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Cellular Enrichment with Polyunsaturated Fatty Acids Induces an Oxidative Stress and Activates the Transcription Factors AP1 and NFκB

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A 48-h incubation of cultured human fibroblasts with 5×10^{-5} M oleic acid or polyunsaturated fatty acids (PUFA) from the (n-6) (linoleic, γ-linolenic and arachidonic acids) or (n-3) (α-linolenic and eicosapentaenoic acids) series resulted in an enrichment of the cells with the introduced fatty acid. Cell enrichment with PUFA initiated a rise in the intracellular level of reactive oxygen species (ROS) and lipid peroxidation products (thiobarbituric reactive substances TBARS). Simultaneously, cell enrichment with all the studied PUFA induced an increase in AP1 and NFκB binding activity measured by electrophoretic mobility shift assay, whereas no significant effect was observed with the monounsaturated oleic acid. Furthermore, the antioxidants vitamin E (α-tocopherol) and *N*-acetyl cysteine prevented both the arachidonic acid-induced increase in intracellular ROS and TBARS, and the activation of AP1 and NFκB. These results indicate that the accumulation of PUFA from (n-6) and (n-3) series elicited an intracellular oxidative stress, resulting in the activation of oxidative stress-responsive transcription factors such as AP1 and NFκB.

Key Words: **polyunsaturated fatty acids; oxidative stress; AP1; NFκB.**

Reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical are constantly formed in all aerobic cells by the mitochondrial electron transport chain and several enzymatic reactions (1, 2). Cellular antioxidant defenses including vitamin E and vitamin C protect cells from their deleterious effects on DNA, proteins and lipids (3). Either an overproduction of ROS or a decrease in cellular defenses result in variation of the redox state, leading to oxidative stress. This phenomenon has been involved in the pathogenesis of diseases such as atherosclerosis and cancer, as well as in aging and in inflammatory disorders (4, 5). It has also been demonstrated that reactive oxygen species can stimulate the expression of early growth-response genes and cause growth in some cell types (6, 7).

The double bonds found in PUFA of cellular lipids are ready targets for ROS. Several laboratories have demonstrated that increasing the amount of double bonds by supplementation of cultured cells with PUFA enhanced their susceptibility to oxidant injury in terms of lipid peroxidation (8, 9). In addition, this effect was prevented by the addition of the antioxidant vitamin E (10). We (11) and others (12) have reported that supplementation of cultured cells with PUFA enhanced the secretion of superoxide anion. Simultaneously, the intracellular level of lipid peroxidation products was increased (9, 11).

The transcription factor AP1 consists of homo- or heterodimers of the proteins encoded by the *fos* and *jun* gene families and is believed to regulate genes involved in the control of cell growth and differentiation (13). More recently, it has been also shown that AP1 activity is under the control of the Redox state of the cell (14, 15). The transcription factor NFκB is present in the cytoplasm as an heterodimer composed of NFκB1 (p50) and Rel A (p65) subunits bound to an inhibitor protein, IκB (16). Following activation, NFκB dissociates from IκB and is translocated to the nucleus (16). NFκB regulates the inducible expression of a variety of genes involved in inflammatory or immune responses and in cell growth control (16, 17). The involvement of ROS such as H_2O_2 in the activation of NFκB has been evoked (18) and it is admitted that NFκB is an oxidative stress-responsive transcription factor in eukaryotic cells (19).

Abbreviations used: PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances (lipid peroxidation end products); NAC, *N*-acetyl cysteine.

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TABLE I

Modification of the Fatty Acid Composition of MRC5 Fibroblasts Cultured in Media Enriched with Fatty Acids

