Stimulatory effect of unsaturated fatty acids on the level of plasminogen activator inhibitor-1 mRNA in cultured human endothelial cells

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Abstract To determine whether unsaturated fatty acids induce changes in the mRNA level of plasminogen activator inhibitor type-1 (PAI-1), Northern analyses were performed on human umbilical vein endothelial cells (HUVEC) and vascular smooth muscle cells that were treated with two common fatty acids. Supplementation of cultured HUVEC with docosahexanoic acid (DHA) or with dihomogamma linolenic acid (DGLA), resulted in a concentration dependent, specific increase of the PAI-1 transcript levels, which was detectable within 2 h. DHA and DGLA treatment of smooth muscle cells did not result in changes in the PAI-1 mRNA levels. Homology search of the upstream regulatory region of the PAI-1 gene sequences identified a consensus nucleotide sequence for a fatty acid-responsive element. Our results indicate that unsaturated fatty acids selectively increase PAI-1 mRNA levels in endothelial cells, the primary source of circulating PAI-1 in vivo.

Key words: Plasminogen activator inhibitor type-1; Fatty acid; Docosahexanoic acid; Dihomogamma linolenic acid; Fatty acid-responsive element; Endothelial cell

1. Introduction

Plasminogen activators are serine proteases with restricted substrate specificity. Their primary substrate is plasminogen, which when cleaved forms plasmin, another serine protease which has a broader spectrum of activity. Plasmin mediates fibrinolysis, which is an important process for limiting the growth of fibrin clots within damaged blood vessels. The major circulating physiologic inhibitor of both tissue-type and urokinase-type plasminogen activators in vivo is plasminogen activator inhibitor type 1 (PAI-1), a member of the serpin class of protease inhibitors (for review see [1]). Serum PAI-1 levels have been found to be elevated in young [2] and old [3] survivors of myocardial infarction and in patients with other thrombotic disorders such as deep vein thrombosis [4]. In addition, there is a reasonably strong positive correlation between triglyceride levels and PAI-1 levels in plasma [2,5]. Furthermore, triglyceride-rich lipoproteins such as very low density lipoprotein have been shown to increase PAI-1 secretion from cultured endothelial cells [6].

It has been suggested that dietary fish oil, consisting mainly of unsaturated ω-3 fatty acids, provides protection from coronary artery disease and may reduce elevated triglyceride levels [7–9]. Consumption of fish oil, or plant seed oils rich in unsaturated ω-6 fatty acids has been shown to improve symptoms in patients with chronic inflammatory diseases, including rheumatoid arthritis [10,11]. Paradoxically, in human trials, fish oil has been demonstrated to significantly increase the level of plasminogen activator inhibitor type-1 (PAI-1) in plasma of patients and healthy humans [12–15]. However, a model that would explain the beneficial clinical effects, or the molecular mechanism by which the fatty acids exert their stimulatory action on PAI-1 level, has not been elucidated.

Endothelial cells readily take up fatty acids which are added to the culture medium [16]. The fatty acid either incorporates into cellular lipids or enters the fatty acid metabolic pathway [16,17]. Exogenously provided fatty acid can serve as a biological effector [18]. Fatty acids can also be directly involved in regulating gene expression by binding to specific, fatty acid-responsive transcription factors [19–25].

As part of our investigation of the plasminogen activator system of endothelial and vascular smooth muscle cells, we undertook the present study to evaluate the role of unsaturated fatty acids in the regulation of PAI-1 mRNA. Our results indicate that dihomogamma linolenic acid (DGLA) and docosahexanoic acid (DHA), increase the steady state PAI-1 mRNA level in cultured human endothelial cells. The stimulatory effect is mRNA and cell specific. A potential fatty acid responsive element in the regulatory region of the PAI-1 gene is also identified.

2. Materials and methods

2.1. Fatty acids

Docosahexanoic acid (DHA; C22:6, ω-3) and dihomogamma linolenic acid (DGLA; C20:3, ω-6), (all from Cayman Chemical, Ann Arbor, MI) were stored in ethanol at 0.4 M concentration, at −20 °C.

