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Propionyl-L-Carnitine is Efficacious in Ulcerative Colitis Through its Action on the Immune Function and Microvasculature

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OBJECTIVES: Microvascular endothelial dysfunction characterizes ulcerative colitis (UC), the most widespread form of inflammatory bowel disease. Intestinal mucosal microvessels in UC display aberrant expression of cell adhesion molecules (CAMs) and increased inflammatory cell recruitment. Propionyl-L-carnitine (PLC), an ester of L-carnitine required for the mitochondrial transport of fatty acids, ameliorates propionyl-CoA bioavailability and reduces oxidative stress in ischemic tissues. The present study aimed to document the efficacy of anti-oxidative stress properties of PLC in counteracting intestinal microvascular endothelial dysfunction and inflammation.

METHODS: To evaluate the efficacy *in vivo*, we analyzed the effects in intestinal biopsies of patients with mild-to-moderate UC receiving oral PLC co-treatment and in rat TNBS-induced colitis; in addition, we investigated antioxidant PLC action in TNF-α-stimulated human intestinal microvascular endothelial cells (HIMECs) *in vitro*.

RESULTS: Four-week PLC co-treatment reduced intestinal mucosal polymorph infiltration and $CD4^+$ lymphocytes, ICAM-1^+ and $iNOS^+$ microvessels compared with placebo-treated patients with UC. Oral and intrarectal administration of PLC but not L-carnitine or propionate reduced intestinal damage and microvascular dysfunction in rat TNBS-induced acute and reactivated colitis. In cultured TNF-α-stimulated HIMECs, PLC restored β-oxidation and counteracted NADPH oxidase 4-generated oxidative stress-induced CAM expression and leukocyte adhesion. Inhibition of β-oxidation by L-aminocarnitine increased reactive oxygen species production and PLC beneficial effects on endothelial dysfunction and leukocyte adhesion. Finally, PLC reduced iNOS activity and nitric oxide accumulation in rat TNBS-induced colitis and in HIMEC cultures.

CONCLUSIONS: Our results show that the beneficial antioxidant effect of PLC targeting intestinal microvasculature restores endothelial β-oxidation and function, and reduces mucosal inflammation in UC patients.

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Subject Category: Inflammatory Bowel Disease

INTRODUCTION

Ulcerative colitis (UC) and Crohn disease are the two major forms of inflammatory bowel disease (IBD).[1] UC is the most common, with an incidence of 1.2–20.3 cases for 100,000 people per year.[1,2] Genetic and environmental factors contribute to the deregulation of mucosal inflammatory response in patients with IBD, and both immune and non-immune regulatory pathways contribute to UC pathogenesis.[1,3] Microvascular endothelium has a crucial role in the initiation and the progression of the inflammatory response and consequent tissue remodeling of UC. Intestinal microvascular endothelial cells regulate the influx of leukocytes through the expression of cell adhesion molecules (CAMs) and chemokine secretion.[4] Chronically inflamed intestinal microvessels in UC patients display aberrant CAM expression and enhanced susceptibility to adherence, migration, and recruitment of leukocytes.[1,5] Our hypothesis was that intestinal mucosal microvascular endothelial dysfunction has a primary role in governing mucosal inflammation in UC patients, thus representing a consistent therapeutic target in UC patients.[6] Experimental models and colonic mucosal biopsies from UC patients documented metabolic changes and an impairment of β-oxidation.[7,8] Propionyl-L-carnitine (PLC) is an ester of L-carnitine that is required for the transport of fatty acids into the mitochondria.[9] PLC has been documented to be capable of reducing membrane lipid peroxidation and the effects of hypoxia in cardiomyocytes as a sulfoxide scavenger.[10,11] PLC has been introduced among non-interventional medical regimens to counteract adverse effects of peripheral arterial disease.[12,13] Oxidative stress is characterized by reactive oxygen species (ROS) overproduction causing cell damage and death.[14] PLC appeared effective in the reduction of endothelial dysfunction induced by ROS accumulation in ischemic rabbit limbs.[15] Consequently, the

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reduction of oxidative stress could explain the clinical advantage of PLC treatment in patients with peripheral arterial disease.[16] Interestingly, plasma levels of PLC but not of L-carnitine are reduced in UC patients.[17] Preliminary data reported the improvement of inflammation in patients with mild UC receiving topical irrigation of PLC.[18] Moreover, a multicentric phase II double-blind trial documented the clinical efficacy of PLC in patients with mild-to-moderate UC under oral stable aminosalicylate (5-ASA) therapy.[19] Here we report that the anti-inflammatory efficacy of adjuvant therapy with PLC in UC patients is mediated by microvascular endothelial dysfunction-targeted antioxidant action.

METHODS

A detailed description is available in the Supplementary Materials and Methods online.

Clinical and microscopic study. A randomized, double-blind, placebo-controlled, multicenter clinical study[19] was approved by the Independent Ethics Committee and conducted in accordance with good clinical practice and the Declaration of Helsinki. From that study, 23 patients, affected from mild-to-moderate UC, were randomly selected (see Supplementary Table 1) from three groups receiving stable oral aminosalicylate therapy (5-ASA; 3.2 g/day) with or without orally PLC co-treatment (Chemical Department of Sigma-Tau S.p.A., Italy) or placebo. Microscopic analysis of intestinal biopsies was performed at baseline and after a 4-week-follow up by using semiquantitative methods (see Supplementary Materials and Methods).

Induction of experimental colitis in rats. Male Sprague–Dawley rats, weighing 200-250 g, were provided by Charles River (Calco, Lecco, Italy). Experiments were performed according to international guidelines for animal experiments and approved by the Company veterinarian and Italian Ministry of Health. A set of experiments were performed to understand the efficacy of PLC by using different administration routes, dosages, and days of treatment and their frequencies (see Supplementary Materials and Methods). Acute colitis was induced by intrarectal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS, 120 mg/ml/kg), under halothane anesthesia. PLC and 5-ASA (120 mg/kg; Sigma-Aldrich, Milan, Italy) were administered by intrarectal instillation. For the reactivated colitis, 42 days after the first intracolon TNBS instillation, 10 mg/kg TNBS was administered subcutaneously twice daily for three consecutive days. Two hours before killing, rats received intraperitoneally 30 mg/kg of Bromodeoxyuridine (BrdU, Sigma-Aldrich).[20] After euthanasia, the colon segments were excised, opened longitudinally, photographed, sampled, and analyzed by morphometric methods (see Supplementary Materials and Methods).

Measurement of plasma carnitine levels. Rat blood venous samples were collected before intrarectal instillation of TNBS to assess the baseline concentration and after 6 days of treatments. On day 6, blood samples were collected (time 0) and 1, 4, and 7 h after the last vehicle or PLC administration. Plasma carnitine concentrations were determined by high-performance liquid chromatography-mass spectrometry (see Supplementary Materials and Methods).

Immunohistochemistry and morphometric analysis. For immunohistochemistry,[21] 4-μm thick serial sections were deparaffinized, rehydrated and, after antigen retrieval and nonspecific peroxidase blocking, incubated with mouse monoclonal anti-ICAM-1 (Pierce, IL, USA), anti-eNOS (Pierce), anti-Ki67 (Ventana Medical Systems, AZ, USA), anti-BrdU (YLEM, Avezzano, Italy), anti-human CD31 (Ventana), anti-CD4 (Ventana), and rabbit polyclonal anti-VCAM-1 (Abcam, CB, UK), anti-iNOS (Pierce), and anti-PlGF (Abcam). For rat tissues, mouse monoclonal anti-rat CD31 (BD Pharmingen, NJ, USA), was used. Morphometric evaluation of immunoreactivity was performed according to defined criteria (see Supplementary Materials and Methods).

Ultrastructural study. For transmission electron microscopy, small rat colon samples were fixed in Karnovsky (2% paraformaldehyde, 2.5% glutaraldehyde) in 0.1 M cacodylate buffer, pH 7.35 for 24 h at 4 °C, post-fixed in 1% OsO4 for 2 h, and dehydrated through an alcohol series and propylene oxide before embedding in EPON 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate, and photographed with a Philips 301 electron microscope.

Cell culture and leukocyte adhesion assay. First-third passage human intestinal microvascular endothelial cells (HIMECs, Innoprot, Spain) and human umbilical vein endothelial cells (HUVECs, Lonza, Italy) were treated with TNF-α (5 ng/ml; Sigma-Aldrich), PLC (1 mM), L-aminocarnitine, an inhibitor of carnitine-palmitoyltransferase-2 (1 μM, Sigma-Tau), or desipramine, inhibitor of the acidic sphingomyelinase (5 μM; Sigma-Aldrich). For *in vitro* assays, cells were pretreated with PLC before adding TNF-α or, in some experiences, PLC was added after TNF-α stimulation. For proliferation assay, HIMECs were incubated with 1 mM PLC, and the number of surviving cells was determined after 72 h.[15] For leukocyte adhesion assay, cells were starved in 0.1% FBS with or without PLC (1 mM) for 24 h and successively stimulated with TNF-α (5 ng/ml) for 4 h. Human leukocytes were incubated with 2 μM 2′7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (Invitrogen, Life Technology, Monza, Italy) for 45 min at 37 °C. After washing, leukocytes were laid on endothelial cells for 1 h on a rocker plate. Afterwards, adhering cells were fixed in 2% glutaraldehyde and counted using a fluorescent microscope (E600 Eclipse, Nikon).

