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Inhibition of Platelet-Activating Factor Synthesis in Human Neutrophils and Platelets by Propionyl-L-Carnitine

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ABSTRACT. Propionyl-L-carnitine (PrC) has been shown to exert beneficial effects in the treatment of myocardial and peripheral ischemia in man. These conditions are associated with the activation of circulating neutrophils and platelets. To determine whether PrC could affect the synthesis of lipid mediators known to influence neutrophil and platelet functions, we explored the effects of PrC on the synthesis of platelet-activating factor (PAF) and arachidonic acid (AA) metabolites. Preincubation (90 min) of human neutrophils with PrC (0.1–100 μM) inhibited the synthesis of PAF and of a PAF analog (1-alkyl-1′enyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine: AEGPE) induced *in vitro* by the calcium ionophore A23187. In contrast, concentrations of PrC up to 100 μM did not influence the uptake of exogenous AA or the A23187-induced release of AA and eicosanoids from neutrophils *in vitro*. PrC (1 μM) also inhibited PAF synthesis from human platelets stimulated *in vitro* with thrombin, but had no effect on thrombin-induced aggregation. Oral administration of PrC (2 g/day for two weeks) to five normal volunteers resulted in a significant inhibition of PAF and AEGPE synthesis by neutrophils stimulated with A23187 *ex vivo*, with no effect on AA or eicosanoid release. These data indicate that PrC selectively inhibits *in vitro* and *ex vivo* PAF synthesis from human neutrophils and platelets without influencing AA metabolism or eicosanoid release. This effect of PrC might represent an additional mechanism by which this molecule can exert protective effects in tissue ischemia and in other inflammatory diseases associated with neutrophil and platelet activation. BIOCHEM PHARMACOL **58**;8:1341–1348, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. eicosanoids; neutrophil; PAF; platelet; propionyl-carnitine

Eicosanoids (prostaglandins, thromboxanes, and leukotrienes) and PAF‖ are lipid mediators involved in inflammatory and immune responses, in the regulation of vascular and bronchial tone, and in the pathogenesis of tissue ischemia and thrombosis [1, 2]. A role for these mediators has been proposed in diseases such as bronchial asthma, peripheral and myocardial ischemia, septic shock, and acute respiratory distress syndrome [3, 4]. The synthesis of eicosanoids and PAF in human cells is biochemically linked. The common initial step is the hydrolysis of 1-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine by cytosolic phospholipase A_2, generating free arachidonate and lyso-PAF [5]. Arachidonate can subsequently be converted to eicosanoids by cyclooxygenases and lipoxygenases, whereas acetylation of lyso-PAF by an acetyl transferase generates PAF [6, 7]. Given this biochemical link, eicosanoids and PAF are often produced simultaneously and participate in the development of inflammation and tissue damage [8]. Neutrophils and platelets are major sources of PAF and eicosanoids in humans. Both cells are activated during thrombosis and ischemia to produce lipid mediators [9, 10]. Increasing evidence suggests that PAF and eicosanoids generated in the ischemic area play a major role in the promotion of tissue damage and the amplification of the local inflammatory response [11, 12]. Furthermore, biochemical and pharmacological interventions aimed at reducing lipid mediator synthesis exert beneficial effects in experimentally induced ischemia [13].

L-Carnitine (3-hydroxy-4-*N*-trimethylamino-butyrate) and its short-chain acyl derivative, propionyl-L-carnitine (PrC), are currently under evaluation as therapeutic agents in the treatment of peripheral tissue ischemia [14, 15]. Carnitine is a natural amino acid that plays a fundamental role in the transport of long-chain fatty acids into mitochondria for subsequent β-oxidation [16]. Given its crucial biochemical role, carnitine can modulate several steps of

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‖ *Abbreviations:* AA, arachidonic acid; AEGPE, 1-alkyl-1′-enyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine; 5-HETE, 5-hydroxyeicosatetraenoic acid; LTB_4, leukotriene B_4; lyso-PAF, lyso-platelet-activating factor, 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocoline; and PrC, propionyl-L-carnitine.

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lipid metabolism, such as the levels of intracellular free fatty acids and their incorporation and distribution within membrane phospholipids [17, 18]. These steps are important in regulating the levels of the common precursor 1-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and, therefore, the biosynthesis of eicosanoids and PAF [6, 8]. However, very little is known on the effect of carnitine or PrC on the metabolism of lipid mediators. It has been reported that carnitine enhances the conversion of linoleic acid to AA [19] and modulates prostaglandin and thromboxane production from rat peritoneal macrophages [20, 21]. In contrast, carnitine and short-chain acyl carnitines have no effect on eicosanoid production from bovine endothelial cells [22]. The present study was designed to determine whether PrC might influence the *in vitro* and *ex vivo* synthesis of PAF and eicosanoids from human neutrophils and platelets.