2.2. Cell culture

Human umbilical vein endothelial cell (HUVEC) were propagated from pooled primary cultures of umbilical veins as previously described [26]. Briefly, endothelial cells were grown on fibronectin coated flasks (Nunclon) to confluence and passaged 2–3 times in complete medium containing Medium 199 (Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, McLean, VA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco), and endothelial cell growth factor (ECGF, partially purified acidic FGF; ~150 ng/ml) and heparin (90 μg/ml). Cells were split 1:2 and fed every 2–3 days. Human vascular smooth muscle cells (SMC) were cultured from umbilical vein using the explant technique [27]. Briefly, the SMC were cultured in DMEM (Gibco) and Ham's F-12 (Gibco) (1:1 ratio) containing medium that was supplemented with

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FCS, penicillin and streptomycin as described above. For any given experiment, cells at second or third passage level were seeded into fibronectin-coated Petri dishes (10 cm in diameter). On the following day, the medium was replaced with fresh medium, and the cells were further cultured. On the next day when the cells reached confluency, the culture medium was supplemented with fatty acid and the cells were further cultured for the indicated time period. Control experiments testing the effect of solvent ethanol, up to a 1.8% final concentration, were also included. In the time course experiments fresh medium was added to the cells at the time of fatty acids supplementation. At the end of the fatty acid treatments cells appeared normal under phase contrast microscopy. Viability was >98% using Trypan blue exclusion test as criterion.

2.3. RNA isolation

Total cytoplasmic RNA was isolated by immediate solubilization of the cells in a one-phase solution of phenol and guanidine thiocyanate (RNazol, Biotecx, Houston, TX) according to the manufacturers recommendation. Briefly, culture medium was removed from the cells and 1 ml RNazol was added to each dish to lyse the cells. A scraper was used to collect the lysate. The lysate was extracted with chloroform, and the RNA in the aqueous phase was precipitated with isopropanol. The RNA was quantified by absorbance at 260 nm. The average yield was ~70–90 μg RNA per dish. The quality of the RNAs was tested on agarose gel by analyzing the integrity of the UV visualized, ethidium bromide stained 28S and 18S rRNAs.

2.4. Northern blot analysis

RNA (10 μg) was denatured and separated on 1% agarose/formaldehyde gel, then transferred to Nytran membrane (Schleicher and Schuell, Keene, NH) according to standard procedures [28]. The RNA was fixed to the membrane with ultraviolet irradiation and baked in a vacuum oven at 70°C for 30 min to eliminate traces of formaldehyde. The membrane was prehybridized in 5 × SSC, 50% (v/v) deionized formamide (Clonetech, Palo Alto, CA), 5 × Denhardt's solution (1 × Denhardt's solution contains 0.02% BSA, 0.02% Ficoll 400, 0.02% Polyvinyl pyrrolidone), 0.1% SDS, 10 mM Tris-HCl (pH 7.5) and 0.1 mg/ml sonicated Salmon sperm DNA (Sigma, St. Louis, MO) at 42°C for 2 h in a hybridization oven (Techne, Princeton NJ). The hybridization was performed at 42°C for 16–22 h in the prehybridization solution which was supplemented with the appropriate radiolabeled and denatured probe. To probe the Northern blots, 25 ng of DNA was labeled using [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) and a random prime labeling kit (Boehringer Mannheim, Indianapolis, IN). The blots were washed three times with 2 × SSC and 0.1% SDS at room temperature for 15 min, then washed once with 0.1 × SSC and 0.1% SDS at 52°C for 10 min. The filters were exposed to Kodak XAR film using an intensifier screen at −70°C for 4–48 h. To obtain exposures in the linear range of the film, the same Northern blots were exposed for different time periods. Before reprobing, the filters were stripped of the previous probe by shaking at 90°C in 0.01% SDS solution for 20 min. They were then re-exposed with films to confirm successful stripping. Autoradiograms of the Northern blots were quantified by densitometry using a Microtech scanner and image analysis program (Image V1.4 NIH). Densitometric values were first adjusted for differences of the background, then normalized to values of the same blot reprobed with GAPD. Some of the Northern blots were also quantitated using storage phosphor technology (PhosphorImager, Molecular Dynamics, Sunnyvale, CA).

