Biochemical Pharmacology, Vol. 38, No. 19, pp. 3261–3266, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

PALMITOYL-L-CARNITINE INCREASES THE RELEASE OF PROSTACYCLIN FROM VASCULAR ENDOTHELIAL CELLS

JEAN-MARIE BOEYNAEMS,* ISABELLE RAMBOER and SABINE PIROTTON
Institute of Interdisciplinary Research, School of Medicine, Free University of Brussels, Campus Erasme, Route de Lennik 808, B-1070 Brussels, Belgium

(*Received* 10 *February* 1989; *accepted* 31 *March* 1989)

Abstract—Prostacyclin biosynthesis is dramatically increased in patients with acute myocardial infarction. As palmitoylcarnitine accumulates during myocardial ischemia, the action of this metabolite on the endothelial production of prostacyclin was studied. Palmitoyl-L-carnitine (10–100 μM) enhanced the release of prostacyclin and free arachidonic acid from bovine aortic endothelial cells. This action was mimicked by lysophosphatidylcholine, but by none of the following compounds: acetylcarnitine, carnitine, palmitic acid, sphingosine, dihydrosphingosine and *N*-stearoyl-dihydrosphingosine. In addition to mobilizing free arachidonate, palmitoylcarnitine induced the release of free choline and phosphorylcholine presumably via the activation of phospholipases C and D. Palmitoyl-L-carnitine had also a cytotoxic effect on the endothelial cells. These data suggest that the increased biosynthesis of prostacyclin in myocardial infarction might be partially explained by the accumulation and release of palmitoyl-L-carnitine.

In various animal models, hypoxia or ischemia activate phospholipase A_2 and prostaglandin biosynthesis in the heart [1–5]. In particular, ischemia increases the release of prostacyclin (PGI_2), which is the major cardiac metabolite of arachidonic acid [5–7]. The biosynthesis of PGI_2 is tremendously increased in patients with acute myocardial infarction [8, 9]. Since this enhancement is unrelated to and exceeds the simultaneous increase of thromboxane A_2 biosynthesis, it is unlikely to result from platelet activation alone, as is the case in unstable angina [8] or severe atherosclerosis of lower limbs [10]. The increase in PGI_2 might derive from the infarcted myocardium itself, from adjacent endocardial cells or from damaged endothelial cells in the infarct zone. One striking biochemical consequence of myocardial ischemia is an increase in the level of long-chain acylcarnitines, mostly palmitoyl-L-carnitine, and of lysophosphoglycerides [11–13]. Normally present in mitochondria, palmitoyl-L-carnitine accumulates at mM levels in the cytosol of ischemic cardiomyocytes: it is also present in the sarcolemma, leaks to the extracellular space and is transported in blood [14, 15]. In this study, we have investigated the action of palmitoyl-L-carnitine on the synthesis of PGI_2 in vascular endothelial cells, in order to determine if this metabolite might contribute to the increased biosynthesis of PGI_2 in myocardial infarction.

MATERIALS AND METHODS

(A). *Preparation and culture of bovine aortic endothelial cells*. Bovine aortic endothelial cells were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow, as previously described [16, 17]. The cells were seeded on 100-mm Petri dishes and incubated at 37°, under an atmosphere of 5% CO_2/95% air, in the following medium: MEM D-valine (80%, v/v), fetal calf serum (20%, v/v), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B. The medium was changed the following day and later on every 3 days. After 4 or 5 days, the primary cultures formed confluent monolayers and could be subcultured. The cells were detached by a 5-min incubation in a Ca^{2+}- and Mg^{2+} free Hanks buffer containing trypsin (10 mg/dl) and EDTA (1 mM). Thereafter, they were seeded on 35-mm Petri dishes and the culture was pursued in the following medium: DMEM (60%, v/v), Ham's F_{12} (20%, v/v), fetal calf serum (20%, v/v) with the same concentrations of penicillin, streptomycin and amphotericin B as mentioned above. For the measurement of PGI_2 release, the cells were washed twice and incubated for 30 min up to 2 hr in DMEM (± 10^6 cells in 1 ml) at 37°, in the presence of the tested agents. All the compounds tested were dissolved in ethanol, which was present at a 0.5% final concentration. It has been checked that, at this concentration, ethanol *per se* has no effect on the release of PGI_2.