MATERIALS AND METHODS

Materials

Labeled PAF (1-^{3}H-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; 60 Ci/mmol), lyso-PAF (1-^{3}H-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine; 45 Ci/mmol), ^{3}H-arachidonic acid (80 Ci/mmol), and ^{3}H-acetic acid (Na salt; 55 Ci/mmol) were purchased from Du Pont. Ficoll, ADP, collagen, fibrinogen, indomethacin, prostaglandin E_1, prostaglandin B_2, creatine kinase, and phosphocreatine were purchased from Sigma. LTB_4, 20-OH-LTB_4, 20-COOH-LTB_4, and 5-HETE were purchased from Biomol. PrC and its D-isomer (D-PrC) were generous gifts from Sigma-Tau. All reagents were HPLC grade and were purchased from Carlo Erba. PIPES buffer is composed of 25 mM piperazine-*N*,*N*′-bis (2-ethanesulfonic acid), 110 mM NaCl, 5 mM KCl, pH 7.4.

Neutrophil Experiments In Vitro

Neutrophils were isolated by dextran sedimentation followed by centrifugation over Ficoll gradients as previously described [23]. The purity of neutrophils in this preparation was greater than 98% and the viability was greater than 95%, as assessed by trypan blue exclusion. Neutrophils were resuspended at a concentration of 10^7 cells/mL in PIPES buffer containing 1 mM $CaCl_2$ and were incubated at 37° with or without various concentrations of PrC for 30 to 120 min. In some experiments, the cells were prelabeled with ^{3}H-AA (0.1 μCi/mL) or with ^{3}H-lyso-PAF (0.1 μCi/mL) for 90 min. Incorporation of precursors, expressed as percent of radioactivity added to the tubes, was 45 ± 16% and 32 ± 14% for ^{3}H-AA and ^{3}H-lyso-PAF, respectively (N = 3). At the end of the incubation, the cells were washed with buffer containing human serum albumin (0.5 mg/mL) to remove the radioactive lipids not incorporated into the cells. In the experiments in which the incorporation of ^{3}H-acetate into PAF was measured, ^{3}H-acetic acid (1 μCi/mL) was added to the cells immediately before stimulation. Neutrophils were then resuspended (10^7 cells/mL) in PIPES buffer containing 1 mM $CaCl_2$ and 0.5 mg/mL albumin and were stimulated with the Ca^{2+} ionophore A23187 (5×10^{-7} M) for 20 min at 37°. The incubation was stopped by the addition of methanol/chloroform (2:1, v/v).

Platelet Experiments In Vitro

Human platelets were obtained from healthy volunteers who had not taken any medication for the preceeding two weeks. Blood was obtained by venipuncture and anticoagulated with 3.8% (w/v) sodium citrate. Blood was centrifuged ($120 \times g$, 20 min, 22°) to obtain platelet-rich plasma that was further centrifuged ($1000 \times g$, 5 min, 22°) to obtain isolated platelets. Platelets were preincubated (30–120 min, 37°) with various concentrations of PrC and subsequently stimulated with thrombin (0.5 U/mL). Aggregation was measured turbidimetrically as previously described [24]. In another set of experiments designed to measure PAF production, platelets were washed twice in PIPES buffer without calcium containing prostaglandin E_1 (0.1 μg/mL) and were resuspended (300,000 cells/μL) in PIPES buffer containing $CaCl_2$ (1 mM) and fibrinogen (1 mg/mL). Five hundred μL of this platelet suspension was aggregated with human thrombin (0.5 U/mL) and when aggregation reached its maximum (usually within 5 min), 1 mL of methanol was added to the cell suspension and the lipid extract of the whole mixture (cells plus supernatant) then processed for PAF assay (see below).

Neutrophil Experiments Ex Vivo

The study protocol was approved by the Ethical Committee of the University of Naples Federico II. Five healthy males (age range 28–47 years) gave their informed consent to be treated with PrC (2 g/day per os) for two weeks. This protocol was selected according to the results of a dose titration study in patients with intermittent claudication [15]. No side effect was reported by any of the participants in this study. The day before the initiation and at the end of treatment, after overnight fasting, 80 mL of venous blood was drawn by venipuncture. Neutrophil isolation and stimulation with A23187 were performed as described for *in vitro* experiments.

