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TRANS FATTY ACIDS INDUCE A PROINFLAMMATORY RESPONSE IN ENDOTHELIAL CELLS THROUGH ROS-DEPENDENT NUCLEAR FACTOR-κB ACTIVATION

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It has been shown that increased intake of *trans* fatty acids (TFAs) is associated with a higher risk of cardiovascular disease. In this study, we have investigated the effects of linoelaidic (LA) and elaidic (EA) acids on the proinflammatory response in endothelial cells, a key step in vascular disease. Human aortic endothelial cells (HAECs) were treated with different concentrations (100 μmol/l in most experiments) of LA or EA for different periods of time. The surface protein and mRNA expression of ICAM-1 and VCAM-1 were determined by flow cytometry and real time RT-PCR, respectively. Adhesion of leukocytes to TFA-treated HAECs was evaluated by an adhesion assay. Activation of nuclear factor-κB (NF-κB) was evaluated by measuring NF-κB p65 phosphorylation using flow cytometry. ROS production was determined by the reduction of fluorescent 2',7'-dichlorofluorescein diacetate (DCFH-DA). LA treatment significantly increased protein and mRNA levels of ICAM-1 and VCAM-1, leukocyte adhesion to HAECs, phosphorylation of NF-κB and ROS generation. Similar effects were achieved for cells incubated with EA. Experiments with HAECs pretreated with pyrrolidine dithiocarbamate, an inhibitor of NF-κB, revealed that both LA and EA-mediated induction of ICAM-1 and VCAM-1 is mainly regulated by NF-κB. The ROS production induced by both of the studied acids was inhibited in the presence of diphenyleneiodonium (DPI), a NADPH oxidase inhibitor, suggesting ROS production through the activation of NADPH oxidase. Furthermore, LA or EA-induced ICAM-1 and VCAM-1 expression, activation of NF-κB and adhesion of leukocytes to HAECs were abolished in the presence of DPI. Conclusion: TFAs present in our diet have a direct proinflammatory effect, which promotes leukocyte adhesion to the endothelium through ROS-dependent NF-κB activation.

Key words: *atherosclerosis, cell adhesion molecules, NADPH oxidase, nuclear factor-κB, reactive oxygen species, trans fatty acids*

INTRODUCTION

Vascular inflammation is a primary event in the pathogenesis of atherosclerosis (1, 2). Monocytes and lymphocytes dominate in the early atheromatous plaque, and their effector molecules accelerate plaque progression. Furthermore, activation of inflammation can elicit acute coronary syndromes. The vascular inflammatory reaction is mediated by complex interactions between the circulating leukocytes and vascular cells (*e.g.*, endothelial cells and smooth muscle cells). Vascular cells regulate the inflammatory process through the expression of adhesion molecules, cytokines, chemokines and growth factors. Various adhesion molecules (CAMs) have been identified on endothelial cells, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (3, 4). These molecules are upregulated in the vascular endothelium in response to inflammatory stimuli, which in turn mediates leukocyte adherence to the endothelium, followed by their extravasation into the vessel wall in the early stages of vascular inflammation, ultimately leading to the progression of vascular diseases. Overexpression of ICAM-1 and VCAM-1 is a common feature of the atherosclerotic process.

Stimulation of vascular endothelial cells by inflammatory stimuli causes the activation of NADPH oxidase, which plays a key role in generating reactive oxygen species (ROS) (5). The NADPH oxidase system has been best characterised in phagocytic cells, in which it is used for the induction of the oxidative burst. It is composed of two cell-membrane subunits: $p22^{phox}$ and $gp91^{phox}$ which form flavocytochrome B558. The transfer of an electron from NADPH to the molecular oxygen - generating the superoxide radical - is catalysed by the oxidase as a result of its activation through the translocation of cytoplasmic regulatory subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac, and the binding with cytochrome B558. A number of NADPH oxidases have been identified based on different $gp91^{phox}$ homologies called NOX 1-5. Those present in endothelial cells are mainly NOX4. ROS contribute to the intracellular signalling cascades associated with the inflammatory response (6, 7). They may function as second messengers by activating certain kinases and transcription factors, notably nuclear factor-κB (NF-κB). NF-κB plays an important role in the development of inflammatory responses by upregulating the expression of many inflammatory mediators (8). In non-stimulated cells, NF-κB is mostly present in association with its inhibitory molecules (IκB). Upon stimulation of endothelial cells by inflammatory stimuli, IκB becomes phosphorylated, permitting NF-κB to translocate into the nucleus, where it binds to the promoter regions of inflammatory target genes, including *ICAM-1* and *VCAM-1*.

The major portion of energy supplied by our diet comes from fatty acids, containing in their molecules one or more double bonds. In most cases, they are present in the cis configuration, *i.e*. the two hydrogen atoms on the carbons adjacent to the double bond are located on the same side of the carbon chain, resulting in a bent shape and liquid state at room temperature. Some fatty acids have one or more double bonds in the *trans* configuration, *i.e*. hydrogen atoms on the carbons adjacent to the double bond are on opposite sides, resulting in a straight shape and solid state at room temperature. These *trans* fatty acids occur naturally in ruminant fats, but most dietary *trans* fatty acids are the result of industrial processing by partial hydrogenation of vegetable or fish oils to produce partially hardened fats and food products, such as margarines, shortenings, baked goods and fast food (9). During the partial hydrogenation of vegetable oils the number of double bonds is reduced, while approx. 30-50% of unsaturated fatty acids are transformed from *cis* into *trans*. The main fatty acid formed in the process of vegetable oil solidification is elaidic acid (C18:1, *trans*-9) (10). However, the process of frying or baking food in vegetable oils results in the generation of linoelaidic acid (C18:2; *trans*-9,12) (11). The high temperature accompanying this process causes the conversion of the double bond from the *cis* configuration into *trans* one.

The studies conducted so far indicate that increased intake of *trans* fatty acids (TFAs) is associated with a higher risk of cardiovascular morbidity and mortality (12, 13). In comparison to a low-calorie diet containing polyunsaturated or saturated fatty acids, a diet including TFAs has resulted in a higher LDL to HDL cholesterol ratio and ApoB to ApoA ratio, *i.e.*, changes recognised as cardiovascular risk factors. Although it might be presumed that this effect is responsible for the increased cardiovascular risk, the previously conducted prospective studies examining the relationship between TFAs and diseases of atherosclerotic origin seem to indicate that the influence of *trans* isomers on those diseases is greater than it might be expected based on the changes in the lipid profile (14, 15). There are published reports from studies suggesting other mechanisms as being responsible for the adverse impact of TFAs on the course and progression of cardiovascular diseases. Increased intake of TFAs has been shown to lead to elevated C-reactive protein (CRP) and interleukin-6 (IL-6) levels (16). In patients with cardiovascular diseases in whom systemic inflammatory markers were elevated as compared to healthy individuals, the levels of TFAs in the erythrocyte cell membrane correlated with IL-6, tumour necrosis factor-α (TNFα) and monocyte chemotactic protein-1 (MCP-1) (17). The above observations seem to suggest that TFAs may play a significant role in the development of cardiovascular disease through their effects on the mechanisms of the inflammatory response. However, no detailed studies have been conducted to evaluate whether they can directly stimulate the inflammatory response.