Protein extraction and western blot analysis. The total protein extracts were isolated using lysis buffer containing phosphatase and proteases. After protein content determination, proteins were blotted onto nitrocellulose membranes[22] and incubated with anti-NADPH-oxidase 4 (Nox4, Abcam), anti-ICAM-1 (Pierce), anti-VCAM-1 (Abcam), anti-iNOS (Pierce), anti-eNOS (Pierce), and anti-α tubulin (Sigma Aldrich) antibodies. Specific complexes were quantified as reported.[22]

Reverse transcriptase and real-time Polymerase Chain Reaction. Total RNA was extracted with the Trizol reagent (Invitrogen). Polymerase chain reaction (PCR) and real-time PCR[22] were performed in triplicate with gene-specific primers (see Supplementary Table 2). Results were normalized against the hypoxanthine-guanine phospho-ribosyltransferase (HPRT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

Detection of intracellular ROS, nitric oxide and β-oxidation activity. ROS were measured by a 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) fluorescence method (Molecular Probes, Eugene, OR) as described,[15] using an oxygen radical absorbance capacity antioxidant assay kit (Zen-Bio Inc., NC, USA). The nitric oxide (NO) content was measured by using a colorimetric assay kit (BioVision, CA, USA). β-Oxidation activity was evaluated by using a flavin adenine dinucleotide colorimetric assay (Sigma-Aldrich) and absorbance expressed in optical density (OD).

Dihydroethidium assay. Superoxide generation in HIMECs were measured by using the dihydroethidium (DHE, Sigma-Aldrich) fluorescence method (Molecular Probes, Eugene, OR, USA). HIMECs were pretreated or not with PLC (1 mM for 24 h) or desipramine (5 μM for 2 h) before TNF-α addiction (5 ng/ml for 4 h). Then HIMECs were incubated with 5 μM DHE for 20 min at 37 °C in the dark, and dihydroethidium fluorescence was measured by using a fluorescence microtiter plate reader (Beckman Coulter, CA, USA). Results were expressed as the mean of three different experiments.

Acidic sphingomyelinase activity assay. The enzymatic hydrolysis of sphingomyelin to ceramide and phosphocholine by acidic sphingomyelinase was measured at pH 5.0 with the Amplex Red reaction kit (Molecular Probes, Eugene, OR). HIMECs were pretreated or not with PLC (1 mM for 24 h) or desipramine (5 μM for 2 h) before TNF-α addiction (5 ng/ml for 4 h), and cell membrane-free supernatant fractions (adjusted to pH 5.0) were assayed for the acidic sphingomyelinase activity in a two-step reaction system (see Supplementary Materials and Methods).

Detection of inflammatory cytokines. Using the same conditions reported above for HIMEC stimulation, cell supernatants were collected by centrifugation at 800 *g* for 5 min at 4 °C and stored at −80 °C. Supernatant samples were thawed once and analyzed for IL-8 and MCP-1 content in duplicate using a commercially available ELISA kit with assay reproducibility greater than 95% (R&D Systems).

Statistical analysis. A detailed description of statistical analysis of clinical study is available in the Supplementary Materials and Methods. For microscopic evaluation and *in vitro* studies, data were expressed as the mean ± s.e.m. and differences analyzed by using Student's *t*-test or one-way ANOVA followed by using Dunnett's test. In case of nonGaussian distribution, the Kruskal–Wallis test was used. When appropriate, data were analyzed by were Fisher's exact test or χ^2 test. Blinded microscopic measurements were performed by two independent researchers, with an interobserver reproducibility >95%. Values of $P<0.05$ were considered statistically significant.

RESULTS

PLC co-treatment is effective and reduces mucosal inflammation and ICAM-1 expression in patients with UC. We investigated the effect of PLC co-treatment in intestinal biopsies of 23 UC patients receiving 5-ASA. Baseline values were similar in all experimental groups (not shown). Co-treatment with PLC-ameliorated clinical/endoscopic response vs. placebo (74.2 + 5.2% vs. 49.3 + 3.1%, respectively; $P<0.05$) as reported;[19] in particular, 76.4 + 6.3% of patients receiving 1 *g* PLC had a clinical/endoscopic response ($P<0.05$). Representative images of biopsies are shown in Figure 1a. The histological index of mucosal damage (Figure 1b) was reduced in PLC-treated compared with placebo (1g/day $P<0.01$; 2g/day $P<0.05$). The number of polymorphs in the lamina propria was decreased as well as intraepithelial polymorphs exudation ($P<0.05$; Figure 1c,d). Immunohistochemistry (Figure 2) showed the reduction in intramucosal $CD4^+$ lymphocytes in PLC compared with placebo patients ($P<0.05$; Figure 2a,e). Anti-inflammatory effect was parallel to the reduction of $iNOS^+$ and ICAM-1^+ microvessels ($P<0.05$; Figure 2b,c and e). Moreover, the percentage of proliferating $Ki67^+$ cryptic cells in PLC-treated patients was reduced ($P<0.05$) and close to that of control (Figure 2d,e); instead, the percentage of $CD31^+$ vessels was unchanged (Supplementary Figure I).

PLC reduces damaged area and inflammation in rat TNBS-induced acute colitis. To better document the microvascular endothelial targeting of PLC, we used different models of rat TNBS-induced colitis. PLC effects were investigated by using different administration routes, dosages and days of treatment (see Supplementary Figure II). In TNBS-induced acute colitis, intrarectal PLC administration (25 mg/kg twice daily, for one week) reduced the extent of macroscopic mucosal damage (Figure 3a,b) compared with TNBS alone ($P<0.05$). The positive effects of intrarectal (50 and 100 mg/kg once daily, for 1 week) and oral (120mg/kg once daily, for 1 week) administration were comparable and similar to that of intrarectal 5-ASA (120 mg/kg once daily, for 1 week; Supplementary Figure II); instead, propionate and L-carnitine treatments were ineffective. Microscopic examination confirmed the marked mucosal inflammation with the necrosis of epithelium, the presence of granulation tissue and intestinal wall thickness in TNBS-treated rats ($P<0.0001$ vs. control groups; Figure 3c–e) that were reduced by PLC ($P<0.001$ and $P<0.05$, respectively). Ultrastructural investigation well documented the endothelial cell swelling and leukocyte recruitment in TNBS-treated inflamed intestinal microvessels (Figure 3f), and the reduction of inflammation and perivascular edema in PLC-treated rats. Intrarectal PLC administration also reduced the damage area ($P<0.05$ at 12.5 and 25 mg/kg; Supplementary Figure IId and e), as well as intestinal wall thickness and inflammation in the TNBS-induced reactivated colitis (data not shown).

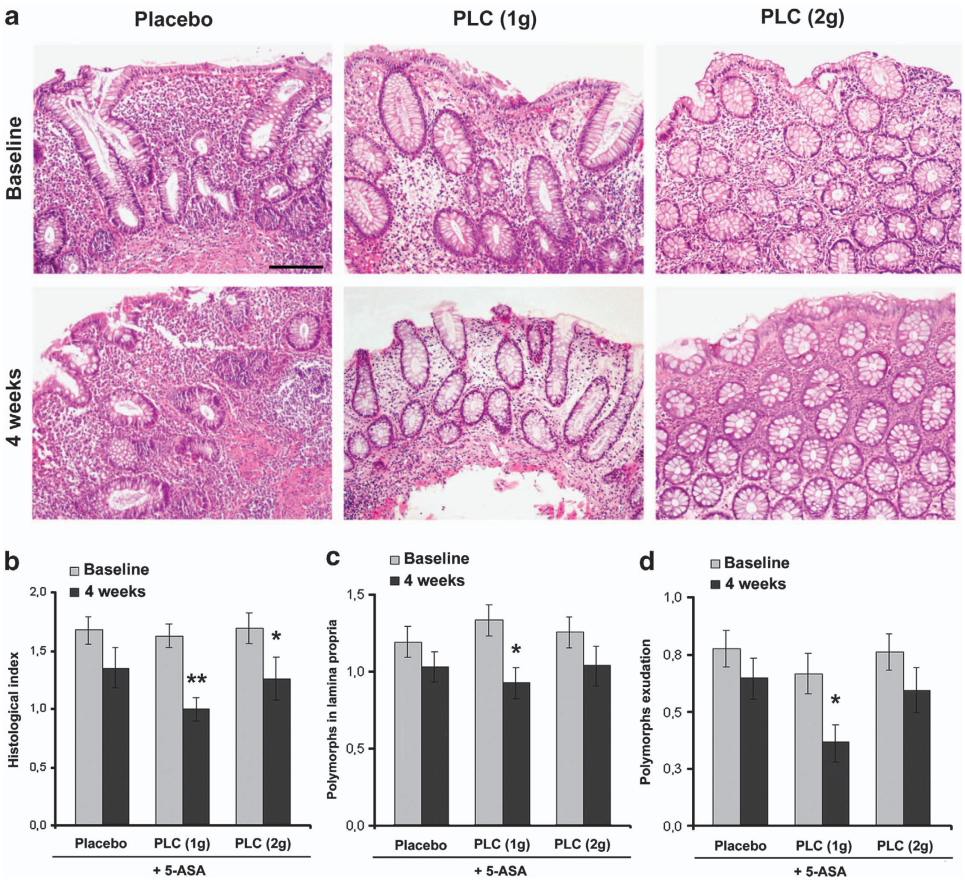

Figure 1 Microscopic amelioration of intestinal mucosa in UC patients co-treated with PLC. (**a**) Representative microphotographs of colon biopsies from UC patients treated with 5-ASA (3.2 g/day) plus PLC (1 g/day and 2 g/day) or placebo. Scale bar = 100 μm. (**b–d**) Bar graphs showing the intestinal histological index, the number of polymorphs in the lamina propria and the polymorphs exudation at baseline and after 4 weeks. Student's *t*-test: * and **, $P<0.05$ and $P<0.01$, respectively. Values are expressed as mean ± s.e.m.