PAF Synthesis and Assay

PAF synthesis in neutrophils was measured as the incorporation of labeled acetate, as previously described [25]. In selected experiments, PAF formation was measured using ^{3}H-lyso-PAF as the labeled precursor. At the end of incubation, the lipids were extracted from cells and supernatants by a modified procedure of Bligh and Dyer [26]. Unlabeled PAF (10 μg/tube) was added as a carrier immediately before the extraction. The lipids were separated by TLC on silica gel G plates developed in chloroform/

methanol/acetic acid/water (50:25:8:3, v/v). This system also separated AEGPE, a PAF analog synthesized by human neutrophils [27]. The radioactivity comigrating on the TLC plates with authentic PAF or AEGPE was detected with a radioactivity scanner (Bioscan, Canberra Packard). The areas of PAF and AEGPE were isolated and the radioactivity was determined by liquid scintillation counting. Intra-assay and interassay variabilities of this procedure were <10% and <15%, respectively. Recovery of PAF and AEGPE standards after extraction and TLC separation ranged from 75 to 85%. PAF production from platelets was measured by a previously described bioassay [24]. Briefly, lipids were extracted from the whole mixture (cells and supernatant) without adding PAF carrier. PAF was isolated by TLC as described above and was extracted from the silica with methanol/water (3:1, v/v). The recovery of authentic PAF with this procedure ranged from 80 to 92%, as determined by extracting parallel samples containing 0.1 μCi of ^{3}H-PAF. The amount of PAF in the samples was then determined using a rabbit platelet aggregation bioassay [24]. Washed rabbit platelets were obtained using the same procedure described above for human platelets. Rabbit platelets were suspended (300,000 cells/μL) in PIPES buffer containing $CaCl_2$ (1 mM), fibrinogen (1 mg/mL), and indomethacin (1 μM) to block thromboxane production, and creatine kinase (13.9 U/mL) and phosphocreatine (0.7 mmol/L) to remove ADP released by aggregating platelets. The amount of PAF in the samples was calculated using standard curves obtained with known amounts of authentic PAF (10 pM–1 μM). The lower limit of detection of this assay was 10 pM.

Separation of 2-Acetyl- and 2-Propionyl-PAF

The two molecular species of PAF (1-alkyl-2-acetyl- and 1-alkyl-2-propionyl-*sn*-glycero-3-phosphocholine) were separated as previously described [25]. In these experiments, neutrophils were prelabeled (90 min, 37°) with ^{3}H-lyso-PAF. The cells were then washed and stimulated (20 min, 37°) with A23187 (5×10^{-7} M). Lipids were extracted from the whole mixture (cells and supernatant) and separated by TLC as described above. The amount of radiolabeled PAF, lyso-PAF, and 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine was determined by liquid scintillation counting. PAF was subsequently isolated by TLC as described above, extracted from the silica with methanol/water (3:1, v/v), and hydrolyzed with phospholipase C [25]. Recovery of initial radiolabeled material with this procedure ranged from 65 to 78%. The resulting diradylglycerols were derivatized with acetic anhydride and separated by TLC on silica gel G plates developed in hexane/diethyl ether/formic acid (90:60:6, v/v). This system clearly separates 1-^{3}H-alkyl-2,3-diacetylglycerol from 1-^{3}H-alkyl-2-propionyl-3-acetylglycerol.

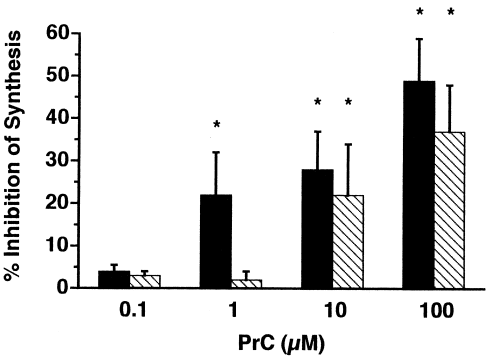

FIG. 1. Effect of different concentrations of PrC on the synthesis of PAF and AEGPE from human neutrophils induced by A23187 (5×10^{-7} M). The data are expressed as percent inhibition of the incorporation of labeled acetate into PAF or AEGPE (N = 6). Mean A23187-induced incorporation was 37,250 ± 8,700 and 19,600 ± 6,300 dpm/10^7 cells for PAF and AEGPE, respectively. *P < 0.01.

