Propionyl-L-carnitine Improves Postischemic Blood Flow Recovery and Arteriogenetic Revascularization and Reduces Endothelial NADPH-Oxidase 4–Mediated Superoxide Production

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Objective—The beneficial effect of the natural compound propionyl-L-carnitine (PLC) on intermittent claudication in patients with peripheral arterial disease is attributed to its anaplerotic function in ischemic tissues, but inadequate information is available concerning action on the vasculature.

Methods and Results—We investigated the effects of PLC in rabbit hind limb collateral vessels after femoral artery excision, mouse dorsal air pouch, chicken chorioallantoic membrane, and vascular cells by angiographic, Doppler flow, and histomorphometrical and biomolecular analyses. PLC injection accelerated hind limb blood flow recovery after 4 days ($P<0.05$) and increased angiographic quadriceps collateral vascularization after 7 days ($P<0.001$) Histomorphometry confirmed the increased vascular area ($P<0.05$), with unchanged intramuscular capillary density. PLC-induced dilative adaptation, and growth was found associated with increased inducible nitric oxide synthase and reduced arterial vascular endothelial growth factor and intracellular adhesion molecule-1 expression. PLC also increased vascularization in air pouch and chorioallantoic membrane ($P<0.05$), particularly in large vessels. PLC increased endothelial and human umbilical vascular cell proliferation and rapidly reduced inducible nitric oxide synthase and NADPH-oxidase 4–mediated reactive oxygen species production in human umbilical vascular endothelial cells; NADPH-oxidase 4 also regulated NF-κB–independent intracellular adhesion molecule-1 expression.

Conclusion—Our results provided strong evidence that PLC improves postischemic flow recovery and revascularization and reduces endothelial NADPH-oxidase–related superoxide production. We recommend that PLC should be included among therapeutic interventions that target endothelial function. (Arterioscler Thromb Vasc Biol. 2010;30:426-435.)

Key Words: arteriogenesis ■ endothelial function ■ oxidative stress ■ vascular function

Peripheral arterial disease (PAD) is the most common clinical consequence of atherosclerosis, affecting $\approx20\%$ of adults older than age 55 years. $^{1}$ Atherosclerotic occlusion of leg arteries induces clinical manifestations, most frequently intermittent claudication. $^{1,2}$ Among the functional vascular changes observed with atherosclerosis, endothelial dysfunction plays a major role. $^{3,4}$ L-carnitine is a natural amino acid that plays a crucial role in the shuttle mechanism of long-chain fatty acids and in fueling β oxidation. $^{5}$ The endogenous L-carnitine pool includes a series of short-chain, medium-chain, and long-chain esters in homeostatic equilibrium. $^{5,6}$ Propionyl-L-carnitine (PLC) is a short-chain L-carnitine ester that has been introduced among emerging noninterventional medical regimens that aim to counteract PAD-related adverse effects. $^{1,2,7}$ PLC activity is classically related to the anaplerotic function of providing substrates for energy expenditure in ischemic tissues. $^{1,8}$ Although some data showed beneficial remodeling after injury, the mechanisms through which PLC influences arterial function remain largely hypothetical. Recently, oxidative stress was implicated in PLC-induced flow-mediated dilatation in PAD patients. $^{10}$ Arterial occlusion leads to 2 distinct adaptive mechanisms, namely angiogenesis via capillary sprouting $^{11}$ and arteriogenesis via dilatation and growth of preexisting collateral vessels. $^{12,13}$ Arteriogenesis provides much more blood flow than angiogenesis and represents a crucial step in vascular function maintenance and tissue reperfusion after vascular occlusion. $^{12,13}$ The latter induces an immediate shear-stress increase in collateral vessels that undergo an early adaptive dilatation, which is subsequently followed by growth through proliferation of arterial cells and extracellular matrix accumulation. $^{12,13}$ To better understand the
vascular machinery, we investigated PLC effects in various models and in vascular cell cultures, with specific reference to the modulation of oxidative stress-induced endothelial dysfunction.

**Materials and Methods**

A detailed methods description is available in the Supplemental Materials (available online at http://atvb.ahajournals.org).

**Rabbit Hind Limb Ischemia**

All animal procedures conformed to the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (1996). Unilateral hind limb ischemia was performed by left femoral artery excision in New Zealand rabbits (Charles River-Italy Spa). After 3 days, PLC (Sigma-Tau SpA; Pomezia) was injected in 1 mL saline into 3 points of the quadriceps muscle. Contralateral vehicle injection was the nonsurgical control.

**Arteriogenesis and Tissue Perfusion Assessment**

A blinded morphometric analysis of the hind limb collateral vessels was performed by angiography after a contrast medium injection through the internal iliac artery. Before the angiography, the laser Doppler flowmetry measurement was reported as ischemic-to-normal limb blood flow ratio.13

**Morphometric Determination of Rabbit Hind Limb Vascular Area and Capillary Density**

Serial quadriceps muscle sections were stained with hematoxylin and eosin and Verhoeef–Van Gieson. Vascular and vessel areas were calculated according to morphometric criteria.9 Intramuscular capillary density per mm² and intramuscular capillary density normalized to muscle fiber diameter were measured on biotinylated isocitrate-stained sections.

**Air Pouch Assay**

The vascular area and the number of vessels were calculated after 7 days in the mouse dorsal cervix sac by subcutaneously injecting 3 mL air. Then, an intrapouch administration of 500 μL of saline alone or with 25 or 50 μg/mouse PLC was performed.

**Chicken Chorioallantoic Membrane Vasculogenesis Assay**

We applied 2 μL of a 390 mmol/L PLC solution, 1-carnitine, or sodium propionate (Sigma-Tau) to a chicken chorioallantoic membrane surface using a gelatin sponge. Fibroblast growth factor-2 (50 ng/embryo) and propionate (Sigma-Tau) to a chicken chorioallantoic membrane surface through the internal iliac artery. Before the angiography, the laser Doppler flowmetry measurement was reported as ischemic-to-normal limb blood flow ratio.13

**Immunohistochemistry**

After antigen retrieval, immunohistochemistry was performed by incubating sections with anti-vascular endothelial growth factor, anti-inducible nitric oxide synthase (iNOS), anti-NF-κBp65 (Santa Cruz Biotechnology), anti-NOS (BD Biosciences), anti-vascular cell adhesion molecule-1, anti-intracellular adhesion molecule-1 (Novus Biologicals), anti-α-actin, anti-ICAM-1, and anti-Ki67 (DakoCyto- mation) using positive and negative controls. The percentages of positive vessels and staining intensity were calculated according to morphometric criteria.

