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ORIGINAL CONTRIBUTION

Annette Schmidt
Sven Geigenmüller
Wolfgang Völker
Eckhart Buddecke

The antiatherogenic and antiinflammatory effect of HDL-associated lysosphingolipids operates via Akt → NF-kappaB signalling pathways in human vascular endothelial cells

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Priv.-Doz. Dr. A. Schmidt (✉)
S. Geigenmüller · W. Völker · E. Buddecke
Leibniz-Institute of Arteriosclerosis Research
Molecular-Cardiology
University of Münster
Domagkstr. 3
48149 Münster, Germany
Tel.: +49-251/835 8626
Fax: +49-251/835 8628
E-Mail: annschm@uni-muenster.de

E. Buddecke
Institute of Physiological Chemistry and Pathobiochemistry
University of Münster
Münster, Germany

Abstract Adhesion of mononuclear cells to the vascular endothelium and their subsequent transmigration into the arterial wall represent key events in the pathogenesis of arteriosclerosis. In previous studies we have shown that high density lipoproteins (HDL) and the HDL-associated sphingosylphosphorylcholine (SPC) have the ability to suppress the TNF-alpha-induced expression of endothelial cell E-selectin. However, the current understanding of the mechanism by which HDL reduces the expression of E-selectin is still incomplete. In the present study we show that interaction of the HDL-associated sphingosylphosphorylcholine and sphingosylgalactosyl-3-sulfate (lysosulfatide, LSF) with the G-protein-coupled EDG receptor initiates a signalling cascade that activates the protein kinase Akt and reduces the E-selectin, ICAM-1 and VCAM-1 expression on protein and mRNA level. This signalling cascade is consistently associated with a reduced translocation of TNF-alpha-activated NF-kappaB into the cell nucleus. The suppressor effect of SPC and LSF is completely reverted by inhibition of the phosphatidylinositol-3-kinase/Akt pathway. We conclude that the antiatherogenic/antiinflammatory effect of lysosphingolipids depends on a competitive interaction of EDG receptor-induced inhibition and TNF-alpha-initiated stimulation of NF-kappaB translocation into the cell nucleus thereby preventing or stimulating inflammatory events in atherogenesis.

Key words HDL – lysosphingolipids – cell adhesion molecules – NF-kappaB – endothelial cells

Introduction

Adhesion of mononuclear cells to the vascular endothelium and their subsequent transmigration into the arterial wall are characteristic features of inflammation phases in the pathogenesis of arteriosclerosis [24]. While normal endothelium maintains a non-adhesive surface the endothelial cells express and secrete cell adhesion molecules (CAMs) in response to inflammatory mediators such as TNF-alpha or IL-1β, which are capable of promoting the recruitment of mononuclear cells across the endothelial barrier [15, 31].

In atherosclerotic plaques TNF-alpha is produced by resident smooth muscle cells and immigrated macrophages. TNF-alpha activates the transcription of proinflammatory gene products including E-selectin, ICAM-1 and VCAM-1 via a translocation of NF-kappaB from the cytoplasm into the cell nucleus and subsequent induction of the cell adhesion molecules promotor reporter genes [7].

Studies *in vivo* provided evidence for beneficial effect of HDL on endothelial function including the ability to attenuate expression of E-selectin in culture of endothelial cells as well as the expression of cytokines promoting leukocyte extravazation such as IL-8 [5, 6]. More recently

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several studies have demonstrated that the HDL-associated lysosphingolipids sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF, sphingosylgalactosyl-3-sulfate) can mimic the HDL effect [10, 19]. SPC and LSF identified as HDL-associated lysosphingolipids [19] were found to bind specifically to and act through the G-protein-coupled EDG cluster [14], a family of at least eight G-protein-coupled receptors. The EDG (endothelial differentiation gene) receptor-1 and -3 (also known as $S1P_1$, $_{-3}$ receptors) are largely expressed in human endothelial cells [12].

In previous studies we [20] and others [19, 21] have shown that SPC in HUVECs (human umbilical vein endothelial cells) may elicit an activation of phosphatidylinositol-3-kinase and the protein kinase Akt resulting in activation of eNOS and antiapoptotic action. Furthermore SPC and other HDL-associated lysosphingolipids were shown to suppress the TNF-alpha-induced and NF-kappaB-mediated expression of E-selectin and cell adhesion molecules [20, 28]. However, recent studies focussed on the interplay between NF-kappaB and phosphatidylinositol-3-kinase/Akt have yield conflicting results. An Akt-depending pathway for activation of NF-kappaB by TNF-alpha has been described [11] but this pathway does not seem to be active in all cell types and was not shown for HUVECs [4, 8, 29]. In HUVECs the phosphatidylinositol-3-kinase inhibitor LY294002 effectively inhibited TNF-alpha-dependent activation of Akt but left the I-kappaB degradation and cell adhesion molecules expression unaffected [1]. From these results it was concluded that phosphatidylinositol-3-kinase/Akt does not contribute to the activation of NF-kappaB in endothelial cells. Although some studies demonstrated a requirement of NF-kappaB for complete expression of CAMs our knowledge of possible interaction of PI-3 kinase/Akt and NF-kappaB pathway is not fully understood.

