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Conjugated linoleic acid exhibits stimulatory and inhibitory effects on prostanoid production in human endothelial cells and platelets

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Abstract

In addition to their reported antitumorigenic properties, various conjugated linoleic acid (CLA) isomers have also been shown to decrease prostanoid synthesis as a result of inhibiting the cyclooxygenase (COX) enzyme. We have previously reported that several CLA isomers inhibited both platelet aggregation and formation of thromboxane A_2 (TXA_2), a proaggregatory and vasoconstrictive agent. Since the interaction between platelets and vascular endothelial cells is essential to maintaining vascular homeostasis, we decided to investigate the effects of various CLA isomers on the production of endothelial prostacyclin (PGI_2), a potent vasodilator and inhibitor of platelet function. Using interleukin 1-β (IL1-β)-stimulated human umbilical vein endothelial cells (HUVECs), we initially established that HUVECs of passage #2 should be used since these cells were most responsive to thrombin-induced conversion of endogenous arachidonic acid to PGI_2, as monitored by the formation of its stable, inactive metabolite, 6-keto$PGF_{1\alpha}$. In the first part of the study, the effects of CLA isomers in the free fatty acid form were tested. The 10(*E*), 12(*Z*)- and 9(*Z*), 11(*E*)-CLA isomers inhibited thrombin-induced 6-keto$PGF_{1\alpha}$ formation with I_{50}'s of 2.6 and 5.5 μM, whereas the 9(*Z*), 11(*Z*)- and 9(*E*), 11(*E*)-CLA were ineffective at concentrations up to 60 μM. The inhibitory effect of the 10(*E*), 12(*Z*)-CLA was irreversible. Next, the effects of CLA incorporation into HUVECs on PGI_2 generation was determined. An average 8-fold stimulation of 6-keto$PGF_{1\alpha}$ formation was obtained with quiescent IL1-β-exposed HUVECs pretreated for 18 h with 25 μM 9(*Z*), 11(*Z*)-CLA, whereas cells preincubated with the 10(*E*), 12(*Z*) isomer enhanced this eicosanoid 3-fold. Such IL1-β-treated HUVECs prelabeled with 25 μM 9(*Z*), 11(*Z*)-CLA became refractory to thrombin stimulation, as measured by 6-keto$PGF_{1\alpha}$ production, whereas a small, statistically insignificant, inhibition was observed upon thrombin treatment of HUVECs prelabeled with the 10(*E*), 12(*Z*) isomer. Qualitative similar results were obtained with resting or thrombin-stimulated platelets containing these esterified CLA isomers indicating that these effects occur with cells that contain either the COX-1 or COX-2 isozymes. The results of this in vitro study indicate that the effects of CLA on cellular prostanoid formation in endothelial cells and platelets can be either inhibitory or stimulatory, and this seems to depend not only on the specific CLA isomer and whether or not the CLA is in the free fatty acid form or esterified into cellular lipids, but also whether cells are in the resting or stimulated state. These findings suggest that in vivo, CLA might have multiple, complex effects on vascular homeostasis.

Keywords: Conjugated linoleic acid; Endothelial prostacyclin; Cyclooxygenase enzyme

1. Introduction

Conjugated linoleic acid (CLA) is a term now used for a group of positional and geometrical isomers of linoleic acid and characterized by the presence of conjugated double bonds. CLA appears to exhibit a plethora of biological activities including its protective effects against various types of cancer and heart disease [1]. Recently, we reported that various CLA isomers inhibited platelet aggregation [2]. In trying to identify some of the mechanisms through which CLA exerts its effects, modulation of eicosanoid metabolism was an attractive hypothesis since eicosanoids are known to be potent modulators of certain cancers and cardiovascular functions [3,4].

Prostanoidal eicosanoids are products of arachidonic acid (AA) metabolism formed via the cyclooxygenase (COX) pathway. Two such eicosanoids, prostacyclin (PGI_2) and

Abbreviations: AA, arachidonic acid; CLA, conjugated linoleic acid; COX, cyclooxygenase; EIA, enzyme immunoassay; FBS, fetal bovine serum; GLC, gas–liquid chromatography; HSA, human serum albumin; PG, prostaglandin; PGI_2, prostacyclin; TLC, thin-layer chromatography; TXA_2, thromboxane A_2

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thromboxane A_2 (TXA_2), play an essential role in maintaining vascular hemostasis [4–7]. PGI_2, mainly produced by endothelial cells under physiological conditions [4,8], is known to inhibit platelet aggregation and to act as a vasodilator. In contrast, TXA_2, the primary prostanoid formed by platelets, induces platelet aggregation and acts as a vasoconstrictor [6]. In view of their counteracting biological effects, the PGI_2/TXA_2 ratio appears to be important in the physiological regulation of vascular hemostasis. For example, a decrease in this ratio has been related to several pathophysiological conditions such as thrombosis and ischemia [4,7]. Thrombin is another important player in vascular homeostasis via its effects on cellular kinases and phospholipases and many studies have shown that thrombin can stimulate resting human umbilical vein endothelial cells (HUVECs) and platelets which results in enhanced formation of PGI_2 and TXA_2, respectively [8,9].

Since we had previously reported that certain CLA isomers affected the formation of platelet TXA_2 (as measured by its inactive metabolite TXB_2 [2]) and in view of the importance of PGI_2 in maintaining normal vascular/platelet homeostasis, studies were undertaken to examine the effects of all pure, commercially available, CLA isomers on PGI_2 production by endothelial cells. In addition, a number of studies have reported on the effects of fatty acid incorporation on prostanoid production in endothelial cells and platelets [10,11]. Using a similar approach, we incorporated several CLA isomers into either endothelial cells or platelets and investigated the effect of such esterification on the formation of PGI_2 and TXA_2 by these modified cells.

