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Regulation of inducible adhesion molecule expression in human endothelial cells by grape seed proanthocyanidin extract

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Abstract

Altered expression of cell adhesion molecule expression has been implicated in a variety of chronic inflammatory conditions. Regulation of adhesion molecule expression by specific redox sensitive mechanisms has been reported. Grape seed proanthocyanidins have been reported to have potent antioxidant properties. We evaluated the effects of grape seed proanthocyanidin extract (GSPE) on the expression of TNFα-induced ICAM-1 and VCAM-1 expression in primary human umbilical vein endothelial cells (HUVEC). GSPE at low concentrations (1–5 μg/ml), down-regulated TNFα-induced VCAM-1 expression but not ICAM-1 expression in HUVEC. Such regulation of inducible VCAM-1 by GSPE was also observed at the mRNA expression level. A cell-cell co-culture assay was performed to verify whether the inhibitory effect of GSPE on the expression of VCAM-1 was also effective in down-regulating actual endothelial cell/leukocyte interaction. GSPE treatment significantly decreased TNFα-induced adherence of T-cells to HUVEC. Although several studies have postulated NF-κB as the molecular site where redox active substances act to regulate agonist-induced ICAM-1 and VCAM-1 gene expression, inhibition of inducible VCAM-1 gene expression by GSPE was not through a NF-κB-dependent pathway as detected by a NF-κB reporter assay. The potent inhibitory effect of low concentrations of GSPE on agonist-induced VCAM-1 expression suggests therapeutic potential of this extract in inflammatory conditions and other pathologies involving altered expression of VCAM-1. (Mol Cell Biochem **216**: 1–7, 2001)

Key words: proanthocyanidins, adhesion molecules, endothelial cell, inflammation, reactive oxygen species, antioxidant

Introduction

Cell adhesion represents a process that is centrally important in immune function and inflammation [1]. Intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD 106) expressed on endothelial cells are major cell surface inducible glycoproteins that contribute to the cell adhesion processes [1, 2]. The ligands for ICAM-1 and VCAM-1 on leukocyte are lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) and very late antigens-4 (VLA-4, CD49d/CD29), respectively. Several stimuli such as pro-inflammatory cytokines [3], phorbol 12-myristate 13-acetate (PMA) [4, 5], oxidants [6] and HIV-1-tat protein [7] are known to activate the expression and function of cell adhesion molecules.

Cell adhesion molecule expression and adhesive properties of cells are greatly modified in several diseased conditions involving redox imbalances such as cancer, atherosclerosis, diabetes, chronic-inflammation and ischemia-reperfusion injury. Therapeutic agents that down-regulate inducible ICAM-1 or VCAM-1 expression and block leukocyte-endothelial interactions have been shown to markedly regulate the progression of inflammatory responses in a number of *in vivo* models [2]. Regulation of both ICAM-1 and VCAM-1 gene

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expression has been related to oxidative stress through specific reduction-oxidation (redox) sensitive transcriptional or posttranscriptional factors [8, 9]. Thus, antioxidants have been suggested to have therapeutic potential in pathologies related to changes in cellular adhesion.

Plants flavonoids are reported to have therapeutic potentials because of their antioxidant, anti-inflammatory, anticancer, anti-allergic or anti-ischemic properties [10–16]. Most of the biological activity of flavonoids is attributed to their antioxidant and protein kinase inhibitory properties. The proanthocyanidins or condensed tannins are a group of biologically polyphenolic bioflavonoids that are synthesized by many plants. Grape seed proanthocyanidin extract (GSPE), has also been reported to have various clinically relevant properties such as antioxidant and anti-cancer activity [17–21]. In the present study, the efficacy of GSPE in regulating inducible ICAM-1 and VCAM-1 expression in human endothelial cells and the mechanisms involved in such regulation were investigated.