(B). *Prostaglandin radioimmunoassay (RIA)*. The production of PGI_2 was measured by the RIA of its stable degradation product, prostaglandin 6-keto-$F_{1\alpha}$ (6-K-$PGF_{1\alpha}$),† performed directly in the incubation medium, without extraction or chromatography. A rabbit antiserum was raised against 6-K-$PGF_{1\alpha}$ coupled to bovine serum albumin, as described [18]: the limit of detection was 16pg and the cross-reactions were 1.2% with $PGF_{2\alpha}$, 0.3% with PGE_2 and 0.1% with thromboxane B_2. 100 μl aliquots of incubation media, [^{3}H]-6-K-$PGF_{1\alpha}$ a(11,000 dpm), anti-6-K-$PGF_{1\alpha}$ antiserum (final dilution: 10^{-4}) and bov-

* Address for correspondence: J. M. Boeynaems, I.R.I.B.H.N., Campus Erasme, Bldg C, Route de Lennik 808, 1070 - Brussels, Belgium.

† Abbreviations used: 6-K-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; GC, gas chromatography; ECD, electron capture detection.

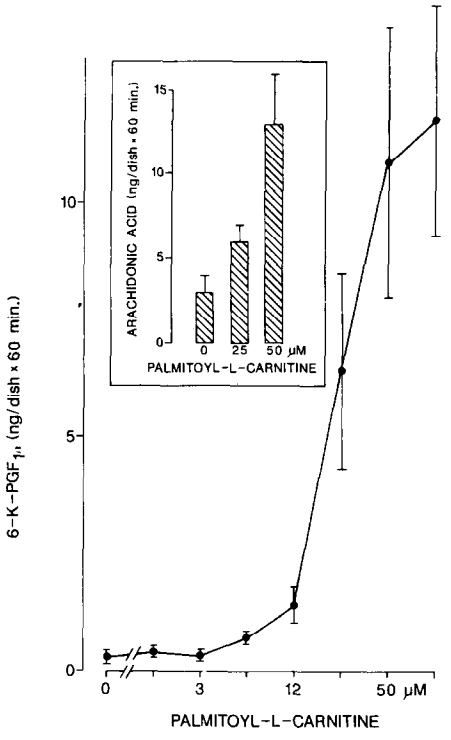

Fig. 1. Stimulatory effect of palmitoyl-L-carnitine on the release of PGI_2 from bovine aortic endothelial cells: concentration–action curve. The cells (10^6/dish) were incubated for 60 min in 1 ml DMEM, containing various concentrations of palmitoyl-L-carnitine. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of this incubation (mean ± SD of triplicate determinations in 1 representative experiment out of 3). Inset: Mobilization of free arachidonic acid in bovine aortic endothelial cells in response to palmitoyl-L-carnitine. The cells were incubated for 60 min in a Krebs–Ringer medium buffered with Hepes, containing BSA (1 mg/ml) and indomethacin (1 μg/ml). Arachidonic acid accumulated in the medium was measured by gas chromatography with electron capture detection, as described in Methods. Results represent the mean ± SD of triplicate determinations in 1 representative experiment out of 3.