PLC ameliorates endothelial dysfunction in rat TNBS-induced colitis. Immunohistochemical evaluation (Figure 4) documented the reduced percentages of mucosal ICAM-1$^+$, VCAM-1$^+$ and iNOS$^+$ vessels in PLC-treated TNBS-induced acute colitis ($P<0.01$ and $P<0.001$, respectively). In addition, PLC treatment induced the increase in CD31$^+$ and PlGF$^+$ vessels compared with TNBS alone group ($P<0.05$). The percentage of BrDU$^+$-proliferating cryptic cells was markedly enhanced in TNBS-inflamed mucosa compared with the vehicle ($P<0.01$; Figure 4). PLC restored an almost normal proliferation rate of colonocytes, as documented from the reduction in BrdU$^+$ cryptic cells ($P<0.01$ vs. TNBS alone). Similar effects of PLC were observed in TNBS-induced reactivated colitis (data not shown). Real-time PCR and blot analysis confirmed the reduction in VCAM-1 and ICAM-1 mRNA and proteins in PLC-treated intestinal tissue compared with TNBS alone (Figure 5a,b; $P<0.05$ and $P<0.01$).

Plasma carnitine levels in rat TNBS-induced colitis. PLC and L-carnitine pharmacokinetic analysis (Supplementary Table 3) revealed that after 6 days in the TNBS group plasma carnitine levels were slightly lower compared with that of the TNBS + PLC and PLC group, and in PLC-treated group, a slight increase in plasma PLC level was observed, with a systemic exposure increasing from 15 to 30% (Supplementary Table 4). In the TNBS + PLC group, carnitine levels were lower compared with control PLC rats, although the difference was not significant for the high inter-individual variability.

PLC reduces intestinal ROS accumulation and iNOS activity in rat TNBS-induced colitis. We observed the increase in ROS levels as fluorescence loss in TNBS-treated intestinal tissue compared with untreated control ($P<0.05$; Figure 5c); in PLC-treated TNBS rats, ROS accumulation was inhibited ($P<0.05$). Blot analysis (Figure 5b) documented that PLC partially counteracted the strong increase of Nox4 protein in the intestinal tissue of the TNBS-treated group ($P<0.05$). In addition, real-time PCR (Figure 5a) showed the increase in iNOS transcripts in TNBS-treated intestinal tissue compared with that of untreated control ($P<0.001$); iNOS transcript increase was partially prevented by PLC ($P<0.01$); instead, eNOS transcript levels were unchanged.

PLC reduces TNF-α-induced leukocyte adhesion in microvascular intestinal endothelial cells. To better understand if the anti-inflammatory effect of PLC is mediated

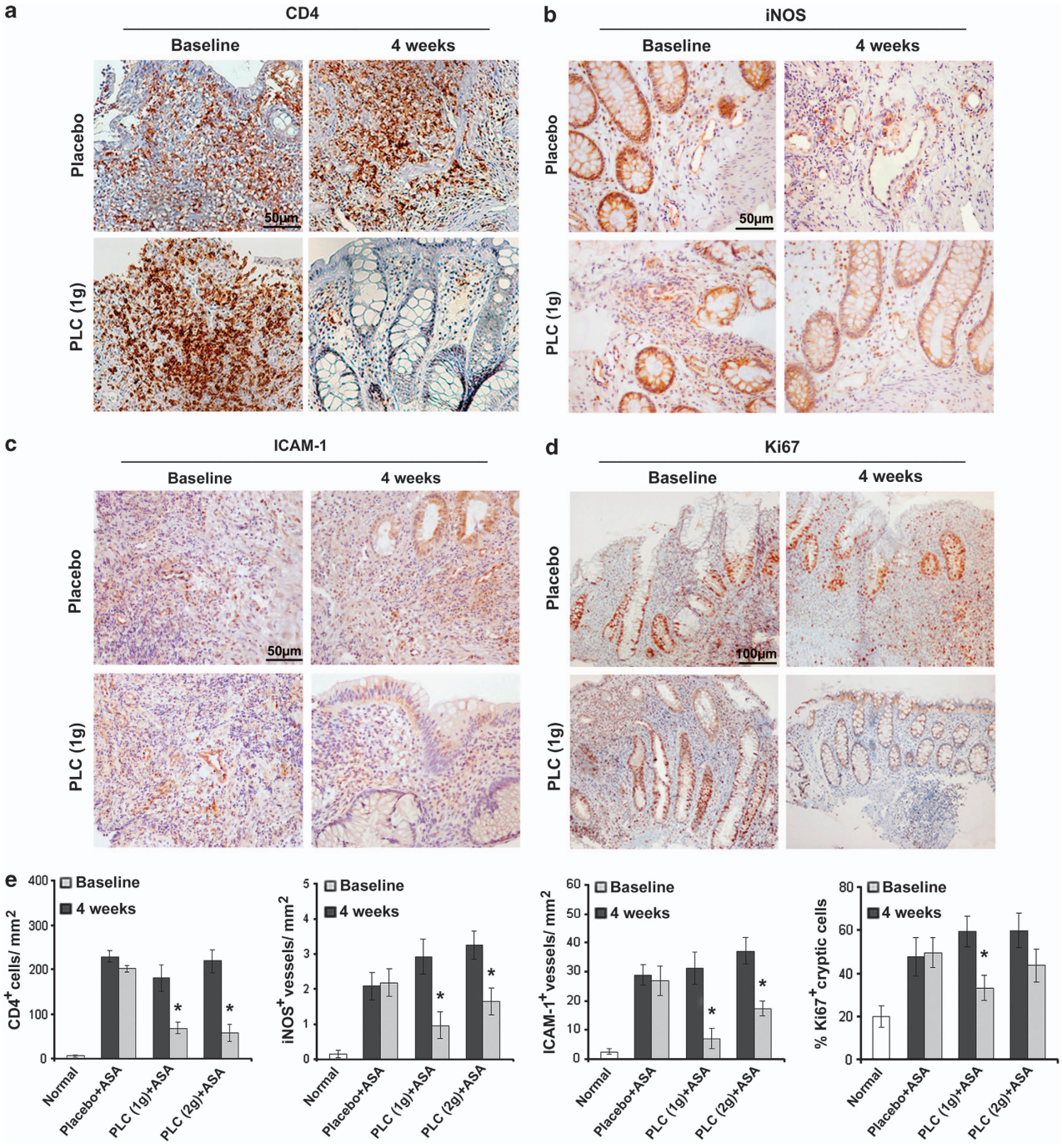

Figure 2 PLC reduces inflammation and endothelial dysfunction in UC patients. (**a–d**) Representative images of CD4$^+$ lymphocytes, iNOS$^+$ and ICAM-1$^+$ microvessels, and Ki67$^+$ cryptic cells in intestinal biopsies from UC patients treated with 5-ASA plus PLC (1 g/day and 2 g/day) or placebo. Scale bar = 50 μm. (**e**) Bar graphs of semiquantitative evaluation of CD4$^+$ lymphocytes, iNOS$^+$ and ICAM-1$^+$ microvessels, and Ki67$^+$ cryptic cells. Student's *t*-test: *, $P<0.05$ vs. placebo. Values are expressed as mean ± s.e.m.

by its protective effect on microvascular intestinal endothelium, we used *in vitro* models of leukocyte adhesion. After TNF-α stimulation, leukocyte adhesion was increased in HIMEC and HUVEC monolayers ($P<0.001$ vs. control; Figure 5d); PLC pretreatment reduced TNF-α-induced leukocyte adhesion in both cultures ($P<0.01$). Blots and real-time PCR (Figure 5e,f) demonstrated in HIMECs that the TNF-α-induced increase in ICAM-1 and VCAM-1 protein and mRNA ($P<0.01$) was reduced by PLC pretreatment ($P<0.05$). TNF-α also increased intestinal specific MAdCAM-1 transcript levels ($P<0.01$; Figure 5f), which was similarly reduced by PLC ($P<0.05$). Similar positive effect on endothelial function was observed in PLC-treated HUVECs after TNF-α stimulation (Supplementary Figure IIIa and b).

