Am J Physiol Heart Circ Physiol 293: H2344–H2354, 2007.
First published June 22, 2007; doi:10.1152/ajpheart.00432.2007.

Homocysteine induces VCAM-1 gene expression through NF-κB and NAD(P)H oxidase activation: protective role of Mediterranean diet polyphenolic antioxidants

Maria Annunziata Carluccio,[1] Maria Assunta Ancora,[1] Marika Massaro,[1] Marisa Carluccio,[1] Egeria Scoditti,[1] Alessandro Distante,[1] Carlo Storelli,[2] and Raffaele De Caterina[3]

[1]*Consiglio Nazionale delle Ricerca Institute of Clinical Physiology and* [2]*Department of Biology, University of Lecce, Lecce; and* [3]*Institute of Cardiology, "G. d'Annunzio" University, Chieti, Italy*

Submitted 7 April 2007; accepted in final form 18 June 2007

Carluccio MA, Ancora MA, Massaro M, Carluccio M, Scoditti E, Distante A, Storelli C, De Caterina R. Homocysteine induces VCAM-1 gene expression through NF-κB and NAD(P)H oxidase activation: protective role of Mediterranean diet polyphenolic antioxidants. *Am J Physiol Heart Circ Physiol* 293: H2344–H2354, 2007. First published June 22, 2007; doi:10.1152/ajpheart.00432.2007.—Hyperhomocysteinemia is a recognized risk factor for vascular disease, but pathogenetic mechanisms involved in its vascular actions are largely unknown. Because VCAM-1 expression is crucial in monocyte adhesion and early atherogenesis, we evaluated the NF-κB-related induction of VCAM-1 by homocysteine (Hcy) and the possible inhibitory effect of dietary polyphenolic antioxidants, such as *trans*-resveratrol (RSV) and hydroxytyrosol (HT), which are known inhibitors of NF-κB-mediated VCAM-1 induction. In human umbilical vein endothelial cells (HUVEC), Hcy, at 100 μmol/l, but not cysteine, induced VCAM-1 expression at the protein and mRNA levels, as shown by enzyme immunoassay and Northern analysis, respectively. Transfection studies with deletional VCAM-1 promoter constructs demonstrated that the two tandem NF-κB motifs in the VCAM-1 promoter are necessary for Hcy-induced VCAM-1 gene expression. Hcy-induced NF-κB activation was confirmed by EMSA, as shown by the nuclear translocation of its p65 (RelA) subunit and the degradation of the inhibitors IκB-α and IκB-β by Western analysis. Hcy also increased intracellular reactive oxygen species by NAD(P)H oxidase activation, as shown by the membrane translocation of its $p47^{phox}$ subunit. NF-κB inhibitors decreased Hcy-induced intracellular reactive oxygen species and VCAM-1 expression. Finally, we found that nutritionally relevant concentrations of RSV and HT, but not folate and vitamin B6, reduce (by >60% at 10^{-6} mol/l) Hcy-induced VCAM-1 expression and monocytoid cell adhesion to the endothelium. These data indicate that pathophysiologically relevant Hcy concentrations induce VCAM-1 expression through a prooxidant mechanism involving NF-κB. Natural Mediterranean diet antioxidants can inhibit such activation, suggesting their possible therapeutic role in Hcy-induced vascular damage.

atherosclerosis; endothelial activation; homocysteine; gene expression; inflammation; nuclear factor-κB

HOMOCYSTEINE (Hcy) is a sulfur-containing amino acid synthesized during the metabolic conversion of methionine to cysteine (Cys) (41, 50). Despite the physiological role of Hcy in methyl-transfer reactions (68), elevated plasma levels of Hcy (>15 μmol/l) lead to endothelial damage in humans (24) and are related to increased cardiovascular risk (2). Hyperhomocysteinemia (HHcy) occurs in ~5–7% of the general population and is considered an independent and graded risk factor for atherothrombotic vascular disease (67). Although intervention trials with folate and other B vitamins have led to inconsistent effects in mild HHcy (7, 35, 56), still few would dispute that moderate-to-severe HHcy plays a causal role in human vascular disease. A direct causal relationship between HHcy and accelerated atherosclerosis has been shown in apolipoprotein E-deficient mice, where HHcy can be induced by dietary (29, 70) or genetic (65) manipulations. Moreover, recent studies have shown increased binding of monocytes to the endothelium and increased expression of adhesion molecules in rats with diet-induced HHcy (64). However, the basic mechanisms through which Hcy affects such intermediate pathogenetic events in early atherogenesis and the identification of inhibitable targets in such a cascade are largely unknown.