	14:0	16:0	16:1	18:0	18:1 n-9	18:1 n-7	18:2 n-6	18:3 n-6	18:3 n-3	20:3 n-9	20:3 n-6	20:4 n-6	20:5 n-3
Control	3.38	26.90	10.70	14.86	28.04	7.06	2.05	n.d.	n.d.	n.d.	0.93	5.28	0.80
18:1n-9	3.56	24.50	8.66	12.80	35.93	6.25	2.86	n.d.	n.d.	n.d.	0.60	4.38	0.46
18:2n-6	2.22	26.75	7.25	15.36	18.95	5.44	15.60	0.60	n.d.	0.31	1.00	6.02	0.50
18:3n-6	0.51	24.38	4.44	17.06	20.26	5.95	2.10	11.83	n.d.	n.d.	7.85	5.62	n.d.
20:4n-6	2.92	30.97	7.33	14.15	20.59	5.75	5.27	0.35	n.d.	n.d.	0.55	10.38	n.d.
18:3n-3	3.14	27.65	6.61	14.95	16.59	4.90	3.31	n.d.	17.17	n.d.	0.33	3.07	2.28
20:5n-3	2.76	30.31	7.29	16.39	19.52	5.53	2.99	n.d.	n.d.	n.d.	0.64	3.22	9.08

Note. Cells were cultured for 48 h in DMEM devoid of serum and supplemented with 0.1% (w/v) BSA and 5×10^{-5} M fatty acids. Results are expressed as means of the percentages of cellular fatty acids (four experimental values from two experiments in duplicate). For the sake of clarity, S.D. values are not given, but did not exceed ±10%. n.d., not detectable.

In the present study, we demonstrate that supplementation of cultured cells with PUFA of the (n-6) and (n-3) series resulted in an oxidative stress, in terms of ROS and lipid peroxidation product generation. Concomitantly, an activation of the transcription factors AP1 and NFκB was observed.

MATERIALS AND METHODS

Materials. DMEM medium and fetal calf serum were from Gibco (Grand Island, NY, USA). The oligonucleotide probes for AP1 (5′-AGC TAG GTG ACT CAC CAA GCT TGG A-3′) and NFκB (5′-GCT TCA GAG GGG ACT TTC CGA GAG G′) were synthesized by Eurogentec, Belgium. [γ-^{32}P]ATP 7000 Ci/mmol was from ICN, CA, USA. Dichlorofluorescein-diacetate was purchased from Molecular Probes, Interchim, France. All other chemicals were of Sigma grade.

Enrichment of cells with PUFA and PUFA analysis. The MRC5 human fibroblasts were purchased from BioMérieux, France and maintained in Dulbecco's MEM supplemented with 10% foetal calf serum. 24 h after seeding, the cells were cultured for 48 h in medium devoid of serum and supplemented with 5×10^{-5} M of different fatty acids introduced in bovine serum albumin 0.1% (final concentration). The modification of the fatty acid content of cellular lipids was assessed by capillary gas chromatography as previously described (20) after extraction of the lipids by the method of Bligh and Dyer (21). In some experiments, the antioxidants vitamin E 10^{-5} M and N-acetyl cysteine 5×10^{-3} M were introduced simultaneously with arachidonic acid.

Quantitation of intracellular ROS with dichlorofluorescein-diacetate and fluorescence microscopy. The increase in fluorescence in living cells in the presence of this probe allows the quantitation of reactive oxygen species (23). After the 48 h incubation with PUFA, the cells were incubated for 30 min with 2×10^{-6} M 2′7′dichorofluoresceine-diacetate in DMEM without phenol red, washed three times with phosphate buffer saline, solubilized in H_2O and sonicated. The fluorescence was determined at 503/529 nm using a Shimadzu RF-5301PC fluorometer, normalized on a protein basis and expressed in percentages of control. Optical and fluorescence microscopy was performed using a Nikon Diaphot inverted microscope equipped with a CH250 CCD camera (Photometrics, Ltd., A, USA). Images were acquired and treated using the V for Windows software (Photometrics, Ltd., A, USA). Fluorescence images were digitized.

Quantitation of intracellular lipid peroxidation products. After the 48 h incubation with PUFA, the cells were washed, harvested and resuspended in H_2O. Intracellular lipid peroxidation end products (thiobarbituric acid reactive substances: TBARS) were determined by the method of Yagi (24). Results, normalized on a protein basis, were expressed in percentages of control.

Preparation of nuclear extracts. After the 48 h incubation period with PUFA, nuclear extracts were prepared according to Dignam *et al.* (22). The cells were resuspended in 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P40 and the protease inhibitors aprotinin 2 mM, antipain, pepstatin, benzamidine and leupeptin 1 mg/ml. After homogenization with a Dounce homogenizer and a 10 min incubation at 4°C, nuclei were collected by centrifugation at 2000×g for 30 min. The pellet was resuspended in 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and the above cited antiproteases. The nuclear proteins were extracted by incubation at 4°C during 30 min. After centrifugation at 13000×g for 15 min, the supernatant was kept at −80°C.