In the present study we address the question by which signalling pathways the HDL-associated lysosphingolipids SPC and LSF suppress the TNF-alpha-induced expression of E-selectin, ICAM-1 and VCAM-1 and which downstream target is activated to reduce the nuclear translocation of NF-kappaB in human vascular endothelial cells.

Methods

Enzyme-linked immunosorbent assay (ELISA) for determination of E-selectin and E-selectin-specific forward and reverse oligonucleotide primers were from R&D Systems, Minneapolis, USA. NF-kappaB/p65 ActivELISA was from IMGENEX corporation, San Diego, USA. Anti-phosphospecific antibodies against Akt^{Ser473} were obtained from New England Biolabs, Schwalbach, Germany. RNeasy RNA isolation kit was from Qiagen, Hilden, Germany. Superscript II polymerase, cell culture media, and bovine pituitary extract were purchased by Gibco Life Technologies, Karlsruhe, Germany. All other reagents were from Sigma, Taufkirchen, Germany, and were of the highest purity available. HDL was isolated from human plasma as described [20].

Cell culture

HUVECs were harvested and characterized as described [20]. Cells (2^{nd}-5^{th} passage) were cultured in gelatine-precoated tissue flasks in RPMI 1640 medium supplemented with FCS (15% v/v), heparin (50 µg/mL), bovine pituitary extract (50 µg/mL), and ciprofloxacin (10 µg/mL). For treatment with agonists and/or inhibitors the medium was replaced by a serum-free medium (SFM, Gibco Life Technologies, Karlsruhe, Germany). Experiments were carried out on different cultures in duplicate or triplicate.

Isolation of RNA and RT-PCR analysis

After treatment with agonists and/or inhibitors HUVECs were washed three times and total RNA was isolated by Qiagen RNeasy kit and further purified by DNase digestion according to the manufacturer's protocol. Two microgram of total RNA was than reverse transcribed into cDNA using the Superscript II Polymerase. The sequence-specific E-selectin forward and reverse oligonucleotide primers for PCR reaction were designed by R&D Systems. PCR products were visualized by agarose gel electrophoresis. The size of the cDNA fragment specific for E-selectin was 485 bp.

Quantitative determination of E-selectin, ICAM-1 and VCAM-1 (CAMs)

For determination of CAMs protein concentration enzyme linked immunosorbent assays (ELISA) recognizing recombinant and natural human soluble and cell surface bound protein at a concentration range of 0.5–10 ng/mL were used as described previously [13, 25]. After treatment with agonists and/or inhibitors according to a protocol described previously [20], HUVECs were washed three times with PBS and solubilized on ice in 0.2–0.3 mL of 0.5% (w/v) $Brij^{35®}$ in PBS containing protease inhibitors. The cell lysate was centrifuged at 10,000 × g for 10 min at 4 °C and the clear supernatant was immediately used for determination of cell adhesion proteins according to the manufacturer's instruction. Absorbances were read at 450 nm in a 96-well microplate reader (Dynatech) against a blank substrate. Cell surface expression of E-selectin was determined from the supernatant of the cell suspension obtained by trypsinization.

Determination of nuclear NF-kappaB concentration

Control and pretreated adherent HUVECs were harvested using a cell scraper in ice cold PBS and centrifuged for 3 min at 800 × g in a microcentrifuge. After careful washing in ice cold PBS the cells were resuspended in 400 µL hypotonic lysis buffer and incubated on ice for 15 min. After adding 30 µL of 10% NP-40 and vortexing for 10 seconds the cell lysate was centrifuged for 30 seconds at 15,000 × g and the supernatant saved for analysis of the cytoplasmic extract. 220 µL ice cold nuclear extraction buffer was added to the pellet (nuclear fraction) followed by an incubation of the extract on ice for 30 min with intermittent vortexing. After centrifuging for 10 min at 10,000 × g at 4 °C the supernatant was transferred to a pre-chilled tube and used for determination of p65 NF-kappaB by an ELISA system according to the manual of the producer or stored at –70 °C.

Western blotting of Akt and p-Akt was performed according to a previous protocol [20].

Immunocytochemical staining of HUVECs for NF-kappaB

For this, 10,000 cells were seeded on gelatine-coated eight-well chamberslides (Nunc, cat. # 177402) grown for about three days to confluence and pretreated with SPC (10 µM) or with vehiculum (ethanol) for 15 min. Following stimulation with TNF-alpha for 30 min, cells were fixed and permeabilized at room temperature with 1% formaldehyde containing 0.1% saponin in PBS followed by treatment with 2% bovine serum albumin, 0.1% saponin and 0.01% sodium azide in PBS. Negative controls were then incubated with NF-kappaB-specific blocking peptide (Santa Cruz, cat. # sc-109 P) 1:20.