2. Materials and methods

Thrombin was purchased from Chronolog Corp. (Havertown, PA) and the 9(*Z*), 11(*E*)-CLA, 9(*E*), 11(*E*)-CLA, 9(*Z*), 11(*Z*)-CLA and 10(*E*), 12(*Z*)-CLA isomers were obtained in >98% purity from Matreya Inc. (Pleasant Gap, PA). Production of cellular 6-keto$PGF_{1\alpha}$ and TXB_2 (the stable hydrolysis products of PGI_2 and TXA_2, respectively) was measured by enzyme immunoassay (EIA) using commercial kits from Assay Designs Inc. (Ann Arbor, MI) and following the manufacturer's instructions. The cross-reactivities of both the 6-keto$PGF_{1\alpha}$ and TXB_2 antisera for other common prostanoids were generally <0.01% range. Silica gel G 60 thin-layer chromatography (TLC) plates (Merck), common chemicals and reagents were obtained from Fisher Scientific and [^{3}H]-TXB_2 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Fatty acid-free human serum albumin (HSA), aspirin and gelatin were purchased from Sigma Chemical Co. (St. Louis, MO).

HUVECs (cryopreserved, obtained from pooled donors) were purchased from Biowhittaker (Walkersville, MD). The cells ($1-4\times10^4$/well in a 12-well plate) were cultured on gelatin-coated plates in modified EGM medium supplemented with growth factors and a final 2% serum concentration as recommended by the manufacturer (Biowhittaker). Cells between passages #2 and #4 were used and allowed to reach confluence (6 days, $\sim10^5$/well). After removal of the medium and washing the cells twice with RPMI 1640, EGM medium containing 1% defatted fetal bovine serum (FBS) and interleukin 1-β (IL1-β, 100 u/ml) was added and the HUVECs were incubated overnight at 37 °C. I_{50} determinations for the free fatty acid form of CLA were carried out as follows: after removal of the medium, the cells were washed twice with RPMI 1640 and then incubated in RPMI (1 ml) containing the appropriate amount of CLA isomer or vehicle (ethanol). After 5 min, thrombin (0.2 u/ml) was added. Cell supernatants were collected 15 min after thrombin addition, acidified with 10% formic acid, neutralized after 15 min and stored at −20 °C until 6-keto$PGF_{1\alpha}$ analysis by EIA.

In the CLA-prelabeled HUVEC experiments, HUVECs (passage #2) were treated with IL1-β and with or without CLA isomers according to the method of Lagarde et al. [11,12]. Briefly, CLA/HSA mixtures were prepared by first evaporating (under nitrogen) solvent from ethanolic CLA isomer stock solutions, then adding EGM buffer, pH 7.2 containing 3% HSA and incubating the mixture under a nitrogen atmosphere for 18 h at 37 °C. Confluent HUVECs (after washing as described above) in EGM medium containing 1% defatted FBS and IL1-β (100 u/ml) were incubated with EGM medium containing either CLA-treated HSA or HSA (control) for 18 h at 37 °C. HUVECs were then either untreated or treated with thrombin (0.2 u/ml) for 15 min. The supernatants were removed, processed and analyzed for 6-keto$PGF_{1\alpha}$ content as described above.

Platelets, obtained from human donors who had not taken any nonsteroidal antiinflammatory drugs during the previous 2 weeks, were isolated according to our previously reported procedure and were resuspended at a concentration of 10^8/ml of Tyrode–HEPES buffer, pH 7.4 [2]. Aggregation studies, using a Chronolog model 440 VS dual aggregometer, were carried out as previously described [2]. Briefly, control platelets (1 ml) were placed in the aggregometer and the thrombin concentration needed to produce 40–50% of maximum response was determined. I_{50}'s of CLA isomers (as the free fatty acids) were determined as previously reported [2]. Incorporation of CLA isomers into platelets was carried out by incubating CLA-precoated HSA- or HSA–Tyrode–HEPES buffer preparations with platelets for 2 h at 25 °C. After removing the HSA or CLA/HSA media by low-speed centrifugation, the platelets were resuspended in Tyrode–HEPES buffer (10^8/ml) and placed in the aggregometer. Platelets were preincubated for 2 min at 37 °C, then either untreated or stimulated with thrombin (0.02–0.05 u/ml, using a concentration selected with control platelets) for 3 min. Platelets were then extracted as previously described [2], but in the presence of 100 nCi [^{3}H]-TXB_2 for final yield recovery determinations. TXB_2 was separated by TLC, extracted from the silicic acid and

quantitated by EIA. Determinations were performed in duplicate and final values adjusted for recovery.

In order to determine the fatty acid content of platelets pretreated with CLA isomers, platelet lipids were extracted using the Bligh and Dyer method [13]. Total lipids were transmethylated using sodium methoxide and the fatty acid methyl esters were analyzed by gas–liquid chromatography (GLC) using a 100 m CP Sil 88 column and a temperature program from 45 to 215 °C [14].

Results are expressed as the mean±S.D. Statistical significance was examined by Student's *t*-test, using $P<0.05$ as the significance level.