Local leukocyte recruitment into the vessel wall is the earliest basic step in atherogenesis (47) and is largely explained by the induction of endothelial activation, featuring the increased expression of endothelial leukocyte adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin (36). These endothelial adhesion molecules mediate the adhesion and transmigration of leukocytes to the vascular endothelial wall and may also promote plaque growth and instability. VCAM-1, in particular, plays a dominant role in the development and progression of atherosclerotic lesions (18). Its expression is regulated by prooxidant mechanisms through the activation of redox-sensitive transcription factors, such as NF-κB and activator protein-1 (AP-1) (15).

NF-κB, in particular, plays an important role in increasing the expression of endothelial proinflammatory genes (10, 14, 23, 26). The VCAM-1 promoter region contains two tandem NF-κB sites that are essential for VCAM-1 induction by several proatherogenic triggers (6, 16, 42, 46). No data on the involvement of NF-κB in Hcy-induced VCAM-1 expression are available. NF-κB activation has been shown in vascular cells (3, 29), but no link with endothelial VCAM-1 induction was demonstrated.

We therefore sought to investigate *1*) the possible direct effects of Hcy on VCAM-1 expression and NF-κB activation by testing the cause-effect relationship between the two via analysis of Hcy effects on various deleted VCAM-1 promoter constructs in promoter activity assays and *2*) the effects of NF-κB inhibitors on VCAM-1 induction by Hcy.

Address for reprint requests and other correspondence: R. De Caterina, Institute of Cardiology, "G. d'Annunzio" Univ.-Chieti, c/o Ospedale S. Camillo de Lellis, Via Forlanini, 50, I-66100 Chieti, Italy.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Hcy is also known to induce oxidative stress by increasing reactive oxygen species (ROS) levels by autoxidation (53) and by interfering with the activity and expression of pro- and antioxidant enzymes (27, 59). Since increased intracellular ROS levels are known to activate NF-κB (8, 16), yielding a plausible putative mechanism for adhesion molecule expression, we also investigated the effects on these phenomena of various dietary polyphenolic antioxidants that were recently reported by us and others to reduce cytokine-induced NF-κB activation (12, 17). If proven effective in this model, such findings would support the possibility that specific dietary interventions modify cardiovascular risk associated with Hcy, which was recently reported to be unaffected by the administration of folate and vitamins B6 and B12 (7, 35, 56).

METHODS

Materials. Hydroxytyrosol (HT) was obtained from Cayman Chemical (Ann Arbor, MI), and carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-H_2DCFDA) was purchased from Molecular Probes (Eugene, OR). When not otherwise specified, all other reagents, including DL-Hcy (99% pure), L-cysteine (Cys), and *trans*-resveratrol (RSV), were obtained from Sigma Chemical (St. Louis, MO).

Cell cultures. Human umbilical vein endothelial cells (HUVEC), a widely used model of vascular endothelium, were harvested and maintained as described elsewhere (11, 12) and used at *passages 1–3* after primary culture. Cell number was assessed by direct counting of adherent cells after trypsin detachment. The cells were stained with trypan blue, and the percentage of cells excluding trypan blue was taken as a measure of cell viability. Monocytoid U937 cells were obtained from American Tissue Culture Collection and grown in RPMI 1640 (GIBCO, Invitrogen, Milan, Italy) containing 10% FCS. At confluence, HUVEC were switched from 10% FCS to 5% FCS ≥5 h before addition of the designated compounds, and the effect of antioxidants or inhibitors was tested. Hcy-induced cellular toxicity (with or without stimuli) was tested for concentrations up to 5 mmol/l by a variety of techniques, including cell counting, morphology, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay, and binding of annexin V-fluorescein isothiocyanate, with detection of the exposure of phosphatidylserine in the outer membrane leaflet.

Detection of cell surface molecules. HUVEC at confluence were treated for specified times with Hcy alone (or Cys, as control) and in combination with selected cytokines with or without preincubation with antioxidants, folate, or vitamin B6. Assays of cell surface molecules were carried out by cell surface enzyme immunoassays (EIA) using primary mouse anti-human monoclonal antibodies against VCAM-1 (Ab E1/6), E-selectin (Ab H18/7), ICAM-1 (HU5/3), or the monoclonal antibody E1/1 against a non-cytokine-inducible and constitutive endothelial cell antigen, as previously described (11).

Isolation of RNA and Northern analysis. RNA was isolated using the method of Chomczynski and Sacchi (13), and Northern analysis was performed as described elsewhere (11).

Transfections and promoter activity assays. Human VCAM-1 promoter constructs containing the chloramphenicol acetyltransferase (CAT) reporter gene were described previously (45). HUVEC were transfected with 10 μg of each reporter plasmid using the GenePORTER II transfection reagent (Gene Therapy Systems, San Diego, CA) according to the supplier's manual. As an internal control for transfection efficiency, 2.5 μg of pRSV β-galactosidase (β-Gal) plasmid was cotransfected in all experiments. HUVEC transfected at ~60% confluence were stimulated 48 h later with Hcy (100 μmol/l) ± TNF-α (1 ng/ml). After 15 h, cellular extracts were prepared, and CAT and β-Gal were assayed as described elsewhere (20).