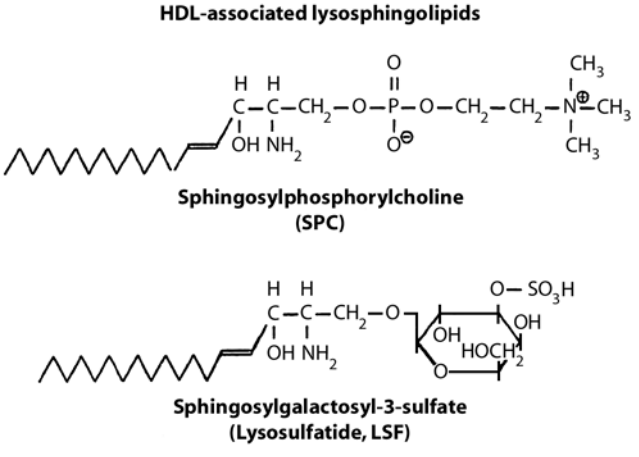

Fig. 1 Chemical structure of HDL-associated lysosphingolipids

Immunostaining was with rabbit polyclonal antibody directed against the p65 subunit of NF-kappaB (Santa Cruz, cat. # sc109) 1:200. Detection was performed with anti-rabbit Cy 3-conjugated secondary antibody (Chemicon, USA) 1:200. Stained cells on cover slips were embedded in mounting medium (DakoCytomation, USA) and examined by fluorescence microscopy.

Statistics

Results are expressed as means ± SD by 1-way ANOVA with Bonferroni's significance correction test. Differences were considered significant at a P value of less than 0.05.

Results

Effect of SPC and LSF on the expression of E-selectin, ICAM-1 and VCAM-1 on protein level

The HDL-mimicking compounds sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF) (Fig. 1) are known to interact with the G-protein-coupled EDG ($S1P_3$) receptor. The action of these lysosphingolipids was investigated by addition to the culture medium of HUVECs. After incubation for 15 min in the presence or absence of SPC (10 µM) and LSF (10 µM) the cells were subjected to a stimulation with TNF-alpha (10 ng/mL) for 6 h followed by an immunoassay of the specified cell adhesion molecules. HDL (1 mg/mL) was used as positive control.

As shown in Fig. 2A SPC and LSF reduce the TNF-alpha-induced expression of E-selectin, ICAM-1 and VCAM-1 to about 60% of control cultures ($p < 0.001$). Basic levels of cells not stimulated with TNF-alpha are below values of 1–5 ng CAM protein/10^5 cells (column C, control). The equal inhibitory effect on other cell adhesion molecules demonstrates that the inhibitory effect of lysosphingolipids is not restricted to E-selectin but comprises all cell adhesion molecules required for transendothelial migration of mononuclear blood cells [30]. The lysosphingolipids (SPC, LSF) are effective only if cells are exposed to them at least 15 min before stimulation with TNF-alpha. A posttreatment did not attenuate the TNF-alpha action. The interaction of the lysosphingolipids with the G-protein-coupled EDG-3 receptor is confirmed by pretreating the cells with suramin (SU) or pertussis toxin (PTX). Suramin uncouples the G-protein from the receptor, PTX inactivates the receptor-coupled G-protein by ADP ribosylation. Figure 2B shows that in the presence of SU (75 µM) or PTX (100 ng/mL) the