HPLC of Eicosanoids

LTB_4, 20-OH-LTB_4, 20-COOH-LTB_4, and 5-HETE released in the supernatant of stimulated neutrophils were separated by reverse-phase HPLC and quantitated using prostaglandin B_2 (250 ng) as internal standard, as previously described [23].

Statistical Analysis

The results were expressed as means ± SE. Multiple comparisons among different groups were performed by one-way ANOVA followed by Student's *t*-test with Bonferroni's correction [28].

RESULTS

Effect of PrC on PAF Synthesis and AA Metabolism in Human Neutrophils In Vitro

In a first group of experiments, we evaluated the effect of PrC on the synthesis of PAF from human neutrophils stimulated *in vitro* with A23187. Initial kinetic experiments indicated that the effect of PrC required a preincubation time of at least 30 min to be evident and that it was maximal with a preincubation ranging from 90 to 120 min (data not shown). Therefore, all experiments were performed with a preincubation time of 90 min. Preincubation of neutrophils with PrC (0.1–100 μM) inhibited PAF synthesis in a concentration-dependent fashion (Fig. 1). The inhibitory effect of PrC was significant ($P < 0.01$) at 1 μM and reached a maximum of 48.3 ± 10.4% inhibition at 100 μM. No further inhibition was observed at higher concentrations of PrC (data not shown). PrC did not influence cell viability at concentrations up to 5 mM. Human neutrophils stimulated with A23187 also produce a PAF analog, AEGPE, that is synthesized through the same biochemical steps of PAF [29] and possesses PAF-like activities [30]. Preincubation of neutrophils with PrC also inhibited the synthesis of AEGPE (Fig. 1). The inhibitory

TABLE 1. Effect of PrC and D-PrC on A23187-induced PAF synthesis in neutrophils labeled with ^{3}H-lyso-PAF

Conditions	Lyso-PAF	PAF	1-alkyl-2-acyl-GPC
	% of total radioactivity		
None	12.5 ± 4.3	2.1 ± 0.6	84.7 ± 6.6
PrC, 10^{-5} M	11.2 ± 3.8	2.2 ± 0.9	85.2 ± 5.8
PrC, 10^{-4} M	14.9 ± 4.5	2.1 ± 0.6	82.0 ± 6.0
None + A23187	16.4 ± 4.1	10.5 ± 2.6	72.1 ± 8.4
PrC, 10^{-5} M + A23187	17.0 ± 5.5	6.9 ± 1.4*	75.0 ± 7.6
PrC, 10^{-4} M + A23187	17.9 ± 5.0	4.4 ± 0.8*	76.5 ± 6.6
D-PrC, 10^{-5} M + A23187	16.8 ± 4.6	10.3 ± 3.2	71.6 ± 7.2
D-PrC, 10^{-4} M + A23187	17.5 ± 6.2	11.0 ± 2.7	70.4 ± 9.5

Neutrophils (10^7/mL) were prelabeled (37°, 90 min) with 1-^{3}H-lyso-PAF either in the absence or presence of the indicated concentrations of PrC, washed, and subsequently stimulated (20 min, 37°) with A23187 (5×10^{-7} M). At the end of the incubation, lipids were extracted from the whole incubation mixture (cells and supernatants) and analyzed by TLC. Radioactivity in the areas of lyso-PAF, PAF, and 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine was determined by liquid scintillation counting. The data are the means ± SE of three experiments. *$P < 0.05$ vs none + A23187.

effect on AEGPE synthesis was significant ($P < 0.01$) at 10 μM and reached a maximum of 36.5 ± 8.6% at 100 μM. It has been reported that only 10–20% of PAF produced by neutrophils stimulated with A23187 is released in the supernatant, the majority remaining cell-associated [31]. To determine whether PrC influences the release of PAF, neutrophil pellets and supernatants were analyzed separately. PAF release, expressed as percent of total PAF produced, was 12 ± 5% and 14 ± 6% in control and PrC-treated cells, respectively. Therefore, PrC did not modify the amount of PAF released and that retained within the cells.