Preparation of nuclear extracts and EMSA. Confluent HUVEC were treated for 1 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml), and nuclear proteins were prepared as described elsewhere (12). Oligonucleotides corresponding to the binding sequences for NF-κB or AP-1 in the VCAM-1 promoter were radiolabeled by a Klenow fill-in reaction, as described elsewhere (12). The DNA binding reaction was performed at 30°C for 15 min in a volume of 20 μl containing 6 μg of nuclear extracts (20). Samples were subjected to electrophoresis on native 5% polyacrylamide gels. We determined the specificity of the assay by including a 50- to 100-fold excess of unlabeled competing wild-type or mutant sequences in the binding mixture. After electrophoresis, gels were dried and directly autoradiographed using Kodak X-AR film.

Total protein extracts. HUVEC were cultured and treated in six-well plates, washed twice with PBS, and lysed in 150 μl of lysis buffer: 150 mmol/l HEPES (pH 7.9), 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, 10% glycerol, 1 mmol/l DTT, 1 mmol/l PMSF, 1 mg/ml aprotinin, and 1 mg/ml leupeptin. Lysates were vortexed three times for 10 s each and incubated on ice for 30 min; then they were centrifuged at 10,000 *g* for 20 min at 4°C. After determination of protein concentration (9), supernatants were stored at −20°C.

Assessment of $p47^{phox}$ translocation. After several 0- to 60-min exposures to Hcy, HUVEC monolayers were washed three times in cold PBS and harvested by scraping. Cells were centrifuged, resuspended in extraction buffer [7.5 mmol/l Tris·HCl (pH 7.5), 2 mmol/l EGTA, 2 mmol/l EDTA, 0.25 mol/l sucrose, 1 mmol/l DTT, 1 mmol/l PMSF, 1 mg/ml aprotinin, and 1 mg/ml leupeptin], and disrupted by sonication on ice with three 15-s bursts. Sonicates were centrifuged at 500 *g* for 10 min, the nuclei-rich pellet was discarded, and the supernatant fluid was centrifuged at 100,000 *g* for 1 h at 4°C. The supernatant fluid (cytosolic fraction) was removed and stored at −20°C, while the pellet, containing the particulate fraction, was resuspended in extraction buffer containing 1% Triton X-100 and centrifuged at 100,000 *g* for 1 h. The resultant supernatant fluid (now membrane-enriched fraction) was removed and stored at −20°C. The plasma membrane and cytosolic fractions were used to detect specific subunit translocation after protein separation by SDS-PAGE and subsequent immunoblotting.

Immunoblotting. Equal amounts of proteins were separated by SDS-PAGE. The resolved proteins were transferred to supported nitrocellulose sheets (Amersham Biosciences, Milan, Italy) and, after saturation of nonspecific binding sites, incubated overnight with specific mono- and polyclonal antibodies against the p65 NF-κB subunit, IκB-α or IκB-β, or $p47^{phox}$ (all from Santa Cruz Biotechnology). Protein bands were visualized by an enhanced chemiluminescence kit (Amersham). Blots were scanned, and digitized images were subjected to densitometry analysis using the ScionImage software.

Quantitative detection of intracellular ROS. Intracellular levels of ROS were monitored quantitatively by flow cytometry using the ROS probe the carboxy derivative of fluorescein, carboxy-H_2DCFDA, as described elsewhere (40, 48). This method is based on the oxidation of carboxy-H_2DCFDA by ROS, resulting in the formation of a fluorescent compound. Confluent HUVEC in six-well plates were preincubated with inhibitors or antioxidants for 60 min before 45 min of stimulation with Hcy (100 μmol/l) ± TNF-α (1 ng/ml) in DMEM without phenol red with 2% FBS. After stimulation, the medium was removed and the cells were washed with PBS and loaded with 10 μmol/l carboxy-H_2DCFDA in PBS for 45 min. Then the cells were washed with PBS, harvested by careful treatment with 0.01% trypsin-EDTA, immediately added to 2 ml of DMEM without phenol red, supplemented with 2% FBS, and placed on ice. Samples were washed twice with PBS containing 2% BSA, and 10^4 cells per sample were analyzed by flow cytometry (Coulter Electronics Epics XL, Hialeah, FL).

TNF-α measurement. HUVEC in six-well plates at confluence and U937 cells (10^{-6} cells/ml) were treated with 0–500 μmol/l Hcy for 24 h, and the conditioned medium was collected for storage at −80°C until the assay, which consisted of an immunometric sandwich EIA permitting TNF-α measurements within the range 1–250 pg/ml, with a detection limit of 1 pg/ml (Cayman Chemical).