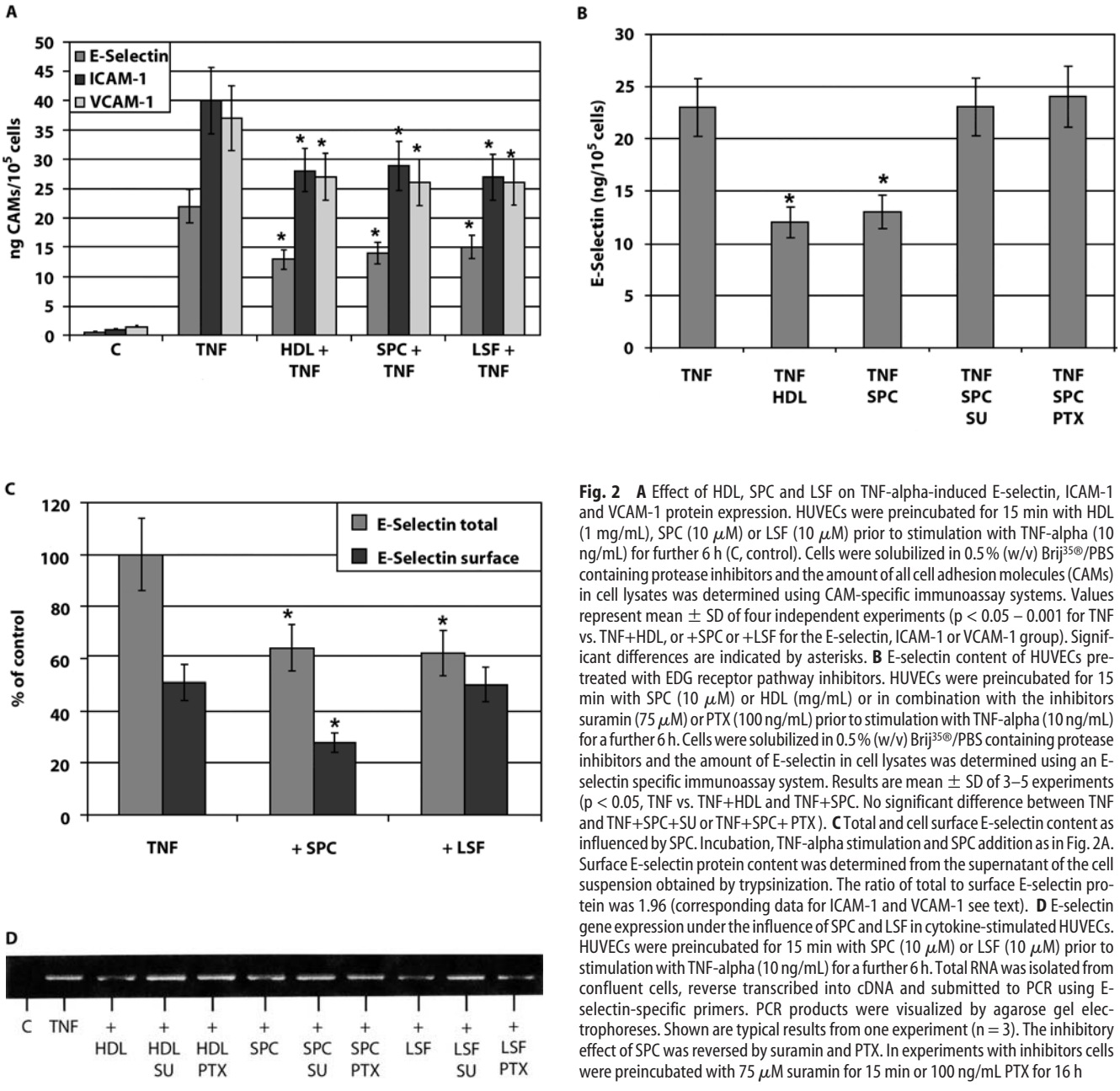

Fig. 2 **A** Effect of HDL, SPC and LSF on TNF-alpha-induced E-selectin, ICAM-1 and VCAM-1 protein expression. HUVECs were preincubated for 15 min with HDL (1 mg/mL), SPC (10 μM) or LSF (10 μM) prior to stimulation with TNF-alpha (10 ng/mL) for further 6 h (C, control). Cells were solubilized in 0.5 % (w/v) Brij$^{35®}$/PBS containing protease inhibitors and the amount of all cell adhesion molecules (CAMs) in cell lysates was determined using CAM-specific immunoassay systems. Values represent mean $\pm$ SD of four independent experiments ($p < 0.05 – 0.001$ for TNF vs. TNF+HDL, or +SPC or +LSF for the E-selectin, ICAM-1 or VCAM-1 group). Significant differences are indicated by asterisks. **B** E-selectin content of HUVECs pretreated with EDG receptor pathway inhibitors. HUVECs were preincubated for 15 min with SPC (10 μM) or HDL (mg/mL) or in combination with the inhibitors suramin (75 μM) or PTX (100 ng/mL) prior to stimulation with TNF-alpha (10 ng/mL) for a further 6 h. Cells were solubilized in 0.5 % (w/v) Brij$^{35®}$/PBS containing protease inhibitors and the amount of E-selectin in cell lysates was determined using an E-selectin specific immunoassay system. Results are mean $\pm$ SD of 3–5 experiments ($p < 0.05$, TNF vs. TNF+HDL and TNF+SPC. No significant difference between TNF and TNF+SPC+SU or TNF+SPC+ PTX). **C** Total and cell surface E-selectin content as influenced by SPC. Incubation, TNF-alpha stimulation and SPC addition as in Fig. 2A. Surface E-selectin protein content was determined from the supernatant of the cell suspension obtained by trypsinization. The ratio of total to surface E-selectin protein was 1.96 (corresponding data for ICAM-1 and VCAM-1 see text). **D** E-selectin gene expression under the influence of SPC and LSF in cytokine-stimulated HUVECs. HUVECs were preincubated for 15 min with SPC (10 μM) or LSF (10 μM) prior to stimulation with TNF-alpha (10 ng/mL) for a further 6 h. Total RNA was isolated from confluent cells, reverse transcribed into cDNA and submitted to PCR using E-selectin-specific primers. PCR products were visualized by agarose gel electrophoreses. Shown are typical results from one experiment (n = 3). The inhibitory effect of SPC was reversed by suramin and PTX. In experiments with inhibitors cells were preincubated with 75 μM suramin for 15 min or 100 ng/mL PTX for 16 h