**Cell Culture**

Second passage human umbilical vein endothelial cells (HUVEC; Cambrex) were starved for 24 hours and then incubated with 2% fetal bovine serum or 0.1% fetal bovine serum and PLC dissolved in phosphate-buffered saline or phorbole 12-myristate 13-acetate (10 nmol/L). Smooth muscle cells (SMC) were obtained from the inner portion of rabbit femoral artery.9 After starvation, SMC were cultured in the presence of 10% or 0.1% fetal bovine serum and PLC at various concentrations. [3H]thymidine incorporation, apoptosis, and cell viability were also determined.15 Results were expressed as the mean of 3 different experiments.

**Adhesion Assay**

Confluent HUVEC were treated with PLC at a 1 mmol/L concentration for 24 hours. Monocytes were separated by Ficoll-Paque and incubated with HUVEC for 1 hour at 37°C. Nonadherent cells were removed during 3 gentle washing steps. Adhering cells were fixed in 2% paraformaldehyde and counted in triplicate.

**Protein Extraction and Western Blot Analysis**

The total protein extracts from multiple quadriceps muscle biopsies or from cultured cells was quantified by Bradford assay. Aliquots were separated by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis, bloated to nitrocellulose transfer membranes, incubated with specific antibodies, and quantified by densitometric analysis in 3 independent experiments.17

**Rac1 Activity Assay**

Rac1 activity was determined by a commercially available kit (Upstate Biotechnology) according to manufacture’s guidelines.

**Reverse-Transcriptase and Real-Time Polymerase Chain Reaction**

RNA was extracted from cells, quadriceps muscle biopsies, and chorioallantoic membranes;11 semiquantitative reverse-transcriptase polymerase chain reaction was performed in triplicate. Primer sequences are available in the Supplemental Materials. Results were normalized on the hypoxanthine guanine phospho-ribosyltransferase or glyceraldehyde-3-phosphate dehydrogenase expression. NADPH oxidase subunits, Rac-1, and intracellular adhesion molecule-1 (ICAM-1) expression was analyzed by real-time polymerase chain reaction and normalized on glyceraldehyde-3-phosphate dehydrogenase level. The copy number of nox isoforms mRNA was also calculated.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts from 2×10⁶ cells were prepared for the electrophoretic mobility shift assay.17 Protein concentrations were determined and electrophoretic mobility shift assay and supershift assays were performed using an NF-κB–specific oligonucleotide (Invitrogen).

**Detection of Reactive Oxygen Species**

The intracellular reactive oxygen species (ROS) level in HUVEC was measured by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester fluorescence method (Molecular Probes). Fluorescence was monitored by analyzing at least 10 000 cells in a flow cytometer.

**Interfering RNA for NADPH Oxidases**

Small interfering RNA (siRNA) for human Nox2 (access NM 000397) and Nox4 (access NM 016931) were designed by using Block-IT RNAi Designer (Invitrogen). The siRNA and scramble sequences are available in the Supplemental Materials. Nox isoforms depletion by siRNA was confirmed by reverse-transcription polymerase chain reaction and blot.

**Statistical Analysis**

Data were expressed as the mean±SEM, and differences were analyzed by a Student t test and 1-way ANOVA or ANCOVA followed by Dennett test. Blinded measurements were performed by 2 independent researchers; the interobserver reproducibility was >95%. Differences were considered statistically significant at a value of P<0.05.
Results

PLC Accelerates Revascularization and Ameliorates Postischemic Blood Flow Recovery in Rabbit Hind Limb

Seven days after PLC injection, iliac arteriography (Figure 1A, B) documented a beneficial effect compared to controls ($P<0.001$) that was still present, although smaller, after 2 weeks. The PLC effect was dose-dependent up to 10 mg and did not further increase at 20 mg (Figure 1C), with an EC$_{50}$ value of 5.86 mg/kg. Three days after femoral artery resection (basal value; Figure 1D), blood flow reduction was $\approx$70%. PLC induced faster flow recovery compared to saline ischemic controls ($P<0.001$; Supplemental Table I, available online at http://atvb.ahajournals.org).

PLC Increases Hind Limb Vascular Area but Not Intramuscular Capillary Angiogenesis

To better-analyze the effects of PLC, we investigated rabbit quadriceps collateral vessels at a 10-mg dose by histomorphometrical analysis. Seven days after PLC injection (Figure 2A), a consistent increase in vascular area compared to saline control ($P<0.001$) was observed, with a 6-fold increase of the lumen area. Intramuscular capillary density and capillary-to-muscle fiber ratio did not vary (Figure 2B, C).

PLC Improves Arteriogenesis of Rabbit Quadriceps Collateral Vessels

We also investigated arteriogenesis$^{12}$ 7 days after PLC injection. Endothelial cell swelling and Ki-67–positive cells were detected in collateral vessels (Figure 2D), as reported.$^{12}$ Contralateral nonischemic vessels were almost Ki-67–negative (not shown). An eccentric intimal thickening from variable rounded SMC accumulation was detected in ischemic collateral vessels. The percentage of vessels showing subendothelial focal intimal SMC accumulation was reduced in PLC compared to saline ischemic controls ($P<0.01$; Figure 2E). Also, Ki-67–positive endothelial cells were increased in PLC-treated rabbits compared to saline ischemic controls ($P<0.05$; Figure 2F). The opposite was observed for intimal SMC ($P<0.05$). Immunostaining also revealed that PLC-induced arteriogenetic remodeling occurs with no significant change in $\alpha$-actin–positive collateral vessel number (not shown).

