determined in these cells by in vitro assays using antibodies specific for src kinase. As indicated in Fig. 4C, PLC significantly activates Src kinase. These data suggest that PLC stimulates tyrosine kinases that are most likely src family kinases, which in turn activate the PI3-kinase-Akt kinase pathway to ultimately lead to eNOS phosphorylation.

3.5. AMPK activation is responsible for PLC-induced tyrosine kinase Src activation

Mitochondrial beta-oxidation is an important system involved in the energy production of various cells. In this system, the function of L-carnitine is essential for the uptake of fatty acids to mitochondria and the conversion of fat to chemical energy [30]. Here, we detected the intracellular energy status after PLC treatment. To our surprise, PLC significantly decreased intracellular ATP/ADP (Fig. 5A). Because AMPK is highly sensitive to small changes in the intracellular ATP/ADP ratio [16], we explored whether PLC activates AMPK. As shown in

![Fig. 1. PLC regulation of endothelial nitric oxide synthase (eNOS) phosphorylation levels is concentration- and time-dependent. (A) HAECs were treated with 0–100 \(\mu\)M PLC for 6 h. (B) HAECs were incubated with 50 \(\mu\)M PLC for 0–8 h before protein and phosphorylation analyses. The blots are representative of three individual experiments \((n = 3)\). \(p < 0.05\) versus control. (C) HAECs were treated with 50 \(\mu\)M PLC for 6 h, and iNOS protein expression was detected with western analysis (upper panel). eNOS activity was assessed by measuring [3H]-l-arginine to [3H]-l-citrulline conversion over 30 min in cells exposed to vehicle alone or PLC (50 \(\mu\)M) for 6 h in the presence or absence of L-NIL, a specific iNOS inhibitor. Data are presented as mean ± SD. \(p < 0.05\) versus control.](Image)
Fig. 5B, PLC significantly increased AMPK phosphorylation at Thr172, which is essential for AMPK activity [14]. Increased AMPK phosphorylation was associated with elevated AMPK activity, as measured by the SAMS peptide assay (Fig. 5C). PLC treatment did not alter total levels of AMPK, suggesting that PLC-induced phosphorylation of AMPK was not due to altered expression of these proteins. Accordingly, PLC dramatically activated Src and eNOS, and these effects were blocked by compound C (Fig. 5B, D), a selective inhibitor of AMPK, suggesting that AMPK activation is responsible for PLC-induced Src and subsequent eNOS activation.

Fig. 2. PLC stimulates eNOS phosphorylation via Akt/PKB activation. 1: HAECs were treated with PLC (0–100 μM) for 6 h. Cell lysates were analyzed by immunoblot using polyclonal anti-phospho-serine 473 Akt/PKB antibody (p-Akt-S473) or polyclonal Akt/PKB (Akt) antibody. 2: HAECs were treated with PLC (50 μM) for 0 to 8 h. Cell lysates were analyzed by immunoblot using polyclonal anti-phospho-serine 473 Akt/PKB (p-Akt-S473) antibody or polyclonal Akt/PKB (Akt) antibody. Data are presented as mean ± SD. *p < 0.05 versus control. 3: HAECs were treated with or without PLC (50 μM) for 6 h and Akt kinase activity was assayed as described in Materials and methods. Data are presented as mean ± SD. *p < 0.05 versus control. 4: ECs were transfected with empty vector (EV) or cDNA for Akt dominant-negative mutant (DN-Akt) for 48 h, or pretreated with MK-2206 (100 nM) for 2 h. The transfected or pretreated cells were treated with PLC (50 μM) for 6 h. The cells lysates were analyzed by immunoblot using anti-phospho-serine 1177 eNOS polyclonal antibody (p-eNOS) or eNOS antibody, and anti-phospho-serine 473 Akt/PKB antibody (p-Akt-S473) or Akt/PKB (Akt) antibody. *p < 0.05 versus control; #p < 0.05 versus PLC-treated group.

Fig. 3. PI3 kinase is involved in PLC-induced, Akt-mediated eNOS phosphorylation and activation. 1: HAECs were pretreated with vehicle alone, or the PI3 kinase inhibitor wortmannin (50 μM) or LY294002 (50 μM) for 30 min. The cells were then incubated with PLC (50 μM) for 6 h and cell lysates were analyzed for p-eNOS, total eNOS, p-Akt and total Akt. *p < 0.05 versus control; *p < 0.05 versus PLC-treated group. 2: After pretreatment with vehicle alone, wortmannin or LY294002, the cells were then incubated with PLC (50 μM) for 6 h and eNOS activity was assessed as described in Materials and methods. *p < 0.05 versus control; *p < 0.05 versus PLC-treated group.
3.6. PLC increases NO liberation through Src/PI3 K/Akt/eNOS pathway in ECs

eNOS generates the vasoprotective molecule nitric oxide (NO•), which is a very well known biological mediator involved in vascular physiology. In the present study, we demonstrated that the effects of PLC to augment eNOS activity were associated with a net increase in NO release from endothelial cells, reaching a four-fold rise following incubation with PLC for 6 h (p < 0.05) (Fig. 6A). NO production following 6 h incubation with PLC was abolished in endothelial cells coincubated with L-NAME, PD173955, LY294002 and MK-2206, suggesting that PLC increases NO liberation through Src/PI3 K/Akt/eNOS pathway (Fig. 6B).