Materials and methods

Materials

A novel IH636 grape seed proanthocyanidin extract (GSPE) (commercially known as ActiVin, batch #AV609016) was obtained from InterHealth Nutraceuticals Inc. (Concord, CA, USA). GSPE is a natural extract of approximately 54% dimeric-, 13% trimeric- and 7% tetrameric proanthocyanidins, and approximately 8% monomeric bioflavonoids [22]. Unless otherwise stated all other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

Cells and cell culture

Primary human umbilical vein endothelial cells (HUVEC) were obtained from Cascade Biologics Inc. (Portland, OR, USA). The cells were cultured in the medium (Medium 200 supplemented with low growth serum supplement, LSGS) supplied by the Cascade Biologics Inc. HUVEC were used between passages 2 and 5. Prior to shipping, each lot of cells is tested by the vendor using immunohistochemical methods for the presence of von Willebrand factor (vWf) and CD31 antigen and for the absence of α-actin. The uptake of DiI-Ac-LDL is also confirmed. Following 5th passage, the presence of CD31 and E-selectin and VCAM-1 antigen was again confirmed. Human Jurkat T cells clone E6-1 (American Type Culture Collection, ATCC, Bethesda, MD, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 110 mg/L sodium pyruvate and 2 mM L-glutamine. Cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37 ± 2°C.

GSPE stock solutions for cell treatment was prepared fresh in dimethyl sulfoxide (DMSO) at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1% (v/v). Respective controls were treated with equal volume of dimethyl sulfoxide.

Determination of cell surface expression of adhesion molecules

HUVEC were washed twice with phosphate buffered saline, pH 7.4 (PBS) and incubated with FITC-labeled ICAM-1 (mouse IgG1, Beckman Coulter Inc., Fullerton, CA, USA) or PE-labeled VCAM-1 (mouse IgG1, Pharmingen, San Diego, CA, USA) monoclonal antibody for 1 h at 4°C. Following incubation, cells were washed twice with PBS and finally resuspended in fresh PBS. Expression of ICAM-1 or VCAM-1 was assayed with a flow cytometer. Appropriate FITC or PE-labeled mouse IgG1 isotypic controls used for background fluorescence in the ICAM-1 or VCAM-1 assay [4].

Flow cytometric analysis

The fluorescence and light scattering properties (forward scatter, FS; side scatter, SS) of the cells were determined by using an EPICS-XL (Coulter, Miami, FL, USA) flow cytometer. Cells immunostained with FITC-conjugated ICAM-1 or VCAM-1 antibody were excited using a 488 nm argon ion laser and the emission was recorded at 525 nm. In each sample, at least 10,000 gated viable cells were examined. A logarithmic scale was used to measure both background and endothelial cell fluorescence. Background fluorescence was then subtracted from endothelial cell fluorescence, allowing linear comparisons of ICAM-1/VCAM-1 expression between various samples.

Adhesion of Jurkat T-cells to HUVEC

Monolayers of HUVEC were seeded (0.25×10^5) onto glass cover-slips. After 24 h of seeding, HUVEC were pretreated with GSPE (2 μg/ml) for 24 h and then activated with TNFα (10 ng/ml) for 16 h. Adhesion assay was performed as described previously [4]. In brief, cells were washed 3 times with PBS and then co-cultured with calcein-labeled Jurkat T-cells (2×10^6 cells/ml) for 1 h in a culture incubator with humidified air containing 5% CO_2 at 37 ± 2°C [4]. GSPE was not present in the incubation medium during co-culture period. Following 1 h of co-culture, non-adherent Jurkat T-cells were washed. Images were obtained using a fluorescence microscope.