In another group of experiments, we evaluated the effect of PrC on the release of AA and its metabolites from human neutrophils. The cells were prelabeled with ^{3}H-AA and then preincubated (90 min) with increasing concentrations of PrC before stimulation with A23187. Initial experiments demonstrated that incubation of neutrophils with PrC did not modify the uptake or distribution of ^{3}H-AA within cellular phospholipids (data not shown). Concentrations of PrC up to 100 μM had no effect on the release of radiolabeled AA from neutrophils stimulated with A23187 (control: 17.8 ± 2.8%; 100 μM PrC: 17.2 ± 2.4% of total cellular AA; N = 5). In addition, HPLC analysis revealed that concentrations of PrC up to 100 μM had no effect on A23187-induced release of the four major eicosanoids produced by neutrophils. Eicosanoid release, expressed as pmol/10^7 cells in control and PrC-treated cells, was: LTB_4: 80 ± 35 vs 74 ± 28; 20-OH-LTB_4: 24 ± 9 vs 27 ± 12; 20-COOH-LTB_4: 20 ± 6 vs 24 ± 14; 5-HETE 15 ± 6 vs 12 ± 5 (N = 3). Once in the cell, PrC is rapidly converted to propionyl-coenzyme A and free carnitine [32], raising the intracellular content of propionyl groups. Propionate can be incorporated instead of acetate at the *sn*-2 position of lyso-PAF, leading to the formation of propionyl-PAF [33]. Such a change would appear in our assay as a reduction of PAF synthesis, since it was based on the incorporation of radiolabeled acetate. To verify this possibility, we used an alternative PAF assay in which the neutrophils were prelabeled for 90 min with ^{3}H-lyso-PAF either in the absence or in the presence of PrC, and subsequently stimulated with A23187. As previously shown [5], under these conditions the majority of labeled lyso-PAF was incorporated into 1-^{3}H-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. PrC had no effect on the incorporation of lyso-PAF into 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (Table 1). Stimulation of neutrophils with A23187 resulted in ^{3}H-PAF formation, which was inhibited by PrC but not by the inactive isomer propionyl-D-carnitine (D-PrC) (Table 1). In addition, radiolabeled PAF produced in these experiments was extracted and the relative proportions of 2-acetyl- and 2-propionyl-PAF were determined by TLC separation. Table 2 shows that 2-propionyl-PAF never exceeded 10% of the total PAF even in samples incubated with the highest concentration of PrC. Therefore, the inhibition of PAF synthesis by PrC was not due to a redirection of the precursor (lyso-PAF) toward the synthesis of 2-propionyl-PAF.

TABLE 2. Effect of PrC on the relative proportion of 2-acetyl- and 2-propionyl-PAF synthesized by A23187-stimulated neutrophils

Condition	2-acetyl-PAF (dpm/10^8 cells)	2-propionyl-PAF (dpm/10^8 cells)
None	8305 ± 256	1020 ± 179
PrC, 10^{-7} M	7345 ± 271	864 ± 202
PrC, 10^{-6} M	6906 ± 307	644 ± 205
PrC, 10^{-5} M	6398 ± 268	492 ± 195
PrC, 10^{-4} M	4358 ± 379	364 ± 173

Neutrophils (10^8/mL) were prelabeled (37°, 90 min) with 1-^{3}H-lyso-PAF either in the absence or in the presence of the indicated concentrations of PrC and subsequently stimulated (20 min, 37°) with A23187 (5×10^{-7} M). At the end of the incubation, lipids were extracted from the whole incubation mixture (cells and supernatants). Radiolabeled PAF was isolated by TLC, digested with phospholipase C and derivatized with acetic anhydride. 2-Acetyl-PAF and 2-propionyl-PAF were separated by TLC and their amounts were determined by liquid scintillation counting.

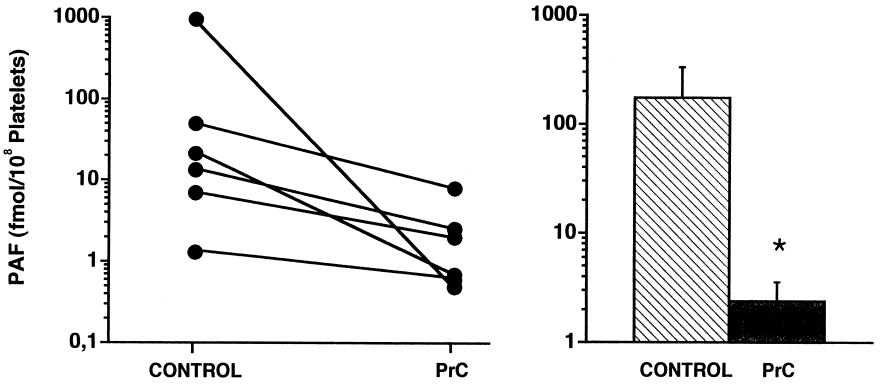

FIG. 2. Effect of PrC (1 μM) on the synthesis of PAF from human platelets induced by thrombin *in vitro*. The data are expressed as fmol of PAF/10^8 platelets in each individual donor (N = 6) (left panel) and as the mean ± SE (right panel). *P < 0.001.