In this study, we examined the hypothesis that TFAs could directly promote a proinflammatory response in endothelial cells. In addition, potential molecular mechanisms, including ROS production and NF-κB activation, were studied. This study provides us with a better understanding of molecular mechanisms by which TFAs exert harmful effects on the cardiovascular system.

MATERIALS AND METHODS

Materials

EBM-2 basal medium, EGM-2 Bulletkit, foetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), trypsin-EDTA and trypsin neutralising solution (TNS) were obtained from LONZA (Switzerland). Diphenyleneiodonium (DPI), oxypurinol, rotenone, pyrrolidine dithiocarbamate (PTDC), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), nonenzymatic cell dissociation solution, fatty acid-free bovine serum albumin (BSA), 2',7'-bis-2-carboxyethyl-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were procured from Sigma (St. Louis, MO, USA). Human recombinant TNFα, Phosflow Buffer I, Phosflow Perm Buffer III, phycoerythrin (PE)-labelled mouse antibody against human ICAM-1, VCAM-1, NF-κB p65, IgG1 (isotype control) and 7-AAD were supplied by Becton Dickinson (San Diego, CA, USA). 2',7'-dichlorofluorescein diacetate (DCFH-DA) were procured from Molecular Probes and Ficoll-Paque from Pharmacia (Sweden). Linoelaidic (*trans* -C18:2) and elaidic (*trans*-C18:1) acids were acquired from Nu-Check Prep Incorporated.

Cells culture and experimental conditions

Human aortic endothelial cells (HAECs) were purchased from LONZA and cultured in the endothelial cell growth medium (EGM-2 Bulletkit = EBM-2 + growth supplements + 2% FBS) according to the manufacturer's instructions. Cells were maintained at 37°C in a 5% CO_2, humidified atmosphere and were used for experiments between passages number 3 and 4. Stock solutions of linoelaidic acid (LA) and elaidic acid (EA) were prepared by complexing with fatty acid-free BSA, as described previously (18). When HAECs reached 95% confluence, they were rested for 5 hrs in EBM-2 basal medium supplemented with 10% FBS, and the cells were treated with different concentration of LA (*trans*-C18:2) or EA (*trans*-C18:1) for the indicated period of time. Cells treated with medium containing only 0.2% BSA were used as control. Cell viability, as determined by trypan blue exclusion and MTT assay, was unchanged under all experimental conditions used.

Measurement of adhesion molecule expression by flow cytometry

Resting HAECs in 12-well plates were treated with 50, 100 and 200 μmol/L of LA (*trans*-C18:2) or EA (*trans*-C18:1) for 16 hours. The cells were then incubated for 15 min with a non-enzymatic cell dissociation solution. This solution is prepared in Hanks' balanced salts solution without calcium and magnesium, and contains EDTA, glycerol and sodium citrate. After the detaching, the cells were centrifuged and washed in PBS containing 1% FBS and resuspended in 100 μl of the labelling buffer. Subsequently, the cells were incubated in the dark for 1 h with PE-conjugated mouse anti-human ICAM-1 and VCAM-1 antibodies. For the isotype control, cells were treated with PE-conjugated mouse anti-IgG_1 antibody. Next, the cells were washed with PBS and analysed (10,000 cells per sample) with a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest Software. After correcting for non-specific binding (from the isotype control), mean fluorescence intensity was measured as an indicator of ICAM-1 and VCAM-1 surface protein expression.

Quantitative real-time RT-PCR for ICAM-1 and VCAM-1 mRNA analysis

Resting HAECs in 6-well plates were treated with 100 μmol/L of LA (*trans*-C18:2) or EA (*trans*-C18:1) for 6 hours. To test the ICAM-1 and VCAM-1 mRNA levels, quantitative real-time RT-PCR was performed using a LightCycler system (ABI PRISM® 7000 Sequence Detection System; SDS Software Version 1.1, Applied Biosystems). Total RNA was extracted from approximately 2 x 10^6 studied cells by the method of the Chomczynski and Sacchi (19) using TRIzol reagent (Invitrogen) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To amplify the target

genes, appropriate primers were synthesised (Institute of Biochemistry and Biophysics, Poland). The sequences of the forward and reverse primers are as follows:
h-ICAM1F: 5'-CTCATCCGCGTGTGTGTGTG-3';
h-ICAM1R: 5'-GGAGGCTGAGGTGGGAGGAT-3';
h-VCAM1F: 5'-TGGGAAGATGGTCGTGATCC-3';
h-VCAM1R: 5'-GCCTGTTGGTGCTGCAAGTC-3'. The *β-actin* gene was used as a reference (housekeeping) gene (forward primer, ActbF: 5'- TGAAGTGTGACGTTGACATCC-3'; reverse primer, ActbR: 5'- GCCAGAGCAGTAATCTCCTTC-3'). Reaction mixtures (20 µl) consisted of SYBR® Green PCR Master Mix (Applied Biosystems), the forward and reverse primers, and an aliquot of the reverse-transcribed samples (cDNA, 2 µl). Negative controls were tested in parallel. The relative mRNA levels of the studied genes were calculated by the comparative cycle threshold (C_T) method (ABI Prism 7000 SDS Software, version 1.1, Applied Biosystems). *β-actin* mRNA level measured in the same RNA sample for the target C_T normalisation was used to compute the relative gene expression ratio.

Cell adhesion assay

Human mononuclear cells (MNCs) were isolated from the blood of healthy volunteers by density gradient centrifugation with Ficoll-Paque. The isolated MNCs were resuspended in serum-free medium and labelled with 10 µmol/L of BCECF-AM for 1 hour at 37°C. Non-fluorescent BCECF-AM is lipophilic, and its methylester is cleaved intracellularly and becomes a highly charged fluorescent BCECF that is retained by viable cells. After the labelling, cells were washed twice with PBS containing 5% FBS and resuspended in EBM-2 medium at a density of 5 x 10^5 cells/ml. Resting HAECs in 24-well plates were treated with 100 µmol/L of LA (*trans*-C18:2) or EA (*trans*-C18:1) for 16 hrs. Then, fluorescently labelled MNCs (2.5 x 10^5 cells/well) were seeded over the HAEC monolayer and incubated for 1 hour at 37°C and in 5% CO_2. After the incubation, the MNC suspension was withdrawn, and HAECs were washed twice with PBS to remove non-adherent cells. The fluorescence intensity of each well was measured with a fluorescence multi-well reader at excitation and emission wavelengths of 485 and 530 nm, respectively.