Electrophoretic mobility shift assay. The double stranded oligonucleotides were end-labeled using T4 kinase and [γ-^{32}P]ATP. 7 μg of nuclear extract was incubated with 100,000 DPM of labeled probe (0.5 ng) in the presence of 1 mg of poly(dIdC) at room temperature for 20 min. Separation of the mixture on a 6% non denaturing polyacrylamide gel was then performed in Tris 89 mM/boric acid 89 mM/EDTA 2 mM. After autoradiography, the radioactivity was quantified by liquid scintillation counting or by quantitative analysis of the autoradiogram using a Molecular Dynamic laser densitometer (Molecular Dynamics, CA, USA). Results are representative of at least 3 independent experiments.

RESULTS

1. Cell Enrichment with PUFA

We first checked the enrichment of cells with PUFA by determination of the fatty acid percentages by capillary gas chromatography. The data from Table I show that a 48 h incubation with 5×10^{-5} M fatty acids induced an enrichment in their respective percentages in cellular lipids. In the case of PUFA, the increase in the introduced PUFA was accompanied by a decrease in the percentage of palmitoleic acid C16:1 and of oleic acid $C18:1_{n-9}$. In addition, preincubation with gamma linolenic acid $C18:3_{n-6}$ enhanced the intracellular content of both gamma linolenic and arachidonic acid $C20:4_{n-6}$, which demonstrates an active conversion of the former to the latter by MRC5 fibroblasts. Similar

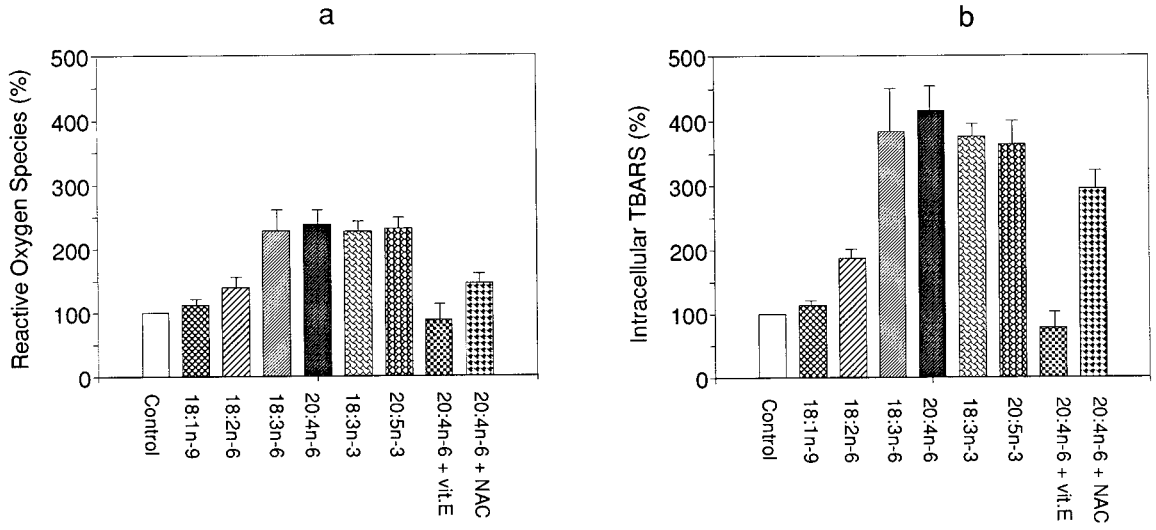

FIG. 1. PUFA enrichment increases the intracellular level of reactive oxygen species ROS (a) and lipid peroxidation products TBARS (b), an effect prevented by antioxidants. Cells were preincubated for 48 h in medium supplemented with 5×10^{-5} M fatty acid in the presence or absence of vitamin E 10^{-5} M or *N*-acetyl cysteine 5×10^{-3} M. For the determination of reactive oxygen species, the cells were further incubated for 30 min with 2×10^{-6} M 2′7′-dichorofluorescein-diacetate (23) in DMEM without phenol red, washed three times with phosphate buffer saline, and solubilized in H_2O. The fluorescence was determined at 503/529 nm, normalized on a protein basis, and expressed in percentages of control. For the determination of lipid peroxidation products, the cells were resuspended in H_2O and the TBARS measured as described (24). The results, normalized on a protein basis, are expressed in percentages of control (100%: 773 pmol eq malondialdehyde/mg protein). Means ± s.d. of 3 experiments in duplicate.

observation can be made concerning alpha linolenic acid C18:3_{n-3}, which was converted to eicosapentaenoic acid C20:5_{n-3}.