Table 1. Specificity of the stimulatory effect of palmitoyl-L-carnitine on the release of PGI_2 from bovine aortic endothelial cells

	6-K-$PGF_{1\alpha}$ (ng/dish × 30 min)
—	0.2 ± 0.1
Palmitoyl-L-carnitine (50 μM)	8.7 ± 1.8
Palmitoyl-DL-carnitine (50 μM)	7.9 ± 3.1
Acetyl-DL-carnitine (50 μM)	1.3 ± 0.8
Acetyl-DL-carnitine (100 μM)	0.5 ± 0.2
Carnitine (50 μM)	0.7 ± 0.2
Carnitine (100 μM)	0.5 ± 0.3
Palmitic acid (50 μM)	1.1 ± 0.5

The cells were incubated for 30 min in the presence of the various compounds tested. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of this incubation (mean ± SD of triplicate determinations in 1 representative experiment out of 2).

Table 2. Specificity of the stimulatory effect of palmitoyl-L-carnitine on the release of PGI_2 from bovine aortic endothelial cells

	6-K-$PGF_{1\alpha}$ (ng/dish × 60 min)
—	0.5 ± 0.1
Palmitoyl-L-carnitine	10.0 ± 4.0
Erythro-dihydrosphingosine	0.9 ± 0.2
Threo-dihydrosphingosine	0.8 ± 0.2
N-stearoyl-DL-dihydrosphingosine	0.4 ± 0.1
Ceramides	0.5 ± 0.4

The cells were incubated for 60 min with the various compounds tested, all at a 100 μM concentration. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of this period (mean ± SD of triplicate determinations in 1 representative experiment out of 3).

ine gamma globulins (0.25 g/dl) in Tris buffer (50 mM, pH 7.4) were incubated in a total volume of 0.4 ml for 60 min at room temperature. Then 0.4 ml of a cold 25% (wt/wt) solution of polyethylene glycol was added to separate bound and free antigen. Results were corrected for the cross-reactions of palmitoyl-L-carnitine and L-α-lysophosphatidylcholine which were, respectively, 0.08% and 0.011%.

(C). *Assay of free arachidonic acid release.* Arachidonic acid was measured by gas liquid chromatography (GLC) with electron capture detection (ECD) as described [19]. Incubation of endothelial cells was performed in a Krebs–Ringer medium, buffered with Hepes (25 mM, pH 7.4), containing BSA (1 mg/ml) and indomethacin (1 μg/ml). After the addition of 1 μg docosahexaenoic acid as an internal standard, the incubation medium was extracted with 1 vol. ethyl acetate. The free fatty acids were converted into pentafluorobenzyl esters by a modification of the method of Wickramasinghe *et al.* [20]. To the dry residue of the extract were added 290 μg pentafluorobenzylbromide (in 5 μl acetonitrile) and 44 μg diisopropylethylamine (in 5 μl acetonitrile). After 5 min at 40° and evaporation of the solvent under nitrogen, the samples were redissolved in 50 μl hexane. The 20-fold reduction in the amounts of reagents used, as compared with the original procedure, made it possible to directly analyse the samples without prior purification. GLC analysis was performed in a Varian instrument (model 3700: Varian Associates, Palo Alto, CA) equipped with a ^{63}Ni ECD. Two-m columns of 3% OV-1 on Gas chrom Q (Applied Science Laboratories, Div. of A.S. Laboratories, Inc., Waltham, MA) were used isothermally at 235°.

(D). *Assay of water-soluble choline metabolites.* Bovine aortic endothelial cells were labelled with [^{3}H]-choline (10 μCi/ml) for the last 24 hr of the culture period. After removal of the culture medium, they were washed twice and incubated in DMEM, in the presence of various concentrations of palmitoyl-L-carnitine. Aliquots of the incubation medium

Table 3. Stimulation of PGI_2 release from bovine aortic endothelial cells: comparison between palmitoyl-L-carnitine and L-α-lysophosphatidylcholine

(μM)	Palmitoyl-L-carnitine	L-α-lyso phosphatidylcholine
0	0.8 ± 0.2	
25	30 ± 2	15 ± 1
50	26 ± 4	30 ± 3
100	ND	28 ± 2

The cells were incubated for 60 min with the tested compounds. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium at the end of this incubation (mean ± SD of triplicate determinations in 1 representative experiment out of 3).