3. Results

Since other reports had indicated that PGI_2 formation (as measured by its stable, inactive metabolite 6-keto$PGF_{1\alpha}$) was maximal after 16 h upon treatment of HUVECs with IL1-β [15,16], these conditions were routinely used. However, in initial experiments with HUVECs of different passage numbers, we observed that thrombin-induced 6-keto$PGF_{1\alpha}$ production was quite variable. We then determined that a crucial parameter in analyzing 6-keto$PGF_{1\alpha}$ formation in HUVECs was the relative amounts of 6-keto$PGF_{1\alpha}$ produced in the presence and absence of thrombin. We designated this as the 'fold stimulation by thrombin' factor or 'fst' factor, i.e. the ratio of the amounts of 6-keto$PGF_{1\alpha}$ formed in the presence and absence of thrombin. In preliminary studies (not shown), we determined that 0.2 units of thrombin yielded optimum amounts of 6-keto$PGF_{1\alpha}$. In addition, HUVECs were routinely monitored with COX inhibitors. Thus, we found that 3 μM NS-398, a COX-2 inhibitor [17], and 200 μM aspirin inhibited HUVEC 6-keto$PGF_{1\alpha}$ formation by 100% and 86%, respectively (results not shown). The results shown in Table 1 indicate that while HUVECs of passage #2 produced 45–3150 pg/ml of 6-keto$PGF_{1\alpha}$, production of this metabolite decreased substantially with cells of passages #3 and #4. This was further confirmed when the 'fst' factor was determined, decreasing from a mean 'fst' factor 3.9 (HUVEC passage #2) to the mean 'fst' factor of 1.6 and 1.8, respectively (cell passages #3 and #4). Furthermore, when the effect of 9(*Z*), 11(*E*)-CLA on 6-keto$PGF_{1\alpha}$ formation as a f(HUVEC passage number) was determined, it was found that this CLA isomer inhibited 6-keto$PGF_{1\alpha}$ formation in six of eight experiments with cells of passage #2, but that cells of higher passage number were essentially refractory to this CLA isomer. Consequently, only HUVECs of passage #2 were used in subsequent experiments.

To determine the relative inhibitory potencies of several CLA isomers on HUVEC PGI_2 production, IL1-β-stimulated HUVECs were pretreated with the CLA isomer for 5 min followed by the addition of thrombin. These CLA isomers were chosen because they were the only ones commercially available in >98% purity. Formation of the inactive PGI_2 metabolite 6-keto$PGF_{1\alpha}$ was monitored by EIA. The results shown in Table 2 indicate that the I_{50} of 10(*E*), 12(*Z*)-CLA was 2.6 μM, which was twice as potent an inhibitor as 9(*Z*), 11(*E*)-CLA, whereas neither the 9(*Z*), 11(*Z*)- nor the 9(*E*), 11(*E*)-CLA isomers were appreciably effective at concentrations up to 60 μM. The inhibitory potency of the nonconjugated LA was comparable to the 10, 12-CLA isomer. In order to determine if the inhibitory effect of 10(*E*), 12(*Z*)-CLA was reversible, IL1-β-stimulated HUVECs were pretreated with 10 μM of this CLA isomer for 5 min, after which the incubation mixture was diluted 10-fold prior to the addition of thrombin. A 64% inhibition of 6-keto$PGF_{1\alpha}$ formation was observed, which was comparable to the 61% inhibitory effect of 10 μM 10(*E*), 12(*Z*)-CLA, but not the 11% decrease found with 1 μM of this CLA isomer (Fig. 1).

Since Spector et al. [10,18] and Lagarde et al. [11,12] had shown that endothelial cells and platelets incorporated unsaturated fatty acids into their lipids and that such incorporation attenuated eicosanoid formation generated from endogenous AA, the effect of CLA incorporation on 6-keto$PGF_{1\alpha}$ production from CLA-enriched HUVECs was examined. Two CLA isomers were used in these studies and were chosen based on the results shown in Table 2 in which the CLA isomers were in the free fatty acid form: i.e. one isomer to be tested was the most effective inhibitor of HUVEC 6-keto$PGF_{1\alpha}$ production [10(*E*), 12(*Z*)-CLA], and the other CLA isomer [9(*Z*), 11(*Z*)-CLA] used was an ineffective inhibitor. First, IL1-β-treated confluent HUVECs were preenriched during an overnight incubation with either HSA (fatty acid-free) or HSA precoated with one of these CLA isomers. After removal of the HSA/CLA-prelabeling medium, the cells were incubated for 15 min and 6-keto$PGF_{1\alpha}$ formation measured. In four separate experiments, control HUVECs produced and released 42–1000

Table 1
Influence of cell passage number on thrombin-induced 6-keto$PGF_{1\alpha}$ formation by IL-1β pretreated HUVECs

Cell passage #	Cell treatment		
		+Thrombin	+9(*Z*), 11(*E*)-CLA +thrombin
	6-keto$PGF_{1\alpha}$ formation		
	Range (pg/ml)	Fold stimulation by thrombin (mean±S.D.)	Inhibitory
2	45–3150 (*n*=9)	1.6–12 (3.9±3.5)	*n*=6 of 8
3	22–250 (*n*=5)	1.0–2.2 (1.6±0.43)	*n*=1 of 4
4	17–210 (*n*=5)	1.1–2.3 (1.8±0.51)	*n*=1 of 3

HUVECs (120,000/well) were treated for 24 h with 100 μ IL-1β, followed either by no treatment or by the addition of 0.2 u thrombin. When the effect of CLA on 6-keto$PGF_{1\alpha}$ formation in HUVECs was tested, the IL-1β-treated cells were first treated with either dimethylsulfoxide (vehicle) or with varying amounts of 9(*Z*), 11(*E*)-CLA. Thrombin (0.2 u) was added after 5 min and after an additional 15 min, the supernatant was removed and assayed for 6-keto$PGF_{1\alpha}$ formation by EIA. *n*=Number of separate experiments.