2. PUFA Increased the Intracellular Level of Reactive Oxygen Species and Lipid Peroxidation Products

In order to demonstrate the PUFA-induced modification of the redox state, we determined the intracellular level of reactive oxygen species by the fluorescence of dichlorofluorescein. The results from Fig. 1a indicate that again, oleic acid had no significant effect whereas PUFA induced an elevation in reactive oxygen species, with a less marked increase for the (n-3) series as compared to the (n-6) series. The TBARS intracellular level was increased in a similar manner (Fig. 1b). Figure 2 shows the fluorescence of cells labeled with dichlorofluorescein-diacetate after enrichment with arachidonic acid in the presence or absence of vitamin E. Arachidonic acid markedly enhanced the intracellular fluorescence (Fig. 2e) as compared to the control (Fig. 2d), and preincubation with vitamin E decreased the intracellular fluorescence to a value close to that of the control (Fig. 2f).

3. PUFA Increased AP1 and NFκB Activity

The effect of PUFA enrichment on AP1 and NFκB DNA binding activity was then studied by electrophoretic mobility shift assay and results from a typical experiment are shown in Fig. 3. It can be noted that whereas the monounsaturated oleic acid had no significant effect, all the studied PUFA induced an increase in AP1 (Fig. 3a) and NFκB (Fig. 3b) activities, with a less marked effect for the diunsaturated linoleic acid C18:2_{n-6}. Furthermore, the two studied PUFA from the (n-6) series, gamma linoleic C18:3_{n-6} and arachidonic C20:4_{n-6} acids, exhibited a somewhat more marked effect than those from the (n-3) series, alpha linolenic C18:3_{n-3} and eicosapentaenoic C20:4_{n-3} acids.

4. The Effect of PUFA Was Prevented by Antioxidants

The next experiment was designed to study the action of the lipophilic (vitamin E) and hydrophilic (N-acetylcysteine) antioxidants on the arachidonic acid-induced increase in AP1 and NFκB activity. Figure 4 shows that both antioxidants prevented the increase in the AP1 (Fig. 4a) and NFκB (Fig. 4b). DNA binding activities, which suggests an involvement of the intracellular redox state in the mechanism of action of PUFA. It is of note that Vitamin E completely prevented the arachidonic acid-induced elevation of intracellular ROS and TBARS whereas N acetyl cysteine partially inhibited this effect (Fig. 1).

DISCUSSION

Our study clearly demonstrate that long term supplementation of cultured cells with PUFA induced a modification in their fatty acid composition (Table I),