Measurement of NF-κB p65 phosphorylation

NK-κB p65 phosphorylation was determined by flow cytometry using a specific antibody that differentiates between the phosphorylated and non-phosphorylated version of a given protein, as described previously (20). Resting HAECs cultured

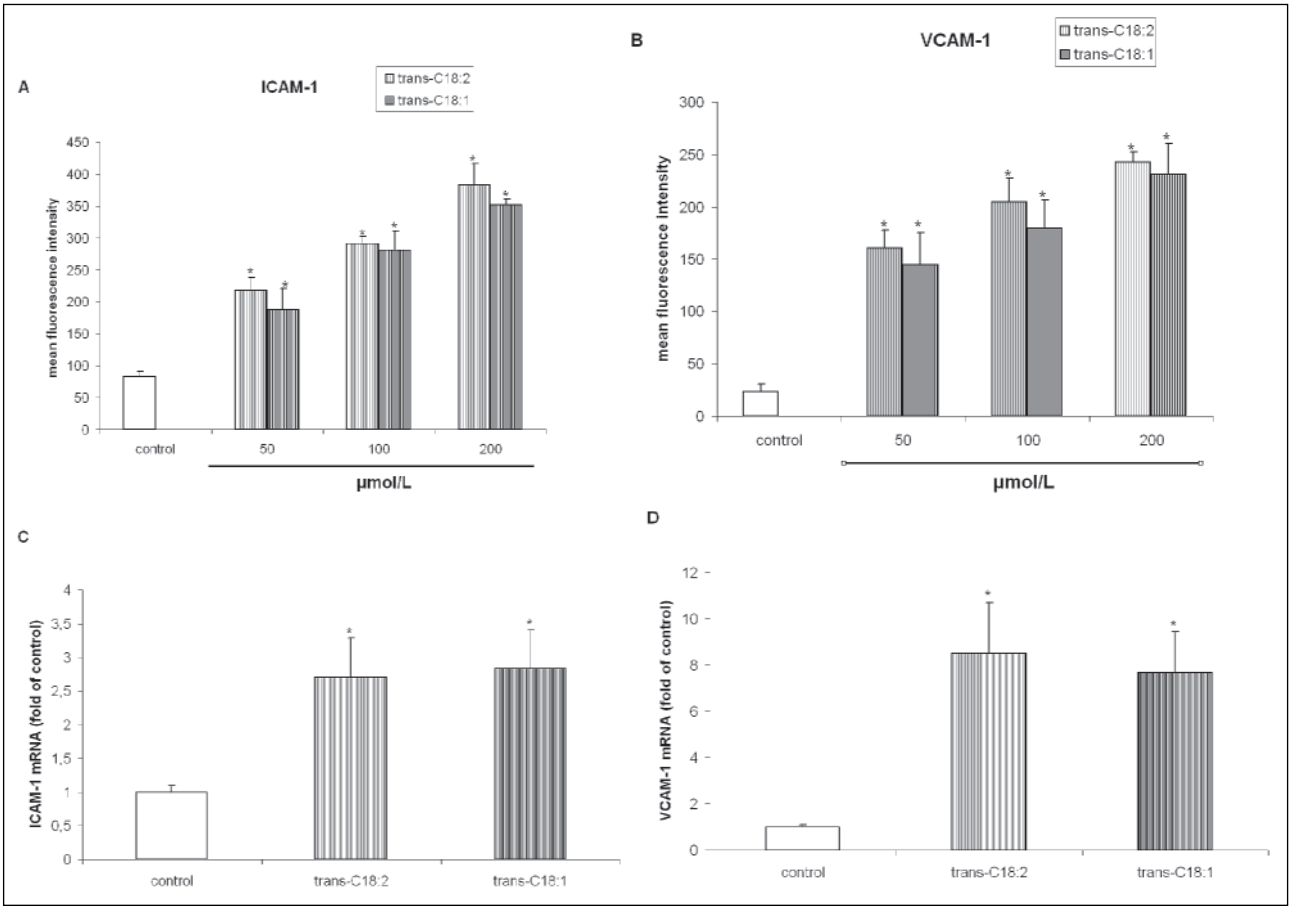

Fig. 1. Effects of *trans* fatty acids on ICAM-1 and VCAM-1 expression in endothelial cells. (*A, B*) Surface protein expression. HAECs were incubated with BSA (control) or with the indicated concentration of LA (*trans*-C18:2) or EA (*trans*-C18:1) for 16 hrs. ICAM-1 (*A*) and VCAM-1 (*B*) expression was measured by flow cytometry. Data are expressed as mean fluorescence intensity corrected for non-specific binding and are shown as means ±S.D. of 6 separate experiments. * $p \leq 0.001$, as compared to the control. (*C, D*) mRNA expression. HAECs were incubated with BSA (control) or with LA (*trans*-C18:2) or EA (*trans*-C18:1) in concentration of 100 µmo/L for 6 hrs. Total RNAs were isolated, and the mRNA levels of *ICAM-1* (C) and *VCAM-1* (D) were analysed by quantitative real-time RT-PCR as described in "Materials and methods". The expression levels of the control were set to 1, and the values are normalised to *β-actin*. Data are expressed as a multiple of the control and are shown as means ±S.D. of 7 separate experiments. * $p \leq 0.001$, as compared to the control.

in 6-well plates were treated with 100 µmol/L of LA (*trans*-C18:2) or EA (*trans*-C18:1) for 45 min. The cells were then detached with a non-enzymatic cell dissociation solution and fixed with Phospho Fix Buffer I for 10 min at 37°C. To stain intracellular phospho-p65, fixed cells were washed in PBS and permeabilised with Phospho Perm Buffer III on ice for 30 min. Next, cells were resuspended in 100 µl of staining buffer (1% FBS and PBS) and stained with PE-conjugated mouse antibody directed against human phospho-p65 for 2 hrs at room temperature in the dark. For the isotype control, cells were treated with PE-conjugated mouse ani-IgG2b,κ antibody. Cell were next washed and analysed (10,000 cells per sample) with a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest Software. After correcting for non-specific binding (from the isotype control), mean fluorescence intensity was measured.

Detection of intracellular reactive oxygen species (ROS) generation

2',7'-dichlorofluorescein diacetate (DCFH-DA) was used to estimate the intracellular generation of ROS. This assay is a reliable method for the measurement of intracellular ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), and hydroperoxides (ROOH) (21). The dye is a non-polar compound that readily penetrates into the cells. Intracellular peroxides oxidise DCFH-DA to a highly fluorescent compound (2',7'-dichlorofluorescein (DCF)). Resting HAECs cultured in 24-well plates were incubated with 50 µmol/L of DCFH-DA in HBSS for 50 min. During the staining, cells were also incubated with or without DPI (10 µmol/L), rotenone (2 µmol/L) or oxypurinol (10 µmol/L). Next, cells were washed twice with PBS, incubated without or with indicated concentration of LA (*trans*-C18:2) or EA (*trans*-C18:1) for 1 hour, and the plates were then read on a fluorescence plate reader at excitation and emission wavelengths of 485 and 530 nm, respectively. All fluorescence measurements were corrected for background fluorescence. Data were expressed as percentages of control values (DCF fluorescence of wells without TFA/inhibitor treatment).