lysosphingolipid-induced suppression of E-selectin is reverted (no significant difference by comparison of TNF vs. TNF+SPC+SU or PTX). The suramin concentration used in our experiments is in the same range as in reports about the purinergic receptor antagonist effect of suramin [9, 32].

Since determination of E-selectin, ICAM-1 and VCAM-1 in cell lysate does not actually represent surface expression further experiments for evaluating the amount of CAMs exposed to the cell surface were performed. As seen from Fig. 2C the expected suppression of E-selectin under the influence of SPC and LSF is apparent also on the cell surface fraction of E-selectin. The E-selectin protein exposed to the cell surface accounts for about 50 % of the E-selectin protein of the cell cytoplasma ($p < 0.05$, n = 3). The ratio of total to surface E-selectin is 1.96. The corresponding data for ICAM-1 and VCAM-1 (not shown) are 1.84 and 1.42 respectively. The ratio of CAMs did not change in the presence of SPC and LSF ($p > 0.1$ compared with TNF-alpha).

Effect of SPC and LSF on E-selectin mRNA level

To verify the effect of SPC and LSF on TNF-alpha-induced E-selectin protein the E-selectin gene expression level was examined (Fig. 2D). As expected after TNF-alpha stimulation a large amount of E-selectin-specific PCR product could be detected in TNF-alpha-stimulated HUVECs but not in untreated cells (control, C). The E-selectin cDNA level was markedly decreased in HUVECs pretreated with SPC or LSF but could be completely reverted by treatment of HUVECs with SU or PTX.

LY294002 as inhibitor of SPC-triggered nuclear NF-kappaB translocation

LY294002 is an inhibitor of phosphatidylinositol-3-kinase thereby preventing or attenuating the phosphorylation of protein kinase Akt at Ser^{473}. To investigate the way in which the phosphorylation of Akt is related to the translocation of NF-kappaB the cells were pretreated with 5 μM LY294002 for 15 min and then incubated in the presence and absence of 10 μM SPC for further 30 min. Cell lysates were submitted to a Western blot analysis. Figure 3A demonstrates that cells not stimulated with TNF-alpha accomplish a formation of phosphorylated Akt (p-Akt) that is clearly enlarged in the presence of SPC. However, a phosphorylation of Akt occurs also upon TNF-alpha stimulation but the Akt phosphorylation is essentially more pronounced in the presence of SPC (not shown). In both cases the phosphorylation of Akt is prevented by the PI-3 kinase inhibitor LY294002.

A requirement of NF-kappaB for complete expression of E-selectin has been described and postulates a positive correlation between the expression of E-selectin protein with the nuclear concentration of NF-kappaB. Therefore