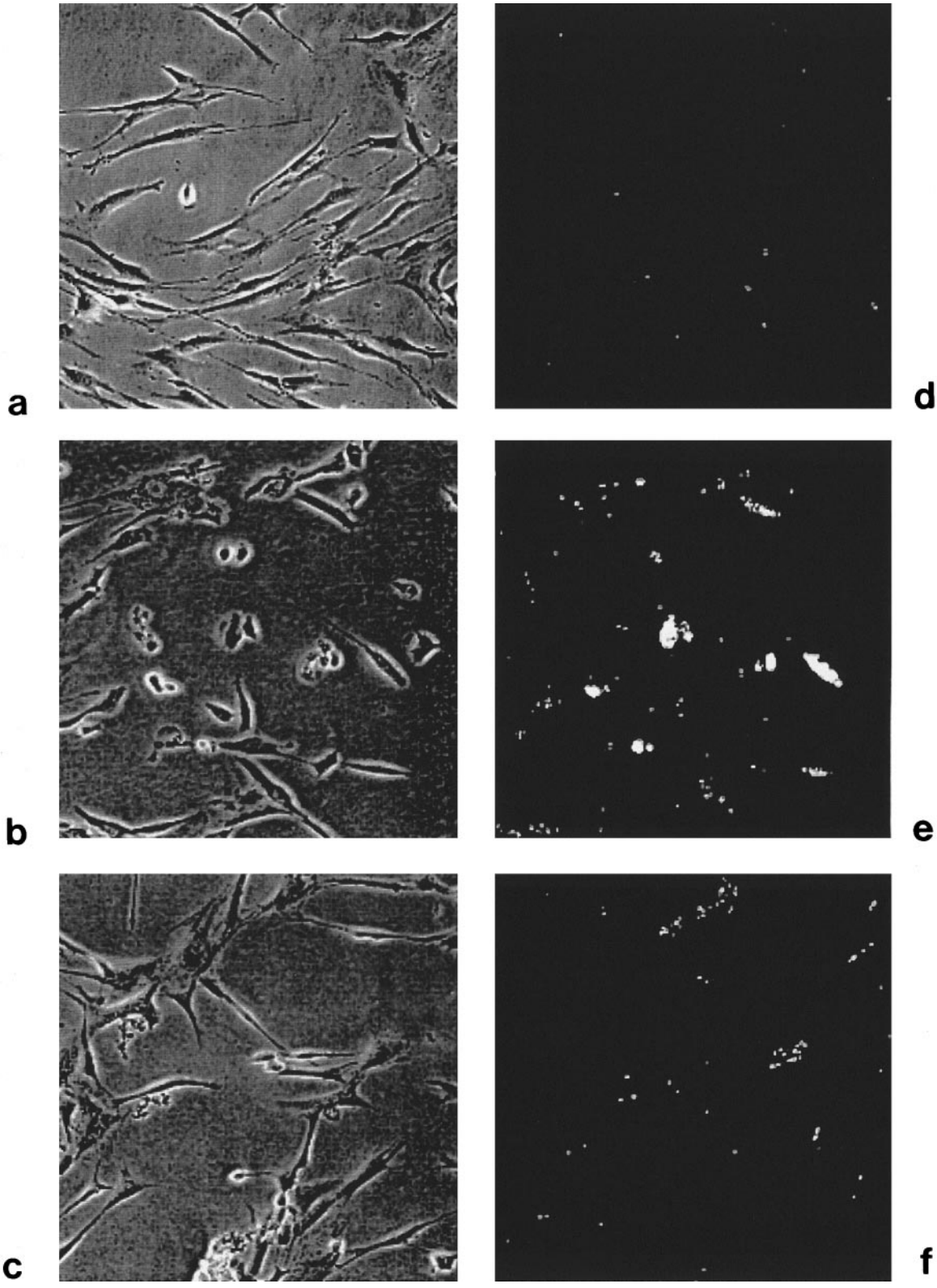

FIG. 2. The antioxidant vitamin E prevents the arachidonic acid-induced increase in ROS intracellular level. Cells were preincubated for 48 h in the absence (a, d) or presence of arachidonic acid (b, e), or arachidonic acid + vitamin E (c, f). Optical and fluorescence microscopy was performed using a Nikon Diaphot inverted microscope equipped with a CH250 CCD camera (Photometrics, Ltd., A, USA). Images were acquired and treated using the V for Windows software (Photometrics, Ltd., A, USA). Fluorescence images were digitized.