2.5. Probes

The following specific cDNAs were used to probe the blot. For uPAR transcripts the probe was a ~1.1 kb insert from pGEMuPAR which contains the full coding sequence for human uPAR [29]. For the detection of PAI-1 mRNA, a purified 1.9 kb human specific cDNA from pUC19-PAI-1 (kindly provided by Dr. T.C. Wun of Monsanto Co.) was used. The glyceraldehyde-3-phosphate dehydrogenase (GAPD) transcripts were detected by using the linearized plasmid pHcGAP (ATCC, Rockville, MD). For α-enolase mRNA detection an RT-PCR amplified ~1.1 kb product was used as probe [30]. The human β-actin specific probe was a 620 bp long cDNA that was obtained in RT-PCR amplification using specific primers and HUVEC mRNA. The primers were designed based on the β-actin sequence obtained from GenBank (Accession Number: X00351). The 5'-primer (5'-CAAGGCCAACCGCGAGAAG-3') corresponds to nucleotides 377–395, the 3'-primer (5'-CAGTGATCTCCTTCTGCATC-3') corresponds to nucleotides 996–977. Probes obtained by PCR amplification were gel purified before labeling.

2.6. Nucleotide sequence analyses

Sequence information was retrieved from GenBank R81.0 by the MacDNASIS V3.2 program (Hitachi, San Bruno, CA) using the following Accession Numbers: human PAI-1 gene, X06692; mouse PAI-1 gene, M33961; rat S14 gene, M33553; mouse fatty acid binding protein (aP2) gene, M84651; [31]. Sequence alignment was performed with the MegAlign program (DNASTAR, Madison, WI).

3. Results

3.1. DGLA and DHA increase the level of PAI-1 mRNA of cultured endothelial cells

It has been shown that culturing HUVEC in medium supplemented with 150 μM of a variety of saturated or unsaturated fatty acids results in dramatic changes in the fatty acid composition of the cells without any detectable effect on cell counts