To determine the contribution of ICAM-1 or VCAM-1 in TNFα-induced adhesion of Jurkat cells to HUVEC a quantitative cell-cell adhesion assay was performed as described previously [4]. Monolayers of HUVEC were seeded at a density of 10^4 cells/well in 96 well tissue culture plates (Falcon 3072, Becton Dickinson, Franklin Lakes, NJ, USA). The rest of the conditions were exactly identical to the conditions described in the previous section. To block the ICAM-1 or VCAM-1 sites, HUVEC were treated with anti-human ICAM-1 (10 μg/ml) or anti-human VCAM-1 (10 μg/ml) antibodies for 30 min before the co-culture assay. The fluorescence intensity of each well is measured using a fluorescence plate reader. The excitation and emission wavelengths for calcein molecule are 480 and 530 nm, respectively. Autofluorescence of GSPE loaded in HUVEC interfered with the quantitative cell adhesion assay, therefore, this assay could not be performed to quantitate the effect of GSPE on TNFα-induced cell adhesion.

RNA isolation and Northern blot analysis

Total RNA was extracted from 2–3 × 10^6 HUVEC using guanidium isothiocyanate following the method of Chomczynski and Sacchi [23]. RNA samples (10 μg) were subjected to electrophoresis in 1% (w/v) formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham, Piscataway, NJ, USA) overnight in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). The RNA was cross-linked to the nylon membrane by 5 min UV exposure. Blots were then prehybridized for at least 1 h at 37°C in 50% formamide, 5 × SSC, 0.1% SDS, 5 × Denhardt's solution (0.1% bovine serum albumin, Ficoll and polyvinylpyrrolidine) and 100 μg/ml denatured sperm DNA (Life Technology, Gaithersburg, MD, USA). Blots were hybridized at 37°C for 16 h using human ICAM-1 or VCAM-1 cDNA probes (R & D systems, Minneapolis, MN, USA). cDNA probes were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The blots were washed twice with wash I solution (1 × SSC, 0.1% SDS) for 10 min at 37°C; followed by two more washes in wash II solution (0.2 × SSC, 0.1% SDS) for 10 min at 60°C. In order to normalize mRNA content, the blots were stripped and reprobed with radio-labelled human β-actin cDNA probe (R & D systems, Mineapolis, MN, USA).

NF-κB driven transactivation of transiently transfected luciferase gene

HUVEC were transiently transfected with pNF-κB-Luc (Stratagene, La Jolla, CA, USA) using standard transfection techniques [24]. SuperFect (Stratagene, La Jolla, CA, USA) reagent was used for the transfection of HUVEC with pNFκB-Luc plasmid. Cells were pretreated with GSPE followed by activation with TNFα for 6 h. After that period of time cells were harvested and the luciferase activity in cell lysate was determined using a commercially available luciferase assay kit (Stratagene, La Jolla, CA, USA) and a luminometer.

Statistical analyses

Mean ± S.D. were derived from at least 3 independent experiments. Differences between means of groups were determined by Student's *t*-test and analysis of variance. The minimum level of significance was set at $p < 0.05$.

Results

Differential regulation of inducible ICAM-1 and VCAM-1 expression by GSPE

The ICAM-1 and VCAM-1 expression levels were very low in non-stimulated HUVEC. Treatment of HUVEC with TNFα markedly increased VCAM-1 and ICAM-1 expression (Fig. 1). Such increase reached a plateau following 12–16 h of TNFα treatment. Pretreatment of the cells with GSPE significantly inhibited TNFα-induced VCAM-1 expression in a dose (1–5 μg/ml)-dependent manner (Fig. 1). A similar pretreatment slightly increased the inducible ICAM-1 expression in HUVEC (Fig. 1). The down-regulatory effect of GSPE on inducible VCAM-1 expression was not due to cytotoxicity, since no significant difference in the viability was observed between non-treated or GSPE treated cells as determined by the propidium iodide exclusion test using a flow cytometer (not shown).