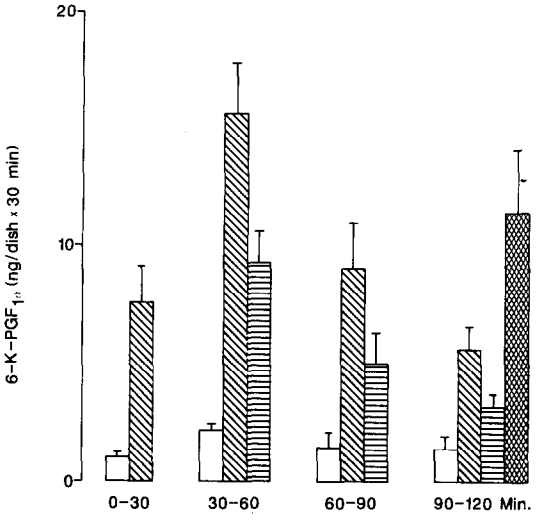

Fig. 2. Time course of the PGI_2 release from bovine aortic endothelial cells stimulated by palmitoyl-L-carnitine. The cells were incubated in 1 ml DMEM for 4 successive periods of 30 min: the medium was collected and changed at the end of each period. Results represent the amount of 6-K-$PGF_{1\alpha}$ accumulated in the medium during each period (mean ± SD in 1 representative experiment out of 3: each condition was tested in triplicate). □: Control; ▨: palmitoyl-L-carnitine (50 μM); ☰: palmitoyl-L-carnitine (50 μM) present only during the 1st period; ▩: palmitoyl-L-carnitine (50 μM) readded during the 4th period, after being present during the 1st one and absent for the 2nd and 3rd periods.

were collected at various times for liquid scintillation counting. At the end of the incubation, the medium was removed and 2 ml TCA (25%) were added to the cell monolayer. Proteins were eliminated by centrifugation and the cell extract was then washed 4 times with 4 ml diethylether. Separation of choline from phosphorylcholine was performed by high-performance liquid chromatography (HPLC), as described [21]. Aliquots of incubation media and cell extracts were injected on a μ Porasil column (3.9 mm × 30 cm, 10 μ particles). Elution was performed with a mixture of acetonitrile, water, ethanol, acetic acid and 0.83 M sodium acetate in water: the composition of this mixture was 300:127:68:2:3 (v/v) in initial conditions (A) and 400:400:68:53:79 (v/v) in final conditions (B). After 5 min in initial conditions, a linear gradient from 0 to 100% B was developed in 30 min: final conditions were maintained for 15 min. The flow rate was 2.7 ml/min and 1 min fractions were collected for liquid scintillation counting. The injector (U6K), the pumps (6000A) and the gradient programmer (600) were from Waters.

(E). *Measurement of cytotoxicity*. Bovine aortic endothelial cells were labelled with [^{3}H] thymidine (5 μCi/ml) for the last 24 hr of the culture period. They were then washed twice and incubated in DMEM, with or without palmitoyl-L-carnitine. Aliquots of medium were collected at various times for liquid scintillation counting. At the end of the incubation, the medium was removed, the cells were lysed in a hypotonic solution of pronase (20 μg/ml) and DNA was measured by fluorimetry, using the ethidium bromide method, as described [22].

Materials. MEM-D-valine, DMEM, Ham's F_{12}, glutamine, streptomycin, penicillin, fungizone were purchased from Flow Laboratories (Irvine, U.K.). Fetal calf serum was obtained from Gibco (Uxbridge, U.K.) and collagenase type II from Cooper Biomedical. Palmitoyl-L-carnitine, palmitoyl-DL-carnitine, acetyl-DL-carnitine, carnitine, palmitic acid, D-sphingosine, erythro- and threo-dihydrosphingosine, *N*-stearoyl-DL-dihydrosphingosine ceramides and L-lysophos-phatidylcholine were obtained from Sigma Chem. Co. (St Louis, MO). [^{3}H]-6-K-$PGF_{1\alpha}$, [^{3}H]-choline, [^{14}C]-phosphorylcholine and [^{3}H]-thymidine were purchased from Amersham (Bucks, U.K.). 6-K-$PGF_{1\alpha}$ was obtained from Upjohn Diagnostics (Kalamazoo, MI).