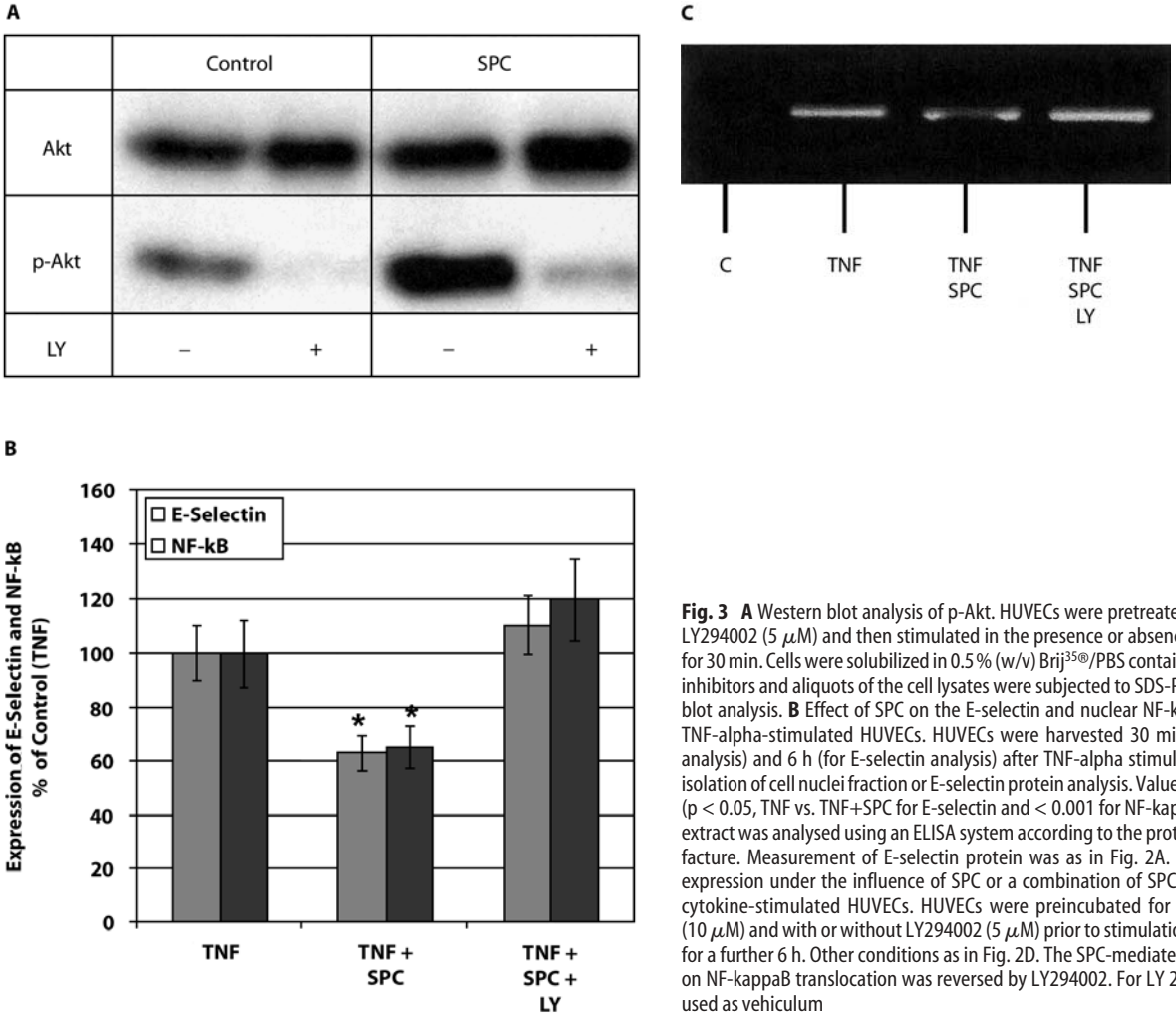

Fig. 3 **A** Western blot analysis of p-Akt. HUVECs were pretreated for 15 min with LY294002 (5 μM) and then stimulated in the presence or absence of SPC (10 μM) for 30 min. Cells were solubilized in 0.5 % (w/v) Brij[35®]/PBS containing phosphatase inhibitors and aliquots of the cell lysates were subjected to SDS-PAGE and Western blot analysis. **B** Effect of SPC on the E-selectin and nuclear NF-kappaB content of TNF-alpha-stimulated HUVECs. HUVECs were harvested 30 min (for NF-kappaB analysis) and 6 h (for E-selectin analysis) after TNF-alpha stimulation followed by isolation of cell nuclei fraction or E-selectin protein analysis. Values are means ± SD ($p < 0.05$, TNF vs. TNF+SPC for E-selectin and < 0.001 for NF-kappaB). The nuclear extract was analysed using an ELISA system according to the protocol of the manufacture. Measurement of E-selectin protein was as in Fig. 2A. **C** E-selectin gene expression under the influence of SPC or a combination of SPC and LY294002 in cytokine-stimulated HUVECs. HUVECs were preincubated for 15 min with SPC (10 μM) and with or without LY294002 (5 μM) prior to stimulation with TNF-alpha for a further 6 h. Other conditions as in Fig. 2D. The SPC-mediated inhibitory effect on NF-kappaB translocation was reversed by LY294002. For LY 294002 DMSO was used as vehiculum

we assessed the p65-fraction of NF-kappaB of the cell nuclei and the expression of E-selectin protein in strictly parallel cultures. Monitoring the kinetics of nuclear translocation of p65-NF-kappaB after TNF-alpha stimulation a maximum translocation was found after 30 min. For quantification of the nuclear fraction of NF-kappaB the adherent cells were harvested and the prepared nuclear fraction (see Methods) was extracted. The extract of control and TNF-alpha-stimulated cells was analysed for p65-NF-kappaB using an ELISA system. In addition the E-selectin protein was determined 6 h after TNF-alpha stimulation in the cell lysate. Figure 3B shows a positive correlation of the nuclear NF-kappaB extract with the E-selectin protein determined in the cell lysate. For better comparison of SPC-induced reduction of both E-selectin and nuclear NF-kappaB concentration, the values are given in percent of control. Under the influence of SPC the E-selectin concentration and the nuclear NF-kappaB concentration were obviously suppressed to values ranging from 63–58 % of the control values ($p < 0.05$). Fig. 3B clearly demonstrates that the SPC-induced suppression of NF-kappaB translocation is completely reverted by LY294002 (TNF+SPC vs. TNF+SPC+LY, $p < 0.05$ for E-selectin and $p < 0.001$ for NF-kappaB). The LY294002 effect is also detectable on the mRNA level of E-selectin (Fig. 3C).