Effects of GSPE on adhesion of Jurkat T-lymphocytes to HUVEC

The lymphocyte adhesion assay was performed to verify whether the inhibitory effect of GSPE on the expression of VCAM-1 was also effective in down-regulating actual cell to cell adhesion. The adhesion of Jurkat T-cells to HUVEC was markedly up-regulated following treatment of HUVEC with TNFα for 16 h (Fig. 2). A decrease in the adherence of Jurkat T-cells to HUVEC was observed following pretreatment of HUVEC with GSPE (Fig. 2). Treatment of HUVEC with anti-ICAM-1 or anti-VCAM-1 blocked ~ 33.8 ± 3.17% or 56.1 ± 6.2%, respectively, TNFα-induced Jurkat cell adhesion to HUVEC (Fig. 3). This data indicates that ICAM-1 and VCAM-1 together account for ~ 90% of the TNFα-induced Jurkat T-cell adhesion to HUVEC suggesting that these two molecules play major role in TNFα-induced Jurkat – HUVEC adhesion.

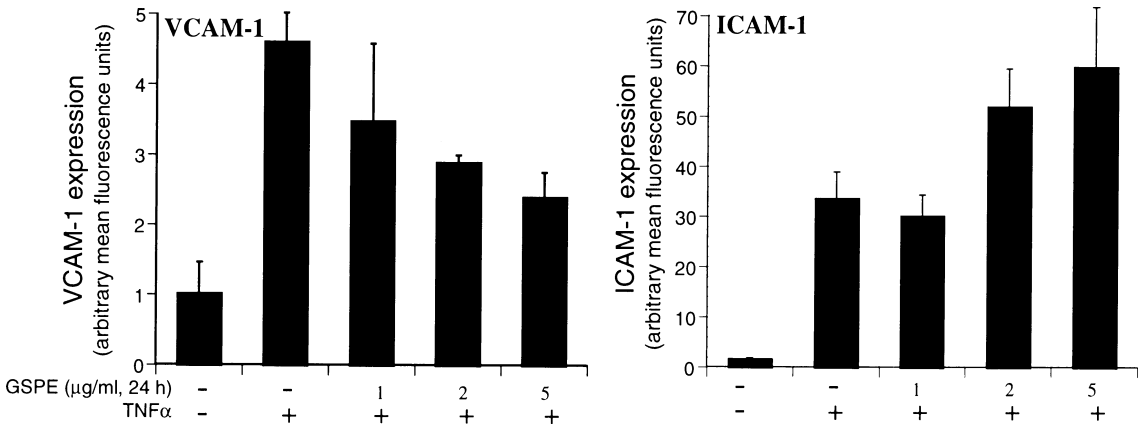

Fig. 1. GSPE down-regulates TNFα-induced VCAM-1 but not ICAM-1 protein expression in human primary umbilical vein endothelial cells (HUVEC). HUVEC were pretreated with GSPE (1–5 μg/ml) for 24 h and then activated with TNFα (10 ng/ml) for 16 h. Expression of VCAM-1 or ICAM-1 was assayed using a flow cytometer. Data are mean ± S.D. of 3 independent experiments.

mRNA levels

To characterize the molecular mechanisms responsible for the down-regulation of TNFα-induced VCAM-1 expression by GSPE, the effect on VCAM-1 or ICAM-1 mRNA levels were investigated by Northern blot analysis. The levels of VCAM-1 or ICAM-1 mRNA reached a plateau following activation of the cells with TNFα for 6 h (data not shown). Cells were pretreated with GSPE, then stimulated with TNFα for 6 h. Consistent with the protein expression data, GSPE inhibited inducible VCAM-1 but not the ICAM-1 mRNA expression (Fig. 4).