PLC Increases Vascularization in Mouse Air Pouch and Chicken Chorioallantoic Membrane

We investigated PLC effects in vasculogenetic models without increase of shear stress. In mouse air pouch (Supplemental Figure I, available online at http://atvb.ahajournals.org), PLC induced an increase of the vascular area compared to vehicle ($P<0.05$). In particular, PLC increased large-vessel recovery. Repeated PLC administration did not amplify the response (not shown). Representative pictures of chorioallantoic membrane (Figure 3A, B) and quantification (Figure 3C) 3 days after PLC treatment (20 $\mu$g/embryo) documented an increase of large vessels compared to control ($P<0.05$), comparable to that obtained with 50 ng/embryo fibroblast growth factor-2. Equimolar concentrations of L-carnitine and propionate were ineffective, indicating that PLC effects were not attributable to these moieties. In addition, evaluation of $\alpha$-actin immunostaining of chorioallantoic membrane (Figure 3D, E) revealed a marked increase of positive vessels in PLC compared to vehicle (Figure 428 Arterioscler Thromb Vasc Biol March 2010

Figure 1. Effects of PLC on postischemic rabbit hind limb revascularization and blood flow. A, Angiographic images show the effects of PLC injection (10 mg/rabbit) on postischemic hind limb revascularization after 7 and 14 days. B, Angiographic score as mean values $\pm$ SEM; t test, $^*P<0.001$ and $^{**}P<0.05$ vs saline. C, Dose-dependent effect of PLC on angiographic score 7 days after injection. One-way ANOVA plus Dennett test of 6 to 9 animals. $^*P<0.001$ and $^{**}P<0.05$ vs saline. D, Laser Doppler flowmetry of dose-dependent effect of PLC 7 days after injection compared to basal and immediately after surgery (day 0) values. One-way ANOVA plus Dennett test, $^{**}P<0.05$ vs saline.
Finally, densitometric evaluation of reverse-transcription polymerase chain reaction (Figure 3G, H) demonstrated that PLC-induced vasculogenetic process was accompanied by a decrease of Nox4 and Nox2 transcripts compared to vehicle ($P<0.01$ and $P<0.001$, respectively).

**PLC Reduces Vascular Endothelial Growth Factor and ICAM-1 and Increases iNOS Expression in Collateral Vessels**

Immunohistochemical investigation (Figure 4) showed that nonoperated hind limb collateral vessels were practically negative for vascular endothelial growth factor, iNOS, ICAM-1, and NF-$\kappa$Bp65 subunit; endothelial nitric oxide synthase immunostaining was faint. Seven days after injection, vascular endothelial growth factor-positive vessels and immunoreactivity were increased in treated groups compared to nonoperated hind limb but were less in PLC compared to controls ($P<0.05$ and $P<0.01$; Figure 4B,C). The same was observed for ICAM-1 ($P<0.01$); however, iNOS increased ($P<0.02$). Although increased compared to nonoperated contralateral limbs, endothelial nitric oxide synthase, p65, and vascular cell adhesion molecule-1 expression did not vary in PLC compared to control groups. RAM11$^+$ macrophages around hind limb collateral vessels were almost absent in nonoperated rabbits. Seven days after injection, their number was slightly reduced in PLC (36.1±4.9/mm$^2$) compared to controls (45.2±6.6/mm$^2$; $P<0.05$). Reverse-transcription polymerase chain reaction showed a PLC-induced reduction of Nox4 and ICAM-1 transcripts in biopic tissue 4 and 7 days after treatment (not shown).

**PLC Exerts Opposite Effects on HUVEC and SMC Proliferation In Vitro**

After 12 hours, PLC did not modify [$^{3}$H]thymidine-incorporated HUVEC and vascular SMC cultures (Supplemental Figure II). At 24 hours, [$^{3}$H]thymidine incorporation was increased in HUVEC by PLC at 1 mmol/L ($P<0.01$) and was reduced at 2 mmol/L concentration ($P<0.05$) in SMC. After 48 hours, PLC also increased the HUVEC number ($P<0.01$) compared to untreated controls, with an IC50 of 1.43 mmol/L at 72 hours. The opposite was true for SMC (IC50 at 72 hours, 2.05 mmol/L), with a slight reduction of cell viability at 1- and 2-mmol/L doses (91.0±1.2 and 89±2.0), partly because of increased apoptosis (4.3%±0.8% and 5.1%±1.0%, respectively) when compared to controls (1.1%±0.4%, $P<0.05$), according to previously reported data.$^{17}$
PLC Increases iNOS Early and Downregulates Nox4 and ICAM-1 Levels in HUVEC

To characterize endothelial downstream pathways activated by PLC treatment, we investigated HUVEC cultures at different times. As reported in Supplemental Figure II, after 4 and 8 hours, only iNOS transcripts increased ($P<0.01$ and $P<0.05$, respectively). After 12 hours, Nox4 and ICAM-1 transcripts decreased ($P<0.01$), whereas endothelial nitric oxide synthase and vascular cell adhesion molecule-1 were unchanged when compared to controls. ICAM-1 downregulation was not preceded by NF-$\kappa$B subunits and IkB modulation. Similarly, SN50-induced NF-$\kappa$B translocation inhibition in HUVEC did not abolish PLC-induced ICAM-1 reduction (Figure 5A).

PLC Reduces ROS Accumulation and Downregulates Nox4, Nox2, and ICAM-1 Expression

In HUVEC, reverse-transcription polymerase chain reaction showed that Nox4 was more abundant than Nox2 transcripts, whereas Nox1 was barely expressed and Nox3 and Nox5 were absent (Figure 5B), confirming previously reported data. Quantitative real-time polymerase chain reaction specified that the RNA copy number/g of Nox4 (15 540 ± 1889) is much more than those of Nox2 (4.1 ± 1.8), whereas remaining Nox isoforms were not quantifiable. As shown in Figure 5C, serum deprivation induced ROS accumulation in HUVEC ($P<0.05$). After 24 hours of treatment, PLC strongly reduced ROS level ($P<0.05$). Real-time polymerase chain reaction also documented that serum deprivation strongly increased Nox4 and Nox2 transcript level in PLC-treated chorioallantoic membranes; original magnification, ×200. F. Percentage of $\alpha$-actin–positive vessels. G and H, Densitometric evaluation of reverse-transcription polymerase chain reaction after normalization on glyceraldehyde-3-phosphate dehydrogenase shows the reduction of Nox4 and Nox2 transcript level in PLC-treated chorioallantoic membranes; ADU indicates arbitrary densitometric units. Data are expressed as mean of 3 independent experiments; $t$ test, *$P<0.01$ and **$P<0.05$ vs vehicle.
Figure 4. PLC-induced phenotypic vascular changes during rabbit hind limb vascularization. A, Vascular endothelial growth factor, iNOS, NF-κBp65, and ICAM-1 immunoreactivity in post-ischemic rabbit collateral vessels 7 days after PLC (10 mg/rabbit), saline (control) injection, and in contralateral (nonoperated) vessels. Diaminobenzidine as chromogen. Original magnification, ×200; fa indicates femoral artery. Percentage of positive vessels (B) and intensity of immunoreaction (C). Values are mean±SEM of 8 animals/group; t test, *P<0.05, **P<0.02, and ***P<0.01 vs control.