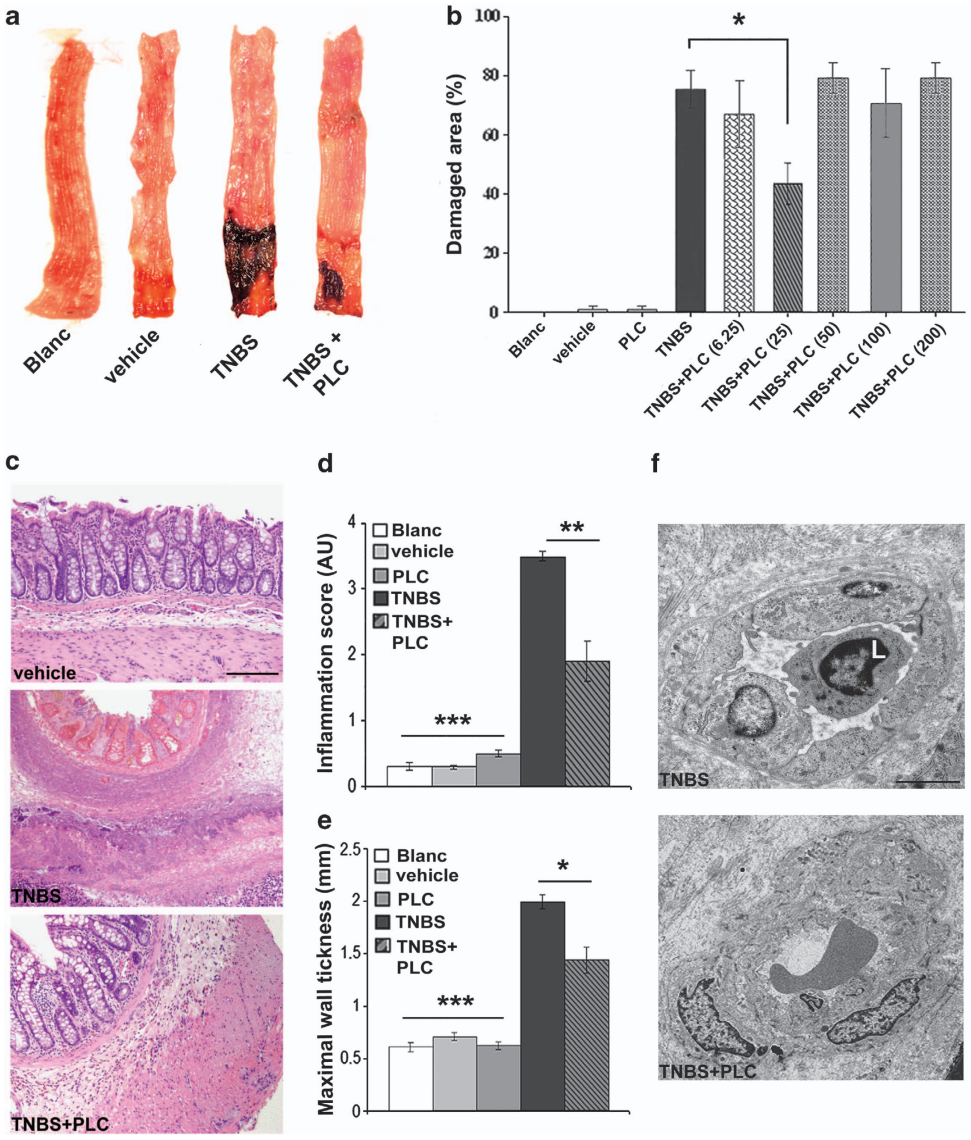

Figure 3 PLC reduces the inflammatory damage in rat TNBS-induced acute colitis. (**a**) Representative photographs showing rat colon segments from the different experimental groups: blank, vehicle (50% v/v ethanol), PLC alone (25 mg/kg intrarectal, twice daily for 1 week), TNBS and TNBS plus PLC (25 mg/kg intrarectal twice daily for 1 week). (**b**) Bar graph shows the percentage of intestinal-damaged area in the different experimental groups. (**c**) Representative microphotographs of intestinal mucosa from vehicle, TNBS and TNBS plus PLC (25 mg/kg, intrarectal administration twice daily for 1 week) groups. Scale bar = 100 μm. (**d**, **e**) Bar graphs showing rat mucosal inflammation score and maximal wall thickness measurement. (**f**) Transmission electron microscopy images from rat intestinal mucosal microvessels of TNBS (left) and TNBS plus PLC (25 mg/kg, right) groups. Scale bar = 10 μm. AU, arbitrary units; L, leukocyte. Student's *t*-test: *, **, and ***$P<0.05$, $P<0.001$, and $P<0.0001$, respectively. Values are expressed as mean ± s.e.m.

The reduction in CAM expression was also observed after 12 h of PLC treatment following the TNF-α exposure (data not shown).

PLC reduces TNF-α-induced NADPH oxidase 4-mediated ROS generation in microvascular intestinal endothelial cells. In order to establish whether the antioxidative effect of PLC mediates its protective effects, we investigated ROS accumulation in microvascular intestinal endothelial cells. TNF-α stimulation induced higher ROS accumulation in both HIMECs and HUVECs ($P<0.05$ compared with 0.1% FBS; Figure 6a,b). PLC decreased ROS levels in both endothelial cell lines ($P<0.05$ and $P<0.001$, respectively; Figure 6a,b). As reported,[15] PLC also reduced serum withdrawal-induced ROS increase in HUVECs but not in HIMECs (Figure 6c), confirming that those endothelial cell populations partially differ in their response.[23] Real-time PCR (Figure 6d) documented that Nox4 is the main NADPH-oxidase subunit in HIMECs. TNF-α increased Nox4 protein and transcripts in HIMECs as documented by blot and real-time PCR ($P<0.01$ vs. control; Figure 6e,f), and these effects were partially counteracted by PLC pretreatment ($P<0.05$). In HIMECs, Nox4 expression was also reduced by 12-h PLC treatment following TNF-α exposure data (not shown). TNF-α-induced

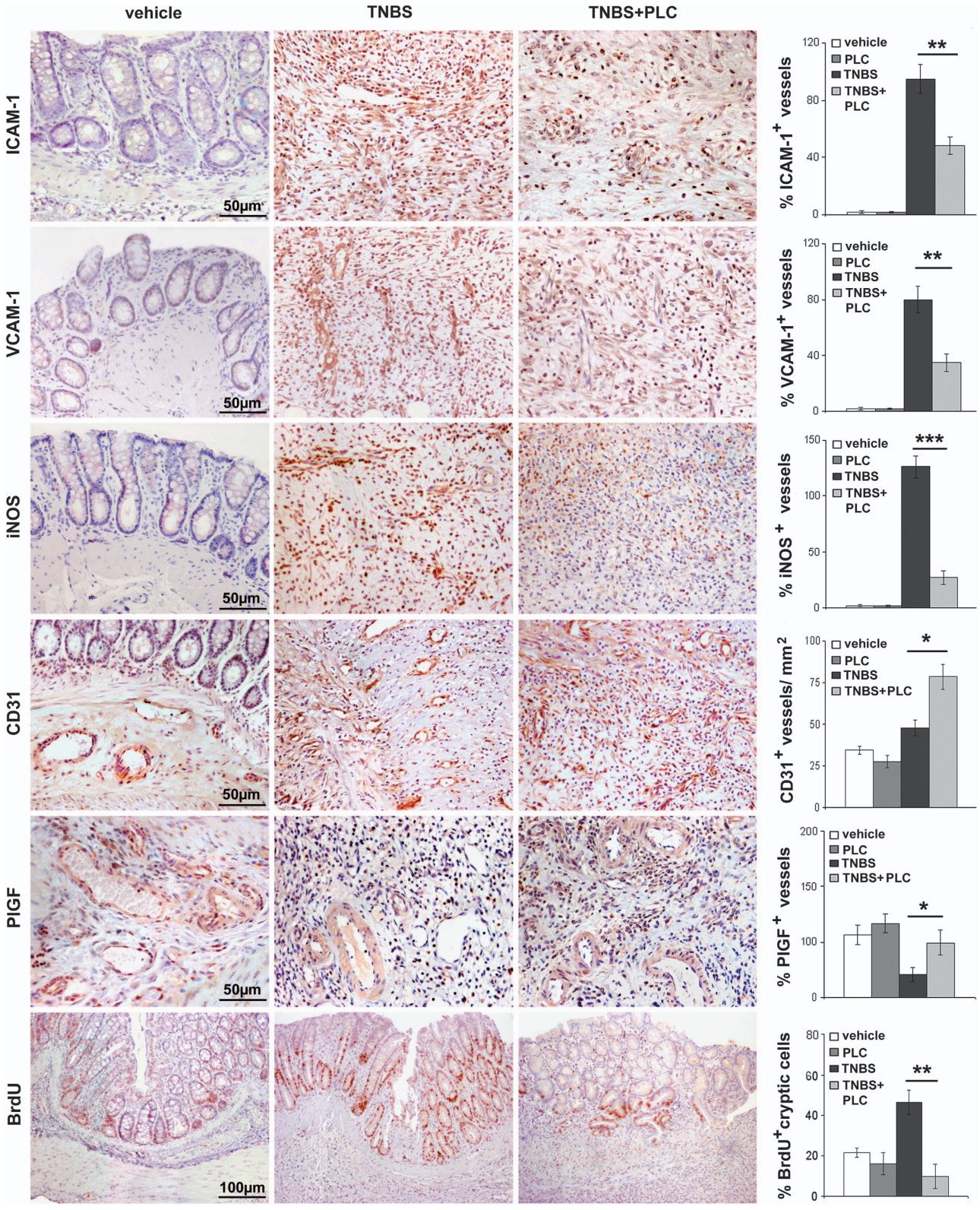

Figure 4 PLC treatment reduces inflammation and endothelial dysfunction in rat TNBS-induced acute colitis. Representative microphotographs and morphometric evaluation of ICAM-1, VCAM-1, iNOS, CD31, PIGF, and BrDU staining of rat colon tissue from the different experimental groups: vehicle (50% ethanol, v/v), PLC (25 mg/kg intrarectal, twice daily for 1 week), TNBS and TNBS plus PLC (25 mg/kg intrarectal, twice daily for 1 week). Student's *t*-test: *, **, and ***$P<0.05$, $P<0.01$, and $P<0.001$, respectively. Values are expressed as mean ± s.e.m.