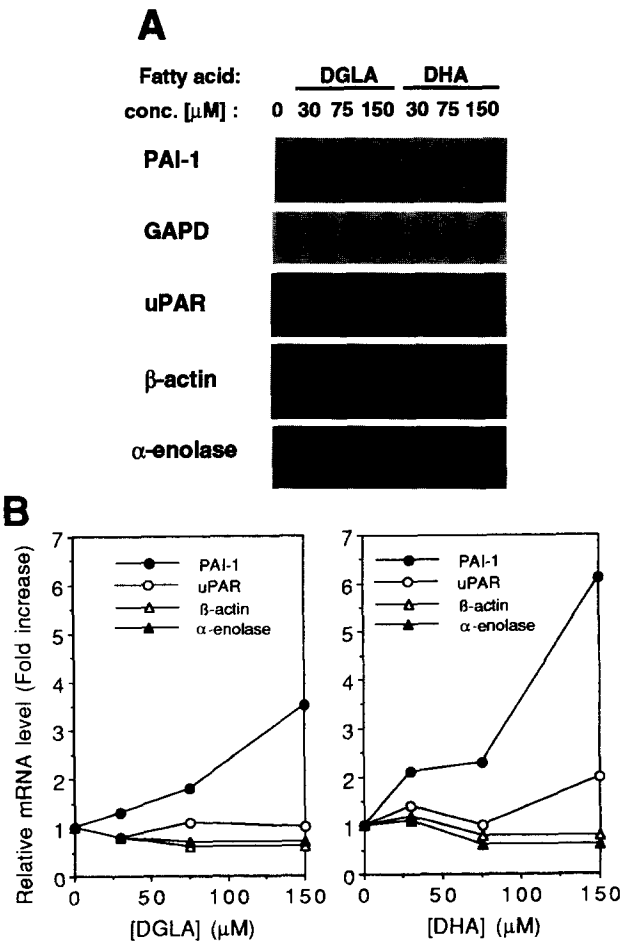

Fig. 1. The effect of DGLA and DHA on mRNA level of human endothelial cells. (A) Human umbilical vein endothelial cells were treated with DGLA and DHA for 22 h. Total RNAs were isolated and 10 μg aliquots were analyzed on Northern blot using the following probes: PAI-1 (plasminogen activator inhibitor-1), GAPD (glyceraldehyde phosphate dehydrogenase), uPAR (urokinase receptor), β-actin and α-enolase. (B) Densitometric analyses of the autoradiograms. The arbitrary values were standardized for GAPD mRNA to correct for uneven sample loading. Data are presented relative to the specific mRNA of the control untreated cells.

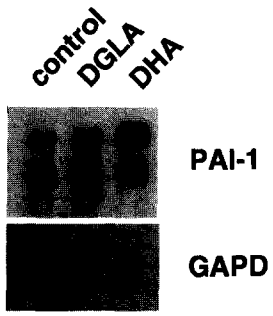

Fig. 2. PAI-1 mRNA induction by DGLA and DHA. Human endothelial cells were treated with 150 μM DGLA or DHA for 2 h. Total RNAs were isolated and 10 μg aliquots were analyzed on Northern blot using PAI-1 and GAPD as probes.

or their viability [32]. To evaluate if the supplementary fatty acids can specifically regulate the level of mRNA in endothelial cells, we selected treatment conditions similar to those used for the metabolic studies [32,33].

Confluent third passage HUVEC were cultured in media with or without DGLA or DHA supplementation. Cells were harvested 22 h later and total RNA prepared. Autoradiograms of the Northern blots and their densitometric analyses are shown in Fig. 1 A and B, respectively. Cultured HUVEC expressed detectable levels of both the 3.4 and 2.4 kb PAI-1-specific mRNA species. Addition of DGLA or DHA to the culture media resulted in concentration dependent increases in the levels of both PAI-1 transcripts. The effect appeared to be specific because steady state levels of mRNA for uPAR, another component of the endothelial cell plasminogen activator system was not substantially changed, nor were transcript levels for β-actin, GAPD or α-enolase. In control experiments, corresponding amounts of solvent ethanol, up to a 1.8% final concentration, had no detectable effect on cell morphology or on mRNA levels of PAI-1 and GAPD. Higher concentrations of fatty acids (~600 μM) were toxic to HUVEC and resulted in decreases in steady state PAI-1 mRNA level (not shown). Stimulation with oleic acid (75 μM) performed in parallel with the experiment presented in Fig. 1, resulted in a 3.4-fold increase in PAI-1 mRNA (not shown). Four independent experiments revealed that the mean fold increase in PAI-1 mRNA levels compared to the untreated control was 2.2 ± 0.5 (±S.E.M., $P < 0.05$) for DGLA (150 μM). In 8 independent experiment, there was a 2.3 ± 0.6-fold increase ($P < 0.0003$) for DHA (150 μM). In time course studies, at the earliest point investigated (2 h), a 1.7-fold increase in PAI-1 mRNA level was detected for both DGLA and DHA (150 μM), as shown in Fig. 2.

Some variability in the magnitude of the stimulatory effect was noted, depending on the baseline absolute level of PAI-1 mRNA. In three additional experiments where baseline PAI-1 mRNA levels were found to be substantially higher (~5-fold) than in the other 12 experiments, no significant further increase in PAI-1 transcript levels could be detected, suggesting that the stimulation of PAI-1 mRNA by fatty acids required no more than moderately high PAI-1 steady state mRNA levels at baseline to observe the effect.