An adhesion assay was performed to check the effects of PLC on the interaction between monocytes and HUVEC in vitro. In control HUVEC, the number of adhering monocytes was extremely low. Serum deprivation induced an increase of monocyte adhesion that was partially inhibited by PLC (71.9%±2.7% of control).

Discussion

The first major finding of this study is that PLC ameliorated postischemic rabbit hind limb vascular perfusion and revascularization by accelerating flow recovery and vasodilative arteriogenetic remodeling in collateral vessels. PLC also increased vascularization in mouse air pouch and chorioallantoic membrane. PLC effects were dose-dependent and, when investigated, not observed using L-carnitine and propionate moieties. Adaptive dilatation and growth of preexisting collateral vessels play the most important role in tissue-saving and organ-saving processes after femoral artery occlusion or dissection.13 The therapeutic ineffectiveness of capillary angiogenesis19 leads researchers to further investigate arteriogenesis to discover new approaches to counteract adverse effects of vascular occlusion.20 Arteriogenesis is a complex phenomenon with different phases.21 Increased shear stress initiates arterial growth and induces marked endothelial activation of collateral vessels, with rapid adjustment of proximal preexisting tone.22 Successively, structural remodeling by active parietal growth occurs.12,22 We documented an early greater blood flow recovery in PLC-treated samples compared to controls, in line with a positive effect on the early vasodilative adaptation phase.23 In fact, blood flow recovery by PLC was significant after 4 days and preceded the vascular area increase on the seventh day. PLC-induced dilative adaptation confirms previous ex vivo experiments using subcutaneous arteries.24 Moreover, PLC effects were found to be associated with increased endothelial cell proliferation but not SMC growth. These findings strongly suggest that endothelium is target for the pharmacological action of PLC.

Healthy endothelium activation by various noxae upregulates vasoactive, inflammatory, and mitogenic factors.21 Endothelial ROS accumulation plays a central role in vascular diseases, including atherosclerosis and restenosis.3 Nox is the primary source of superoxide anions in the arterial wall.21 In addition to structural (gp91phox, p22phox) and regulatory components (p40phox, p47phox, p67phox), endothelial cells express Nox4 as a main active subunit.18 Increased hemodynamic forces activate endothelial Nox.25 We documented that PLC reduced ROS production, and this effect was mediated by downregulation of Nox4 activity in HUVEC. Endothelial machinery counteracting physiopathological changes by oxidative stress appropriately explains PLC-induced effects in patients with PAD.26 Similar to other endothelial stress conditions, ROS generation triggers endothelial synthesis or release of inflammatory mediators and modulates downstream signaling pathways, including selectin ICAM-1.27 In patients with PAD, elevated inflammatory biomarkers were found to be associated with impaired vascular function.28 Postischemic ICAM-1 expression in PLC rabbit collateral depleted transcript level (P<0.01), which was reduced by PLC after 4 hours (P<0.05; Figure 5H). Remaining Nox isoforms were not modulated by PLC (data not shown).

PLC-Induced Reduction of ROS and ICAM-1 Is Nox4-Dependent

To check the contribution of Nox isoforms to PLC-mediated ROS reduction in HUVEC, we used downregulation by siRNA. Nox4 siRNA inhibited serum deprivation-induced ROS and ICAM-1 increase and abolished PLC effects (Figure 6A, B). Instead, Nox2 downregulation by siRNA did not significantly modulate ROS production or PLC effects (Figure 6C), confirming that the PLC antioxidative effect is mainly Nox4-mediated. Moreover, Nox4 siRNA did not influence the Nox2 level; siNox2 did not influence Nox4 (Figure 6D) and ICAM-1 expression (not shown). Rac1 activity was not detected in HUVEC after serum deprivation and PLC treatment (Figure 6E). Finally, PLC-induced NF-κB–related gene reduction in serum-deprived HUVEC occurred only after 12 hours (Figure 6G–I).
vessels was less than in controls, but it was still increased compared to sham-operated rabbits. This apparently contradicts ICAM-1–driven arteriogenesis, because this selectin mediates leukocyte attachment or transmigration and favors the growth of collateral vessels. Nevertheless, other substances, such as leptin, have shown the capacity to stimulate arteriogenic remodeling in the absence of a significant inflammatory macrophage-driven arterial growth. The early beneficial effect of PLC on blood flow recovery, despite the relatively lower Nox4-mediated increase of ICAM-1, are in line with a mechanotransduction mechanism leading to the direct activation of protective biophysical, biochemical, and gene regulatory responses in endothelial cells. Excessive oxidative stress by ROS generation impairs endothelium-derived nitric oxide bioactivity. Nitric oxide is a potent vasodilator released by endothelial cells, and dilatation of preexisting vessels plays a major role in early adaptation favoring flow recovery. In patients with PAD, nitric oxide bioavailability and flow-mediated dilatation appear re-
The increased iNOS activity we documented with PLC likely increases vascular nitric oxide availability in the postischemic acute phase, counteracting inactivation by ROS, with the latter also reduced. Although referred as detrimental in atherosclerosis, iNOS expression may be protective, as reported for allograft arteriosclerosis. Altogether, our data provide further evidence of an alternative pathway for the early increase of postischemic collateral flow and explain the clinical advantage with PLC in PAD patients.

At present, medical therapeutic opportunities of PAD still present more advantages and better safety compared to recently described methods, including therapeutic angiogenesis by stem cells. Although trials documented that PLC improves walking capacity in severe PAD, its use is still unapproved in the United States. Hiatt associated the pharmacological activity of PLC with its property of providing further substrates for energy expenditure in ischemic tissues, particularly by improving muscle performance through a positive inotropic effect on mitochondria. Nevertheless, PLC reduces cell stress occurring during passage through the capillaries and exerts vasculoprotective effects. In line with the different regulatory pathway of selectins we documented.