Effect of PrC on PAF Synthesis in Human Platelets In Vitro

Platelets are a major source of PAF in humans [34]. In a series of six experiments, we explored the effect of PrC on PAF synthesis from washed human platelets stimulated with thrombin *in vitro*. Preincubation (90 min) of platelets with PrC (1 μM) completely inhibited the synthesis of PAF induced by thrombin (Fig. 2). Mean percentage inhibition of PAF synthesis was 98.2 ± 7.5% ($P < 0.001$). Even though PAF synthesis was almost completely suppressed, PrC had no effect on thrombin-induced platelet aggregation (data not shown).

Effect of PrC on PAF Synthesis and AA Release from Human Neutrophils Ex Vivo

This part of the study was designed to determine whether PrC at pharmacological concentrations obtained *in vivo* was able to inhibit PAF synthesis from neutrophils *ex vivo*. PrC (2 g/day) was given orally for two weeks to five healthy volunteers. This protocol raised the plasma level of PrC from 0.6 ± 0.4 to 25 ± 9 μM (N = 4). Neutrophils were isolated before and at the end of treatment period and were stimulated with A23187. PAF and AEGPE synthesis was then measured as the incorporation of radiolabeled acetate. Figure 3 shows the kinetics of PAF and AEGPE production in neutrophils from an individual donor before (control) and after PrC administration. The formation of PAF (Fig. 3A) and AEGPE (Fig. 3B) induced by A23187 was reduced at any time point explored after treatment with PrC. As shown in Fig. 3, the synthesis of PAF and AEGPE reached a plateau 20 min after stimulation with A23187. Therefore, this time point was selected for subsequent analysis in each individual donor. Oral administration of PrC caused a reduction in the amount of PAF produced by neutrophils from all volunteers participating in the study (Fig. 4A). Average PAF production before and after administration of PrC (in dpm of ^{3}H-PAF/10^7 cells) was 44,600 ± 8,200 and 30,100 ± 6,700, respectively ($P < 0.05$). The mean percentage inhibition was 33.8 ± 9.5%. Oral administration of PrC also inhibited A23187-induced synthesis of AEGPE (Fig. 4B). Average AEGPE production before and after two weeks of PrC administration (in dpm of ^{3}H-AEGPE/10^7 cells) was 18,500 ± 3,200 and 12,100 ± 2,900, respectively ($P < 0.05$). Mean percentage inhibition of AEGPE synthesis was 38.4 ± 10.2.

In agreement with the data obtained *in vitro*, oral

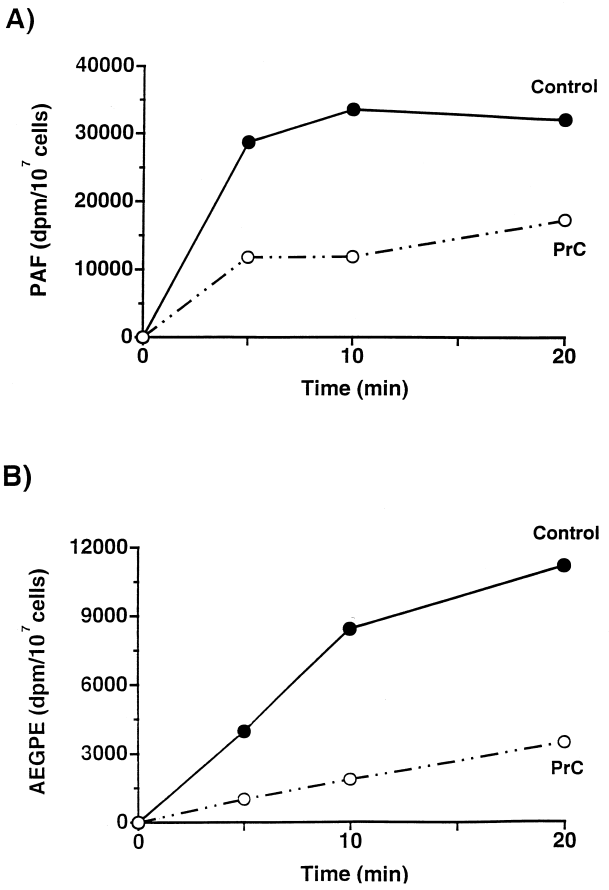

FIG. 3. Kinetics of PAF (A) and AEGPE (B) synthesis from neutrophils obtained *ex vivo* from an individual donor before (control) and after oral administration of PrC (2 g/day for 2 weeks). The data are expressed in dpm of labeled acetate incorporated into PAF or AEGPE/10^7 cells. Similar results were obtained with four other donors.