Table 2
Relative inhibitory potencies (I_{50}) of various conjugated linoleic acids on thrombin-induced (a) 6-ketoPGF$_{1\alpha}$ formation by IL-1β treated HUVECs and (b) TXB_2 production by platelets

Fatty acid	I_{50} (μM)	
	6-ketoPGF$_{1\alpha}$ formation	TXB_2 production
10(*E*), 12(*Z*)-CLA	2.6±1.5 (6)[a]	2.1±1.7 (3)
9(*Z*), 11(*E*)-CLA	5.5±3.1 (7)[a,*]	nd
9(*Z*), 11(*Z*)-CLA	>60 (5)	>30 (4)
9(*E*), 11(*E*)-CLA	>60 (5)	nd
9(*Z*), 12(*Z*)-CLA	3.3±2.7 (4)[b]	nd

(a) These experiments were carried out with HUVEC of passage #2 except where indicated. Protocol was the same as indicated for Table 1. (b) Platelets (10^8/ml) were placed in an aggregometer at 25 °C and were pretreated with different concentrations of CLA in ethanol (or ethanol alone) for 2 min. Thrombin (0.02 u) was added and the reaction terminated after 3 min. Products were extracted and quantitated as described in Materials and methods. *n*=Number of separate experiments and are indicated in parentheses. Values given are mean±S.D. nd=Not determined.
[a] Includes one experiment with HUVEC of passage #4.
[b] Includes one experiment with HUVEC of passage #3.
* Statistically different from the I_{50} of 10(*E*), 12(*Z*)-CLA, $P<0.06$.

pg/ml of 6-ketoPGF$_{1\alpha}$. When the HUVECs were prelabeled with 25 μM 9(*Z*), 11(*Z*)-CLA, an average 8-fold *increase* (range was 1.9–23-fold increase) in endogenous 6-ketoPGF$_{1\alpha}$ formation was observed, whereas 25 μM 10(*E*), 12(*Z*) isomer was not quite half as effective (range was 1.5–7.5-fold increase, Fig. 2). Next, the effect of thrombin on 6-ketoPGF$_{1\alpha}$ formation from CLA-prelabeled HUVECs was investigated. Thrombin stimulation of control cells generated and released 110–2500 pg/ml of 6-ketoPGF$_{1\alpha}$, which represented a 2.6-fold stimulation ('fst' factor) in the formation of this metabolite relative to that observed in the absence of thrombin. Thrombin stimulation using HUVECs preenriched with either 10, 25 or 100 μM 10(*E*), 12(*Z*)-CLA resulted in a decrease in the 'fst' factor from 2.6 (control) to 1.8, 1.9 or 2.2 (respectively) but this decrease was not statistically significant (Table 3). HUVECs preenriched with either 10 or 25 μM 9(*Z*), 11(*Z*)-CLA appeared to be refractory to thrombin since the 'fst' factor decreased to 1.2 ($P<0.05$) and 0.92 ($P<0.01$, respectively).

To determine whether both stimulatory and inhibitory effects of these CLA isomers could also be observed with a different cell type, we examined the interaction of these CLA isomers with platelets in view of the importance of vascular-platelet homeostasis. The effects of 10(*E*), 12(*Z*)-CLA or 9(*Z*), 11(*Z*)-CLA, either as the free fatty acid or esterified into platelet lipids, were tested on both platelet aggregation and on TXA_2 formation (as measured by its stable, inactive metabolite TXB_2). As shown in Table 2, when the free fatty acid forms of these CLA isomers were tested, only the 10, 12-CLA isomer was an effective inhibitor of platelet TXB_2 generation. When the effects of CLA-enriched platelets on the generation and release of TXB_2 were investigated, the results were similar to those obtained with CLA-prelabeled HUVECs. COX activity in control platelets (either untreated or HSA-treated) was monitored both in the absence and presence of 400 μM aspirin which typically decreased TXB_2 formation by 90–95% (results not shown). Prelabeling platelets with HSA precoated with 25 μM 9(*Z*), 11(*Z*)-CLA resulted in a 2.3-fold increase in the formation and release of TXB_2 but only a 30% increase was observed with platelets enriched with

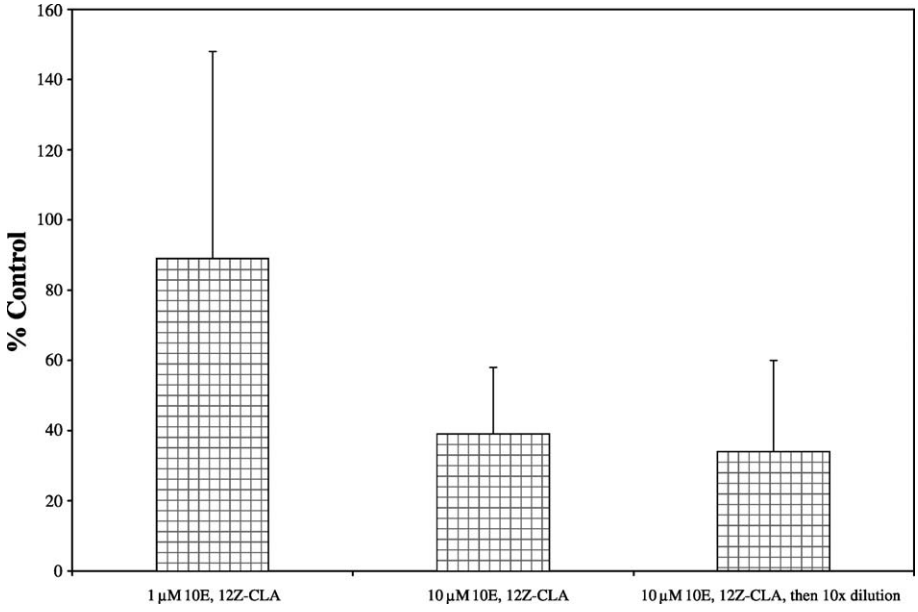

Fig. 1. Nonreversibility of the inhibitory effect of 10(*E*), 12(*Z*)-CLA on HUVEC 6-ketoPGF$_{1\alpha}$ formation. The HUVEC experiments were carried out as described in the legend of Table 1. In the third incubation, HUVECs were pretreated at 37 °C with 10 μM CLA for 5 min, after which the incubation mixture was diluted 10-fold prior to thrombin addition. The values are given as the mean percent of control ± S.D. from three separate experiments. The results for the 10 μM CLA and diluted 10 μM CLA samples are statistically different from the untreated controls, $P<0.01$.