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**Figure 6.** PLC effect on ROS accumulation is Nox4-mediated. A, DCFH fluorescence intensity shows the effects of siNox4 on ROS accumulation in HUVEC. Data are means±SEM of 3 independent experiments; t-test, *P<0.05 comparing 0.1% fetal bovine serum plus scramble and 2% fetal bovine serum and comparing siNox4 vs 0.1% fetal bovine serum plus scramble. B, Reverse-transcription polymerase chain reaction shows that Nox4 downregulation by siRNA prevents PLC-induced ICAM-1 reduction after 8 hours; t test, *P<0.05. C, Effects of siNox2 on ROS accumulation; t test, *P<0.05 and **P<0.01, respectively. D, The siNox4 does not influence Nox2 protein expression and vice versa. E, Real-time polymerase chain reaction of Rac1 mRNA level; t test, *P<0.05. F, Rac-1 activity assay of PLC-treated cells. G to I, Downregulation of NF-κB-related genes after 12 hours in HUVEC by reverse-transcription polymerase chain reaction; t test, *P<0.05, **P<0.01 vs 0.1% fetal bovine serum.
Conclusion

Although well-detailed, the present study has some limitations. The major limitation is that 3-month-old rabbit hind limb acute ischemia is not fully representative of abnormalities observed in patients with PAD, in whom atherosclerotic occlusion of leg arteries is chronically established.1 These patients generally display advanced age and risk factors that cause atherosclerotic progression and adverse clinical outcomes.2 Altered lipid metabolism and aging influence endothelial proliferation and motility;3 moreover, arteriogenetic adaptation is impaired in hypercholesterolemic mice.4 Nevertheless, our results align well with the reduction of impaired endothelial function observed in PAD patients receiving PLC.5 Data concerning pharmacokinetics of PLC suggest a rapid plasmatic clearance but a slow but progressive accumulation of radioactivity in heart and striated muscles in normocholesterolemic mice.44 Nevertheless, our results align well with the reduction of impaired endothelial function observed in PAD patients receiving PLC.10 Data concerning pharmacokinetics of PLC suggest a rapid plasmatic clearance but a slow but progressive accumulation of radioactivity in heart and striated muscle, with a progressive increase of tissue-to-plasma ratio, consistent with the therapeutic efficacy of PLC.45 Of course, the effects of a single-dose intramuscular injection cannot be representative of chronic therapy; nevertheless, our findings helps clarify the beneficial effects of PLC.7 The lowering of atherosclerotic risk factors remains a goal of medical PAD treatment.2 PLC administration reduces aortic and coronary atherosclerosis and plasma triglycerides in hyperlipemic aged rabbits.46 Our data cannot exclude that improvement of postischemic hind limb revascularization derives also from PLC-induced changes in shear stress; nevertheless, the similarity of findings in the different vasculogetic models shows support against this possibility. Finally, further studies are needed to investigate PLC effects in regions on femoral artery occlusion, where profound ischemia prevails and capillary proliferation and tissue repair occur.47

In conclusion, our results indicate that PLC accelerates blood flow recovery and the restoration of vascular function by sustaining a positive dilative arteriogenetic remodeling and by reducing oxidative stress-induced impaired endothelial function. The latter involves mainly Nox4 activity and downstream pathways, including ICAM-1 and iNOS expression. The present findings better-address PLC as a therapeutic strategy aimed at counteracting manifestations of PAD and suggest additional pharmacological studies targeting impaired endothelial function occurring during cardiovascular diseases.

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Disclosure

None.

References


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**Figure I**

### Table: Number of vessels according to the size

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<th>Treatment mg PLC/mouse</th>
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<th>Air pouch vascular area (mm²)</th>
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<td>6.7 ± 0.3*</td>
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<td>50</td>
<td>14</td>
<td>7.3 ± 0.4*</td>
<td>8.4 ± 0.9*</td>
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Figure II
Supplemental Materials

METHODS

Animals. All experimental procedures conformed to the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals.* Rats were housed for at least seven days before start of experiment (period of acclimatization), in Makrolon cages with stainless steel feed racks and sterilized, dust-free bedding cobs. The animal rooms had the following environmental conditions: temperature 22±2°C, relative humidity 55± 10%, about 15-20 filtered air exchanges/hour and 12-hour circadian cycle of artificial light (7 a.m.-7 p.m.). Diet (in pellets) coded GLP 4RF 21 was produced by Mucedola S.r.l. of Settimo Milanese (Milan, Italy) for Charles River–Italy Spa. The animals were randomized using a statistical program (Sigma Stat, SPSS, Chicago, IL; USA). Male New Zealand rabbits weighing 2.5-3.0 kg were housed for at least 7 days before the experiment as period of acclimatization, inside stainless steel cages. The animal room monitored environment conditions were: 18±3°C temperature, 55±10% relative humidity, about 15-20 filtered air exchanges/hour and a 12-hour circadian cycle of artificial light (7 a.m.–7 p.m.). Animals were fed and watered ad libitum, drinking water given through bottles.

Rabbit hindlimb ischemia. Rabbits were anesthetized with ketamine (25 mg/kg i.m.) (Ketavet 100, Demos Siena Italy) and medetomidine (0.5 mg/kg i.m.; Domitor, Orion Pharma, Espoo, Finland). A longitudinal incision was performed in the thigh of left hindlimb, extending distally from the inguinal ligament to a point just above the knee. The femoral artery was dissected free along its entire length, as well as its major branches including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries. After further dissection of the popliteal and saphenous arteries distally, the external iliac artery, as
well as all the above arteries, were ligated with 5.0 silk. Then the femoral artery was completely excised from its proximal origin as a branch of the external iliac artery to the point distally, where it bifurcates into the saphenous and popliteal arteries. After surgery, animals received subcutaneous injection of baytril (Bayer, Shawnee Mission, KS) for 5 days. Three days after femoral artery resection, rabbits were anesthetized again with ketamine/medetomidine. An incision was made on the skin of operated hindlimb and substances were injected with 1-ml syringe in three separate points along the adductor muscle. Animals were sacrificed after 4, 7 or 14 days from femoral artery resection. A first preliminary experience PLC dose-response (from 2.5-to 20 mg/rabbit) experiment at seven days from injection was performed to assess the more appropriate dose of to use in the following experiments. Higher doses than 20 mg did not significantly increase efficacy or prolonged effects. Thus, the dose of 10 mg/rabbit was chosen to better investigate PLC activity.