Statistical analysis

Results are expressed as means ±S.D. from the indicated number of experiments. Comparisons were made using the Student's *t*-test. Differences between the experimental groups were considered to be statistically significant at $p<0.05$.

RESULTS

Effect of trans fatty acids on adhesion molecule expression in aortic endothelial cells

Treatment of HAECs with both TFAs increased the surface expression of ICAM-1 and VCAM-1 in a dose-dependent manner, with concentration of 100 µmol/L inducing over a 3-fold increase in ICAM-1 expression (*Fig. 1A*) and an 8-fold increase in VCAM-1 expression (*Fig. 1B*). To test whether the upregulation of ICAM-1 and VCAM-1 surface expression was caused by *de novo* synthesis of these adhesion molecules, we performed quantitative real-time RT-PCR of mRNA isolated from HAECs incubated without or with LA and EA. As in the case of surface expression, treatment of HAECs with both TFAs for 6 hrs induced an approximately 2-fold increase in specific

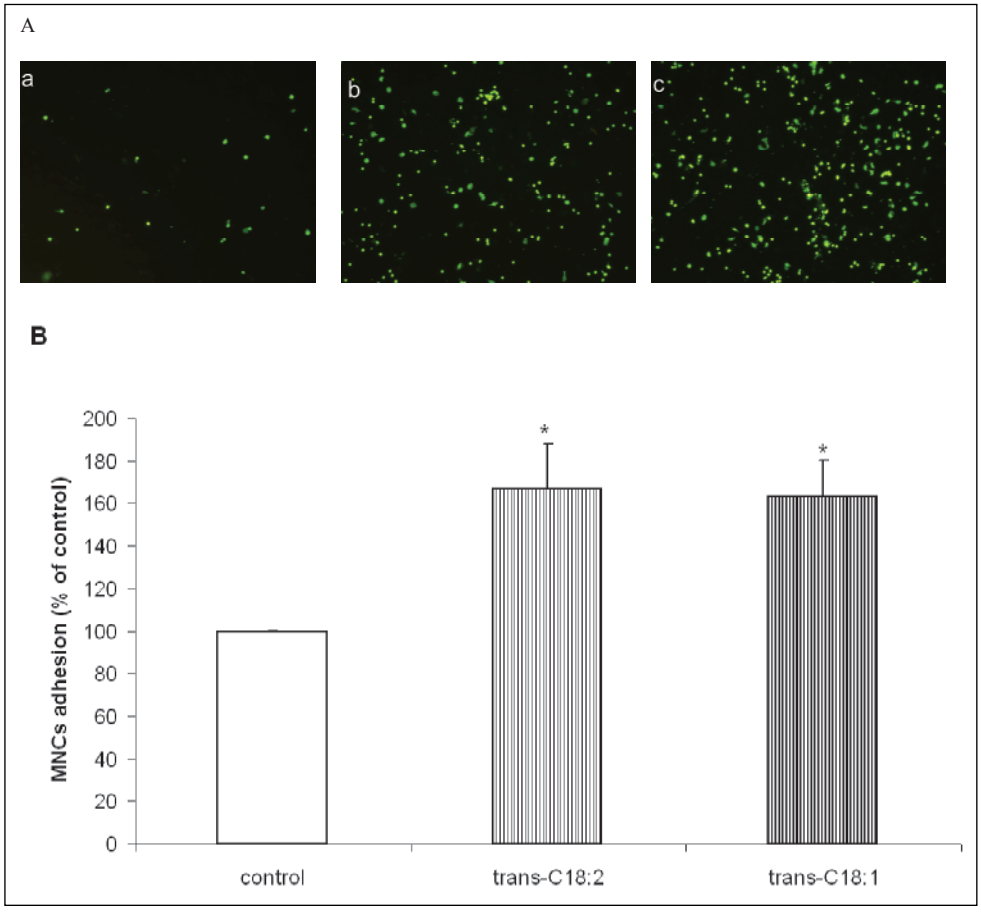

Fig. 2. Mononuclear cell adhesion to HAECs treated with *trans* fatty acids. HAECs were treated with BSA (control) or with LA (*trans*-C18:2) or EA (*trans*-C18:1) in concentration of 100 µmo/L for 16 hrs. Mononuclear cells (MNCs) were labelled with BCECF-AM, and binding to the endothelial cells was quantified as described in "Materials and methods". (*A*) Photographic view of MNCs to HAECs. (a) control; (b) *trans*-C18:2 (LA); (c) *trans*-C18:1 (EA). (*B*) Data were summarised and plotted. Values are expressed as % of control (set at 100%) and are shown as means ±S.D. of 5 separate experiments, each performed in triplicate. * $p\leq0.001$, as compared to the control.