3.2. *DGLA and DHA effect on the level of PAI-1 mRNA of cultured vascular smooth muscle cells*

To determine if the fatty acid stimulatory effect was present in other vascular cells known to secrete PAI-1 in culture, we tested the effect of DGLA and DHA on steady state PAI-1 mRNA levels in cultured human umbilical vein smooth muscle cells in a similar fashion as described above for HUVEC. Autoradiograms of the Northern blots and their densitometric analyses are shown in Fig. 3 A and B, respectively. Four independent experiment revealed that DGLA and DHA treatment of SMC did not result in a significant change in PAI-1 transcript levels. The mRNA levels for uPAR, GAPD and α-enolase were also unchanged. The absolute PAI-1 levels in these experiments were similar to levels in HUVEC studied in parallel that had demonstrated significant enhancement with fatty acid. This suggest that lack of effect in SMC was not due to very high baseline PAI-1 mRNA levels. Finally, as shown in Fig. 4 (which depicts the individual data for experiments performed with DGLA and DHA at 150 μM in both cell types), the stimulatory effect is mainly and consistently seen only in endothelial cells, although the degree of the effect is somewhat variable.

4. Discussion

Results of these experiments demonstrate that DGLA and DHA enhance steady state levels of PAI-1 mRNA in cultured

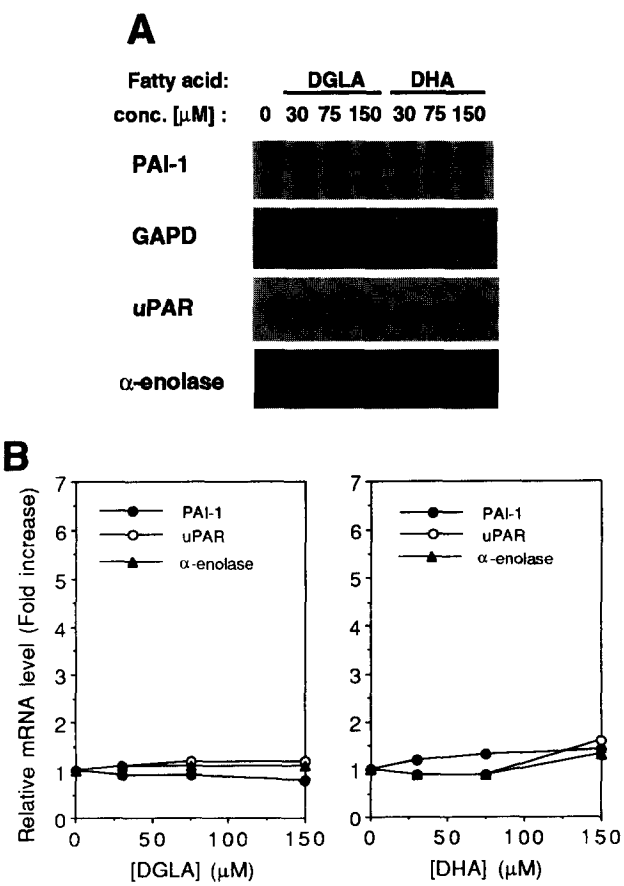

Fig. 3. The effect of DGLA and DHA on mRNA level of human vascular smooth muscle cells. (A) Human vascular smooth muscle cells were treated with DGLA and DHA for 24 h. Total RNAs were isolated and 10 μg aliquots were analyzed on Northern blot using the probes described in the legend of Fig. 1. (B) Densitometrical analyses of the autoradiograms. The arbitrary values were standardized for GAPD mRNA to correct for uneven sample loading. Data are presented relative to the specific mRNA of the control untreated cells.

human endothelial cells. DGLA and DHA treatment increases PAI-1 mRNA levels of HUVEC in a concentration dependent fashion, but does not alter steady state levels of β-actin, α-enolase and GAPD transcripts. Tissue specificity of these effects are suggested by the observation that DGLA and DHA treatment of cultured vascular smooth muscle cells have no effects on levels of PAI-1, uPAR, α-enolase and GAPD mRNAs. These results support the basic finding obtained in clinical trials based on dietary studies [12–15], that polyunsaturated fatty acids increase circulating PAI-1 levels.