Table 3
Effect of thrombin on 6-ketoPGF$_{1\alpha}$ formation by IL-1β-treated HUVECs prelabeled with either 10(*E*), 12(*Z*)-CLA or 9(*Z*), 11(*Z*)-CLA

Treatment of cells	Prelabeling concentration of CLA isomer (μM)	6-ketoPGF$_{1\alpha}$ formation, fold stimulation by thrombin
None	–	2.6±0.42 (4)
10(*E*), 12(*Z*)-CLA	10	1.8±0.53 (4)
	25	1.9±1.1 (5)
	100	2.2±1.1 (5)
9(*Z*), 11(*Z*)-CLA	10	1.2±0.50 (3)*
	25	0.92±0.49 (5)**

HUVECs were treated overnight at 37 °C with IL-1β and either with HSA complexed with the indicated amount of CLA isomer or with HSA alone according to the procedure outlined by Lagarde et al. [11,12]. Insignificant differences in cell viabilities (as measured by LDH release) were observed between control cells and cells pretreated with CLA. After washing, the cells were stimulated with thrombin (0.2 u), and after 15 min, the supernatants were removed and assayed for 6-ketoPGF$_{1\alpha}$ formation by EIA. The values are the mean±S.D. and the number of separate experiments are indicated in parentheses.

* Results that are statistically different from control, $P<0.05$.

** Results that are statistically different from control, $P<0.01$.

Table 4
Effect of thrombin on TXB_2 formation by human platelets prelabeled with either 10(*E*), 12(*Z*)-CLA or 9(*Z*), 11(*Z*)-CLA

Treatment of platelets	Prelabeling concentration of CLA isomer (μM)	TXB_2 formation, fold stimulation by thrombin (mean±S.D.)
None	–	3.3±1.5 (5)
10(*E*), 12(*Z*)-CLA	25	3.2±2.3 (5)
9(*Z*), 11(*Z*)-CLA	25	1.3±0.2 (5)*

Human platelets were treated for 2 h at 37 °C with either HSA complexed with 25 μM of the indicated CLA isomer or with HSA alone according to the procedure outlined by Lagarde et al. [11,12]. After washing, the platelets were incubated for 2 min, stimulated with 0.2 u thrombin for an additional 3 min. The supernatants were then removed and after TLC, assayed for TXB_2 formation by EIA. The values are the mean±S.D. and the number of separate experiments are indicated in parentheses.

* Results that are statistically different from control, $P<0.05$.

the 10, 12 isomer (Fig. 2). When platelets were preenriched with HSA precoated with 25 μM LA, no appreciable increase in TXB_2 production was observed relative to controls (results not shown). The data in Table 4 indicate that thrombin-induced TXB_2 formation from platelets prelabeled with HSA and 9(*Z*), 11(*Z*)-CLA was reduced as their 'fst' factor was significantly lower than that of control platelets (1.3 vs. 3.3). Prelabeling platelets with HSA and 10(*E*), 12(*Z*)-CLA did not have any appreciable effect on the 'fst' factor. No substantial differences were observed in thrombin-induced aggregation between platelets incubated only with HSA and platelets enriched with either the 9, 11- or 10, 12-CLA isomer (results not shown).

To confirm that the CLA isomers were indeed incorporated into cells, platelets were first pretreated with either HSA (control), 25 μM 9(*Z*), 11(*Z*)-CLA/HSA or 25 μM 10(*E*), 12(*Z*)-CLA/HSA, followed by extraction of platelet

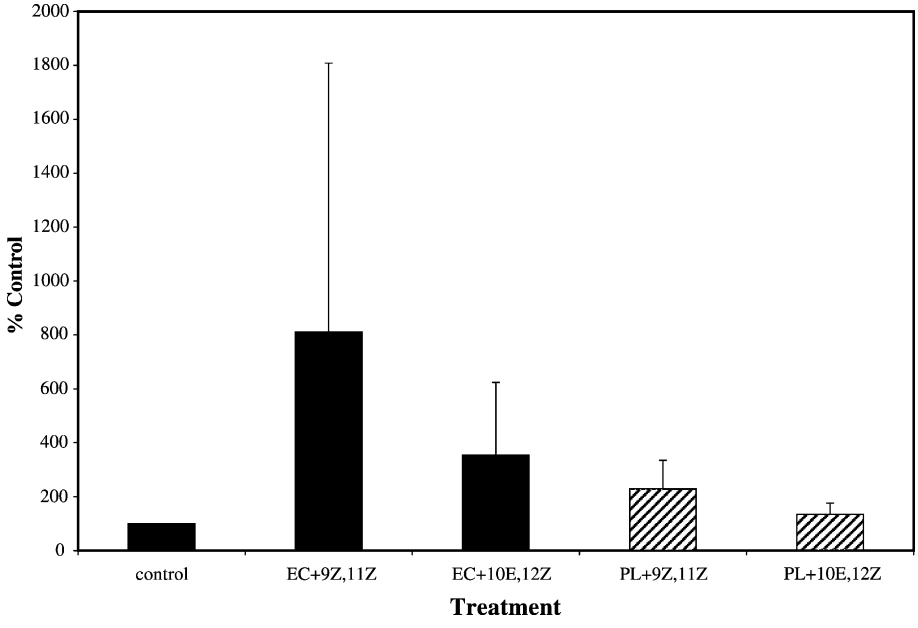

Fig. 2. CLA-prelabeling of HUVECs and platelets increased generation and release of 6-ketoPGF$_{1\alpha}$ and thromboxane B_2, respectively. HUVECs were treated for 18 h at 37 °C with IL-1β and either with HSA complexed with 25 μM of the indicated CLA isomer or with HSA alone according to the procedure outlined by Lagarde et al. [11,12]. Insignificant differences in cell viabilities (as measured by LDH release) were observed between control cells and cells pretreated with CLA. After washing, the cells were incubated for 15 min, the supernatants were removed and assayed for 6-ketoPGF$_{1\alpha}$ formation by EIA. Human platelets were treated for 2 h at 37 °C with either HSA complexed with 25 μM of the indicated CLA isomer or with HSA alone. After washing, the platelets were maintained at room temperature for 5 min. The supernatants were then removed and after TLC, assayed for TXB_2 formation by EIA. The values are the mean±SD and are obtained from four (HUVECs or platelets) separate experiments.