Immunofluorescence staining for p65 subunit of NF-kappaB

In order to confirm the reversal of TNF-alpha-induced NF-kappaB translocation in response to SPC we performed immunofluorescence analysis using polyclonal antibodies directed against the p65 subunit of NF-kappaB. In control cells (Fig. 4a) a moderate perinuclear cytoplasmic fluorescence is observed. In TNF-alpha-stimulated cells the fluorescence is predominantly localized in the cell nuclei (Fig. 4b) while in the SPC-cells upon TNF-alpha-stimulation a substantial part of the fluorescence remains in the cytoplasmic compartment preferably around the cell nuclei (Fig. 4c). Since the antibody recognizes both the inactive form of p65 bound to p50 and I-kappaB we demonstrated that both the cytoplasmic and nuclear immunoflorescence could be quenched completely by blocking antibodies in control and NF-

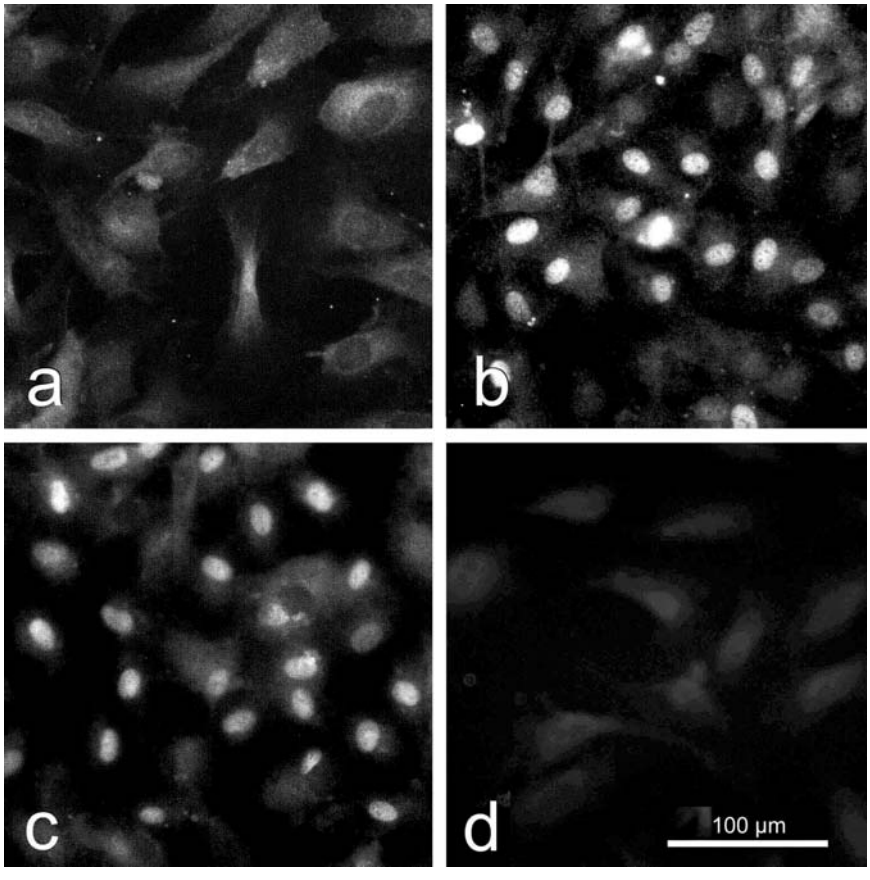

Fig. 4 Immunofluorescence analysis of NF-kappaB in HUVECs. Cells were grown in gelatine-coated chamberslides, pretreated with SPC (10 μM) for 15 min and stimulated with TNF-alpha (10 ng/mL) for 30 min. Subsequently, the cells were fixed, treated with a cell permeant and immunostained with NF-kappaB-specific polyclonal antibodies directed against the p65 subunit (Rel A) and with a corresponding secondary fluorescence-labelled antibody. **a** Non-stimulated cells. **b** Stimulation with TNF-alpha resulted in translocation of p65 into the cell nucleus (arrows) with reduction of cytoplasmic fluorescence. In cells pretreated with SPC (**c**) nuclear localization of NF-kappaB is markedly reduced. In control cells pretreated with NF-kappaB blocking peptide the NF-kappaB-induced fluorescence is completely quenched by the blocking peptide (**d**). Images are representatives of three independent experiments and are printed in black/white. Bar, 100 nm

kappaB stimulated cells (Fig. 4d). In all experiments nonspecific fluorescence was reduced by bovine serum albumin.