RESULTS

Palmitoyl-L-carnitine increased the release of PGI_2 from bovine aortic endothelial cells. This effect was characterized by a steep concentration–action curve (Fig. 1). At 50 μM palmitoyl-L-carnitine, the stimulation was 27 ± 5 fold the control value (mean ± SE of 15 experiments; range: 4–76). Indomethacin (28 μM) produced a 88% inhibition of the release of PGI_2 induced by palmitoyl-L-carnitine (mean of 2 experiments). Palmitoyl-L-carnitine also mobilized free arachidonic acid in bovine aortic endothelial cells (Fig. 1, inset). The release of PGI_2 was not enhanced by carnitine, palmitic acid or acetyl-carnitine (Table 1). Other long-chain bases like sphingosine (not shown) or dihydrosphingosine and related compounds were also inactive (Table 2). The stimulatory action of palmitoyl-L-carnitine was however mimicked by L-α-lysophosphatidylcholine (Table 3). During a continuous exposure of endothelial cells to palmitoyl-L-carnitine, the release of PGI_2 declined spontaneously, but remained higher than the control value for at least 2 hr (Fig. 2). Removal of palmitoyl-L-carnitine from the medium accelerated that decline, while its readdition induced a new wave of PGI_2 release (Fig. 2). Palmitoyl-L-carnitine increased the intracellular level of choline in bovine aortic endothelial cells labeled with [^{3}H]-choline, as well as the extracellular release of both