Table 5
CLA incorporation into total platelet lipids

Pretreatment	CLA (%)			AA (%)
	9(*Z*), 11(*Z*)-	10(*E*), 12(*Z*)-	9(*Z*), 11(*E*)	
HSA (control)	0.03	0.03	0.14	22.91
+25 μM 9(*Z*), 11(*Z*)-CLA	0.23	0.03	0.12	22.73
+25 μM 10(*E*), 12(*Z*)-CLA	0.03	0.66	0.13	22.16

Human platelets were treated for 2 h at 37° with either HSA complexed with 25 μM of the indicated CLA isomer or with HSA alone. After washing, the platelets from six donors ($1-3\times10^9$/donor) were pooled, extracted by the Bligh and Dyer method [13] and transmethylated using sodium methoxide. Fatty acid methyl esters were analyzed by GLC as described in Materials and methods.

lipids. After transmethylation, the fatty acid methyl esters were analyzed by GLC. As shown in Table 5, the amount of 9(*Z*), 11(*Z*)-CLA incorporated (of the total fatty acid content) increased from 0.03% (control) to 0.23%, whereas the amount of 10(*E*), 12(*Z*)-CLA incorporated increased from 0.03% (control) to 0.66%. No appreciable differences were observed in either the 9(*Z*), 11(*E*)-CLA isomer (typically the major CLA isomer present in human tissues) or AA content of the platelet lipids.

4. Discussion

In the present study, we first analyzed PGI_2 generation (as measured by its stable hydrolysis product 6-ketoPGF$_{1\alpha}$) by IL1-β-treated HUVECs that were stimulated by thrombin. Under these conditions, PGI_2 is the primary product formed from the COX-2 isozyme pathway [15]. The ability of thrombin to stimulate 6-ketoPGF$_{1\alpha}$ production in IL1-β-treated HUVECs appeared to be dependent on the HUVEC passage number used. Thrombin potentiated 6-ketoPGF$_{1\alpha}$ formation in passage #2 cells about 4-fold compared to less than 2-fold increases with cells of passage #3 or greater (Table 1). Consequently, cells of passage #2 were routinely used. Several studies have appeared which report similar findings of reduced PGI_2 production with increased HUVEC passage number though different conditions were used in these experiments [19,20]. Whether this phenomenon is due to cell age-related downregulation of receptors, changes in the COX isozymes or other events remains to be established.

When the effects of various CLA isomers were tested, two isomers, the 10(*E*), 12(*Z*)- and the 9(*Z*), 11(*E*)-CLA inhibited 6-ketoPGF$_{1\alpha}$ formation by thrombin-stimulated HUVECs with I_{50}'s of 2.6 and 5.5 μM, respectively, whereas the 9(*Z*), 11(*Z*)- and 9(*E*), 11(*E*)-CLA were ineffective at concentrations up to 60 μM (Table 2). Comparing these values to the reported I_{50}'s of CLA isomers on COX-catalyzed production of eicosanoids, it appears that the inhibitory CLA isomers were much more effective on HUVECs which contain the COX-2 system than on cells (e.g. platelets) containing COX-1. We have previously reported that both the 10(*E*), 12(*Z*)- and 9(*Z*), 11(*E*)- isomers of CLA inhibited platelet TXB_2 with I_{50}'s of 13–16 μM [2]. Bulgarella et al. [22] reported that three of these CLA isomers had I_{50}'s of about 20 μM on the ram seminal vesicle COX-1, with the 9(*Z*), 11(*Z*) isomer the least potent (I_{50} about 100 μM). These differences in I_{50}'s could be due to different sensitivities of the COX isozymes to CLA isomers, the use of diverse cellular sources of these enzymes and/or experimental conditions or inherent dissimilar mechanisms of eicosanoid production. Similar explanations could be advanced to explain the lack of reversibility of the inhibitory effect of 10(*E*), 12(*Z*)-CLA (Fig. 1) which contrasts with that observed with the 9(*Z*), 11(*E*)-CLA isomer on platelet TXB_2 formation [2].