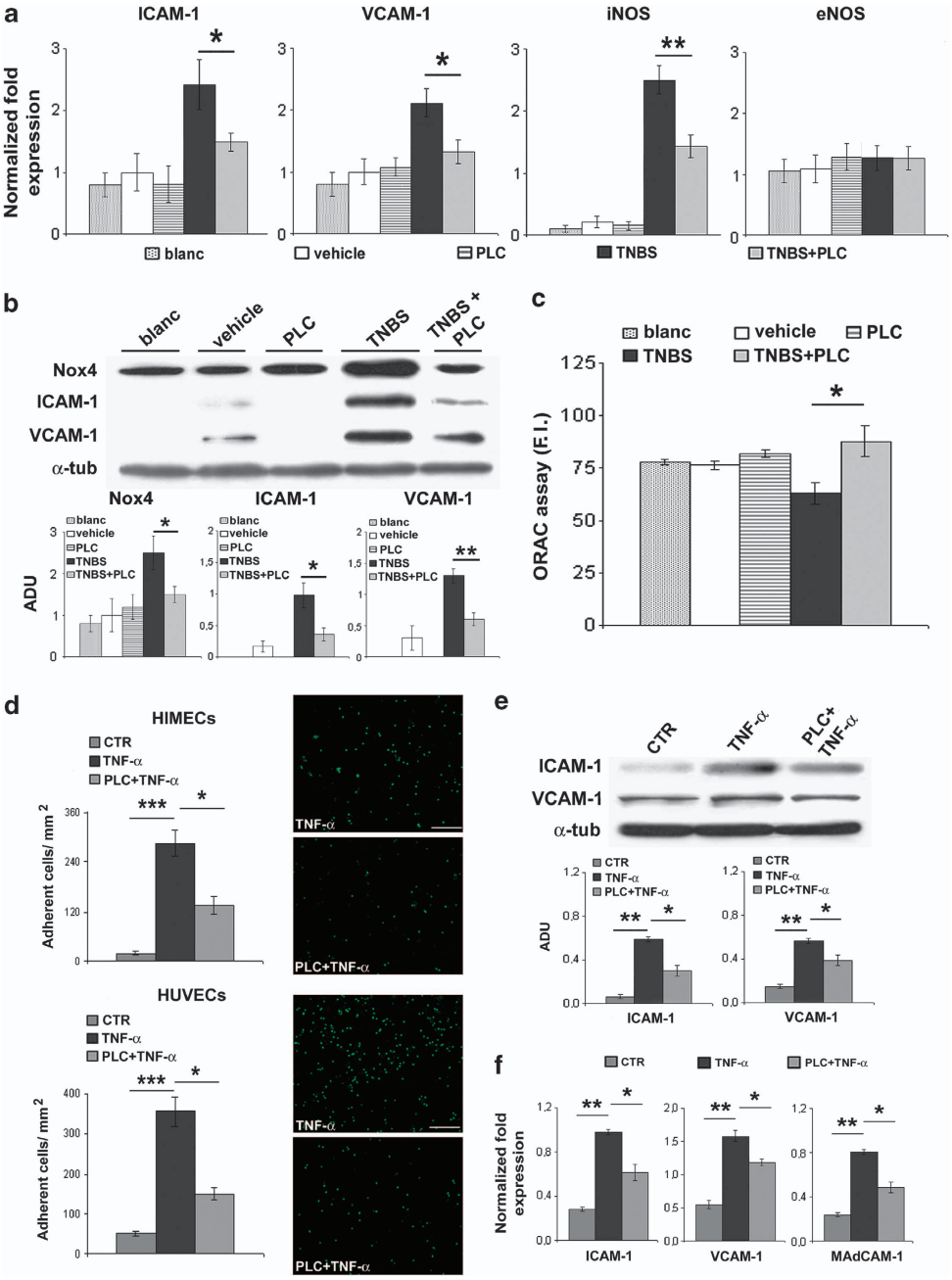

Figure 5 PLC treatment reduces the expression of inflammatory and oxidative stress markers in rat TNBS-induced acute colitis and prevents TNF-α-induced endothelial cell activation. (**a**) Real-time PCR bar graphs for ICAM-1, VCAM-1, iNOS, and eNOS of rat intestinal tissue from the different experimental groups: blank, vehicle (50% ethanol v/v), PLC (25 mg/kg intrarectal, twice daily for 1 week), TNBS and TNBS plus PLC. (**b**) Blot analysis for Nox4, ICAM-1, and VCAM-1 of rat colon tissue extracts. (**c**) Bar graph showing ROS levels, inversely proportional to fluorescence intensity (F.I.), in rat intestinal tissues. PLC restores fluorescence in TNBS-treated colonic mucosa. (**d**) Representative microphotographs and morphometric evaluation of leukocyte adhesion assay of TNF-α-activated HIMECs and HUVECs in the presence of 0.1% FBS (CTR) or with PLC pretreatment (1 mM; PLC + TNF-α). Scale bar = 50 μm. (**e**) Blot analysis for ICAM-1 and VCAM-1 of TNF-α-activated HIMECs compared with 0.1% FBS or PLC pretreatment. (**f**) Bar graphs for ICAM-1, VCAM-1, and MAdCAM-1 transcript levels by real-time PCR. Student,s *t*-test: *, **, and ***$P<0.05$, $P<0.01$, and $P<0.001$, respectively. Values are expressed as mean ± s.e.m.

increase of Nox2 and Rac1 transcription was counteracted by PLC (Figure 6f; $P<0.01$ and $P<0.05$, respectively), whereas Nox1 expression did not change (data not shown). PLC pretreatment also reduced the TNF-α-induced increase of Nox4 transcripts in HUVECs ($P<0.05$; Supplementary Figure IIIc). Finally, real-time PCR showed that PLC pretreatment also increased HO-1 but not HO-2 transcripts compared with TNF-α alone in HIMEC cultures ($P<0.05$; Supplementary Figure IV). Instead, in HUVEC cultures the PLC pretreatment also induced the increase of HO-2 transcripts compared with TNF-α alone ($P<0.05$; Supplementary Figure IV).

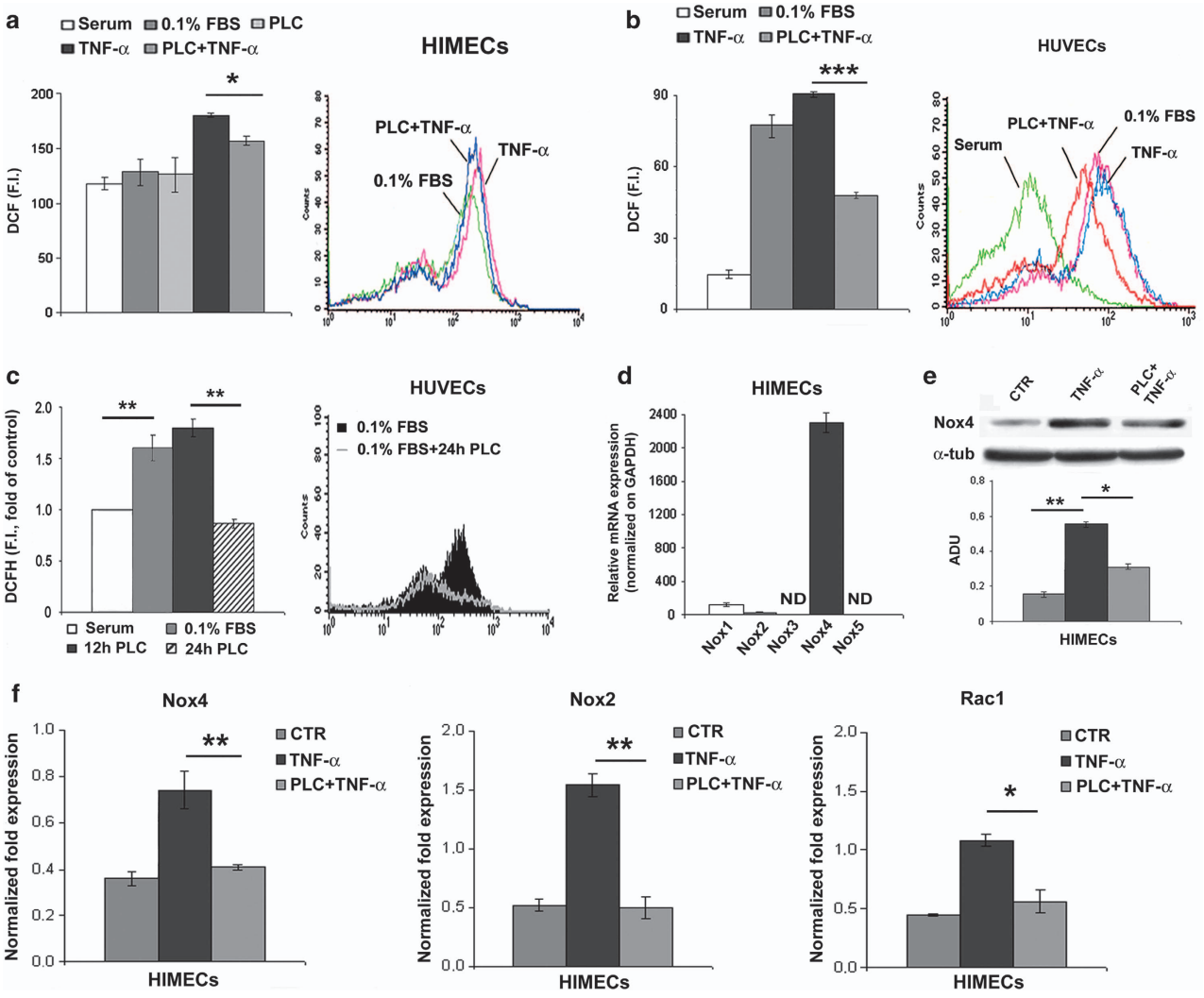

Figure 6 PLC prevents TNF-α-induced ROS generation and Nox4 activity in intestinal endothelial cells. (**a–c**) Histograms show dichlorodihydrofluorescein fluorescence intensity (DCF F.I.) of TNF-α-activated HIMECs and HUVECs compared with serum (2% and 5%, respectively), 0.1% FBS and PLC pretreatment (1 mM; PLC + TNF-α). (**d**) Real-time PCR bar graph for Nox subunits reveals that Nox4 is the major isoform in serum-cultured HIMECs. (**e**) Blot analysis shows the increase of Nox4 expression in TNF-α-treated HIMECs compared with control (CTR; 0.1% FBS) that is partially prevented by PLC pretreatment. (**f**) Real-time PCR bar graphs for Nox4, Nox2, and Rac1 of TNF-α-treated HIMECs compared with 0.1% FBS control (CTR) and with PLC pretreatment. Values are expressed as mean ± s.e.m. Student's *t*-test: *, **, and ***$P<0.05$, $P<0.01$, and $P<0.001$, respectively.

The restoration of endothelial β-oxidation is required for the anti-inflammatory effect of PLC. As β-oxidation is impaired in the inflamed mucosa of UC patients,[7] we investigated the effects of TNF-α stimulation and PLC treatment on β-oxidation of intestinal microvascular endothelial cells. As shown in Figure 7a, the TNF-α-induced strong reduction of mitochondrial β-oxidation in HIMECs ($P<0.01$ vs. control) was counteracted by PLC pretreatment ($P<0.05$). L-aminocarnitine strongly reduced mitochondrial β-oxidation ($P<0.05$ vs. control) and prevented PLC-induced restoration of β-oxidation in TNF-α-treated HIMECs ($P<0.01$). To understand whether β-oxidation regulates endothelial oxidative stress, we measured ROS level in the presence of L-aminocarnitine. As shown in Figure 7b, L-aminocarnitine increased ROS production ($P<0.05$ vs. control) and prevented the effect of PLC on TNF-α-induced ROS generation ($P<0.05$). Finally, L-aminocarnitine strongly increased leukocyte adhesion ($P<0.01$ vs. control; Figure 7c,d) and prevented the inhibitory effect of PLC on TNF-α-induced leukocyte adhesion ($P<0.05$). Altogether, these data strongly support that the reduction in the oxidative stress-mediated intestinal microvascular leukocyte adhesion well explains the anti-inflammatory effect of PLC.