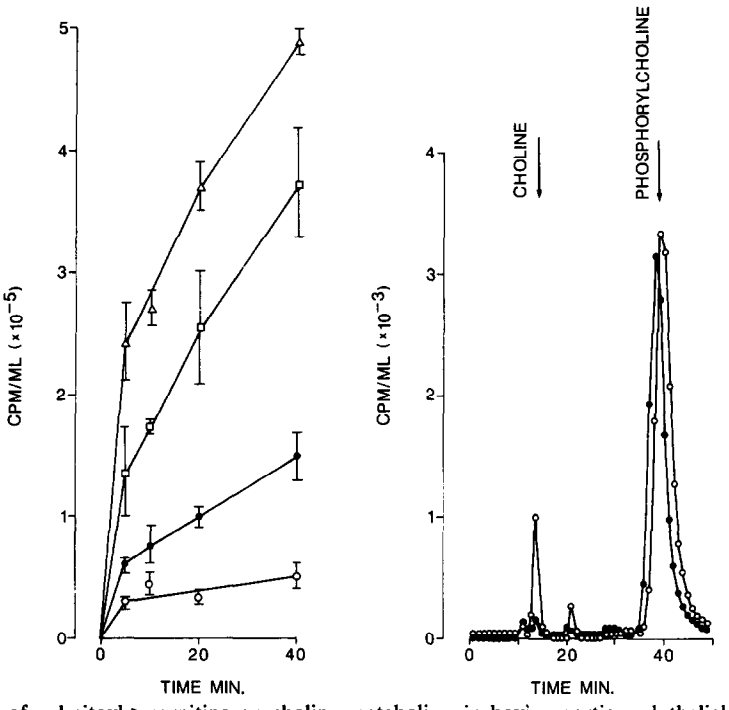

Fig. 3. Effect of palmitoyl-L-carnitine on choline metabolism in bovine aortic endothelial cells. (A) Time course of the accumulation of choline metabolites in the incubation medium of bovine aortic endothelial cells. The cells were labeled with [^{3}H]-choline for 24 hr. After removal of the culture medium and 2 washings, they were incubated in DMEM containing various concentrations of palmitoyl-L-carnitine. Aliquots were collected at different times for liquid scintillation counting. Results are the mean ± SD of triplicate determinations in 1 representative experiment out of 2. Aliquots of the same media were analysed by HPLC to separate choline from phosphorylcholine: results are presented in Table 4. -○-: Control; -●-: 25 μM palmitoyl-L-carnitine; -□-: 50 μM palmitoyl-L-carnitine; -△-: 100 μM palmitoyl-L-carnitine. (B) HPLC separation of water-soluble choline metabolites in extracts of bovine aortic endothelial cells challenged by palmitoyl-L-carnitine. Following the incubation described in section A, the cells were lysed in TCA (25%) and the cell extract was submitted to HPLC on a silica gel column in order to separate water-soluble choline metabolites. The extraction and HPLC procedures are described in the Methods section. -●-: Control; -○-: palmitoyl-L-carnitine (25 μM). The arrows indicate the retention times of choline and phosphorylcholine standards.

Table 4. Effect of palmitoyl-L-carnitine on the levels of choline and phosphorylcholine in cell extracts and incubation media of bovine aortic endothelial cells

	Cell extracts		Medium	
	Choline	Phosphorylcholine	Choline	Phosphorylcholine
Control	3.7 ± 0.1	240 ± 4	47 ± 5	15 ± 3
Palmitoyl-L-carnitine (25 μM)	20.6 ± 1.1	271 ± 14	75 ± 6	72 ± 19

Following a 24 hr-labelling with [^{3}H]-choline, the cells were incubated for 40 min with or without palmitoyl-L-carnitine. The incubation media and cell extracts were then analysed by HPLC, as described in the Methods section. Results represent the amount of radioactivity per dish in cpm × 10^{-3} (mean ± SD of triplicates in 1 representative experiment).

choline and phosphorylcholine (Fig. 3 and Table 4). Palmitoyl-L-carnitine exerted a cytotoxic action on endothelial cells, reflected by an increased release of radioactivity from [^{3}H]-thymidine prelabeled cells and by a loss of cellular DNA (Fig. 4).

DISCUSSION

Long-chain acylcarnitines, mostly palmitoyl-L-carnitine, and lysophosphoglycerides accumulate in the ischemic myocardium [11–13]. *In vitro* they induce electrophysiological and biochemical alterations similar to those seen in the ischemic myocardium *in vivo* [14, 23–24]. During ischemia palmitoyl-L-carnitine accumulates primarily in the cytosol of cardiomyocytes, but it leaks to the extracellular space [14, 15] and could thus influence endothelial cells in the ischemic zone or endocardial cells in its vicinity.