Monocytoid cell adhesion assays. HUVEC were grown to confluence in six-well plates and then treated with the selected antioxidant polyphenols HT and RSV (0.1–100 μmol/l) or their combination for 60 min or with folic acid and vitamin B6 (0.1–100 μmol/l) or their combination; then endothelial adhesion molecule expression was induced by addition of Hcy (100 μmol/l) ± TNF-α (1 ng/ml) for an additional 16 h. Adhesion assays were performed with U937 monocytoid cells as described elsewhere (12).

Statistical analysis. Two-group comparisons were performed by Student's *t*-test for unpaired values. Comparisons of means of three or more groups were performed by ANOVA, and the existence of individual differences, in the case of significant *F*-values at ANOVA, was tested by Scheffé's multiple contrasts. Distribution of fluorescence intensities by flow cytometry was compared using Kolmogorov-Smirnov statistics.

RESULTS

Hcy induces adhesion molecule expression in HUVEC and potentates effects of other inducers of endothelial activation. We treated HUVEC with 10–500 μmol/l Hcy, corresponding mild-to-severe HHcy (28). At these concentrations, Hcy ± TNF-α was not cytotoxic throughout the experimental durations used in the present study, as assessed by morphological analysis, cell number, viability, MTT assay, lactate dehydrogenase release, and annexin V staining (data not shown). Cytotoxicity appeared at ≥1 mmol/l Hcy. Within this concentration range, Hcy, but not Cys, another sulfur-containing amino acid, significantly increased VCAM-1 expression, with a plateau at ≥100 μmol/l (Fig. 1*A*). This effect was relatively modest compared with the effect of TNF-α (Fig. 1*C*). The effect was identical in the presence of polymixin B, thus excluding its dependence on endotoxin contamination (data not shown).

Since Hcy is known to increase plasma levels of TNF-α (29), we investigated whether Hcy induces VCAM-1 expression indirectly by stimulating the endothelial release of TNF-α. Exposure of HUVEC to 10–500 μmol/l Hcy for 24 h did not induce any appreciable TNF-α release (which was always below EIA detection limits), ruling out an effect dependent on the autocrine production of TNF-α. In the same conditions, treatment of U937 monocytoid cells with 100 and 500 μmol/l Hcy induced TNF-α release of 20 and 70 pg/ml, respectively, after 24 h, indicating monocytes-macrophages as a likely source of TNF-α in vivo in HHcy. For this reason, we assessed