PLC acts at the mithocondrial level by reducing ROS generation and downstream-regulated inflammatory cytokine secretion. It is well-known that, after TNF-α stimulation of endothelial cells, the acidic sphingomyelinase is activated and produces ceramide that causes mitochondrial dysfunction and ROS generation.[24,25] We analyzed the sphingomyelinase activity and the effect of PLC on TNF-α-stimulated HIMECs. As shown in Figure 8a, TNF-α induced a

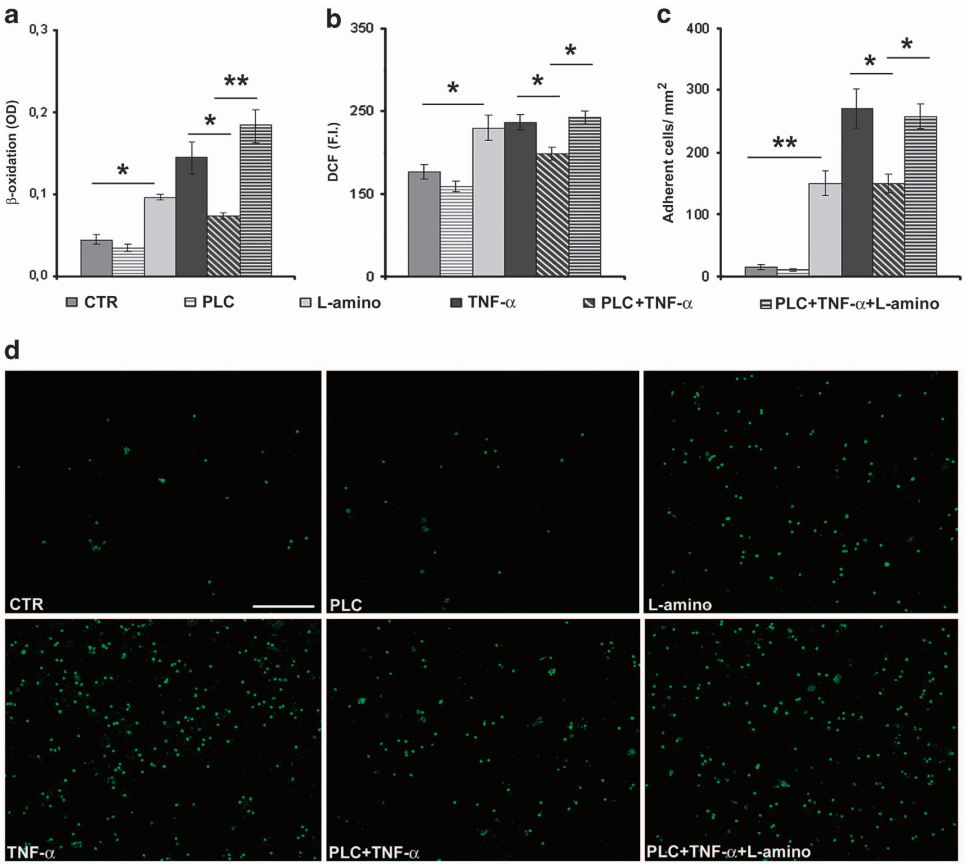

Figure 7 PLC prevents TNF-α-induced mitochondrial β-oxidation dysfunction. (**a**) Histogram shows β-oxidation assay (OD, optical density) of TNF-α-activated HIMECs compared with control (CTR; 0.1% FBS), PLC alone (1 mM), L-aminocarnitine (L-amino, 1 μM) and PLC pretreatment (1 mM; PLC + TNF-α). (**b**) Dichlorodihydrofluorescein fluorescence intensity (DCF, F.I.) of TNF-α-activated HIMECs compared with 0.1% FBS (CTR), PLC alone (1 mM;), L-aminocarnitine and PLC pretreatment. (**c**) Bar graph of leukocyte adhesion evaluation of TNF-α-activated HIMECs compared with 0.1% FBS control (CTR), L-aminocarnitine and PLC pretreatment. (**d**) Representative microphotographs of leukocyte adhesion assay on TNF-α-activated HIMECs compared with 0.1% FBS control (CTR), L-aminocarnitine and PLC pretreatment. Scale bar = 50 μm. Data are shown as mean ± s.e.m. Student's *t*-test: * and ** $P<0.05$ and $P<0.01$, respectively.

strong increase in sphingomyelinase activity ($P<0.001$ vs. control) that was counteracted by the sphingomyelinase inhibitor desipramine pretreatment ($P<0.01$). PLC treatment did not counteract TNF-α-induced sphingomyelinase activity (Figure 8a), supporting that PLC acts at the mitochondrial level by reducing superoxide generation ($P<0.05$ vs. TNF-α; Figure 8b) and inhibiting the downstream-regulated IL-8 and MCP-1 cytokine secretion ($P<0.05$ vs. TNF-α; Figure 8c,d).

PLC decreases TNF-α-induced endothelial iNOS expression and nitric oxide production. TNF-α increased nitric oxide (NO) level in the supernatant of HIMEC and HUVEC cultures ($P<0.05$; Figure 9a,b); that increase was counteracted by PLC pretreatment ($P<0.05$). In HIMECs, TNF-α-induced NO accumulation was parallel to that of iNOS protein and mRNA, and both were prevented by PLC ($P<0.05$; Figure 9c,d). PLC treatment alone did not significantly change NO level and iNOS expression compared with control. Finally, TNF-α and PLC did not increase eNOS expression.

DISCUSSION

Although the etiology of UC, the major form of IBD, remains unknown,[1] the pathogenic role of the intestinal microvascular involvement has been recently highlighted.[4] As a nonimmune regulatory pathway, microvascular endothelium has a central role in the homeostasis of intestinal mucosa.[4] Chronically inflamed intestinal microvessels of UC patients display an enhanced susceptibility to leukocyte adherence, migration, and recruitment, and the aberrant expression of CAMs.[5,6] Recently, Plevy and Targan,[26] reviewing about future therapeutic approaches, speculated that the discovery of therapies targeting specific intestinal defects, at the site of inflammation, is a goal of the future therapy for IBD.[26] In this light, the intestinal microvascular dysfunction in patients with UC represents a reasonable therapeutic target.[4] Biologics targeting inflammation-induced angiogenesis in IBD patients have been recently introduced and demonstrated beneficial anti-inflammatory effects.[27] Rectal administration of a non-replicating IL-10 expressing adenoviral vector reduced symptoms and tissue inflammation in IL-10 knock-out mice.[28] Unfortunately, many concerns still exist about the safety of the use of viral vectors in humans.[26] A trial with ICAM-1 siRNA[29] in patients with mild to moderate UC seemed to give promising results, but still technical problems and elevated cost, appear far to be resolved. Since it has been reported that UC patients have unchanged plasma free L-carnitine and lower PLC levels compared with healthy controls,[17] a specific PLC

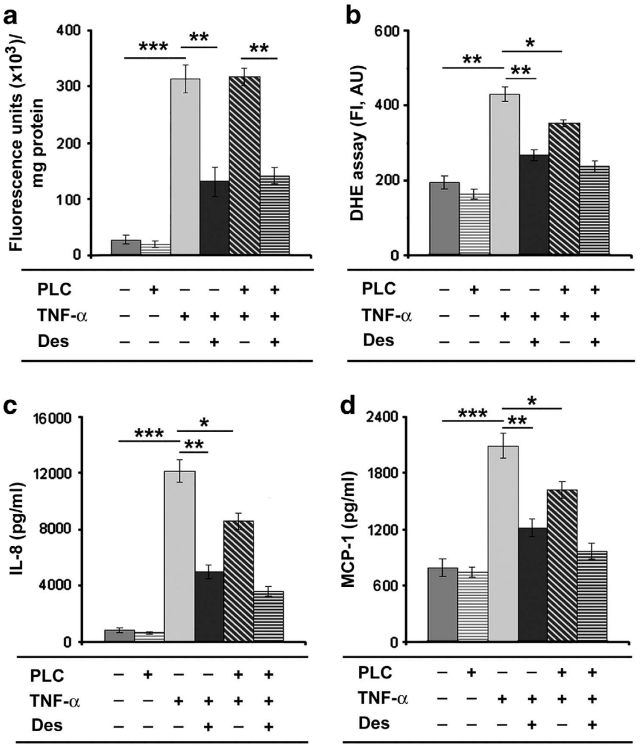

Figure 8 PLC acts at the mitochondrial level reducing ROS generation and downstream-regulated cytokine secretion. (**a**) Histogram shows sphingomyelinase activity assay (fluorescence units) of TNF-α-activated HIMECs compared with control (CTR; 0.1% FBS), PLC alone (1 mM), desipramine pretreatment (Des, 5 μM), and PLC pretreatment (1 mM). (**b**) Histogram shows ethidium bromide fluorescence (DHE oxidation) of TNF-α-activated HIMECs compared with CTR, PLC alone, desipramine pretreatment, and PLC pretreatment. (**c**, **d**) Bar graphs show IL-8 and MCP-1 level of TNF-α-activated HIMECs compared with CTR, PLC alone, desipramine pretreatment, and PLC pretreatment. Values are expressed as mean ± s.e.m. Student's *t*-test: *, **, and ***$P<0.05$, $P<0.01$, and $P<0.001$, respectively. AU, arbitrary units; FI, fluorescence intensity.

