Lipid peroxidation products upregulate c-fos and TF expression in human vascular smooth muscle cells

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Background. Regulation of vascular smooth muscle cells (VSMC) by lipid peroxidation products play an important role in the development of atherosclerosis. Proliferation and thrombosis are two relevant atheromatous events related to VSMC response to oxidative stress.

Objective. The aim of this study is to evaluate the effect of lipid peroxidation on c-fos, c-jun and TF expression in human VSMC (hVSMC).

Methods. We tested the effect of different degrees of LDL modification (native, minimally modified and extensively oxidized), two apolar aldehydes: hexanal and 2,4-decadienal (2,4-DDE) and the hydroxyaldehyde, 4-hydroxynonenal (4-HNE) on some gene expressions in hVSMC. c-fos, c-jun and TF mRNA expression were estimated by RT-PCR and TF antigen was determined by ELISA.

Results. 2,4-DDE and hexanal, two apolar aldehydes that are present in extensively oxidized LDL (oxLDL), increased c-fos mRNA expression in HVSMC, while 4-HNE showed a slight decrease. None of the studied molecules were able to produce a significant change on c-jun mRNA levels. The major effect on c-fos expression was observed by 2,4-DDE, an aldehyde that also increased TF mRNA and protein expression.

Conclusion. The results of this study show that 2,4-DDE and hexanal upregulate c-fos expression in HVSMC. 2,4-DDE also increases TF expression in these cells. Our findings suggest that apolar aldehydes could contribute to the development of atherosclerotic lesions due to the prothrombotic properties observed.

Key words:
Lipid peroxidation. TF. c-fos. c-jun. hVSMC.

LOS PRODUCTOS DERIVADOS DE LA PEROXIDACIÓN LIPÍDICA ESTIMULAN LA EXPRESIÓN DE C-FOS Y TF EN CÉLULAS MUSCULARES LISAS HUMANAS

Introducción. La regulación de las células musculares lisas por productos derivados de la peroxidación lipídica tiene un papel importante en el desarrollo de la arteriosclerosis. La proliferación y la trombosis son dos acontecimientos ateromatosos relacionados con la respuesta de las células musculares lisas (CML) al estrés oxidativo.

Objetivo. El objetivo de este estudio es evaluar el efecto de la peroxidación lipídica sobre la expresión de c-fos, c-jun y TF en CML humanas.

Métodos. Hemos probado el efecto de diferentes grados de LDL modificada (nativa, mínimamente modificada y extensamente oxidada), dos aldehídos apolares: hexanal y 2,4-decadienal (2,4-DDE) y el hidroxialdehído, 4-hidroxinonenal (4-HNE) sobre la expresión de determinados genes en CML humanas. La expresión de mRNA de c-fos, c-jun y TF se estimó por RT-PCR y el antígeno del TF se determinó por ELISA.

Resultados. El 2,4-DDE y el hexanal, dos aldehídos apolares presentes en la LDL extensamente oxidada (oxLDL), aumentaron la expresión de mRNA de c-fos en CML, mientras que el 4-HNE mostró un ligero descenso. Ninguna de

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las moléculas estudiadas fueron capaces de producir un cambio significativo sobre los valores de mRNA de c-jun. El principal efecto sobre c-fos se observó con el 2,4-DDE, aldehído que también incrementó la expresión de mRNA y de proteína del TF.

Conclusión. Los resultados de este estudio muestran que el 2,4-DDE y el hexanal estimulan la expresión de c-fos en CML. El 2,4-DDE, además, aumenta la expresión de TF en estas células. Estos resultados sugieren que los aldehídos apolares podrían contribuir al desarrollo de las lesiones de arterosclerosis debido a las propiedades protrombóticas observadas.

Palabras clave:
Peroxidación lipídica. TF. c-fos. c-jun. CML.

Introduction

Lipid peroxidation plays an important role in the pathogenesis of atherosclerosis[1]. The presence of oxidized lipids in atherosclerotic plaques have been demonstrated both in humans and experimental animals[2]. The oxidative modification of LDL within the arterial wall leads to the formation of a wide range of oxidative products[3]. Among them, aldehydes, end products of PUFA oxidation, could modulate the functionality of the different cell types present in the intima. It has been demonstrated that oxidized LDL (oxLDL) and reactive oxygen species (ROS) enhanced vascular smooth muscle cell (VSMC) proliferation[4,5]. Furthermore, hydroperoxyoctadecadienoic acids (HPODEs) have mitogenic effects, specifically increasing c-fos and c-jun mRNA expression[6]. In more advanced stages of the lesion, the VSMC secretory activity became more important. *In vitro* studies have shown that oxLDL produce thrombogenic effects in VSMC increasing tissue factor (TF) expression and these effects seem to be modulated by oxysterols[7].