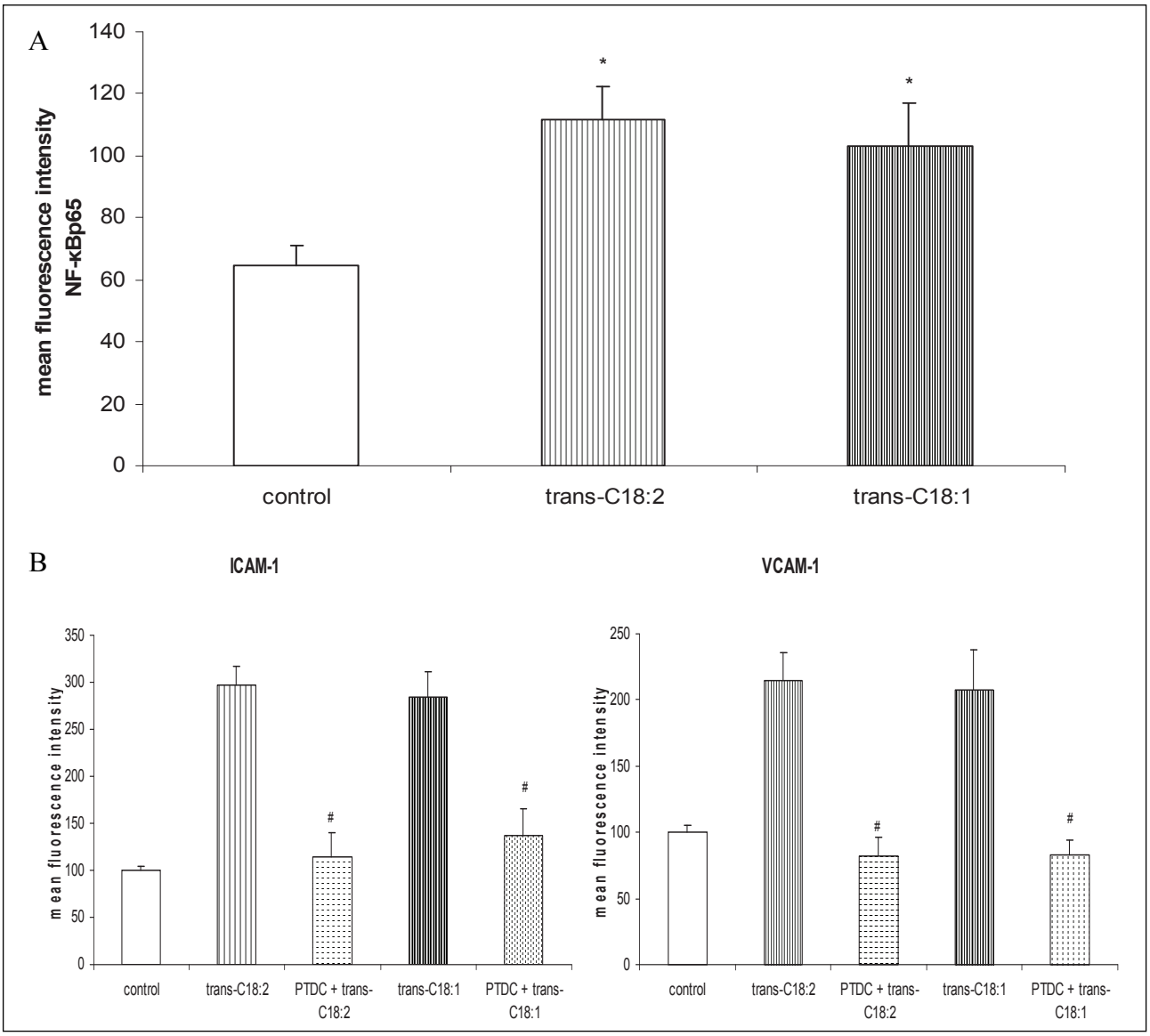

Fig. 3. Trans fatty acids increase ICAM-1 and VCAM-1 expression through NF-κB. (A) *Trans* fatty acids induce phosphorylation of NF-κB p65. HAECs were treated with BSA (control) or LA (*trans*-C18:2) or EA (*trans*-C18:1) in concentration of 100 μmo/L for 45 min. Phosphorylation levels of NF-κB p65 were measured by flow cytometry. Data are expressed as mean fluorescence intensity corrected for non-specific binding and are shown as means ±S.D. of 8 separate experiments. * $p \leq 0.001$, as compared to the control. (*B*) Pre-treatment with PTDC (inhibitor of NF-κB) inhibits both LA (*trans*-C18:2) and EA (*trans*-C18:1)-induced ICAM-1 and VCAM-1 expression as measured by flow cytometry. HAECs were pretreated for 30 min with PTDC (100 μmol/L) before 16 hrs of treatment with 1000 μmol/L of LA (*trans*-C18:2) or EA (*trans*-C18:1). The surface expression of ICAM-1 and VCAM-1 was measured by flow cytometry. Values are shown as mean fluorescence intensity corrected for non-specific binding and are expressed as means ±S.D. of 6 separate experiments. # $p \leq 0.001$, as compared to LA (*trans*-C18:2) or EA (*trans*-C18:1) treated cells.

ICAM-1 (*Fig. 1C*) and an 8-fold increase in specific *VCAM-1* (*Fig. 1D*) PCR products.

Effect of trans fatty acids on mononuclear leukocyte adhesion to aortic endothelial cells

In an attempt to determine the functional importance of stimulatory properties of LA and EA on ICAM-1 and VCAM-1 surface expression, we studied the adhesion of freshly isolated monocytes and lymphocytes to endothelial cells incubated with these TFAs. Results are shown in *Fig. 2.* MNCs showed minimal binding to control HAEC. Adhesion of MNCs was significantly increased when HAECs were treated with LA or EA.

Trans fatty acids stimulate NF-κB activation in aortic endothelial cells

Optimal activation of NF-κB requires the phosphorylation of p65. Therefore, we examined the effects of LA and EA on the phosphorylation of NF-κB p65 using flow cytometry. Treatment of HAECs with both of the TFAs for 45 min induced a significant increase in the phosphorylation levels of NF-κB p65 (*Fig. 3A*).

To determine whether TFA-mediated activation of NF-κB is involved in the upregulation of ICAM-1 and VCAM-1, surface expression of these molecules was studied in HAECs pretreated with PTDC, which is commonly used as an inhibitor of NF-κB