Effect of GSPE on NF-κB driven gene transactivation

The binding motif of NF-κB has been identified in ICAM-1 and VCAM-1 promoter and shown to be involved in the induction of ICAM-1/VCAM-1 gene expression. To evaluate whether the inhibitory effect of GSPE on inducible VCAM-1 expression was mediated by a NF-κB-dependent pathway, HUVEC were transiently transfected with NF-κB-Luc plasmid (Fig. 5). TNFα treatment for 6 h significantly increased NF-κB driven luciferase gene expression in HUVEC as measured by luciferase activity (Fig. 5). The concentration (2 μg/ml) of GSPE that significantly inhibits inducible VCAM-1 protein and mRNA expression, did not affect TNFα-induced NF-κB driven luciferase gene transactivation (Fig. 5).

Discussion

The present study shows that a natural proanthocyanidin extract from grape seed with known antioxidant [19–21] prop-

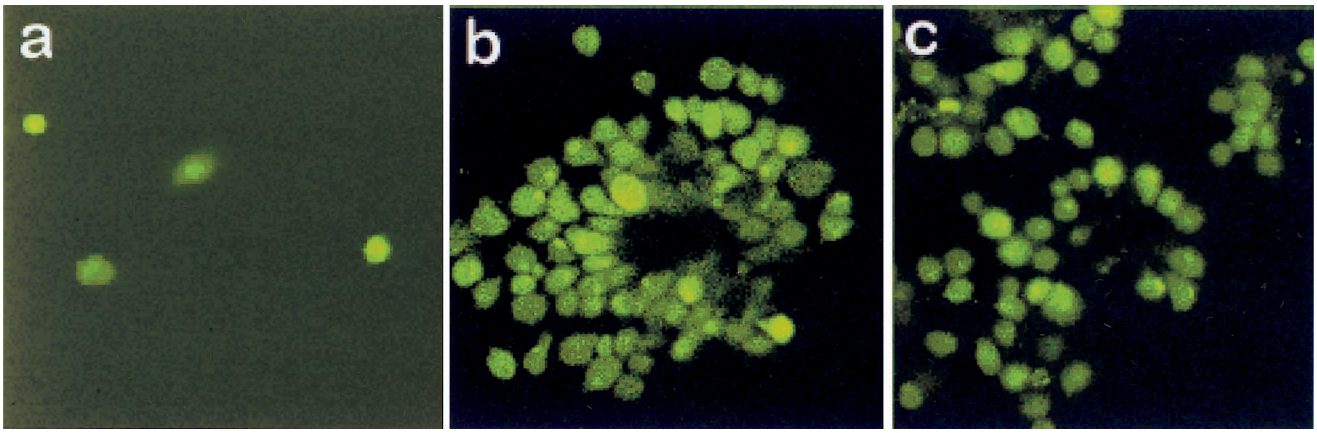

Fig. 2. Effect of GSPE on the adherence of human Jurkat T-cells to TNFα-activated human primary umbilical vein endothelial cells (HUVEC). HUVEC were pretreated with GSPE (2 μg/ml) for 24 h and then activated with TNFα (10 ng/ml) for 16 h. Cells were washed 3 times with phosphate buffered saline and then co-cultured with Jurkat T-cells for 1 h. Following 1 h of the co-culture, non-adherent Jurkat T-cells were washed. Images were obtained using a fluorescence microscope. (a) control (0.1% v/v DMSO); (b) 10 ng/ml TNFα, 16 h; (c) 2 μg/ml GSPE + 10 ng/ml TNFα, 16 h. Results shown are representative of at least 3 independent experiments.