In the present report, we have studied the effect of lipid peroxidation on the expression of genes that regulate cell proliferation and thrombosis in human VSMC (hVSMC). We have focused on the effects of different degrees of LDL oxidation and two types of aldehydes: apolar aldehydes (hexanal and 2,4-decadienal (2,4-DDE)) and hydroxyaldehydes (4-hydroxynonenal (4-HNE)) on c-fos, c-jun and TF mRNA expression.

Material and methods

Cell culture

HVSMC from human uterine artery were a kind gift from Dr. Eva Hurt-Camejo, Göteborg University (Sweden). Cells were grown in Waymouth medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2% (v/v) sodium pyruvate, 1% (v/v) L-glutamine and antibiotics (Invitrogen, USA). Cells were used at passages 3 to 6 and all experiments were done in the absence of FBS and at 70-80% confluence. The concentrations of aldehydes used are in the 0.5-25 µM range. Cells incubated with vehicle alone (ethanol less than 0.5%) were designated as untreated cells. Cytotoxicity was evaluated by means of LDH leakage in the medium and cells were also visualized under phase-contrast microscopy to assess morphological changes.

Determination of oxidative parameters

LDL particles were isolated from a pool of human plasma-EDTA by sequential preparative ultracentrifugation[8]. Minimally modified LDL (mmLDL) was obtained by storing the LDL in PBS at 4 ºC for up to 4 months and extensively oxidized LDL (oxLDL) were performed by direct oxidation with copper ions[9].

The degrees of LDL oxidation were confirmed by the evaluation of their electrophoretic mobility (Lipofilm, SEBIA, France) and by measuring the thiobarbituric acid reactive substances (TBARS)[10] and the hydroperoxides level using a colorimetric kit (Wak-Chemie Medical GMBH, Germany).

The aldehydes content in LDL preparations were evaluated by thin layer chromatography to separate the different types present[11].

Analysis of mRNAs levels by RT-PCR

HVSMC were incubated in 12-well dishes in the presence of native LDL (natLDL), mmLDL and oxLDL (50 µg/ml) and 4-HNE, 2,4-DDE and hexanal (0.5, 5 and 25 µM) for up to 2 h in the dose response experiments for studying c-fos and c-jun genes. The time response experiments were done at 30 min, 1 and 2 h and 5 µM of 2,4-DDE and hexanal. TF was detected in the samples of 2,4-DDE time-response experiments. Total cellular RNA was isolated from the cells by the Ultraspec RNA isolation reagent (Biotecx Lab., Inc., USA). A semiquantitative RT-PCR method was used to determine c-fos, c-jun and TF mRNA using the SuperScript one-step RT-PCR system (Life Technologies, USA). β-actin was used as a constitutive expression gene to normalize the results of the genes of interest. Results are expressed as the % of variation of the net intensity of each condition with respect to the untreated cells after normalization to β-actin results.

Determination of TF antigen

Cell lysates were prepared from HVSMC in 10 cm dishes previously incubated in the presence of 5 µM 2,4-DDE for 30 min and 2 h. Briefly, cells were scraped in 10 mM Tris pH 7.4, containing antiproteases, disrupted by repeated steps of freezing-thawing, cellular debris were sedimented by centrifugation and supernatants were equilibrated in PBS. TF antigen was measured in cell lysates using a commercial enzyme-linked immunoabsorbent assay (American Diagnostica Inc., USA). The concentration of TF was calculated as ng/l using human TF at a concentration between 50 and 1000 ng/l. Results are calculated as ng/mg protein and expressed as percentage of those obtained with the blank.

Results

Analysis of oxidative parameters

Table 1 shows that the levels of hydroxyperoxides, as markers of mildly modified LDL, were higher in mmLDL (127.8 nmol/g protein) than in

Table 1. Lipid peroxidation markers in LDL preparations

	Md (cm)	TBARS (nmol/mg prot.)	LOOH (nmol/g prot.)
nat LDL	0.95 ± 0.05	< 0.5	3.3 ± 0.5
mm LDL	1.23 ± 0.08	3.7 ± 0.7	127.8 ± 11.0
ox LDL	2.10 ± 0.10	45.0 ± 3.0	7.9 ± 0.9

MM-LDL was obtained by storing LDL at 4 ºC for 4 months in antioxidant free-PBS and represents a LDL particle slightly modified. Extensively oxidized LDL was obtained by incubation with 10 µM $CuCl_2$. Migration distance (Md) of lipoproteins represent the characteristic increase of electronegativity observed in ox-LDL by agarose-acrylamide gel electrophoresis.
TBARS: thiobarbituric reactive substances. LOOH: lipid hydroperoxides. Results are expressed as the mean ± SEM.