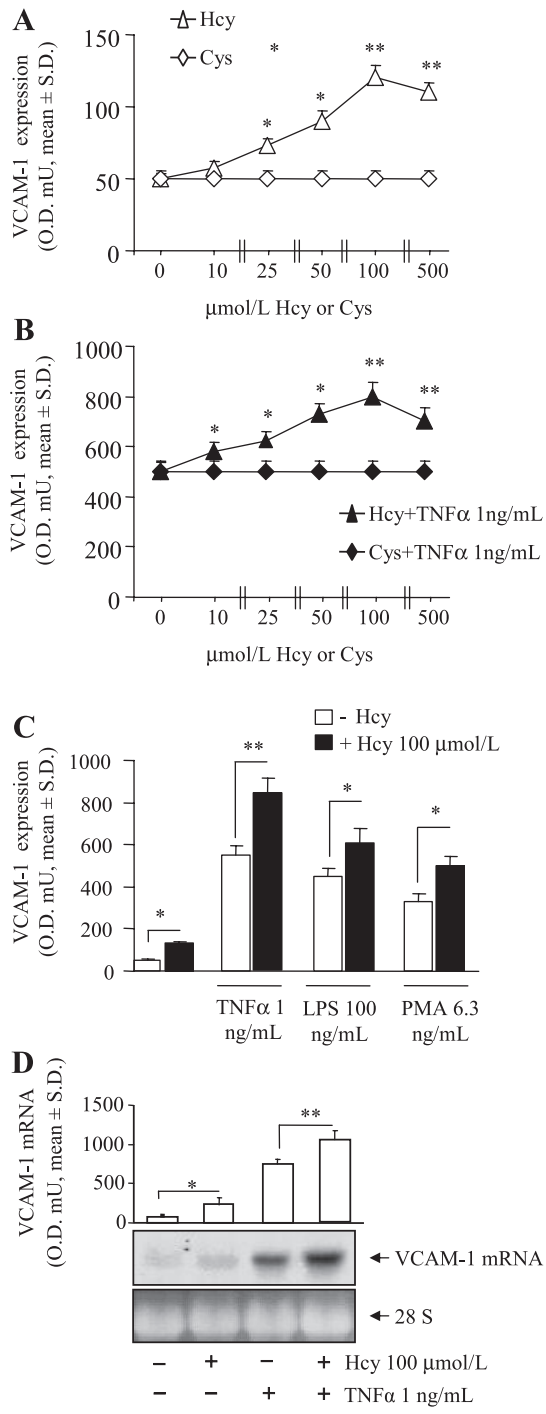

Fig. 1. Effect of homocysteine (Hcy) on VCAM-1 protein and mRNA expression in endothelial cells. *A*: Hcy, but not cysteine (Cys), induces VCAM-1 surface expression. Human umbilical vein endothelial cells (HUVEC) at confluence were stimulated for 20 h with Hcy (0–500 μmol/l) or Cys, and VCAM-1 expression was assessed by cell surface enzyme immunoassay (EIA). Hcy alone induces a moderate, but significant, concentration-dependent increase in VCAM-1 surface expression, reaching a plateau at 100 μmol/l, whereas Cys had no effect. *B*: Hcy, but not Cys, potentiates TNF-α-induced VCAM-1 surface expression. HUVEC were stimulated for 20 h with Hcy ± TNF-α (1 ng/ml). Costimulation resulted in a more-than-additive and concentration-dependent effect on VCAM-1 surface expression. OD, optical density. Data in *A* and *B* are based on 6 different experiments, each consisting of ≥8 repeats for each condition. $*P < 0.05$; $**P < 0.01$ vs. non-Hcy-stimulated cells. *C*: Hcy potentates VCAM-1 surface expression induced by unrelated stimuli such as TNF-α, LPS, or PMA. HUVEC at confluence were treated for 20 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml), LPS (100 ng/ml), or PMA (6.3 ng/ml), and VCAM-1 expression was measured by EIA. All stimuli affected VCAM-1 surface expression, but the effect was greater with TNF-α. Data are based on 4 different experiments, each consisting of ≥8 repeats for each condition. $*P < 0.05$; $**P < 0.01$ vs. LPS, TNF-α, or PMA alone. *D*: Hcy ± TNF-α increases VCAM-1 mRNA steady-state levels. HUVEC at confluence were stimulated for 4 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml), and VCAM-1 mRNA was assessed by Northern analysis. Hcy significantly increased VCAM-1 mRNA steady-state levels. As a control for equal loading of lanes, 28S rRNA was stained with ethidium bromide. Quantitative analysis by densitometry of specific bands shows results from 3 independent experiments.

Table 1. *Effects of Hcy ± TNF-α on surface expression of E-selectin, ICAM-1, and E1/1 in HUVEC*

Stimuli	E-Selectin	ICAM-1	E 1/1
None	30±2	400±43	450±40
Hcy			
10 μmol/l	42±5	410±30	430±30
25 μmol/l	58±5*	420±30	435±30
50 μmol/l	70±8*	440±45	450±35
100 μmol/l	74±5*	450±20	440±20
500 μmol/l	81±7*	430±40	430±20
TNF-α (1 ng/ml)	300±24	800±50	450±50
TNF-α + Hcy (10 μmol/l)	320±31	860±40	450±35
TNF-α + Hcy (25 μmol/l)	370±30	868±40	460±40
TNF-α + Hcy (50 μmol/l)	400±35†	900±38	470±39
TNF-α + Hcy (100 μmol/l)	470±29†	1,103±54†	450±30
TNF-α + Hcy (500 μmol/l)	450±30†	1,095±61†	460±30

Values are means ± SD based on 4 different experiments, each based on 8 repeats for each condition and expressed as milliunits of optical density. Human umbilical vein endothelial cells (HUVEC) at confluence were stimulated for 20 h with Hcy (10–500 μmol/l) ± TNF-α (1 ng/ml), and E-selectin, ICAM-1, and E1/1 expression was assessed by enzyme immunoassay using specific monoclonal antibodies. *$P < 0.05$ vs. without Hcy. †$P < 0.05$ vs. TNF-α alone.

the combined effect of Hcy and TNF-α on VCAM-1 expression in HUVEC. We found that the combination of Hcy and a low concentration of TNF-α (1 ng/ml) boosted VCAM-1 expression in a concentration-dependent fashion, at least additively with TNF-α, with a significant increase at 10 μmol/l Hcy (Fig. 1*B*).

Similarly, the simultaneous stimulation of HUVEC with the combination of 100 μmol/l Hcy and low concentrations of other classical proinflammatory stimuli, such as LPS (100 ng/ml) or PMA (6.3 ng/ml), increased VCAM-1 expression in a more-than-additive fashion (Fig. 1*C*). The optimal concentrations for a robust induction of adhesion molecules and a substantial costimulation with Hcy are 1 ng/ml (among 0.1, 1, and 10 ng/ml) TNF-α, 100 ng/ml (among 10, 100, and 1,000 ng/ml) LPS, and 6.3 ng/ml (among 0.63, 6.3, and 63 ng/ml) PMA. The maximum induction of VCAM-1 was obtained with TNF-α cotreatment, resulting in >150% VCAM-1 induction compared with TNF-α alone.