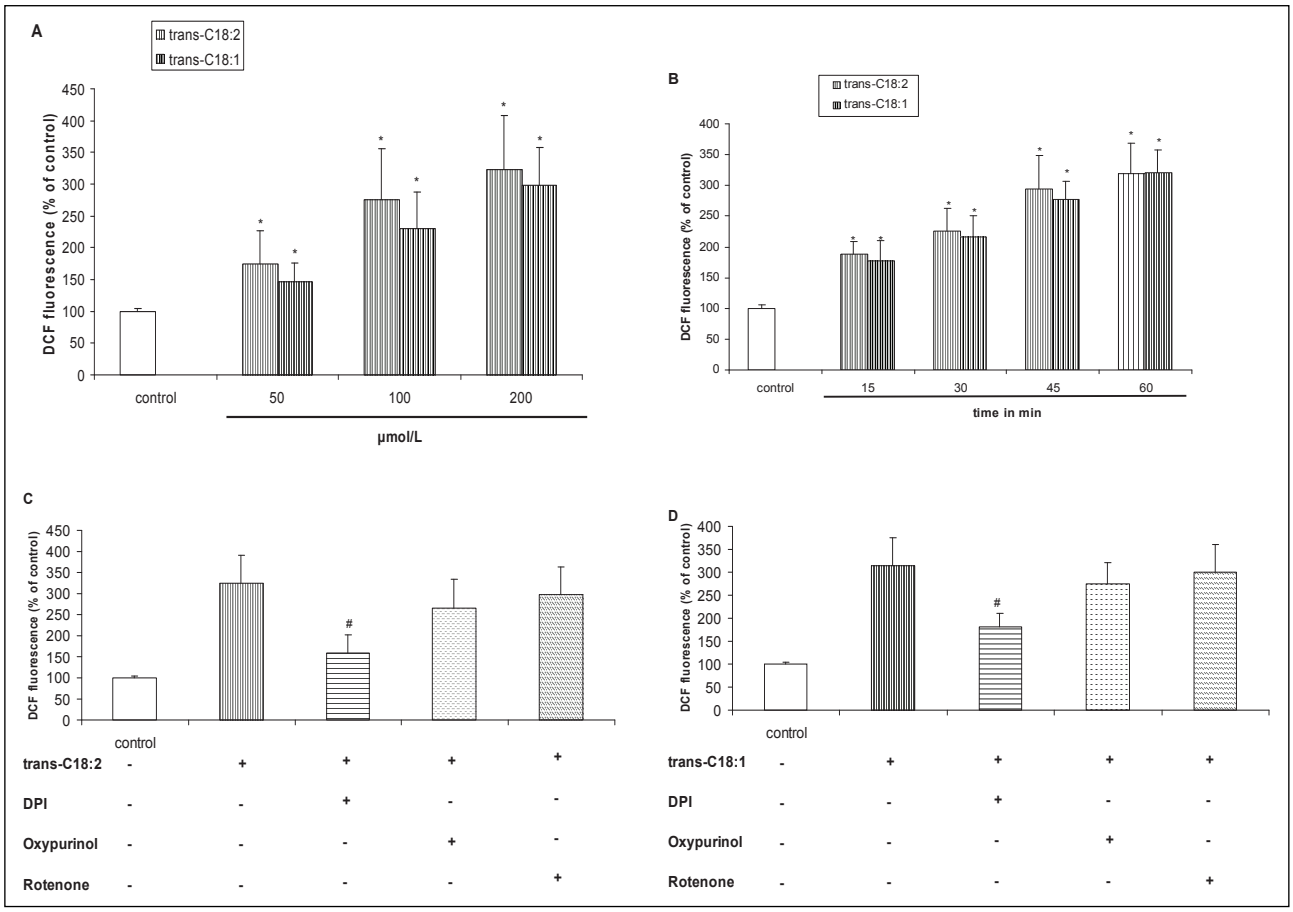

Fig. 4. Effects of *trans* fatty acids on intracellular ROS generation in endothelial cells. HAECs were pre-treated with or without the NADPH oxidase inhibitor, DPI (10 µmol/L), the xanthine oxidase inhibitor oxypurinol (10 µmo/L), or the mitochondrial NADH oxidase inhibitor rotenone (2 µmol/l) for 30 min, and then cells were loaded with DCFH DA (50 µmol/L) for 1 hour and treated with indicated concentration of LA (*trans*-C18:2) or EA (*trans*-C18:1) for indicated periods of time. The conversion of DCFH to DCF was measured as described in "Materials and methods". Values are expressed as a % of control DCF fluorescence (set at 100%) and are expressed as means ±S.D. of 10 separate experiments. (*A*) Effect of increasing concentration of LA (*trans*-C18:2) and EA (*trans*-C18:1) on intracellular ROS generation in HAECs. (*B*) Time dependent effect of LA (*trans*-C18:2) and EA (*trans*-C18:1) in concentration of 100 µmol/L on intracellular ROS generation in HAECs. * $p \leq 0.001$, as compared to untreated cells. Effects of used inhibitors on ROS generation in cells treated with *trans*-C18:2 (*C*) or *trans*-C18:1 (D). # $p \leq 0.001$, as compared to LA (*trans*-C18:2) or EA (*trans*-C18:1)-treated cells.

activation. As shown in *Fig. 3B*, 30 min pre-treatment with PTDC markedly decreased TFA-induced surface expression of ICAM-1 and VCAM-1.

Trans fatty acid-induced NF-κB activation and cell adhesion molecule expression are associated with reactive oxygen species generation: involvement of NADPH oxidase

As shown in *Fig. 4A* both TFAs significantly increased the intracellular ROS production in a dose-dependent manner, with a concentration of 100 µmo/L inducing over a 2-fold increase in ROS generation. In addition both TFAs in concentration of 100 µmol/L increased the intracellular ROS production in a time dependent manner (*Fig. 4B*). To evaluate the role of NADPH oxidase, the effects of DPI, a NADPH oxide inhibitor, on the DCF fluorescence were examined. Pre-treatment of HAECs with DPI prior to the treatment with LA or EA significantly inhibited ROS generation (*Fig. 4C* and *4D*). In contrast, pre-treatment with oxypurinol (inhibitor of xanthine oxidase) or rotenone (inhibitor of mitochondrial NADH oxidase) did not affect the increased DCF fluorescence. These results suggest that both TFAs may stimulate ROS production through the activation of NADPH oxidase.

We further investigated whether ROS generated through the NADPH oxidase activation are necessary for the effects of TFAs leading to an increased surface expression of ICAM-1 and VCAM-1. Pre-treatment of HAECs with DPI significantly inhibited TFA-induced ICAM-1 and VCAM-1 expression (*Fig. 5A* and *5B*). Similarly, pre-treatment with DPI significantly inhibited the stimulatory effect of LA and EA on the phosphorylation of NF-κB (*Fig. 5C*) and adhesiveness of HAECs to monocytes and lymphocytes (*Fig. 5D*). These results suggest that ROS generation is required for the TFA-induced proinflammatory response in aortic endothelial cells, and that TFA-induced ROS production occurs, at least in part, through NADPH oxidase activation.

DISCUSSION

In the present study, we investigated the effects of TFAs on the proinflammatory response of endothelial cells. We found that both LA and EA induce expression of *ICAM-1* and *VCAM-1* on the surface of aortic endothelial cells in a concentration-dependent manner. In addition, real time RT-PCR analysis showed that this fatty acid-induced surface expression correlated with overexpression of ICAM-1 and VCAM-1 mRNA. Thus, it