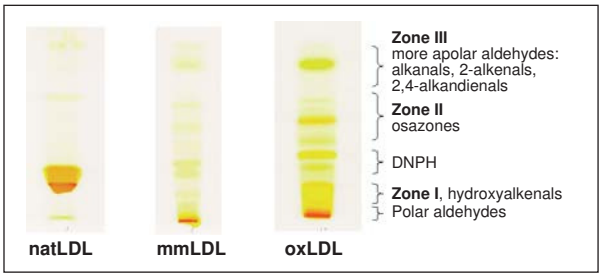

Figure 1. Analysis of aldehydes in LDL preparations. Native, minimally and oxidized LDL were derivatized with 2,4-dinitrophenylhydrazin (DNPH). The hydrazone derivatives were extracted in dichloromethane and subjected to thin layer chromatography to separate the different types of aldehydes by their polarity. 4-hydroxynonenal as a hydroxyaldehyde belongs to zone I and 2,4-decadienal, and hexanal as apolar aldehydes belong to zone III.

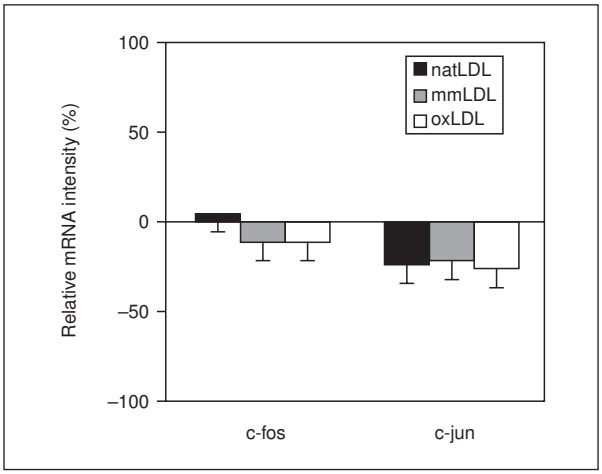

Figure 2. Effect of LDL particles on c-fos and c-jun mRNA levels. HVSMC were incubated with 50 µg/ml of natLDL, mmLDL or oxLDL for 2 h. Cells incubated with vehicle alone were designated as untreated cells. RNA levels were quantified as described in Methods and the data represent the percentage of the relative mRNA intensity with respect to untreated cells. The data are shown as the mean ± SEM, n = 3.

oxLDL (7.9 nmol/g prot). In contrast, TBARS levels, as markers of highly modified LDL, were increased in oxLDL (45 nmol/mg protein) with respect to natLDL and mmLDL. There is an increase in electronegativity associated to the degree of LDL modification. OxLDL showed higher amounts of aldehydes than mmLDL and these compounds were undetected in natLDL. A clear appearance of apolar aldehydes is shown in oxLDL while it is not observed in nat- and mmLDL (fig. 1).

Effect of LDL oxidation and aldehydes on c-fos and c-jun genes in HVSMC

RT-PCR analysis showed a slight decrease in mRNA levels of c-fos and c-jun genes after HVSMC incubations with LDL preparations (50 µg/ml) (fig. 2).

HVSMC incubated for 2 hours with 4-HNE showed a concentration dependent decrease in c-fos and c-jun mRNA levels that began at 0.5 µM. The highest effect was observed at 5 µM 4-HNE in c-fos mRNA expression (30%) (fig. 3).

Apolar aldehydes: 2,4-DDE and hexanal increased c-fos mRNA levels in a dose dependent manner after 2 h of incubation. Low levels of hexanal (0.5 µM) were sufficient to increase c-fos transcription (68%) and the maximum effect was achieved at 5