**Assessment of arteriogenesis and tissue perfusion.** Angiography of the hindlimb was performed to assess collateral vessels growth in the proximal portion of the upper limb (the original location of the femoral artery) following the induction of ischemia. At the day of sacrifice, under anesthesia by intraperitoneal injection of 1.5 ml zoletil 100 (Virbac, Milan, Italy), the abdominal aorta of the animal was exposed and cannulated. The hindlimb vascular bed was washed with 5 ml of heparinized saline (1000 U/ml) and 2 ml of 2% xylocaine (Astra Pharmaceutics Spa. Milan Italy) as local myorelaxation to reduce muscular movements. Contrast medium (Visipaque,320 mg/ml, Nycomed Amersham Sorin S.r.l., Italy) was injected by means of an infusion pump at a rate of 15 ml/min for a total volume of 6-8 ml. Angiographic images were stored and using Adobe Photoshop 6.0 software, a grid overlay, which comprised 240 squares of 1.5 cm each, was placed over the angiographic image. Grid
was positioned among anatomic reference points (i.e., femoral and iliac artery and knee-cap). The number of contrast-opacified arteries crossing over circles and the total number of lines encompassing the medial thigh area were counted in a blinded fashion.\(^3\) The angiographic score was calculated as the ratio of overlying opacified arteries divided by the total number of lines in the ischemic thigh. This angiographic score reflects vascular density in the medial thigh. Angiographic score was analyzed by one-way Anova followed by Dunnett's test. To provide functional evidence for ischemia-induced changes in vascularization, we measured limb blood flow on plantar zone using a laser Doppler flowmeter (periflux system 5000, Perimed AB, Italy). Briefly, a beam of laser light, carried by a fiber-optic probe, is widely scattered and partly absorbed by the tissue being studied. Light hitting moving blood cells undergoes a change in wavelength (Doppler shift) while light hitting static objects is unchanged. Measurements were expressed as arbitrary perfusion units.\(^2\) Three registrations of blood flow for each animal were taken. The first measurement was registered before artery excision as basal value, the second three days after surgery to control the ischemic limb, and the last at the sacrifice to assess flow recovery. The percentage of flow blood recovery was calculated from basal to final values and analyzed by one-way ANOVA followed by Dennett’s or \(t\)-test.

**Rabbit hindlimb tissue sampling.** After euthanasia by intravenous injection of thiopental (150 mg/kg), vessels were washed by saline and then perfused by 10% neutral buffered formalin through a catheter in the distal abdominal aorta, as previously reported.\(^4\) After hip joints disarticulation, both hindlimbs were removed, and five-mm-thick transverse sections were post-fixed in formalin for 24 h and embedded in paraffin. Serial four \(\mu\)m sections were cut for Haematoxylin&Eosin staining or employed for immunohistochemistry (see after).
**Immunohistochemistry and morphometric determination of vascular area and capillary density.** For immunohistochemistry, after deparaffinization, endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide–methanol at room temperature. Antigen retrieval, except for smooth muscle actin (α-actin), was performed by incubating sections in 10 mM sodium citrate buffer (pH 6.0) at 98 °C for 30 min. Nonspecific antibody binding was blocked by incubation with normal goat serum (Ylem, Avezzano, Italy, 1:20 in BSA 5%) for 30 min at room temperature. Sections were incubated for 1 h at room temperature with mouse monoclonal anti-eNOS (BD Biosciences, Franklin Lakes, NJ USA; 1:100), mouse monoclonal anti-VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:20), mouse monoclonal anti-iNOS (Santa Cruz; 1:100), mouse monoclonal anti-NFKBp65 (Santa Cruz), mouse monoclonal anti-ICAM-1 (Novus Biologicals, Littleton, CO, USA; 1:100), mouse monoclonal anti-VCAM-1 (Novus Biologicals; 1:100), mouse monoclonal α-actin (DakoCytomation, Denmark; 1:200), anti-rabbit RAM11 (1:100) and anti-Ki67 (clone MIB-1, Dako; 1:50 1h 30’) with controls. After washing in phosphate-buffered saline (PBS), sections were incubated with biotinylated goat anti-mouse for 30 min, followed by treatment with peroxidase-conjugate streptavidin. Bound antibody was revealed with the use of the substrate 3,3-diaminobenzidine and counterstaining performed with Haematoxylin. Semiquantitative iNOS, eNOS, VEGF, p65, ICAM-1 and VCAM-1 immunoreactions was estimated at X200 magnification by two the authors. The percentage of positive vessels was determined and an average value for staining intensity, at least 10 positive vessels, was obtained for each sample. The authors used a grading system in arbitrary units as follows: absent (0), low and focal (0.5), positive (weakly positive, 1+; moderately positive, 2+; strongly positive, 3+). The interobserver reproducibility was >95%. The effect of PLC on revascularization was assessed on with Haematoxylin&Eosin and Verhoeff-Van Gieson stained sections of adductor longus, adductor magnus and vastus intermedius muscles.
under light microscopy. Images were acquired with a digital camera (KY-F55B, JVC Italia Spa, Milan, Italy) and analyzed using Scion Image software (Scion Corporation, Maryland, USA). RAM11+/mm² cells were calculated at 200x magnification. Total vessels area (lumen area + wall area), lumen and wall area and the ratio vessel area/vessel wall area of vessels greater of 20 µm diameter were measured for each animals at X200 magnification, using outer elastic lamina of the tunica media as peripheral edge of the arterial wall. Ki-67 positive cells and the mean percentage of collateral vessels showing intimal thickening was calculated at X100 magnification. For the evaluation of capillary density, sections were incubated with anti-biotinylated isolectin (Vector Laboratories, Inc., Burlingame, CA, USA; 1:100) and blinded measurement performed by under light microscopy at X200 magnification in five-ten randomly selected fields for each animal by two researchers, with interobserver reproducibility >95%. To ensure that the capillary density was not overestimated as a consequence of myocytes atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was also determined. Values were given as mean ± standard error of mean (s.e.m.) and were analyzed by means of Student’s t-test. The differences were considered statistically significant for value of $P<0.05$.