sequestering in inflamed tissues could be hypothesized. In the present study, we documented that the co-treatment with PLC is effective in patients with mild-to-moderate UC with stable conventional 5-ASA therapy; PLC co-treatment associated with the reduction of intestinal acute and chronic inflammation. The reduction of mucosal inflammation was parallel to that of ICAM-1$^+$ and iNOS$^+$ microvessels compared with the placebo group. Our results strengthen the clinical efficacy of PLC in UC patients and do not contrast those of Mikhailova *et al.*[19] In the latter study, primary histological endpoint was the amelioration of the activity level of UC by comparative analysis, but being the majority (more than 80%) of enrolled UC patients affected from a mild disease, it was difficult to hypothesize a complete remission only after 4 weeks. In contrast with that comparative system,[19] we documented a beneficial effect of PLC co-treatment on intestinal mucosal inflammation in UC patients, in accordance with preliminary reported data.[18] The second question we tried to solve is to understand through which pathways PLC reduces inflammation and ameliorates intestinal microvascular endothelial dysfunction. Oxidative stress from ROS accumulation has a central role in vascular diseases.[30] Intestinal microvascular endothelial dysfunction in UC patients associates with the increase in oxidative stress, normally absent in intestinal vessels.[31] PLC has already been shown to be a superoxide scavenger[11] that reduces oxidative stress in endothelial cells and cardiomyocytes.[32,33] Experimental models and biopsies from UC patients showed that metabolic changes occur in the diseased colonic mucosa, with an impaired β-oxidation and an energy shortage.[8] This finding led to consider colonocyte metabolism as the therapeutic target of PLC.[19] Our present results strongly support that the adjuvant antioxidant action targeting the endothelial dysfunction mediates the reduction of the inflammation, confirming the intestinal microvasculature as a reliable therapeutic target.[4–6] Endothelial dysfunction determines an increase in permeability that is expected to perpetuate intestinal inflammation by allowing increased passage of microbial products and antigenic proteins into the inflamed mucosa.[34] Although we cannot exclude that PLC also counteracts peroxidative damage in colonocytes, our experimental data show that the clinical improvement in UC patients is driven by the ameliorated microvascular function. In fact, in the rat of TNBS-induced colitis model, acute inflammation induces almost total necrosis of epithelium, which is replaced by granulation tissue. Consequently, it is likely that PLC targets subendothelial tissue cells, in particular the microvascular endothelium. Preliminary experiments performed on PLC-treated intestinal epithelial-microvascular endothelial cell co-cultures did not display further beneficial effects compared with PLC-treated endothelial cell monocultures (Orlandi *et al.*, unpublished results). Moreover, we could not exclude a positive effect of PLC on circulating bone marrow-derived endothelial/hematopoietic progenitor cells, which can be locally recruited and contributing to inflammation, as already reported for vascular diseases.[35] We also reported that the restoration of mitochondrial β-oxidation counteracts intestinal microvascular endothelial dysfunction *in vitro*. In fact, inflammatory stimuli (such as inflammatory cytokines, hypoxia and pathogenic bacteria) induce cellular oxidative stress by mitochondrial ROS generation and activating the downstream-regulated inflammatory response.[36] We demonstrated that anti-inflammatory effect of PLC depends on its anti-oxidant efficacy at the mitochondrial level. In fact, *in vitro* assays with L-aminocarnitine and desipramine showed that PLC did not change TNF-α-activated upstream signals, such as sphingomyelinase activity.[24,25] PLC action counteracted TNF-α-induced mitochondrial dysfunction by the inhibition of ROS and downstream-regulated cellular molecule adhesion expression, with the consequent reduction of leukocyte adhesion and secretion of inflammatory cytokines. Although PLC was initially retained to have an anaplerotic function of providing energy substrates in ischemic tissues,[12] the modulation of oxidative stress better explains the clinical efficacy of PLC in patients with peripheral arterial disease.[16] Ameliorated post-ischemic blood flow recovery and arteriogenesis were also documented in PLC-treated rabbit limb ischemic tissues,[15] in line with the vascular target of PLC. The latter also influenced positively post-injury rabbit carotid remodeling.[20] PLC efficacy was macroscopically and microscopically documented in rat TNBS-induced colitis with various experimental settings, dosages, and routes of

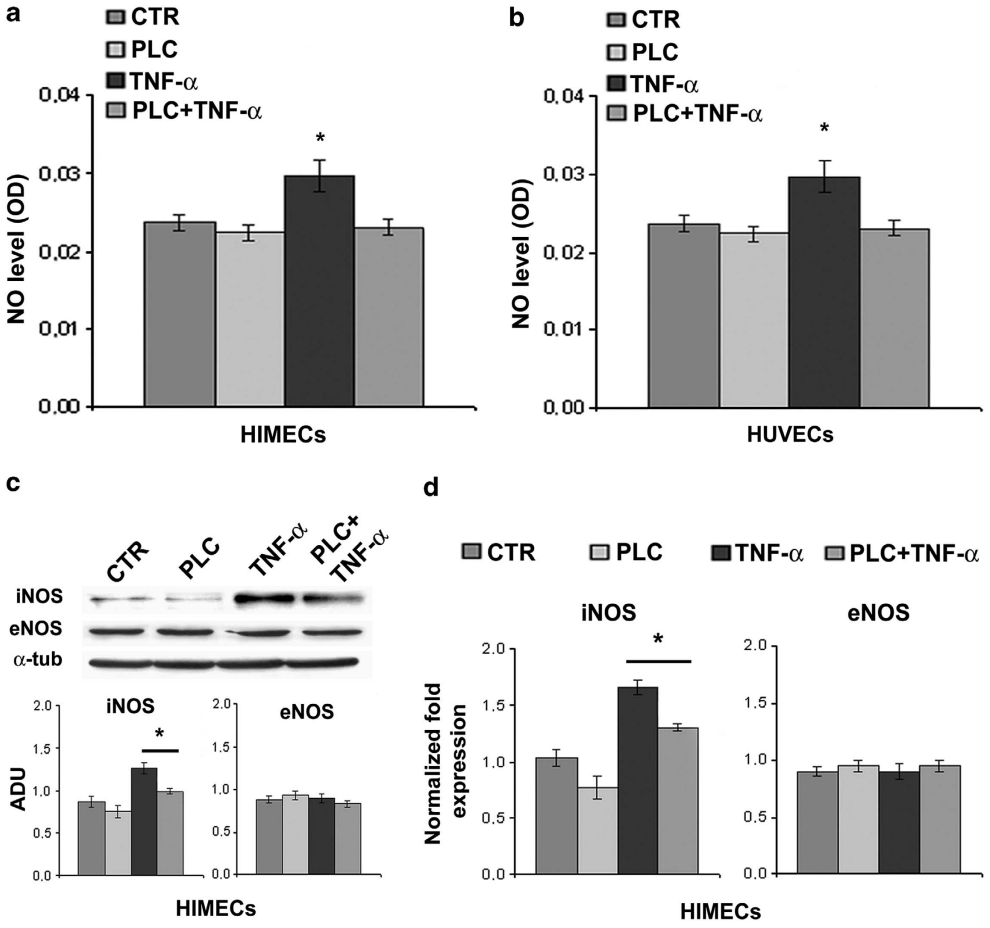

Figure 9 PLC treatment prevents TNF-α-induced increase in iNOS activity. (**a**, **b**) Histograms show nitric oxide (NO) level in TNF-α-treated HIMECs and HUVECs compared with control (CTR; 0.1% FBS), PLC alone (1 mM), and PLC pretreatment. (**c**) Blot analysis for iNOS and eNOS expression in TNF-α-treated HIMECs compared with control (CTR; 0.1% FBS), PLC alone (1 mM), and PLC pre-treatment. (**d**) Real-time PCR for iNOS and eNOS of TNF-α-treated HIMECs compared with control (CTR; 0.1% FBS), PLC alone (1 mM), and PLC pretreatment. Data are shown as mean ± s.e.m. Student's *t*-test: $^*P < 0.05$. ADU, arbitrary densitometric units; OD, optical density; α-tub, α-tubulin.

administration, with effects in some cases comparable to that of 5-ASA. The efficacy in the reduction of mucosal inflammation and CAM expression also in rat TNBS-induced reactivated colitis further supports a potential use of PLC in UC patients who failed to have substantial beneficial effects with classic therapeutic regimens.

Inflammation-induced activation of healthy endothelium determines the up-regulation of vasoactive and mitogenic factors.[30] Nox-derived superoxide has a relevant role in the inflammation-induced vascular dysfunction.[37] In fact, inflammatory stimuli induce an increase in cellular oxidative stress that is driven by mitochondrial and NADPH oxidase-dependent ROS generation.[38,39] The evidence of a interplay between mitochondrial and NADPH oxidase-derived ROS constitutes a feed-forward cycle in which mitochondrial ROS increase NADPH oxidase-dependent ROS production that in turn increases mitochondrial ROS generation, in a vicious cycle.[39] Nox4 is the primary source of superoxide anions in the arterial wall and the main active subunit in HUVECs.[40] ROS generation triggers endothelial synthesis and release of inflammatory mediators, and activates downstream signaling pathways, including ICAM-1, VCAM-1, and the intestinal-specific MAdCAM-1 adhesion molecule expression.[41,42] We documented that PLC-induced reduction of ROS in HIMECs was also mediated by downregulation of Nox4 activity. Moreover, excessive oxidative stress by ROS generation impairs endothelium-derived NO bioactivity. NO is a potent vasodilator that is physiologically produced in endothelial cells by eNOS activity.[43] Inflammation-driven endothelial dysfunction causes the induction of iNOS activity and the consequent abnormal NO production and peroxynitrite accumulation.[44] The increase in iNOS activity and the peroxynitrite accumulation in biopsies from IBD patients, with no change of eNOS expression, has been previously documented.[45] Here we showed that PLC counteracts iNOS expression in intestinal mucosal inflamed vessels of UC patients and in TNF-α-stimulated HIMECs *in vitro*, with no change of eNOS activity. Further studies are needed to verify whether PLC also influences vascular flow and oxygen supply in inflamed intestinal areas.[16]

In conclusion, we demonstrated that PLC efficacy in UC patients is mediated by the ameliorated microvascular dysfunction, with the consequent reduction in inflammatory cell recruitment, through the restoration of endothelial

β-oxidation. Our data suggest that the microvascular targeting of PLC offers further opportunities for pharmacological strategies aimed to counteract the physiopathological changes induced by oxidative stress in other tissues and organs.