Hcy, alone or in addition to TNF-α, also induced expression of E-selectin, with a plateau at 50 μmol/l Hcy (Table 1). Hcy alone did not affect basal ICAM-1 expression but significantly increased TNF-α-induced ICAM-1 expression (Table 1). The effect of Hcy on endothelial activation was not, however, an overall increase in expression of all endothelial antigens, since it did not affect expression of the constitutive antigen recognized by the E1/1 antibody (Table 1).

Hcy increases VCAM-1 mRNA steady-state levels. Because of the dominant role of VCAM-1 in early atherogenesis (18), we next focused our attention on the regulation of VCAM-1 expression by Hcy. We investigated Hcy effects on VCAM-1 mRNA steady-state levels. Densitometric analysis of autoradiographic bands by Northern blot showed an induction of VCAM-1 mRNA on incubation with Hcy (Fig. 1*D*) and a potentiation of TNF-α induction, in agreement with data on surface protein expression, indicating that VCAM-1 induction by Hcy occurs at a pretranslational level.

Hcy increases human VCAM-1 promoter transcriptional activity. Since VCAM-1 is transcriptionally regulated, we sought to determine whether Hcy regulates VCAM-1 promoter transcriptional activity and to identify the promoter regions involved. HUVEC were transiently transfected with several deletional VCAM-1 promoter constructs linked to the CAT reporter gene (Fig. 2*A*) and then stimulated with Hcy (100 μmol/l) ± TNF-α (1 ng/ml). Hcy induced the activity of F0.CAT, the full-length functional VCAM-1 promoter containing AP-1, GATA, and two tandem NF-κB sites, by ~2-fold and the activity of F3.CAT, which contains only the two tandem NF-κB binding sites, by 1.8-fold compared with unstimulated cells, but did not influence the activity of F4.CAT, which lacks also the two tandem NF-κB binding sites as well (Fig. 2*B*), indicating the essential role of the two tandem NF-κB sites in Hcy-induced VCAM-1 expression. Similar results were obtained using Hcy + TNF-α. In such experiments, the F3.CAT construct preserved ~80% of F0.CAT activity. These results indicate that activation of VCAM-1

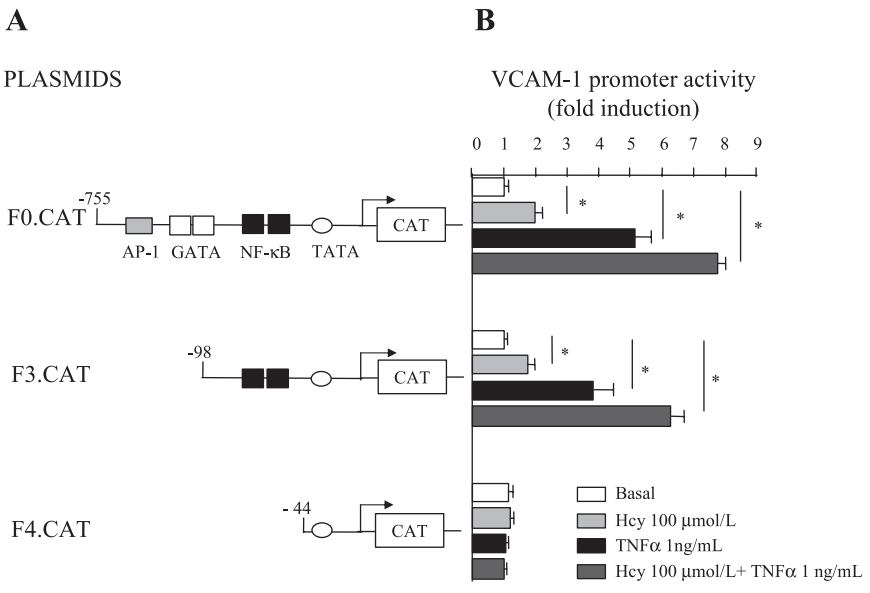

Fig. 2. Effects of Hcy on VCAM-1 promoter activity. *A*: structure of plasmids that contain deletional constructs of VCAM-1 promoter upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. Binding sites for transcription factors and distance from transcription starting site are shown. *B*: plots of VCAM-1 promoter activity with promoter constructs after 20 h of stimulation with Hcy (100 μmol/l) ± TNF-α (1 ng/ml). Promoter activity is expressed as ratio of CAT to β-galactosidase (β-Gal). Values are means ± SD of 3 replicates for each condition. *$P < 0.01$ vs. unstimulated control (basal).

expression by Hcy occurs at the transcriptional level and is mainly due to the two NF-κB DNA binding elements in the VCAM-1 promoter.