**Air pouch assay.** The air pouch model was evaluated in CD-1 mice, weighing 26-30 g. Under 2% halothane anesthesia, an air sac was produced in the dorsal cervix area by subcutaneously injection of 3 ml sterile air, followed, immediately afterwards, by intra-pouch administration of 500 µl of saline or 5% or 10% PLC solution (corresponding to 25 or 50 mg/mouse of PLC). PLC was dissolved in saline and brought to a pH of 5.0 with NaOH 5M. After seven days, the mice were sacrificed and the skin areas of the back in correspondence to the air sac were trimmed off and acquired through a digital camera (JVC, model TK-C1380E) with a 55 mm objective (Nikon), connected to a computer utilizing Image Pro Plus software (Media
The total vascular area and the number and types of vessels were counted and automatically classified, according to their size. Data were expressed as means ± s.e.m. and statistically evaluated by means of one-way ANOVA followed by Dennett’s test.

**Chicken chorioallantoic membrane angiogenesis assay and morphometric analysis.**

Fertilized chicken eggs were placed into an incubator as soon as embryogenesis started and kept under constant humidity at 37°C. On day 4 of incubation a square window was opened into the shells after removing 2-3 ml of albumen to detach the developing chorioallantois from the shell itself. The opening was closed with a glass and sealed with paraffin. On day 9 of incubation, 1 mm³ of sterile gelatin sponge was placed upon the chicken chorioallantoic membrane, immediately followed by topical administration of 2 µl of a 390 mM solution of PLC, L-carnitine and sodium propionate, corresponding to 200, 187 and 74.9 µg/embryo of PLC, L-carnitine and propionate. Fibroblast growth factor-2 (FGF-2) at the dose of 50 ng/embryo and phosphate buffered saline (PBS) were used as positive and negative control, respectively. The quantification was performed by counting the number of vessels converging toward the implant under a stereomicroscope at the time of drug administration (t₀) and 72 hours later (t₇₂h). The difference (Δ) in the number of vessels between t₇₂h and t₀ was then calculated for each sample. Six independent experiments were performed. Differences in response caused by treatments were tested for statistical significance using ANCOVA followed from Dennett’s test. The differences were considered statistically significant for value of \( P<0.05 \). For arteriogenesis assay, CAMs samples were excised and fixed in Bouin’s fixative. Four µm paraffin-embedded sections were cut for Haematoxylin&Eosin staining or employed for immunohistochemistry as reported above. The morphometric analysis was performed on α-actin stained sections in triplicate. Measurement
of α-actin positive vessels, identified from continuous α-actin positive layer, was assessed under light microscopy at X200 magnification by two researchers, with interobserver reproducibility >95%. Values were given as mean ± s.e.m and analyzed by means of Student’s t-test. The differences were considered statistically significant for value of $P<0.05$.

**Cell Culture.** Human umbilical vein endothelial cells (HUVECs, Cambrex Milan, Italy) were grown in endothelial basal medium (EBM-2) supplemented with endothelial growth factors (EGM-2 bullet kit, Cambrex); 2nd passage HUVECs were serum-free harvested in 6-well plates in EBM-2 for 24h and then incubated in the medium containing 2 or 0.1% fetal bovine serum (FBS, Biological Industries, Beit-Haemek, Israel) and PLC dissolved in PBS; in some experiences, phorbol 12-myristate 13-acetate (PMA, 10nM, Sigma-Aldrich) was successively added. Smooth muscle cells (SMCs) were obtained from the inner portion of rabbit femoral artery\(^{10}\) one week after legation alone.\(^{2,10}\) SMCs appeared elongated and growing in hills and valleys and proliferated more compared to those obtained from uninjured arteries; α-smooth muscle actin confirmed the myocytic phenotype. After 24h of serum starvation, sparse SMCs were cultured in the presence of 10% FBS. Medium with or without PLC was changed every two days; \([\text{³H]}\text{thymidine incorporation and cell viability were determined as previously reported.}\(^{5}\) Results were expressed as the mean of three different experiments. To test the effect of PLC on cell growth, the mentioned cell lines were seeded in 96-well plates in complete medium. Twenty-four hrs after plating, scalar concentrations of drug were then added to the cells. The drug was removed after 72h and the number of surviving cells was determined by staining with Sulphorhodamine B dye.\(^{11}\) Optical density was measured at 564 nm. Results, expressed as concentration that represents increment or inhibition of efficacy in the 50% of cell growth (EC\(_{50}\) and IC\(_{50}\)), respectively, were calculated by the ALLFIT program.
Apoptosis. To evidence SMC apoptosis in vitro, apoptosis of adherent SMCs was investigated by calculating the percentage of nuclei showing apoptotic features by Hoechst staining in triplicate. Assigning a serial number to each slide ensured the objectivity of all measurements.

In vitro adhesion assay. Confluent HUVECs cultured in 24-well plates with or without serum starvation were treated with PLC at 1 mM concentration for 24 h. Human peripheral blood mononuclear cells were isolated by using Ficoll-Paque Plus (Amersham Pharmacia Biotech) according to manufacture’s guidelines. The adhesion assay was performed after addition of 500 µL of monocyte suspension at a concentration of 1x10^6/m for 1 hour incubation at 37°C. Non-adherent cells were removed by three times gentle washing with medium. Adhering cells were fixed in 2% paraformaldehyde and then counted. The number of adhering cells was calculated as ratio to the control in triplicate.

Western blot analysis. Total protein extracts from multiple muscle biopsies or from cultured cells was quantified by Bradford-assay. Aliquots were separated by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to nitrocellulose transfer membranes (0.2 µm; Amersham Biosciences) and incubated with rabbit polyclonal anti-Nox4 (1:200 1h at room temperature), mouse monoclonal anti-Nox2 (1:200 1h at room temperature, Santa Cruz Biotechnology Inc., CA, USA), rabbit polyclonal anti-Nox1 (1:200 1h at room temperature, Santa Cruz Biotechnology Inc., CA, USA), mouse monoclonal anti-Rac (1:100 overnight at 4°C, Upstate, NY, USA) and α-tubulin (1:5000, 1h at room temperature). Immunoblots were visualized by enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometric analysis in three independent experiments.
**Rac1 activity assay.** Rac1 activity was determined using a commercially available kit (Upstate Biotechnology, Upstate Biotechnology, NY, USA). Briefly, HUVECs were grown in 10-cm dishes at 70-80% confluence and serum deprived for 24 hours. After exposure to PLC (1.0 mM with 0.1% FBS) for 4 and 12h, cells were lysed according to manufacture’s guidelines. A PAK-GSH fusion protein bound to agarose beads was added, and active Rac1 separated by repetitive centrifugation and washing. After boiling in reduced sample buffer, samples were subjected to 12% polyacrylamide gel SDS-PAGE, and Rac quantified by Western blot analysis with controls. The experiment was repeated twice.