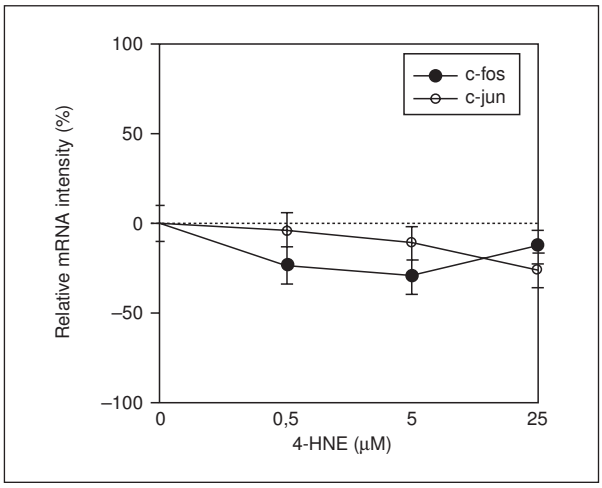

Figure 3. Effect of 4-HNE on c-fos and c-jun mRNA levels. HVSMC were incubated with 4-HNE at concentrations of 0.5, 5 and 25 µM for 2 h. The commercial pure aldehyde (Aldrich, Spain) was dissolved in ethanol and the final concentration of ethanol in the cell culture media was less than 0.5%. Cells incubated with vehicle alone were designated as untreated cells. RNA levels were quantified as described in Methods and the data represent the percentage of the relative mRNA intensity with respect to untreated cells. The data are shown as the mean ± SEM, n = 3. cells.

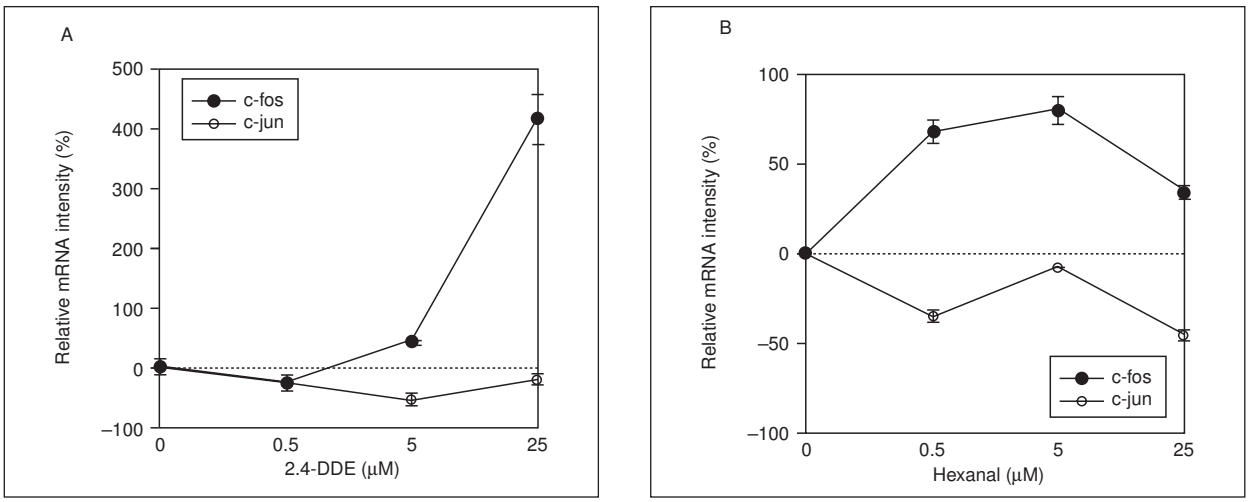

Figure 4. 2,4-DDE and hexanal up-regulate c-fos mRNA levels. HVSMC were incubated with 2,4-DDE (A) or hexanal (B) at concentrations of 0.5, 5 and 25 μM for 2 h. Each commercial pure aldehyde (Aldrich, Spain) were dissolved in ethanol and the final concentration of ethanol in the cell culture media was less than 0.5%. Cells incubated with vehicle alone were designated as untreated cells. RNA levels were quantified as described in Methods and the data represent the percentage of the relative mRNA intensity with respect to untreated cells. The data are shown as the mean ± SEM, n = 3.

μM (80%) (fig. 4A). The effect of 2,4-DDE started at 5 μM (37%) and was maximum at 25 μM. DDE at 25 μM (414%) produced the strongest effect seen in the study. Results from dose response experiments of c-jun did not show any differences (fig. 4B).

Time dose experiments indicated that the upregulation of c-fos mRNA levels were evident at 30 minutes for 2,4-DDE and at 2 h for hexanal (data not shown).

The concentrations of modified LDL and aldehydes used in all the experiments did not produce any morphological changes or any LDH leakage in HVSMC (data not shown).

2,4-DDE induces TF expression

Our data (fig. 5A) revealed that between 30 min and 1 h 2,4-DDE (5 μM) incubations produced an increase in TF mRNA levels and then returned to baseline.