Hcy activates the redox-sensitive transcription factor NF-κB but not AP-1 through a proteasome-dependent pathway. Since promoter activity studies showed a key role for NF-κB in Hcy-induced VCAM-1 expression, we next analyzed the mechanisms of NF-κB activation by Hcy. In endothelial cells, NF-κB consists of heterodimeric proteins, the most prominent being p50 (NF-κB1) and p65 (RelA), sequestered in the cytoplasm in resting conditions through the association with inhibitory molecules (IκB). When cells are exposed to proinflammatory cytokines and other pertinent stimuli, IκB is phosphorylated, ubiquitinated, and then degraded by the 26S proteasome complex, which allows the heterodimeric active moiety to pass through the nuclear envelope and bind to consensus sequences in the promoter region of NF-κB-sensitive genes (4, 57). To verify the involvement of the classical NF-κB signaling pathway in VCAM-1 induction by Hcy, we treated HUVEC with Hcy ± TNF-α in the presence of the well-known proteasome inhibitor lactacystin (1 and 10 μmol/l) (32) and then assessed VCAM-1 expression by EIA. As shown in Fig. 3*A*, the VCAM-1-inducing effect of Hcy was almost totally abolished in the presence of lactacystin, without cellular toxicity (not shown), which indicates the importance of the classical NF-κB/IκB pathway in this induction.

To directly assess the effects of Hcy on NF-κB activation, we used an oligonucleotide corresponding to the tandem κB sites on the VCAM-1 promoter to perform EMSA. We found that 100 μmol/l Hcy induced NF-κB activation, as indicated by the increased intensity of the shifted band of the protein-DNA complex corresponding to NF-κB in Hcy-treated HUVEC (Fig. 3*B, lane 2* vs. *lane 1*), and markedly increased the NF-κB activation induced by TNF-α (Fig. 3*B, lane 4* vs. *lane 3*). Correspondingly, immunoblots for RelA, performed on nuclear extracts, confirmed that Hcy ± TNF-α increased the nuclear translocation of RelA (Fig. 3*C*), which was reduced by lactacystin treatment (not shown).

To further examine the mechanisms by which Hcy induces NF-κB activation, we investigated whether Hcy affects the phosphorylation and the subsequent degradation of the two IκB subunits (α and β) most commonly found in HUVEC (57). We observed that Hcy, in addition to the rapid degradation of IκB-α, which is maximal after 15 min, and its resynthesis after 60 min (Fig. 3*D*) induce a retarded, but prolonged, IκB-β degradation, which is maximal after 60 min (Fig. 3*D*) and persists for up to 120 min (not shown), suggesting a possible mechanism for the persistent NF-κB activation in Hcy-challenged endothelial cells (31).