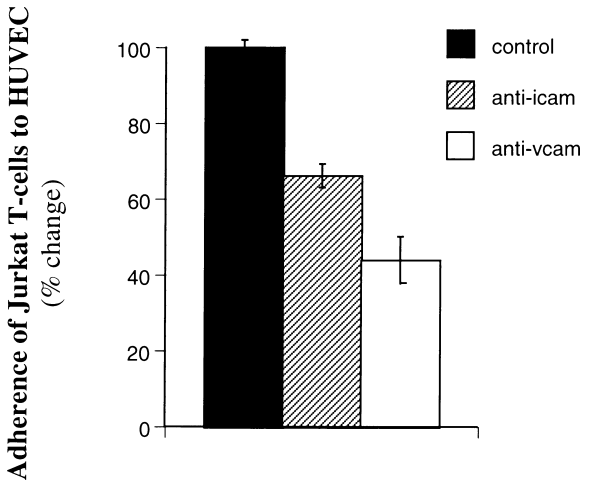

Fig. 3. Relative contributions of ICAM-1 and VCAM-1 molecules in TNFα-induced adhesion of Jurkat cells to HUVEC. HUVEC cells were treated with or without 10 ng/ml TNFα for 16 h. Following activation, the HUVEC were treated with anti- ICAM-1 (10 μg/ml) or anti-VCAM-1 (10 μg/ml) antibodies for 30 min prior to co-culture with Jurkat cells. Adhesion assay was performed with calcein-AM labeled Jurkat T-cells for 1 h as described in the Materials and methods section. Data are presented as % change compared to TNFα-induced cell adhesion (solid bar, control). Data are mean ± S.D. of at least 3 experiments.

erties differentially regulate TNFα-induced VCAM-1 but not ICAM-1 protein expression. VCAM-1 plays a major role in TNFα-induced adhesion of Jurkat cells to HUVEC contributing to ~ 60% of such adhesion. The inhibition in protein expression by GSPE was also observed at the mRNA level suggesting a transcriptional or post-transcriptional regulation of inducible VCAM-1 expression by this extract.

Redox-active properties of GSPE have been elucidated in previous studies [17–21, 25]. The extract provided significant protection against H_2O_2-induced oxidative stress in cultured macrophage J774A.1 cells and in pheochromocytoma PC-12 cells [26]. Protective activity of GSPE against oxidative stress was also observed in a number of *in vivo* studies [17, 20, 27]. GSPE conferred protection against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced liver and brain lipid peroxidation and DNA fragmentation [20]. Reactive oxygen species have been implicated in regulation of a variety of genes including ICAM-1 and VCAM-1 [28, 29]. The reactive oxygen scavenging properties of this natural extract may account for its inhibitory effects on the VCAM-1 gene expression.

In vitro as well as *in vivo* studies have shown that bioflavonoids and flavonoid-rich plant extracts with potent radical scavenging activity markedly inhibit inducible cell adhesion molecule expression and function [30–33]. Apigenin is a potent flavone that has been reported to inhibit adhesion molecule expression in endothelial cells in a dose- and time-dependent manner. An effect of this flavone at the transcriptional level has been demonstrated [34]. Apigenin is also known to inhibit TNFα-induced ICAM-1 expression *in vivo* [32]. Cell adhesion regulatory effects of flavonoids have been consistently evident in other independent studies [30, 35]. The flavonoid delphinidin chloride (CAS 528-53-