CONFLICT OF INTEREST

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Specific author contributions: Maria Giovanna Scioli: conception and design, data analysis and interpretation; Maria Antonietta Stasi: conception and design, data analysis and interpretation; Daniela Passeri: collection and assembly of data; Elena Doldo: collection and assembly of data; Gaetana Costanza: collection and assembly of data; Roberto Camerini: conception and design, data analysis and interpretation; Paolo Fociani: conception and design, data analysis and interpretation; Gaetano Arcuri: collection and assembly of data; Katia Lombardo: collection and assembly of data; Silvia Pace: collection and assembly of data; Franco Borsini: conception and design, data analysis and interpretation; Augusto Orlandi: conception and design, data analysis and interpretation, writing and final approval of the manuscript.
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Potential competing interests: Maria Antonietta Stasi, Roberto Camerini, Katia Lombardo, Silvia Pace and Franco Borsini are employees of Sigma-Tau SpA. All the other authors declared no conflict of interest.

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- Microvascular endothelial dysfunction characterizes ulcerative colitis.
- Propionyl-L-carnitine (PLC) ameliorates propionyl-CoA bioavailability and reduces oxidative stress in ischemic tissues.

WHAT IS NEW HERE

- PLC co-treatment ameliorates mucosa homeostasis by reducing inflammation and intestinal microvascular endothelial dysfunction in ulcerative colitis (UC) patients.
- In the rat TNBS-induced colitis, PLC treatment reduces mucosal inflammation and colonic damage.
- PLC reduces TNF-α-induced intestinal microvascular endothelial cell inflammation improving β-oxidation and preventing oxidative stress.

1. Danese S, Fiocchi C. Ulcerative Colitis. *N Engl J Med* 2011; **365**: 1713–1725.
2. Loftus EV. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* 2004; **126**: 1504–1517.
3. MacDonald TT, Monteleone I, Fantini MC *et al.* Regulation of homeostasis and inflammation in the intestine. *Gastroenterology* 2011; **140**: 1768–1775.
4. Binion DG, West GA, Volk EE *et al.* Acquired increase in leucocyte binding by intestinal microvascular endothelium in inflammatory bowel disease. *Lancet* 1998; **352**: 1742–1746.
5. Ishiguro Y. Mucosal proinflammatory cytokine production correlates with endoscopic activity of ulcerative colitis. *J Gastroenterol* 1999; **34**: 66–74.
6. Hatoum OA. The intestinal microvasculature as a therapeutic target in inflammatory bowel disease. *Ann N Y Acad Sci* 2006; **1072**: 78–97.
7. Roediger WE. The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet* 1980; **2**: 712–715.
8. Chapman MA, Grahn MF. Ileal and colonic epithelial metabolism in quiescent ulcerative colitis. *Gut* 1994; **35**: 1152–1153.
9. Bremer J. Carnitine metabolism and function. *Physiol Rev* 1983; **63**: 1420–1479.
10. Li P, Park C, Micheletti R *et al.* Myocyte performance during evolution of myocardial infarction in rats: effects of propionyl-L-carnitine. *Am J Physiol* 1995; **268**: 1702–1713.
11. Vanella A, Russo A, Acquaviva R *et al.* L -propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector. *Cell Biol Toxicol* 2000; **16**: 99–104.
12. Hiatt WR. Medical treatment of peripheral arterial disease and claudication. *N Engl J Med* 2001; **344**: 1608–1621.
13. Hankey GJ, Norman PE, Eikelboom JW. Medical treatment of peripheral arterial disease. *JAMA* 2006; **295**: 547–553.
14. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension* 2004; **44**: 248–252.
15. Stasi MA, Scioli MG, Arcuri G *et al.* Propionyl-L-carnitine improves postischemic blood flow recovery and arteriogenetic revascularization and reduces endothelial NADPH-oxidase 4-mediated superoxide production. *Arterioscler Thromb Vasc Biol* 2010; **30**: 426–435.
16. Loffredo L, Marcoccia A, Pignatelli P *et al.* Oxidative-stress-mediated arterial dysfunction in patients with peripheral arterial disease. *Eur Heart J* 2007; **28**: 608–612.
17. Bene J, Komlósi K, Havasi V *et al.* Changes of plasma fasting carnitine ester profile in patients with ulcerative colitis. *World J Gastroenterol* 2006; **12**: 110–113.
18. Gasbarrini G, Mingrone G, Giancaterini A *et al.* Effects of propionyl-L-carnitine topical irrigation in distal ulcerative colitis: a preliminary report. *Hepatogastroenterology* 2003; **50**: 1385–1389.
19. Mikhailova TL, Sishkova E, Poniewierka E *et al.* Randomised clinical trial: the efficacy and safety of propionyl-L-carnitine therapy in patients with ulcerative colitis receiving stable oral treatment. *Aliment Pharmacol Ther* 2011; **34**: 1088–1097.
20. Orlandi A, Marcellini M, Pesce D *et al.* Propionyl-L-carnitine reduces intimal hyperplasia after injury in normocholesterolemic rabbit carotid artery by modulating proliferation and caspase 3-dependent apoptosis of vascular smooth muscle cells. *Atherosclerosis* 2002; **160**: 81–89.
21. Ferlosio A, Arcuri G, Doldo E *et al.* Age-related increase of stem marker expression influences vascular smooth muscle cell properties. *Atherosclerosis* 2012; **224**: 51–57.
22. Campagnolo L, Costanza G, Francesconi A *et al.* Sortilin expression is essential for pro-nerve growth factor-induced apoptosis of rat vascular smooth muscle cells. *PLoSOne* 2014; **9**: e84969.
23. Haraldsen G, Kvale D, Lien B *et al.* Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human microvascular endothelial cells. *J Immunol* 1996; **156**: 2558–2565.
24. Corda S, Laplace C, Vicaut E *et al.* Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor-alpha is mediated by ceramide. *Am J Respir Cell Mol Biol* 2001; **24**: 762–768.
25. Marino MW, Dunbar JD, Wu LW *et al.* Inhibition of tumor necrosis factor signal transduction in endothelial cells by dimethylaminopurine. *J Biol Chem* 1996; **271**: 28624–28629.
26. Plevy SE, Targan SR. Future therapeutic approaches for inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1838–1846.
27. Sands BE. Inflammatory bowel disease: past, present, and future. *J Gastroenterol* 2007; **42**: 16–25.
28. Lindsay JO, Ciesielski CJ, Scheinin T *et al.* Local delivery of adenoviral vectors encoding murine interleukin 10 induces colonic interleukin 10 production and is therapeutic for murine colitis. *Gut* 2003; **52**: 363–369.
29. van Deventer SJ, Wedel MK, Baker BF *et al.* A phase II dose ranging, double-blind, placebo-controlled study of alicaforsen enema in subjects with acute exacerbation of mild to moderate left-sided ulcerative colitis. *Aliment Pharmacol Ther* 2006; **23**: 1415–1425.
30. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000; **87**: 840–844.
31. Owczarek D, Cibor D, Mach T. Asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), arginine, and 8-iso-prostaglandin F2alpha (8-iso-PGF2alpha) level in patients with inflammatory bowel diseases. *Inflamm Bowel Dis* 2010; **16**: 52–57.
32. Van Hinsbergh VW, Scheffer MA. Effect of propionyl-L-carnitine on human endothelial cells. *Cardiovasc Drugs Ther* 1991; **5**: 97–105.
33. Calò LA, Pagnin E, Davis PA *et al.* Antioxidant effect of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. *Int J Cardiol* 2006; **107**: 54–60.
34. Stein J, Ries J, Barrett KE. Disruption of intestinal barrier function associated with experimental colitis: possible role of mast cells. *Am J Physiol* 1998; **274**: G203–G209.
35. Orlandi A, Bennett M. Progenitor cell-derived smooth muscle cells in vascular disease. *Biochem Pharmacol* 2010; **79**: 1706–1713.

36. Goossens V, Grooten J, De Vos K *et al.* Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc Natl Acad Sci USA* 1995; **92**: 8115–8119.
37. Lassègue B, San Martín A, Griendling KK *et al.* Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res* 2012; **110**: 1364–1390.
38. Dikalov S. Cross talk between mitochondria and NADPH oxidases. *Free Radic Biol Med* 2011; **51**: 1289–1301.
39. Santos CX, Tanaka LY, Wosniak J *et al.* Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxid Redox Signal* 2009; **11**: 2409–2427.
40. Ago T, Kitazono T, Ooboshi H *et al.* Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation* 2004; **109**: 227–233.
41. Chen YH, Lin SJ, Chen YL *et al.* Anti-inflammatory effects of different drugs/agents with antioxidant property on endothelial expression of adhesion molecules. *Cardiovasc Hematol Disord Drug Targets* 2006; **6**: 279–304.
42. Ogawa H, Binion DG, Heidemann J *et al.* Mechanisms of MAdCAM-1 gene expression in human intestinal microvascular endothelial cells. *Am J Physiol Cell Physiol* 2005; **288**: C272–C281.
43. Gunnett CA, Lund DD, McDowell AK *et al.* Mechanisms of inducible nitric oxide synthase-mediated vascular dysfunction. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1617–1622.
44. McCafferty DM. Peroxynitrite and inflammatory bowel disease. *Gut* 2000; **46**: 436–439.
45. Dijkstra G, Moshage H, van Dullemen HM *et al.* Expression of nitric oxide synthases and formation of nitrotyrosine and reactive oxygen species in inflammatory bowel disease. *J Pathol* 1998; **186**: 416–421.

Supplementary Information accompanies this paper on the Clinical and Translational Gastroenterology website (http://www.nature.com/ctg)