**Reverse transcriptase and Real-Time Polymerase Chain Reaction.** RNA was extracted from cells, frozen quadriceps muscle biopsies and CAM tissue with the Trizol and 1 µg total RNA was reverse transcribed. Using 1µl of the product as a template, polymerase chain reaction was performed in triplicate with gene-specific primers (Table 1). Results were normalized against the hypoxanthine-guanine phospho-ribosyltransferase (HPRT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and expressed as arbitrary densitometry units (ADU). The mRNA levels of Nox isoforms were determined by RT-PCR and quantitative Real-Time PCR performed twice for each of independently obtained total RNAs. The copy numbers were standardized by those of GAPDH and β-actin housekeeping genes. Gene expression of Nox4, Rac-1 and ICAM-1 was analyzed by real-time PCR and GAPDH as housekeeping gene. The results were reported as normalized fold expression of triplicate experiments. Values were given as mean ± s.e.m and analyzed by means of Student’s t-test. The differences were considered statistically significant for value of P<0.05.
Electrophoretic Mobility Shift Assay. To perform the electrophoretic mobility shift assay (EMSA), nuclear extracts from 2x10^6 cells were obtained. Protein concentrations were determined and EMSA analysis and supershift assays were performed using an NF-κB-specific oligonucleotide (Invitrogen). The double-stranded nucleotides were end-labeled with γ^32P-ATP using T4 polynucleotide kinase. NF-κB antibodies used for supershift EMSA were anti-p50 and anti-p65 (Santa Cruz). Blocking of NF-κB nuclear translocation was performed by SN50 and its ineffective analog SM50 30 min before PLC treatment. Results were expressed as the mean of three different experiments.

Detection of intracellular reactive oxygen species. Reactive oxygen species (ROS) levels in HUVECs were measured by 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) fluorescence method (Molecular Probes, Inc., Eugene, OR, USA). After serum starvation cells (3x10^5/mL) were treated with PLC, harvested by trypsinization and incubated with 20μM CM-H2DCFDA for 30 min at 37°C. Cells were resuspended in ice-cold PBS and placed on ice in the dark. Dichlorofluorescein fluorescence was monitored by analyzing at least 10,000 cells in a flow cytometer (Beckman Coulter, CA, USA). Results were expressed as the mean of three different experiments.

Small interfering RNA for gp91phox/Nox2 and Nox4 constructions and transfection. A 19-nucleotide small interfering RNA (siRNA) 3'-overhanged for human gp91phox/Nox2 (gp91phox/Nox2, access NM_000397) and human Nox4 (Nox4, access NM_016931) were designed by using Block-IT™ RNAi Designer (Invitrogen). The sequences sense 5′-UGCCUGAAUUUCAACUGCAdTdT-3′ and antisense 5′-UGCAGUUGAAAUUCAGGCAdTdT-3′ for Nox2 and sense 5′-CCUCAGCAUCUGUUCUUAAdTdT-3′ and antisense 5′-
AAUUGUUCUGUAGCAGUGdTdT-3’ for Nox4 were transfected using Oligofectamine (Invitrogen) according to manufacture’s guidelines. Control siRNA was a scramble sequence without any specific target: 5’-CCTTACGTGTCTCTACTAAdTdT-3’ and 5’-AATGATGTGTCTCGATTGGdTdT-3’. Depletion of Nox4 and Nox2 by siRNA was confirmed by RT-PCR and Western blot.

**Statistical analysis.** Data were expressed as mean±standard error of mean (s.e.m.) and differences analyzed by means of Student’s t-test and one-way ANOVA or ANCOVA followed by Dennett’s test. Blinded measurements were performed by two independent researchers; the interobserver reproducibility was >95%. Differences were considered statistically significant for value of $P<0.05$. 


References

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<th>Gene</th>
<th>Primer Sequence</th>
<th>Temperature of Annealing</th>
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Table I. Effects of PLC on blood flow ratio in rabbit hindlimb vessels

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<th>Group</th>
<th>Dose (mg/rabbit)</th>
<th>Days after injection</th>
<th>Before femoral artery resection</th>
<th>% of recovery</th>
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<td>4</td>
<td>1.09 ± 0.05</td>
<td>46.4 ± 5.3</td>
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<tr>
<td>PLC</td>
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<td>4</td>
<td>1.07 ± 0.06</td>
<td>62.2 ± 5.7 §</td>
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<td>0.91 ± 0.05</td>
<td>56.4 ± 5.1</td>
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<tr>
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<td>7</td>
<td>1.04 ± 0.06</td>
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<td>77.8 ± 4.6</td>
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<tr>
<td>PLC</td>
<td>10</td>
<td>14</td>
<td>1.06 ± 0.03</td>
<td>85.2 ± 3.4 §</td>
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</table>

Blood flow ratio evaluated by laser doppler flowmetry in rabbits receiving propionyl-L-carnitine (PLC) injection three days after ischemia induction by left femoral artery resection. Values are means ± SEM of the ratio of ischemic to controlateral blood flow; *t*-test §: *P*<0.05 vs. vehicle (saline solution).
Legends of supplemental Figures

Figure I. Effects of PLC on vascularization in mouse air pouch. (A) Effects of PLC on mouse air pouch seven days after PLC (25 and 50 mg/mouse) and saline (vehicle) injection. (B) Air pouch vascular area and number of vessels classified according to size; mean ± s.e.m. of 12-18 mice/group from three experiments; One-way ANOVA+Dennett’s test: *, P<0.05.

Figure II. Effect of PLC on vascular cell proliferation. (A) Number of PLC-treated HUVECs and arterial SMCs as percentage of control with an IC50 of 1.43 and 2.05 mM, respectively; t-test: *, P<0.05. (B) [3H]Thymidine incorporation in HUVECs and SMCs as mean of three different experiments; t-test: **, P<0.01, 0.1 mM vs. 1mM PLC in HUVECs. SMC incorporation decreases at 2.0 mM PLC at 24 and 48h; t-test: *,**, P<0.05 and P<0.01, 0.2 vs. 2.0 mM PLC. (C) RT-PCR analysis in normalized (HPRT) arbitrary densitometric units (ADU) 4, 8 and 12h after PLC treatment (1mM) compared to 2% FBS; t-test: *,**, P<0.05 and P<0.01 vs. control.