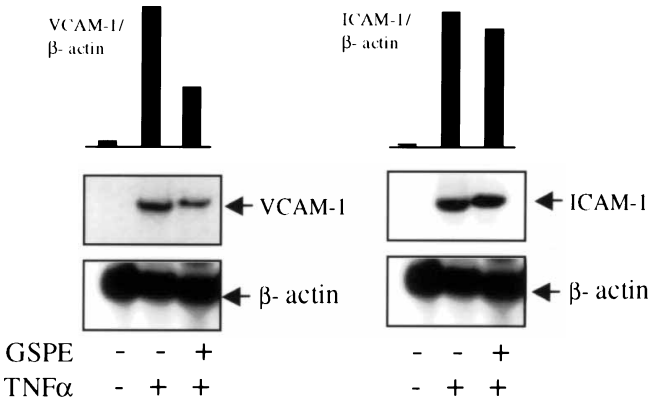

Fig. 4. Differential regulation of TNFα-induced VCAM-1 and ICAM-1 mRNA expression by GSPE in human primary umbilical vein endothelial cells (HUVEC). HUVEC were pretreated with GSPE (2 μg/ml) for 24 h and then activated with TNFα for 6 h. Lane 1, control (0.1% v/v DMSO); lane 2, 10 ng/ml TNFα, 6 h; lane 3, 2 μg/ml GSPE 24 h + 10 ng/ml TNFα 6 h. The levels of mRNA were determined by Northern blot analysis using VCAM-1 or ICAM-1 (top) or β-actin probes (bottom). The bars (densitometry) are expressed as arbitrary units for VCAM-1 or ICAM-1 mRNA expression and normalized to the respective amount of β-actin mRNA using NIH Image 1.58b29 software. Results shown are representative of at least 3 independent experiments.

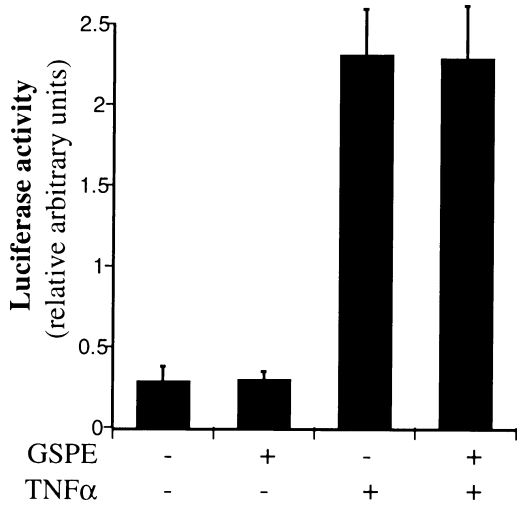

Fig. 5. Effect of GSPE on TNFα-induced NF-κB driven transactivation of transiently transfected luciferase gene in human primary umbilical vein endothelial cells (HUVEC). HUVEC transiently transfected with NF-κB consensus sequence linked to a reporter (luciferase) gene. Following 24 h of transfection, the cells were pretreated with GSPE (2 μg/ml) for 24 h and then activated with TNFα (10 ng/ml) for 6 h. Data are mean ± S.D. of 3 independent experiments.

0, IdB 1056) inhibited acetylcholine and sodium nitroprusside-induced adherence of leukocytes to the venular endothelium in diabetic hamsters [35]. The flavonoid 2-(3-amino-phenyl)-8-methoxy-chromene-4-one (PD 098063) selectively blocks TNFα-induced VCAM-1 expression in endothelial cells in a concentration-dependent manner but had no effect on ICAM-1 expression. This selective inhibition of agonist-induced VCAM-1 protein and gene expression by PD 098063 were also through NF-κB-independent mechanism(s) [30].

Although several studies have postulated NF-κB as the molecular site where redox active substances act to regulate agonist-induced ICAM-1 and VCAM-1 gene expression, inhibition of inducible VCAM-1 gene expression by GSPE was not through NF-κB-dependent pathway. In many cases, the mechanisms that mediate the suppressive effect of flavonoids on cell adhesion have been reported to be independent of the activation of NF-κB [30, 31, 34]. Recently, it has been observed that the flavonoid quercetin significantly and dose dependently down-regulated agonist-induced adhesion molecule expression via AP-1/ c-Jun NH2-terminal kinase (JNK) pathway and not through NF-κB activation pathway [31].

In summary, low concentrations of GSPE significantly inhibited TNFα-induced adhesion of Jurkat cells to human endothelial cells by inhibiting inducible VCAM-1 expression. GSPE did not affect TNFα-induced ICAM-1 expression in endothelial cells. The regulation of VCAM-1 expression by GSPE was at the transcriptional or post-transcriptional level. Furthermore, the inhibitory effect of GSPE on VCAM-1 mRNA was not through NF-κB-dependent mechanisms. Further studies are needed to elucidate the molecular mechanisms underlying the differential regulation of inducible VCAM-1 and ICAM-1 gene by GSPE.

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