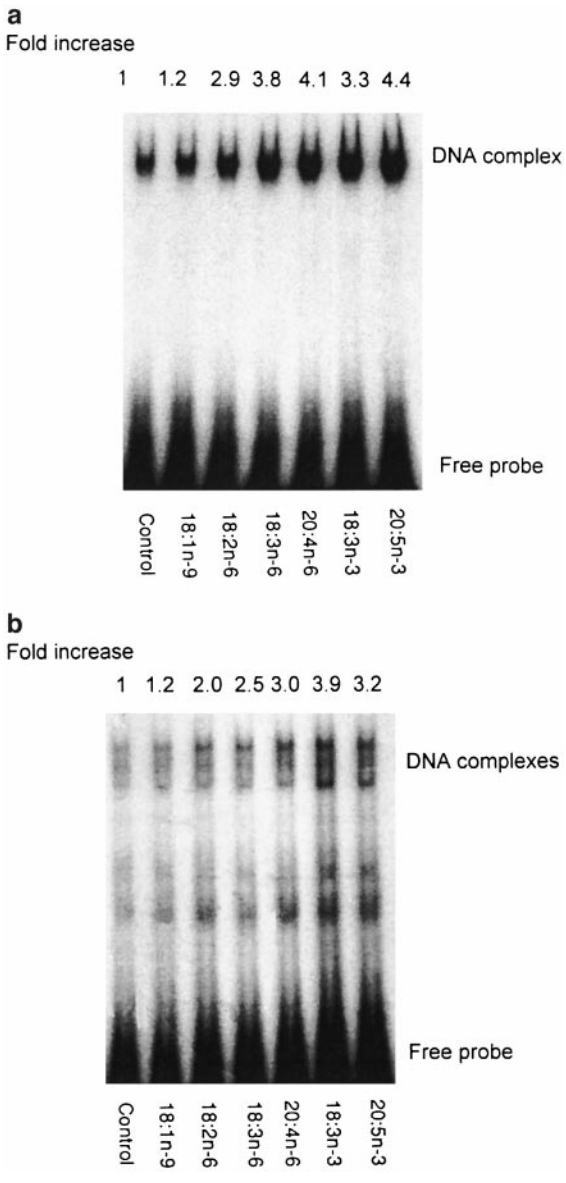

FIG. 3. PUFA-enrichment increases AP1 (a) and NFκB (b) activity. MRC5 human fibroblasts were preincubated for 48 h in medium supplemented with 5×10^{-5} M fatty acid introduced in bovine serum albumin solution. Nuclear extracts were then prepared and AP1 and NFκB binding activities were determined by electrophoretic mobility shift assay. The autoradiogram shown is from a representative experiment. This experiment was repeated 3 times with similar results.

which in turn caused an oxidative stress, as indicated by the increase in the ROS and lipid peroxidation product intracellular levels (Figs. 1 and 2). This is in accordance with our previous report demonstrating that in the same experimental conditions, PUFA enhanced the secretion of superoxide anion by endothelial cells (25). In addition, we have also noted that the amount of TBARS secreted in the culture medium were increased by PUFA enrichment (results not shown). Furthermore, the fact that this phenomenon was observed not only with PUFA from the (n-6) series but also with PUFA from the (n-3) series indicates that this effect is most probably due to the increase in the number of double bonds and that it is independent of the cyclooxygenase, lipooxygenase or Cyt P450 monooxygenase pathways, which only concern PUFA of the (n-6) series.