4. Discussion

PLC is formed via carnitine acetyltransferase from propionyl-CoA, a product of methionine, threonine, valine, and isoleucine, as well as of odd-chain fatty acids [9]. A multicenter and international study on the effects of PLC on exercise duration [1] demonstrates clearly that patients with some degree of deconditioning and relatively preserved myocardial function are likely to benefit from PLC treatment. Moreover, several studies suggest that PLC has beneficial effects of peripheral artery disease [9]. In the present study, we have demonstrated for the first time that PLC can increase eNOS activity through Akt kinase pathway. By activating Src kinase, PLC causes eNOS phosphorylation at Ser-1177 via PI3 kinase-mediated activation of Akt kinase.

eNOS plays a critical role in maintaining vascular homeostasis by exerting a plethora of anti-inflammatory and anti-thrombotic effects while actively promoting endothelial repair, regeneration, and postnatal neovascularization [19]. The phosphorylation of eNOS regulates the activation of the enzyme by various stimuli including VEGF, estrogen and shear stress [7,12,13,20]. Here, we hypothesized that PLC may directly upregulate eNOS expression or activity and, via this mechanism, may serve to regulate endothelial cell function. We make the novel observation that PLC causes the phosphorylation of eNOS at Ser-1177 without changing eNOS protein levels in human aortic endothelial cells. In contrast to Ser-1179, PLC had no effect on the phosphorylation state of Thr-495, suggesting that PLC does not regulate eNOS activation through the dephosphorylation of that residue. Because Akt is critical for phosphorylation of Ser-1177 within eNOS [23], we investigated the effects of PLC in this regard. Our studies clearly indicate that PLC activates protein kinase Akt, leading to posttranscriptional activation of eNOS via phosphorylation of the amino acid Ser1177. Moreover, several studies suggest that PLC has beneficial effects of peripheral artery disease [9]. In the present study, we have demonstrated the role of the PI3-kinase pathway, which is known to play an important role in the regulation of eNOS synthesis by Akt. Inhibition of PLC kinetic pathway by wortmannin or LY294002 inhibited eNOS expression or activity and, via this mechanism, may serve to regulate endothelial cell function. We make the novel observation that PLC causes the phosphorylation of eNOS at Ser-1177 without changing eNOS protein levels in human aortic endothelial cells. In contrast to Ser-1179, PLC had no effect on the phosphorylation state of Thr-495, suggesting that PLC does not regulate eNOS activation through the dephosphorylation of that residue. Because Akt is critical for phosphorylation of Ser-1177 within eNOS [23], we investigated the effects of PLC in this regard. Our studies clearly indicate that PLC activates protein kinase Akt, leading to posttranscriptional activation of eNOS via phosphorylation of the amino acid Ser1177. Next, we probed the role of the PI3 kinase pathway, which is known to play an important role in the regulation of eNOS synthesis by Akt. Inhibition of PI3 kinase by wortmannin or LY294002 inhibited PLC-induced phosphorylation of Akt and eNOS, demonstrating the specific effect of PLC on the activation of the PI3 kinase/Akt pathway. These cumulative results indicate that PI3 kinase stimulation of Akt leading to eNOS phosphorylation at Ser-1177 is critically involved in the activation of the enzyme by PLC (Fig. 5). We have also demonstrated additional proximal signaling events. We demonstrate that a src family tyrosine kinase is a further upstream stimulator of the PI3 kinase/Akt pathway by PLC.

Next, we sought to explore the link between PLC and src. Mitochondrial β-oxidation is an important system involved in the energy production of various cells. In this system, the function of l-carnitine is
induces the activation of Akt, which is an upstream kinase of eNOS and phosphorylates Ser1177 of eNOS, leading to NO production.

PLC-treated group. 2: Proposed signaling pathway mediating PLC-induced eNOS activation and NO liberation. PLC activates Src kinase that causes PI3 kinase activation. PI3 kinase

AMPK (Fig. 5B, C), subsequently leading to Src activation. It is well

known that AMPK acts as a metabolic master switch regulating several intracellular systems including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of mitochondria [8,24,29]. Thus, it is very likely that the improvement of fatty acid β-oxidation by PLC is at least partially due to its ability to activate AMPK. Together, our results suggest that AMPK mediates PLC-induced Src activation.

5. Conclusions

Taken together, the present study demonstrates, for the first time, a novel effect of PLC to modulate eNOS phosphorylation and activity in human endothelial cells, leading to increase in NO bioavailability. This effect may occur via an upstream AMPK and src-mediated signaling cascade that leads to activation of PI3 kinase and Akt kinases. We suggest that approaches aimed at augmenting PLC bioavailability may prevent and limit endothelial dysfunction and cardiovascular diseases.

References