In contrast to the above described effects of CLA isomers in the free fatty acid form, studies were conducted to explore the possibility that incorporation of CLA into HUVEC lipids also might influence 6-ketoPGF$_{1\alpha}$ formation. Cells were prelabeled with either the 10(*E*), 12(*Z*)- or 9(*Z*), 11(*Z*)-CLA. These two isomers were chosen because in the free fatty acid form, they represented the most and least effective inhibitors, respectively, of HUVEC PGI_2 formation. Unexpectedly, an 8-fold increase in 6-ketoPGF$_{1\alpha}$ production was observed in quiescent, IL1-β-treated cells that had been prelabeled with the 9(*Z*), 11(*Z*) isomer, whereas less than half that increase was obtained with the 10(*E*), 12(*Z*) isomer (Fig. 2). A number of groups have reported that incorporation of unsaturated fatty acids, such as linoleic acid, eicosapentaenoic and docosahexaenoic acid, into HUVECs and platelets decreased cellular eicosanoid formation from endogenous AA (e.g. Refs. [10–12,18]). In contrast to these results, a 2- to 3-fold increase in 6-ketoPGF$_{1\alpha}$ formation was observed *during* a PUFA enrichment process of HUVECs (untreated with any inducer of COX-2) with either 22:6 or 20:5, but the authors of this study did not report on the ability of these cells to stimulate 6-ketoPGF$_{1\alpha}$ production *after* removal of the PUFA-containing medium [21]. Most recently, Urquhart et al. [23] tested the effects of the incorporation of 9(*Z*), 11(*E*)- and 10(*E*), 12(*Z*)-CLA isomers on COX-1 activity in human saphenous vein endothelial cells. These authors reported that 50 μM of either CLA isomer inhibited $PGF_{2\alpha}$ formation in resting cells although major differences in their protocol and experimental conditions can readily explain the different results obtained in their work and ours. The results of our study also indicate that the ability of certain CLA isomers to stimulate endogenous prostanoid formation was not limited to HUVECs since enhanced TXB_2 production was also observed in nonstimulated platelets pretreated with 9(*Z*), 11(*Z*)-CLA (Fig. 2). Furthermore, structural differences between CLA isomers can account for the different stimulatory potencies. It is possible that the presence of a conjugated diene functionality is important (but not sufficient) as no stimulatory effect on platelet TXB_2 production was observed with platelets enriched with a nonconjugated PUFA, i.e. LA. The much larger (8-fold) increase in prostanoid formation observed with prelabeled HUVECs compared to that

obtained with prelabeled platelets (2-fold) might be due to either cell differences or to the different CLA pretreatment times used as the HUVECs were pretreated with CLA for 18 h compared to a 2 h pretreatment of platelets.

Short-term (i.e. minutes) exposure of cells to thrombin is known to stimulate cellular phospholipases to release endogenous AA which results in increased formation of 6-ketoPGF$_{1\alpha}$ [8]. Thrombin appeared to have similar stimulatory effects on HUVEC 6-ketoPGF$_{1\alpha}$ formation due to either COX-1 or COX-2 (data not shown). When the effects of thrombin on CLA-enriched HUVECs prestimulated with IL1-β were studied, a statistically significant decrease in 6-ketoPGF$_{1\alpha}$ production was observed (relative to nonenriched cells) when the cells were prelabeled with the 9(*Z*), 11(*Z*)-CLA isomer, whereas a small (not significant statistically) decrease was obtained with cells enriched with 10(*E*), 12(*Z*) isomer (Table 3). Similar results have been reported by Bordet et al. [11] who used HUVECs that had been preenriched with PUFAs such as 18:2 (*n*-6) or 18:3 (*n*-3) though these studies did not involve COX-2 induction. It is possible that the inability of thrombin to stimulate 6-ketoPGF$_{1\alpha}$ formation in HUVECs prelabeled with the 9(*Z*), 11(*Z*) isomer might be due to the fact that incorporation of this CLA isomer into cellular lipids resulted in a changed microenvironment of endogenous esterified AA so that lipids containing AA became less accessible to the phospholipases activated by thrombin. Alternatively, the COX-2 in resting HUVECs prelabeled with the 10(*E*), 12(*Z*)-CLA was in a less active state compared to HUVECs prelabeled with the 9(*Z*), 11(*Z*) isomer so that thrombin could still have a stimulatory effect on the former cells.

Dairy and meat products are the main dietary sources of CLA and the major CLA isomer present is 9(*Z*), 11(*E*)-CLA [24]. This isomer has been reported to be a potent anticarcinogenic agent [25] and inhibited the growth of human mammary cancer cells [26]. A number of studies indicate that other CLA isomers, though minor components of the naturally occurring CLA isomeric mix, also exhibit biological properties, e.g. 10(*E*), 12(*Z*)-CLA reduced leptin secretion [27] and inhibited the growth of preadipocytes [27] and the 9(*Z*), 11(*Z*)-CLA isomer was a potent inducer of gene expression in COS-1 cells [28]. Our findings confirm potent biological effects of these 'minor' CLA isomers on HUVECs and platelets, two cell types essential in vascular homeostasis. Our observation that 9(*Z*), 11(*Z*)-CLA incorporated into HUVEC lipids stimulated PGI_2 formation but had no effect as the free acid suggest that the use of this specific CLA isomer in the diet might have beneficial antithrombogenic effects. Furthermore, the greater stimulation observed in HUVEC PGI_2 production than platelet TXA_2 formation seems to suggest that this CLA isomer increases the PGI_2/TXA_2 balance. As separate cell populations were used in these studies, more work needs to be done using mixed HUVEC-platelet populations in order to delineate the optimum conditions under which 9(*Z*), 11(*Z*)-CLA could play an antithrombotic role.

In summary, our in vitro findings indicate that CLA isomers appear to have multiple and complex effects on COX-catalyzed prostanoid formation in endothelial cells and platelets. These effects depend on the specific CLA isomer, whether the isomer is in the free fatty acid form or esterified into cellular lipids and whether the target cell is in a quiescent or active state. Since it is quite likely that the in vivo CLA effects are even more complex, our results coupled with the increased use of CLA isomer mixtures as dietary supplements point up the need for new dietary studies to explore the use of specific CLA isomers as antithrombogenic agents.