Discussion

High density lipoproteins (HDL) are complex molecules known to induce a multitude of intracellular signals for which different components of HDL have been made responsible [2, 15, 22]. In the present study we demonstrate that the HDL-associated sphingosylphosphorylcholine (SPC) and lysosulfatide (LSF) initiate a signalling cascade involving an activation of PI-3K and Akt (also known as PKB) that attenuates the TNF-alpha-induced expression of E-selectin up to 50%. The reduction of E-selectin expression by SPC is dose-dependent [20] and strictly correlated with a repression of the nuclear level of NF-kappaB. This has been shown by three independent experiments: (I) the decreased NF-kappaB concentration in the cell nuclei isolated by sequential centrifugation of SPC-pretreated HUVECs (Fig. 3B), (II) by reverting the suppressing effect of E-selectin by LY294002 (Fig. 3C) and (III) by immunofluorescence staining of cultured cells *in vitro* using specific anti-p65 subunit antibodies (Fig. 4a–d). From these results we conclude that NF-kappaB is a direct or secondary downstream target of phosphorylated Akt and that SPC is capable to counterbalance the TNF-alpha-induced inflammatory events in atherogenesis.

Cell adhesion molecules are regulated primarily at the level of transcriptional activation of NF-kappaB that after translocation into the cell nucleus binds to the promotor domain of E-selectin and other cell adhesion molecules [1] and induces transcription [17, 27]. Our data allow no conclusion on the mechanism by which phosphorylated Akt causes a retention of NF-kappaB in the cytosol. The pleiotropic effects of TNF-alpha stimulation involve numerous additional signalling pathways including MAPK, SAP kinase [15], GM-CSF, cylcooxygenase 2 [8], ROS generation and proteasome activation [23] and the activation of enzymes of the sphingomyeline cycle [15, 18]. The resulting network of synergisms and antagonisms still needs clarification.

The signalling flow from the EDG-activated PI-3 kinase to phosphorylation of Akt seems not to be unidirectional. Thus, Akt was found to be phosphorylated by NF-kappaB although inhibition of PI-3 kinase by LY294002 did not affect the ability of p65 to stimulate Akt activation in general [16]. These conflicting results may be explained in this way that the presence of an EDG activator drives the signalling flow downstream from Akt to NF-kappaB, while in the absence of HDL or SPC the signalling flow is reversed, thereby providing the anti-apoptotic effect [26] and eNOS activation [22] by Akt.

In our previous studies [20] we found that SPC and LSF have an inhibitory effect on the TNF-alpha-induced expression of CAMs comparable to that of HDL in a concentration range of 0.5–2.0 mg/mL. However, as the extent of HDL-mediated inhibition depends largely on the phospholipid composition and varies markedly within HDL specimens collected from a number of different human subjects [1] we used SPC as a standard HDL-mimicking lysosphingolipid that gives reproducible inhibitory effects of the cytokine-induced E-selectin expression. Nevertheless the contribution of SPC to the HDL action should be accessed with care because the SPC concentration needed for a HDL-like effect is significantly higher than that present in HDL. According to the previously reported data [22] 1 mg HDL contains 290 ± 20 pM SPC. Significantly higher concentrations we used in our experiments. However, *in vivo* additional approaches may be relevant: several further lysosphingolipids contained in HDL (e.g. LSF, S1P) that have also the ability to suppress the E-selectin expression would work together in achieving a reduced E-selectin expression. Moreover, different phosphatidylcholine species containing lineoyl and arachidonyl in the sn-2 position inhibited VCAM-1 expression in activated HUVECs by 95 and 70% [3] and HDL particle shape, size and apolipoprotein composition are important determinants of the ability of HDL to inhibit the TNF-alpha-induced expression of vascular cell adhesion molecules [1]. All these factors would contribute to the assumption that a 10 μM concentration of SPC could be equivalent to a 1 mg dose of HDL we found to be effective in suppressing the expression of E-selectin in previous experiments [20]. Cockerill et al. [6] used a porcine model of inflammation and provided evidence that also reconstituted HDL (recHDL) is able to reduce expression of E-selectin *in vivo*. The recHDL contained apolipoprotein A1 as the sole protein and soybean phosphatidylcholine as the sole phospholipid. However, the suppressive effect of recHDL was not observed when HDL was reconstituted by lipid-free apo-A1 [21]. Furthermore, the *in vivo* effect of recHDL by use of a bolus injection should be accessed with care because a secondary uptake of plasma sphingolipids such as S1P by the recHDL can not be excluded. S1P is present in the blood plasma after release from platelets.

In summary we conclude that the antiatherogenic/antiinflammatory effect of lysosphingolipids depends on a competitive interaction of EDG receptor-induced inhibition and TNF-alpha-initiated stimulation of NF-kappaB translocation into the cell nucleus thereby preventing or stimulating inflammatory events in atherogenesis.

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