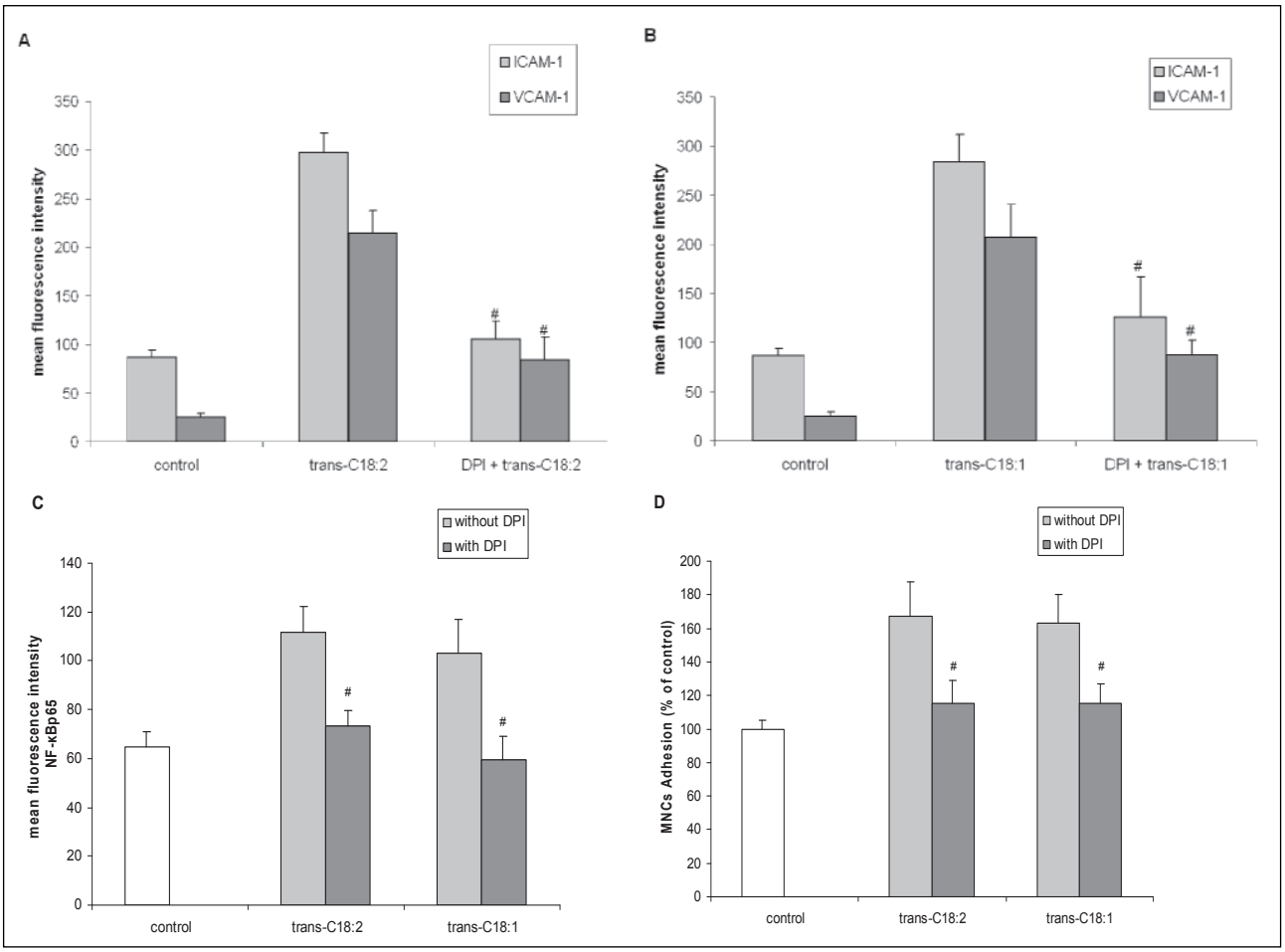

Fig. 5. Involvement of ROS generated by NADPH oxidase in *trans* fatty acid-induced ICAM-1 and VCAM-1 expression, NF-κB activation and adherence of endothelial cells to MNCs. HAECs were pre-incubated with the NADPH oxidase inhibitor, DPI (10 μmol/L) for 30 min, and then were incubated with LA (*trans*-C18:2) (*A*) or EA (*trans*-C18:1) (*B*) in concentration of 100 μmol/L for 16 hrs. Surface expression of ICAM-1 and VCAM-1 was measured by flow cytometry. Values are shown as mean fluorescence intensity corrected for non-specific binding and are expressed as means ±S.D. of six separate experiments. # $p \leq 0.001$, as compared to LA (*trans*-C18:2) or EA (*trans*-C18:1)-treated cells. (*C*) HAECs were pre-treated with DPI (10 μmol/L) for 30 min and then were treated with LA (*trans*-C18:2) or EA (*trans*-C18:1) in concentration of 100 μmo/L for 45 min. Phosphorylation levels of NF-κB p65 were measured by flow cytometry. Data are expressed as mean fluorescence intensity corrected for non-specific binding and are shown as mean ±S.D. of 8 separate experiments. # $p \leq 0.001$ compared to LA (*trans*-C18:2) or EA (*trans*-C18:1)-treated cells. (*D*) HAECs were pre-incubated with DPI (10 μmol/L) for 30 and then were incubated with LA (*trans*-C18:2) or EA (*trans*-C18:1) in concentration of 100 μmol/L for 16 hrs. MNCs were labelled with BCECF-AM, and binding to the endothelial cells was quantified as described in "Materials and methods". Values are expressed as % of the control (set at 100%) and are shown as means ±S.D. of 5 separate experiments, each performed in triplicate. # $p \leq 0.001$ compared to LA (*trans*-C18:2) or EA (*trans*-C18:1)-treated cells.

may be suggested that the observed effects of TFAs may have their origin in their influence on the mechanisms regulating the transcription of genes encoding these molecules.

The impact of linoelaidic acid on ICAM-1 expression observed in this study is in line with the results obtained by others (22, 23). The previous authors, however, have not studied the mechanisms responsible for the stimulating effect of LA on ICAM-1 expression.

In the present study, the higher surface expression of ICAM-1 and VCAM-1 caused by both TFAs results in an increased adhesion of human monocytes and lymphocytes to endothelial cells. Because adhesion is one of the earliest steps in inflammation and atherosclerosis leading to the subsequent migration of blood leukocytes into the sub-endothelial space, the increase in adhesive properties of endothelial cells may be an important mechanism by which dietary TFAs exert their proinflammatory and proatherogenic effects.

To further elucidate the mechanisms of action of TFAs, we investigated their effects on the phosphorylation of NF-κB, which is a critical event in the activation of this transcription factor (8). Activation of NF-κB is the critical process for the regulation of a variety of genes participating in the inflammatory response, including genes encoding ICAM-1 and VCAM-1. Our findings showed that, when compared to non-stimulated cells, treatment with TFAs induced higher levels of NF-κB phosphorylation in HAECs, an indication that NF-κB was activated. Our results suggest that TFA-induced ICAM-1 and VCAM-1 expression is mediated, at least in part, by the modulation of NF-κB activation. This suggestion is supported by the finding that pre-incubation of HAECs with PTDC, which is commonly used as an inhibitor of NF-κB activation, reduced TFA-induced ICAM-1 and VCAM-1 expression.

ROS are an important participant in the redox signalling pathways in cells and, when present in excess, are implicated in

the development of vascular diseases such as hypertension, atherosclerosis or vascular diabetic complication (6, 7). Meanwhile, as shown in an animal model, the inhibition of ROS generation by eplerenone improves vascular function and reduces platelet activation in diabetic rats (24). It is known that endothelial cell apoptosis plays an important role in atherosclerotic plaque development and stability (25). As transpires from recent studies, increased superoxide generation leads to the activation of caspase-3, the main apoptosis executor (26). ROS influence a number of cellular responses by turning on several intracellular cascades in vascular cells, *e.g.* by influencing the redox-sensitive kinases, and among them the mitogen-activated kinases (MAPKs) (5). These kinases are involved, among other things, in the angiogenesis process stimulated by VEGF, BFGF or PPARγ (27). It has been suggested that dietary fatty acids and their metabolites may regulate gene transcription by binding to and modulating PAPRs (28). In addition, NF-κB activation is controlled by the cellular redox status (7). It has been suggested that ROS interfere with the signalling pathways leading to the IκB phosphorylation and degradation.