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References

[1] M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza, G.J. Nelson (Eds.), Advances in Conjugated Linoleic Acid Research, vol. 1, AOCS, Champaign, IL, 1999.
[2] A. Truitt, G. McNeil, J.Y. Vanderhoek, Antiplatelet effects of conjugated linoleic acid isomers, Biochim. Biophys. Acta 1438 (1999) 239–246.
[3] C.A. Carter, R.J. Milholland, W. Shea, M.M. Ip, Effect of the prostaglandin synthetase inhibitor indomethacin on 7,12-dimethylbenz[a]-anthracene-induced mammary tumorigenesis in rats fed different levels of fat, Cancer Res. 43 (1983) 3559–3562.
[4] S. Bunting, S. Moncada, J.R. Vane, The prostacyclin–thromboxane A_2 balance: pathophysiological and therapeutic implications, Br. Med. Bull. 39 (1983) 271–276.
[5] S. Moncada, A.G. Herman, E.A. Higgs, J.R. Vane, Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the antithrombotic properties of vascular endothelium, Thromb. Res. 11 (1977) 323–344.
[6] M. Hamberg, J. Svensson, B. Samuelsson, Thromboxanes, a new group of biologically active compounds derived from prostaglandin endoperoxides, Proc. Natl. Acad. Sci. U. S. A. 72 (1975) 2294–2298.
[7] J.A. Oates, G.A. Fitzgerald, R.A. Branch, E.K. Jackson, H.R. Knapp, L.J. Roberts, Clinical implications of prostaglandin and thromboxane A_2 formation, N. Engl. J. Med. 319 (1988) 689–698.
[8] B.B. Weksler, C.W. Ley, E.A. Jaffe, Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and the ionophore A 23187, J. Clin. Invest. 62 (1978) 923–930.
[9] K. Watanabe, J. Tanaka, F.Q. Wen, M. Yoshida, Effect of cytokines on thrombin-stimulated increases in intracellular calcium and PGI_2 production by cultured human umbilical vein endothelial cells, Cell. Signal. 8 (1996) 247–251.
[10] A.A. Spector, J.C. Hoak, G.L. Fry, G.M. Denning, L.L. Stoll, S. Bryant, Effect of fatty acid modification on prostacyclin production by cultured endothelial cells, J. Clin. Invest. 65 (1980) 1003–1012.
[11] J.C. Bordet, M. Guichardant, M. Lagarde, Modulation of prostanoid formation by various polyunsaturated fatty acids during platelet–endothelial cell interactions, Prostaglandins Leukot. Essent. Fat. Acids 39 (1990) 197–202.

[12] C. Benistant, F. Achard, G. Marcelon, M. Lagarde, Platelet inhibitory functions of aortic endothelial cells. Effects of eicosapentaenoic and docosahexaenoic acids, Atherosclerosis 104 (1993) 27–35.
[13] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[14] J.K.G. Kramer, V. Fellner, M.E.R. Dugan, F.D. Sauer, M.M. Mossoba, M.P. Yurawecz, Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids, Lipids 32 (1997) 1219–1228.
[15] G.E. Caughey, L.G. Cleland, P.S. Penglis, J.R. Gamble, M.J. James, Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective upregulation of prostacyclin by COX-2, J. Immunol. 167 (2001) 2831–2838.
[16] M. Camacho, J. Lopez-Belmonte, L. Vila, Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity, Circ. Res. 83 (1998) 353–365.
[17] N. Futaki, S. Takahishi, M. Yokoyama, I. Arai, S. Higuchi, S. Otomo, NS-398—a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX)-2 activity in vitro, Prostaglandins 47 (1994) 55–59.
[18] A.A. Spector, T.L. Kaduce, P.H. Figard, K.C. Norton, J.C. Hoak, R.L. Czervionke, Eicosapentaenoic acid and prostacyclin production by cultured endothelial cells, J. Lipid Res. 24 (1983) 1595–1604.
[19] N. Hasegawa, K. Yamamoto, A step in the process of prostacyclin production whose decline leads to the age-related decrease in production by human umbilical vein endothelial cells in culture, Mech. Ageing Dev. 69 (1993) 167–178.
[20] K. Neubert, A. Haberland, I. Kruse, M. Wirth, I. Schimke, The ratio of formation of prostacyclin/thromboxane A_2 in HUVEC decreased in each subsequent passage, Prostaglandins 54 (1997) 447–462.
[21] B. Sicard, M. Lagarde, Incorporation of some eicosaenoic acids into endothelial cells—effect on platelet inhibitory activity and prostacyclin production, Thromb. Haemost. 53 (1985) 264–267.
[22] J.A. Bulgarella, D. Patton, A.W. Bull, Modulation of prostaglandin H synthase activity by conjugated linoleic acid (CLA) and specific CLA isomers, Lipids 36 (2001) 407–412.
[23] P. Urquhart, S.M. Parkin, J.S. Rogers, J.A. Bosley, A. Nicolaou, The effect of conjugated linoleic acid on arachidonic acid metabolism and eicosanoid production in human saphenous vein endothelial cells, Biochim. Biophys. Acta 1580 (2002) 150–160.
[24] P.W. Parodi, Conjugated octadecadienoic acids of milk fat, J. Dairy Sci. 60 (1977) 1550–1553.
[25] P.A. Masso-Welch, S.F. Shoemaker, W.K. Shea-Eaton, C. Ip, Conjugated linoleic acid inhibits proliferation and induces apoptosis of normal rat mammary epithelial cells in primary culture, Exp. Cell Res. 250 (1999) 22–34.
[26] M. O'Shea, R. Devery, F. Lawless, J. Murphy, C. Stanton, Milk fat conjugated linoleic acid (CLA) inhibits growth of human mammary MCF-7 cancer cells, Anticancer Res. 20 (2000) 3591–3602.
[27] K. Kang, M.W. Pariza, trans-10, cis-12-conjugated linoleic acid reduces leptin secretion from 3T3-L1 adipocytes, Biochem. Biophys. Res. Commun. 287 (2001) 377–382.
[28] S.Y. Moya-Camarena, J.P. Vanden Heuvel, S.G. Blanchard, L.A. Leesnitzer, M.A. Belury, Conjugated linoleic acid is a potent naturally occurring ligand and activator of PPARα, J. Lipid Res. 40 (1999) 1426–1433.