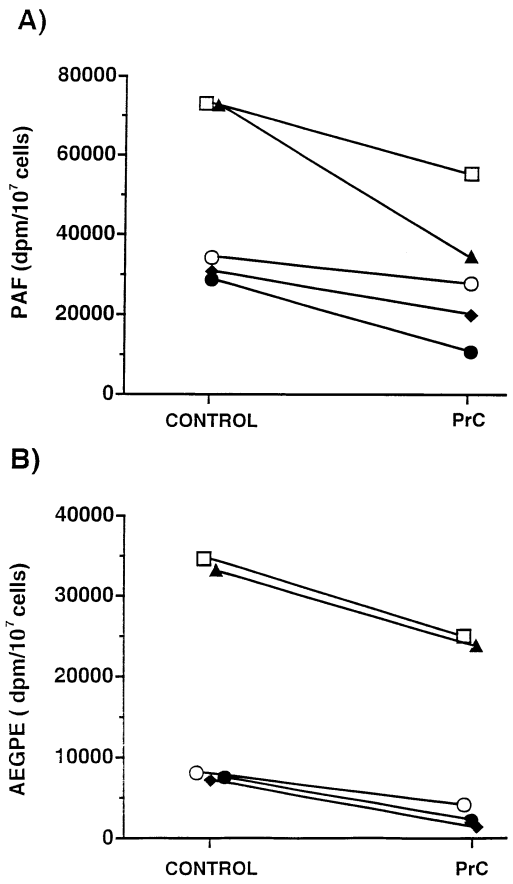

FIG. 4. Effect of oral administration of PrC on the synthesis of PAF (A) and AEGPE (B) from human neutrophils induced by A23187 *ex vivo*. The data of each individual donor (N = 5) are expressed in dpm of labeled acetate incorporated into PAF or AEGPE/10^7 cells. Each symbol represents an individual donor.

administration of PrC had no effect on the metabolism of AA in neutrophils obtained *ex vivo*. The amount of LTB_4, 20-OH-LTB_4, 20-COOH-LTB_4, and 5-HETE produced after A23187 stimulation was not significantly different in neutrophils before and after PrC administration (data not shown). The profile of eicosanoids produced by neutrophils *ex vivo* was similar to that reported in the experiments *in vitro*.

DISCUSSION

Increasing evidence suggests a major role of lipid mediators in the pathogenesis of ischemic vascular diseases [11, 12, 35]. In addition to prostacyclin and thromboxane, other molecules produced by neutrophils and platelets within the ischemic area, such as the 5-lipoxygenase metabolites and PAF [36, 37], can produce local vasoconstriction and induce further recruitment and activation of leukocytes and platelets [38]. A number of experimental and clinical studies have shown that leukocytes, particularly neutrophils, circulating within an ischemic area display morphological and functional features of activation, i.e. ruffling, degranulation, and expression of adhesion molecules [10, 39]. Blockade of neutrophil influx and/or activation exerts a protective effect in experimental models of ischemia and reperfusion [38, 40]. Human and animal studies indicate that carnitine and its derivative PrC exert a beneficial effect on peripheral ischemia by increasing the metabolic efficiency of the skeletal muscle [32, 41]. Recent experimental evidence also suggests that carnitines might influence the function of inflammatory cells with pharmacological characteristics that are not explained by their effect on oxidative metabolism [42]. The physiological plasma level of carnitine in humans is approximately 40 μM, 80% of which is free carnitine and the rest short-chain acylcarnitines including acetylcarnitine and PrC [43]. Carnitine is mostly synthesized in the liver and in the kidney, but a significant proportion derives from dietary intake, predominantly from animal food, and it is absorbed from the small intestine [44]. Carnitine levels are reduced in a number of pathological conditions such as systemic infections, septic shock, chronic inflammatory diseases, peripheral ischemia, and liver or kidney insufficiency. This secondary deficiency is due to a variable combination of increased metabolic need, malnutrition, reduced intestinal absorption, and increased urinary elimination [45]. Under these conditions, oral or parenteral supplementation is effective in maintaining tissue and plasma levels of carnitine [14]. PrC is a short-chain acylcarnitine that is absorbed and concentrated in various cells and tissues more readily than carnitine [32, 46]. Upon entering the cells, PrC, but not its D-isomer, is rapidly hydrolyzed to free carnitine and propionate [32]. Thus, PrC is an effective pharmacological means to raise intracellular concentrations of carnitine.