As suggested from the studies by Cassagno and colleagues (29), a diet rich in TFAs may intensify oxidative stress. In mice fed with a diet rich in TFAs, lowering of plasma vitamin E levels with a concomitant increase in F_2-isoprostanes was observed. In the present study, it was demonstrated for the first time that, in an *in vitro* setting, both LA and EA induce intracellular ROS production in time and concentration dependent manner. Thus, it should be suspected that the observed stimulating effect of selected *trans* fatty acids on the expression of ICAM-1 and VCAM-1 is partially mediated through the enhancement of intracellular ROS production. In support of this thesis may be the observed here more pronounced stimulating effect of LA and EA on the expression of VCAM-1 than on ICAM-1. It has been suggested that the oxidative-stress-dependent NF-κB activation is crucial for VCAM-1 expression, but is not the only factor in ICAM-1 expression (30). Often, various inhibitors of cytokine-stimulated ICAM-1 and VCAM-1 expression inhibit VCAM-1 expression to a larger extent than ICAM-1 expression (31, 32).

In the vascular wall, ROS may be produced by many enzymes, including NADPH oxidase, xanthine oxidase or enzymes of the respiratory chain (33). In certain conditions, *i.e.* in the absence of L-arginine or BH4, which is termed eNOS uncoupling the important source of ROS may be enothelial NO synthase. From the later-stage studies conducted by us, it transpires that the increase in ROS production stimulated by the above acids is inhibited by DPI, an NADPH oxidase inhibitor, but not by the other used inhibitors. Although DPI can inhibit other flavoproteins, such as xantine oxidase or mitochondrial electoron transport chain, the present study showed that specific inhibitors of these enzymes were ineffective for reducing the increase in ROS production induced both by LA and EA. The above results seem to suggest that LA and EA stimulate free radical production through the activation of NADPH oxidase.

NADPH oxidase is the main source of ROS in endothelial cells (5). The known stimulators of NADPH oxidase include angiotensin II, thrombin, cytokines, growth factors, hypoxia/reoxygenation and haemodynamic disturbances. Numerous studies indicate that NADPH oxidase acts as a redox mediator of sensitive signalling pathways upon endothelial cell activation. It has been shown that the stimulation of surface ICAM-1 and VCAM-1 expression by haemodynamic disturbances, TNFα, activation of the RAA system or hypercholesterolaemia is dependent on ROS, the source of which is oxidase and which act through, among other things, the activation of NF-κB (5, 34). The present study confirms these observations because it was demonstrated that pre-incubation of endothelial cells with DPI inhibits, albeit not completely, the stimulating effect of TFAs on the surface expression of ICAM-1 and VCAM-1, endothelial cell adherence to monocytes and lymphocytes, and on the levels of the phosphorylated form of NF-κB p65.

Thus, together with the above findings, the results presented here seem to suggest that the increase in ROS production in endothelial cells, dependent on NADPH oxidase and induced by TFAs, may be responsible for the NF-κB activation with the subsequent induction of ICAM-1 and VCAM-1 expression.

NADPH oxidase-mediated ROS production is regulated at two levels: gene expression of NADPH oxidase subunits and enzymatic activity (35). Recently, it has been shown that resveratrol, which is postulated to explain some of the cardioprotective effects of red wine, decreases the expression of NOX4 (36). Activation of NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits with the membrane-bound cytochrome b558. Despite the lack of direct evidence, we suspect that the studied TFAs enhance the ROS production dependent on this oxidase through assembling its membrane bound components with the components present in the cytoplasm. This suggestion is based on the assumption that one of the possible explanations for how the studied TFAs induce the pro-inflammatory changes in endothelial cells are their effects on the cell membrane components and thus on its structure and properties. As shown by the studies of Harvey and colleagues (22), incubation of HAECs with *trans* 18:2 and *cis* 18:2 leads to the incorporation of those acids into the cell membrane phospholipids. The incorporation of *trans* acid was twice as high as that of the cis form. The studies by Niu and colleagues (37) indicate that phospholipids containing acyl chains in the *trans* form take on a configuration allowing better interactions with cholesterol. Indeed, it has been shown that the level of cholesterol in membrane phospholipids containing TFAs was 40-80% higher than in membranes containing *cis* fatty acid-phospholipids (37). Cholesterol content in the cell membrane affects many important properties of the cell membrane, such as permeability, transport functions, membrane enzyme activity, availability of membrane components as substrates as well as conformation changes of membrane proteins (38, 39). Cholesterol present in the cell membrane has a significant influence on the formation of lipid rafts - regions in the cell membrane rich in cholesterol and sphingolipids, which under the influence of various stimuli undergo clustering or aggregation and thus provide a signalling platform for intracellular signalling pathways (40). Different pro-oxidative stimuli may activate NADPH oxidase through assembling or aggregating its components bound to lipids rafts with the components present in the cytoplasm (41).

One of the better-studied consequences of a rise in the cholesterol content in the cell membrane is the increased inflow of Ca^{2+} ions through the Ca^{2+} channels present in the membrane. In the studies by Kummerow and colleagues (42), incorporation of TFAs into the HAEC cell membrane resulted in an increased inflow of Ca^{2+}. It is known that changes in the intracellular Ca^{2+} ion homeostasis may initiate many processes and act as a universal intracellular messenger (43). Among others, Ca^{2+} ions may, in a direct and phospholipase-D-dependent manner, activate protein kinase C - the main activator of NADPH oxidase. There have been published reports indicating the presence of an association between increased ICAM-1 or VCAM-1 expression and elevated intracellular calcium ions levels (44, 45).

The results discussed above seem to suggest the potential molecular mechanisms of action of TFAs. Still, the issue of whether they have any impact on the properties of cell membrane and the inflow of calcium ions into the cell and whether such potential changes are responsible for the observed proinflammatory effects on endothelial cells requires further investigation.

The doses of studied acids, used by us, are the most commonly used ones *in vitro* studies evaluating the effects of individual acids. It is difficult to say whether the achievement of such serum levels of studied acids is possible, since there are no data published on the subject. Indirect evidence is provided by recent reports indicating that a diet rich in various trans-18:1 forms results in an increase in the level of all *trans* forms from 40 μmol/l to 120 μmol/L (46). Also, it should be remembered that because of the time constraints of *in vitro* tests, the reaction threshold is usually obtained at higher concentrations. Moreover, long-term increased intake of these trans fatty acids may lead to accumulation, and thus a longer-lasting exposure of cells and the possibility of obtaining the same effect at lower doses.

In conclusion, our study demonstrated that TFAs present in our diet have a direct proinflammatory effect, which promotes leukocyte adhesion to the endothelium through the ROS-dependent NF-κB activation. Our findings provide a mechanistic insight into the role of TFAs in the pathogenesis of endothelial dysfunction that could trigger the development of a variety of vascular disorders.

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