Our results indicate that DGLA and DHA selectively increase the steady state level of PAI-1 mRNA. The mechanism by which these unsaturated fatty acids exert this stimulatory effect is not known. One potential explanation could be that the fatty acids are directly involved in transcriptional regulation of the PAI-1 gene. An interesting feature of the polyunsaturated fatty acids is their ability to regulate the expression of genes by binding to fatty acid responsive factors [22,23,25]. Sloots et al. identified consensus nucleotide sequences in the upstream region of five oleic acid-inducible genes of the diploid yeast *Candida tropicalis*. By using mutational analyses they confirmed these sequences to be fatty acid-responsive regulatory elements [19]. Searching for sequence homology in the 5′-flanking region of the human PAI-1 gene, we could identify sequences that are highly homologous to the consensus fatty acid-responsive element (Fig. 5). We could also localize these consensus sequences in the promoter region of the murine PAI-1 gene, as well as in the regulatory regions of the rat S14 and murine aP2 genes, both of which have been reported to be transcriptionally regulated by fatty acids [23,25] (Fig. 5). These findings suggest a potential mechanism for the direct involvement of the unsaturated fatty acids in the stimulation of PAI-1 transcription.

In conclusion, the present study demonstrates that unsaturated fatty acids can induce PAI-1 mRNA in endothelial cells, which are the primary source for PAI-1 production in vivo [34]. Based on the identification of a fatty acid-responsive regulatory element in the PAI-1 gene we can hypothesize that DHA and DGLA might induce PAI-1 transcription directly. These findings suggest a mechanism whereby circulating PAI-1 are increased in humans consuming unsaturated fatty acids.

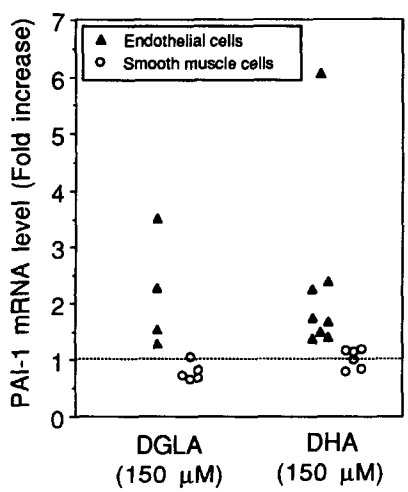

Fig. 4. Comparison of the effect of DGLA and DHA on PAI-1 mRNA levels in endothelial and smooth muscle cells. Human endothelial or smooth muscle cells were treated for 22h with DGLA or DHA (150 μM) and the level of PAI-1 mRNA asssessed relative to untreated control cells as described in Fig. 1. Each symbol represent data from an independent experiment.

consensus[a]:	C/T	G	G	T	T	A/G	T	T	A/G/C	
yeast HDE	C	G	G	T	T	A	T	T	A	-347
yeast POX4	C	G	G	T	T	A	T	T	C	-351
yeast CATL	T	G	G	T	T	A	T	T	A	-687
yeast POX18	C	G	G	T	(A)	G	T	T	A	-215
yeast P450alk	T	G	G	T	T	G	T	T	G	-261
mouse aP2	T	G	G	T	T	(C)	T	T	C	-4892
mouse PAI-1	T	G	G	T	T	(C)	T	T	G	-773
human PAI-1	T	G	G	(C)	T	G	T	T	G	-533
rat S14	T	G	G	(G)	T	G	T	T	G	-717

Fig. 5. Homology alignment of established and potential fatty acid-responsive gene regulatory elements. Conserved fatty acid-responsive sequences of oleic acid inducible genes of yeast (*HDE*, peroxisomal trifunctional enzyme hydratase-dehydrogenase-epimerase; *POX4*, fatty acyl-CoA oxidase; *CATL*, catalase; *POX18*, peroxisomal 18-kDa protein; *P450ALK*, alkane-inducible cytochrome P450) [19] are aligned with highly homologous regulatory sequences of human and murine PAI-1, rat S14 and murine aP2 genes. Numbering indicates the nucleotide distance upstream from the transcription start site (+1). Residues that differ from the consensus are circled. [a]Consensus fatty acid-responsive regulatory sequence proposed by Sloots et al. [19] based on the analyses of the shown five yeast genes.

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