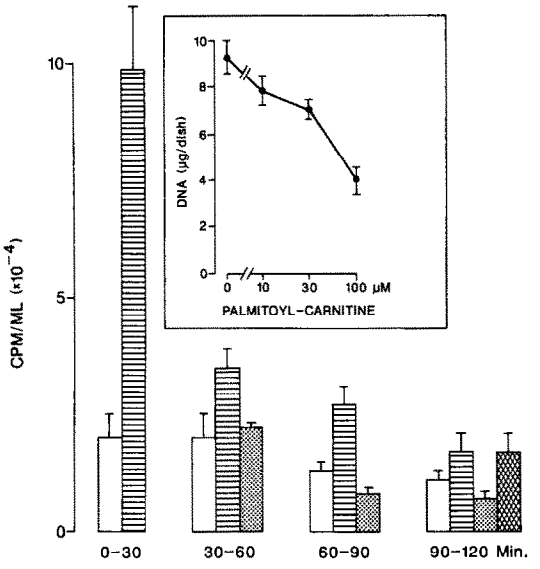

Fig. 4. Cytotoxicity of palmitoyl-L-carnitine for bovine aortic endothelial cells. Cells prelabelled with [^{3}H]-thymidine were incubated in 1 ml DMEM for 4 successive periods of 30 min: the medium was collected and changed at the end of each period. Results represent the radioactivity released during each of these periods (mean ± SD, in 1 representative experiment out of 2: each condition was tested in triplicate). □: Control; ▤: palmitoyl-L-carnitine (50 µM); ▩: palmitoyl-L-carnitine (50 µM) present only during the 1st incubation period; ▨: palmitoyl-L-carnitine (50 µM) readded during the 4th period, after being present during the 1st one and absent during the 2nd and 3rd periods. Inset: Loss of endothelial cells induced by palmitoyl-L-carnitine. Cells were incubated for 2 hr with various concentrations of palmitoyl-L-carnitine. The amount of DNA per dish was then determined. Results represent the mean ± SD of triplicate determinations in 1 representative experiment out of 3.

The stimulation of endothelial PGI_2 synthesis by palmitoyl-L-carnitine is obtained in the same range of concentrations (10–100 µM) as other biochemical and cellular effects previously reported: modulation of the Ca^{2+}–ATPase of sarcoplasmic reticulum [25], inhibition of the Na^+, K^+–ATPase of myocardial sarcolemma [25, 26], inhibition of cardiac cyclic AMP phosphodiesterase and myosin light chain kinase [27], inhibition of phorbol-12-myristate, 13-acetate binding to human HL-60 leukemia cells [28], inhibition of protein kinase C [29], activation of voltage-dependent Ca^{2+} channels [30]. It is likely that palmitoyl-L-carnitine produces these various actions by acting as a "naturally occurring detergent", a suggestion made by Adams *et al.* [25], rather than as a ligand for receptor sites. The steepness of the concentration–action curve (Fig. 1) and the toxicity for the endothelial cells would be consistent with such a mechanism. As with other stimuli, like ATP [16], the increased release of PGI_2 from endothelial cells exposed to palmitoyl-L-carnitine results from an enhanced mobilization of free arachidonic acid, presumably as a consequence of phospholipase A_2 activation. The increased levels of choline and phosphorylcholine suggest that phospholipases C and D are also activated in response to palmitoyl-L-carnitine, but other studies are needed to establish which enzymatic pathway is responsible for these effects. An increase in membrane permeability might explain why the release of phosphorylcholine was much more pronounced that the increase of its cellular level. Similar changes have been observed recently in a variety of cell systems, in response to multiple agonists [31–33].

The biosynthesis of PGI_2 is increased in various diseases involving the intravascular activation of platelets, like severe atherosclerosis of lower limbs [10] and unstable angina, during the episodes of chest pain [8]. *In vitro*, several factors released from activated platelets increase the synthesis of PGI_2 in vascular endothelial and smooth muscle cells: ADP and ATP [16, 34], prostaglandin endoperoxides [35, 36], serotonin and platelet-derived growth factor [37]. The enhanced production of PGI_2 in response to platelet activation constitutes a compensatory mechanism which can limit the extent of platelet aggregation and help to localize thrombus formation to areas of endothelium damage. The enhancement of PGI_2 biosynthesis in acute myocardial infarction [8, 9] might be beneficial, since PGI_2 has a cytoprotective [38] and antiarrythmogenic [39] action. As this increase in PGI_2 exceeds the augmentation of thromboxane A2 production, it cannot be explained by platelet activation alone [8]. The present study suggests that it might partially result from the accumulation of palmitoyl-L-carnitine.

Acknowledgements—We wish to thank Prof. J. E. Dumont for his interest in this work and his continuous support. We are grateful to N. Galand and C. Lagneau for their excellent technical assistance. We thank D. Leemans for typing the manuscript. This work was performed under contract of the "Ministère de la Politique Scientifique" ("Action Concertée") and was supported by a grant of the "Fonds de la Recherche Scientifique Médicale". S. Pirotton was supported by IRSIA.

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