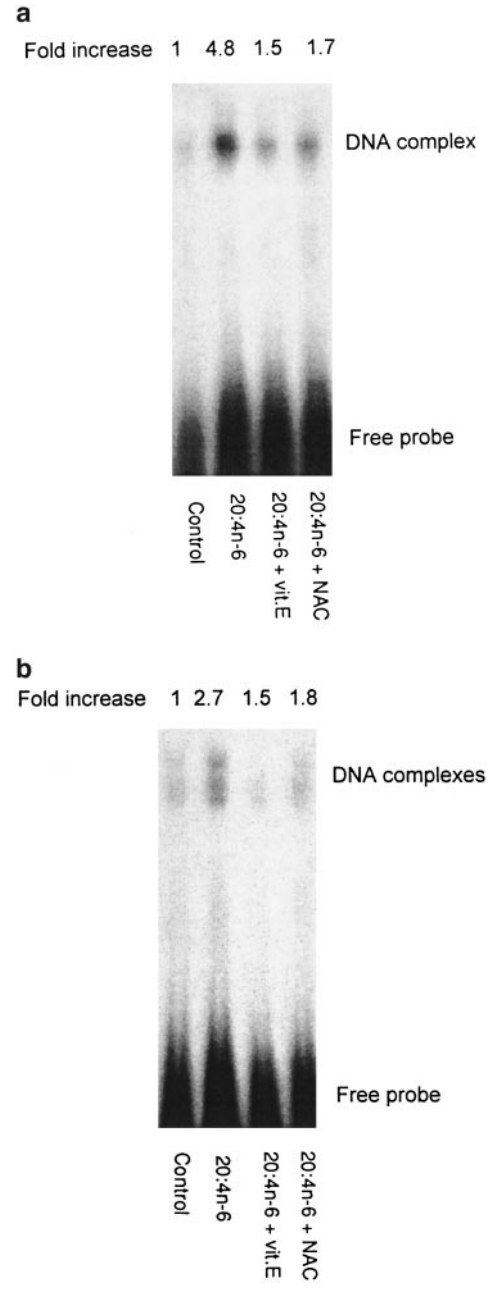

FIG. 4. The antioxidants vitamin E and *N*-acetyl cysteine prevented the arachidonic acid-induced increase in AP1 (a) and NFκB (b) activity. Vitamin E (dl-α tocopherol) 10^{-5} M and *N*-acetyl cysteine 5×10^{-3} M were introduced during the 48 h incubation period with 5×10^{-5} M arachidonic acid. The autoradiogram shown is from a representative experiment. This experiment was repeated 3 times with similar results.

It is of note that our experiments were designed to investigate the effects of an increase the intracellular percentages of PUFA induced by a by a 48 h supplementation on AP1 and NFκB transcription factors. Numerous previous works have been concerned with the short term action of arachidonic acid and its derivatives. In particular, the role of this fatty acid and related PUFAs as second messengers in cell signal transduction events such as Ca^{2+} mobilization (26), G_i protein activity (27) or p38 MAPK phosphorylation (28) has been reported. But these short term effects appear to be mediated by the arachidonic acid molecular species released by activation of phospholipase A_2. Metabolites of arachidonic acid from the Cyt P450 epoxygenase pathway such as epoxyeicosatrienoic acids also play a role as second messengers in response to Epidermal Growth Factor (29) or in mediating the recovery of Ca^{2+} pools (30). In our experimental model, we found no short term (4 h) effect of arachidonic acid on the intracellular level of ROS (data not shown).

The PUFA-induced oxidative stress is accompanied by an increase in AP1 and NFκB activity (Figs. 3 and 4). Concerning the activation of AP1 activity, it has been shown that ROS such as H_2O_2 (31) or hydroxyl radical $OH^{\cdot}$ (31, 32) can increase the DNA binding activity of this transcription factor. In addition, the role of ROS in mediating the agonist-induced activation of AP1 has also been reported: in this case, ROS mediate the increase in ERK, JNK and p38 MAPK activity as well as c-Fos, c-Jun and JunB expression (15). 4 hydroxynonenal, one of the main end products of lipid peroxidation, can also activate stress signaling pathways including JNK, p38 MAPK and ERK activity together with c-Jun expression, an effect which involves an intracellular ROS production (33).

Concerning the NFκB activation, besides the fact that superoxide anion and H_2O_2 have been evoked to be involved in the activation of this transcription factor (34, 35), it has been also demonstrated that N-acetylcysteine and other thiol compounds block the increase in NFκB activity induced by TNF-alpha, Interleukin 1 or lipolysaccharides (18). Vitamin E derivatives also prevent NFκB activation by TNF-alpha (36). It was further reported that the oxidant status of the cytosol increases the phosphorylation and degradation of the inhibitor Iκ B (37, 38). A short term activation of NFκB by PUFA such as linoleic acid (39) or arachidonic acid (40) has also been described. However, in the latter case, the effect was attributed to arachidonate metabolites such as prostaglandines and leukotrienes, since the (n-3) PUFA eicosapentaenoic acid were ineffective. By contrast, arachidonic acid and its non metabolizable analogue eicosatetraynoic acid both inhibit the degradation of IkappaB and thus the translocation of NFκB to the nucleus (41).

Our report demonstrates that the accumulation of the double bonds included in PUFA obviously initiates an oxidative stress, leading to the activation of the stress-responsive transcription factors AP1 and NFκB and regulation of specific genes. It must be noted that other transcription factors, kinases and phosphatases are most probably concerned. Our study also highlights the fact that oxidative stress might have an endogenous origin, arising from the number of double bonds included in the fatty-acid of the cellular lipids. This endogenous stress will then in turn play a regulatory role by means of the activity of stress-responsive transcription factors and enzymes involved in cellular metabolic regulation. The possible consequences of the changes in the activities of oxidative stress-related transcription factors with the diet composition must therefore be kept in mind.

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