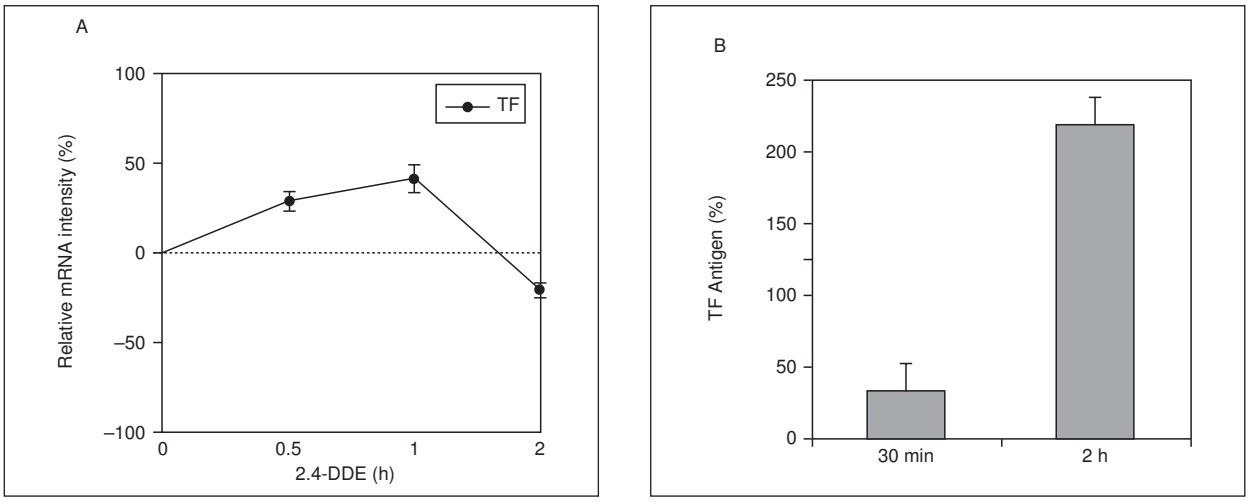

Figure 5. 2,4-DDE modulate TF expression. HVSMC were incubated with 2,4-DDE at 5 μM for up to 2 h. TF mRNA (A) and TF protein (B) expression were estimated by RT-PCR and ELISA respectively. Cells incubated with vehicle alone were designated as untreated cells. RNA and antigen levels were quantified as described in Methods and the data represent the percentage of the relative mRNA intensity or the percentage of TF antigen levels with respect to untreated cells at each time point. The data are shown as the mean ± SEM, n = 3.

The aldehyde consistently increased TF antigen over time (40% at 30 min and 220% at 2 h) (fig. 5B), results that correlated with mRNA studies.

Discussion

The present study shows that apolar aldehydes significantly increase c-fos mRNA expression in HVSMC. Of the studied aldehydes, 2,4-DDE produces the strongest effect on c-fos transcription and this aldehyde also increases TF expression.

Several reports have detected the presence of oxidized lipids *in vivo*[2]. OxLDL used in the study contains apolar aldehydes which could impair biological actions. Previous reports have shown that 4-HNE and 2,4-DDE could modulate chronic inflammation in vascular cells[12,13]. 4-HNE as well as linoleic acid and its oxidative metabolites, the HPODEs, are potent mitogens in VSMC increasing c-fos and c-jun mRNA[6,14]. Our data demonstrate that the apolar aldehydes studied, in contrast to hydroxyaldehydes, are able to induce c-fos expression in HVSMC. The main effect was observed with 2,4-DDE, an aldehyde that has a highly biological activity usually associated with cytotoxicity at high concentrations[15]. However, oxLDL used in our experiments did not significantly modify the studied genes, probably due to the low concentration and exposition time used. At the concentration used, oxLDL probably has low levels of apolar aldehydes and due to the low polarity of 2,4-DDE, it can remain inside the particle and this could block its action. In our study we observed that 2,4-DDE also has a prothrombotic effect by inducing TF expression. The finding that 2,4-DDE increases c-fos mRNA correlates with an induction of TF mRNA and protein expression. The proliferation gene c-fos codes for a protein that belongs to the AP-1 family of transcription factors. We hypothesize that the increase observed in c-fos could imply an upregulation of TF transcription through AP-1 because it has been described that the TF gene is regulated by this transcription factor[16]. c-Fos must heterodimerize with c-Jun to bind to DNA. Although c-jun expression is not increased by apolar aldehydes, preexisting c-Jun could be enough to form the heterodimer. It has been shown that c-fos and c-jun are constituvely expressed in human monocytes[17].

Our findings could have important pathophysiological implications, because TF is expressed in atherosclerotic plaques, because c-fos, c-jun are expressed during SMC proliferation in vascular injury progression and because oxidized compounds have been detected in atherosclerotic lesions. If the studied aldehydes *in vivo* showed similar effects to our findings, they could contribute to the development of atherosclerotic lesion due to the prothrombotic properties observed.

Acknowledgements

This work was supported by a grant from the Fundación Española de Arteriosclerosis.

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