In the present study, we investigated the effects of PrC on the synthesis of lipid mediators from human neutrophils and platelets. Incubation of neutrophils with PrC reduces the production of PAF and of AEGPE, an analog with PAF-like properties [30], in response to A23187. In addition, PrC almost completely suppressed the synthesis of PAF in human platelets stimulated with thrombin *in vitro*. PrC had no effect on platelet aggregation induced by thrombin and, therefore, its inhibitory effect on PAF synthesis in platelets was not due to an interference with the mechanisms of aggregation. In contrast, PrC did not modify the incorporation of AA within neutrophils during short-term labeling, nor did it influence the release of AA or the profile of eicosanoids produced during neutrophil activation. Inhibition of PAF synthesis from neutrophils and platelets *in vitro* occurred at concentrations of PrC that can be achieved *in vivo* during oral supplementation. To confirm this, the effect of PrC was also evaluated in experiments in which the drug was administered *in vivo* to healthy volunteers. Oral administration of PrC at the therapeutic doses used in the treatment of peripheral ischemia resulted in a significant inhibition of PAF and AEGPE synthesis from neutrophils with no effect on AA or eicosanoid release. These data confirm that inhibition of PAF synthesis may be achieved *in vivo* during treatment with PrC. In agreement with previous studies [23, 47], there

was a large variability in the amount of PAF produced by neutrophils and platelets in our experiments. However, PrC consistently reduced the formation of this mediator in all experimental preparations and in all volunteers undergoing treatment *in vivo*.

A series of experiments was designed to identify the biochemical step(s) inhibited by PrC. Most of the currently available inhibitors of PAF synthesis in neutrophils are inhibitors of PLA_2 [48]. However, PrC did not influence PLA_2 or 5-lipoxygenase activities, since it did not modify the release of AA and eicosanoids. At least two mechanisms could explain the inhibitory effect of PrC: (i) an enhanced rate of catabolism of PAF and (ii) an increased removal of precursors (lyso-PAF and/or acetyl-coenzyme A). The simultaneous, although less effective, inhibition of AEGPE production can be explained by either one of these mechanisms. Both PAF and, to a lesser extent, AEGPE are catabolized by acetyl hydrolase [49], an enzyme widely distributed in plasma and in various mammalian cells including neutrophils and platelets. Acetyl hydrolase is irreversibly inactivated by oxygen free radicals [50]. Although not a direct scavenger, PrC has been reported to reduce oxygen radical formation in human neutrophils [51]. Thus, the possibility exists that PrC may increase the catabolism of PAF by preventing inactivation of neutrophil acetyl hydrolase by endogenous oxygen radicals. This hypothesis is supported by the observation of a protective effect of PrC reported in an experimental model of PAF-induced inflammation [52].

An alternative mechanism to explain the inhibitory effect of PrC is the subtraction of acetate groups necessary for conversion of lyso-PAF to PAF. Free carnitine is a rapid acceptor of acetate groups from acetyl-coenzyme A [16]. Therefore, it is conceivable that free carnitine derived from PrC may remove an important precursor of PAF. This hypothesis is supported by a number of observations. First, the effect of PrC is not immediate, but requires a preincubation time of 90 to 120 min. Second, the D-isomer of PrC, which enters the cells but is not hydrolyzed, does not inhibit PAF synthesis (Table 1). Third, more than 80% of PrC incubated with neutrophils is converted to acetylcarnitine.* Therefore, although our data do not conclusively prove the mechanism by which PrC reduces the synthesis of PAF and AEGPE, they do strongly support the hypothesis that PrC acts by inducing a depletion of acetyl-coenzyme A in neutrophils and platelets.

In conclusion, our results indicate that an additional anti-inflammatory activity may contribute to the beneficial effect of PrC in peripheral ischemia. PrC appears to inhibit a biochemical step selective for PAF synthesis without influencing AA metabolism. These data provide a rationale to further explore the effects of PrC in other inflammatory diseases such as systemic infections, septic shock, and the adult respiratory distress syndrome.

* Triggiani M, Oriente A, Brevetti G and Marone G, unpublished observations.

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