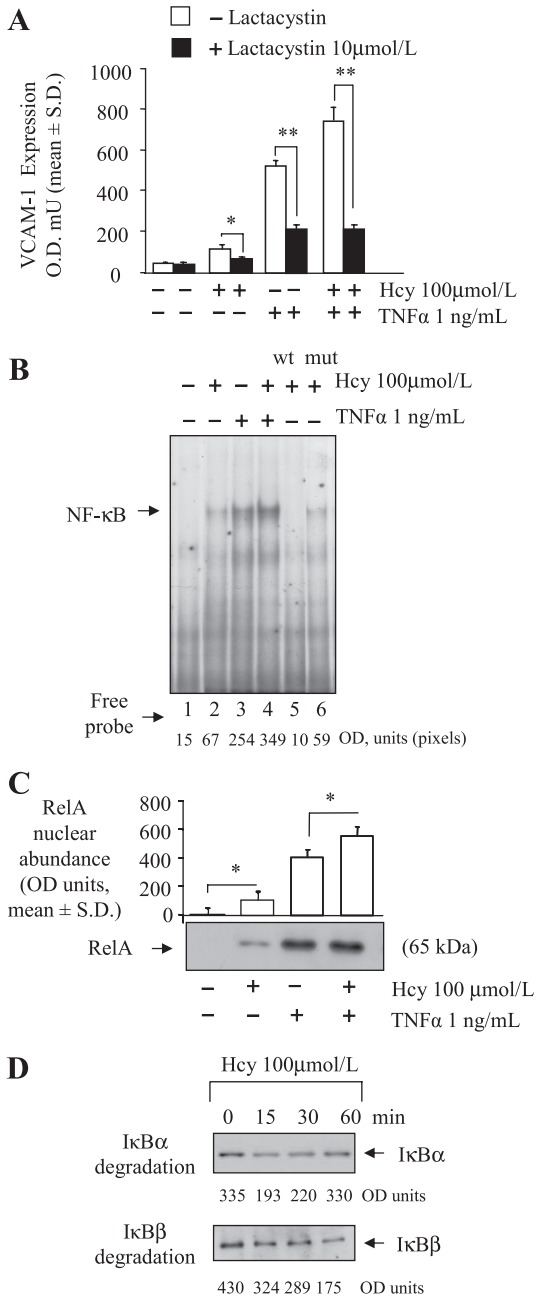

Fig. 3. Effects of Hcy on NF-κB activation in endothelial cells. *A*: the proteasome inhibitor lactacystin reduces Hcy-stimulated surface expression of VCAM-1 in HUVEC. HUVEC were treated for 60 min with lactacystin (10 μmol/l) and then stimulated for 20 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml). After incubations, VCAM-1 expression was assessed by cell surface EIA. Values are means ± SD for 8 replicates in each of 3 separate experiments. $*P < 0.05$; $**P < 0.01$ vs. control (without lactacystin). *B*: results from EMSA showing Hcy-induced increase in NF-κB activation. Nuclear proteins were isolated from HUVEC treated for 1 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml), and EMSA was performed to determine NF-κB DNA binding. Autoradiography shows bands of ^{32}P-labeled NF-κB-binding oligonucleotides in the VCAM-1 promoter shifted in their electrophoretic run by their binding to nuclear protein extracts. Hcy induced appearance of a shifted band of labeled oligonucleotide, indicating formation of DNA-protein complexes. Attribution of shifted band to NF-κB was determined by competition with unlabeled wild-type (wt) or unlabeled mutant (mut) NF-κB oligonucleotide (*lanes 5* and *6*, respectively), whereby only unlabeled wild-type oligonucleotide effectively competes with binding, leading to the disappearance of the band. Results are representative of 3 separate experiments. *C*: Hcy induces nuclear translocation of p65/RelA subunit of NF-κB. RelA/NF-κB nuclear abundance in nuclear extracts of HUVEC treated with Hcy (100 μmol/l) ± TNF-α (1 ng/ml) for 1 h was evaluated by Western analysis. Blot is representative of 3 separate experiments. *D*: Hcy affects IκB-α and IκB-β degradation in HUVEC. HUVEC were treated with Hcy (100 μmol/l) for 0–60 min, and total protein extracts were separated by electrophoresis, transferred to a nitrocellulose membrane, and probed with antibodies for total IκB-α and IκB-β. Blots are representative of 3 different experiments.

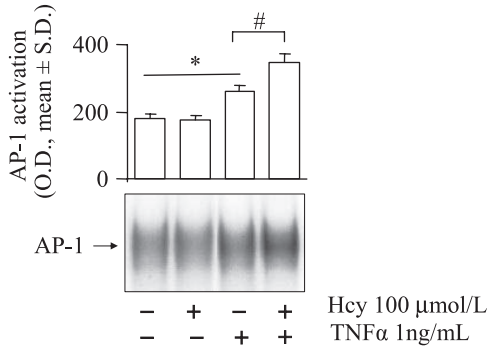

Fig. 4. Effects of Hcy on activator protein-1 (AP-1) activation. EMSA (gel shift) shows bands of ^{32}P-labeled oligonucleotides encoding for AP-1 consensus sequence in the VCAM-1 promoter shifted by coincubation with nuclear protein extracts from HUVEC treated for 1 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml). Hcy alone does not influence baseline AP-1 activation but does increase density of shifted band induced by TNF-α. Values are based on 3 different experiments. *$P < 0.05$; #$P < 0.05$ vs. unstimulated control and TNF-α stimulation, respectively.

Hcy induces NF-κB, but not other transcription factors. For example, AP-1 (Fig. 4), which is activated by TNF-α + Hcy, was not activated by Hcy alone.

Hcy induces VCAM-1 expression through an increase of intracellular ROS: involvement of NAD(P)H oxidase. It is known that the NF-κB/IκB pathway is sensitive to oxidative stress (34) and that Hcy induces oxidative stress in various systems (3, 59). To test whether Hcy treatment affects intracellular ROS generation and to characterize the main enzymatic sources of ROS involved in Hcy-induced VCAM-1 induction, HUVEC were first pretreated with the NAD(P)H oxidase inhibitor diphenyliodonium (DPI, 1 and 10 μmol/l), the xanthine oxidase inhibitor allopurinol (1 and 10 μmol/l), or the mitochondrial NADH dehydrogenase inhibitor rotenone (1 and 10 μmol/l) for 30 min before stimulation with Hcy (100 μmol/l) ± TNF-α (1 ng/ml) for 50 min, and then ROS levels where assessed with the redox-sensitive fluorescent probe carboxy-H_2DCFDA. We observed that only DPI reduced Hcy-induced ROS formation (Fig. 5*A*), whereas the other inhibitors had no effect (not shown). Correspondingly, only DPI reduced Hcy-induced VCAM-1 expression (Fig. 5*B*), suggesting the causal link between stimulation of NAD(P)H oxidase activity and VCAM-1 expression by Hcy.

Activation of endothelial NAD(P)H oxidase requires the assembly of cytosolic subunits (p47^{phox} and p67^{phox} proteins) with the membrane-integrated *b*-type cytochrome, cytochrome *b*-558 (consisting of p22^{phox} and gp91^{phox}) (30). We found that

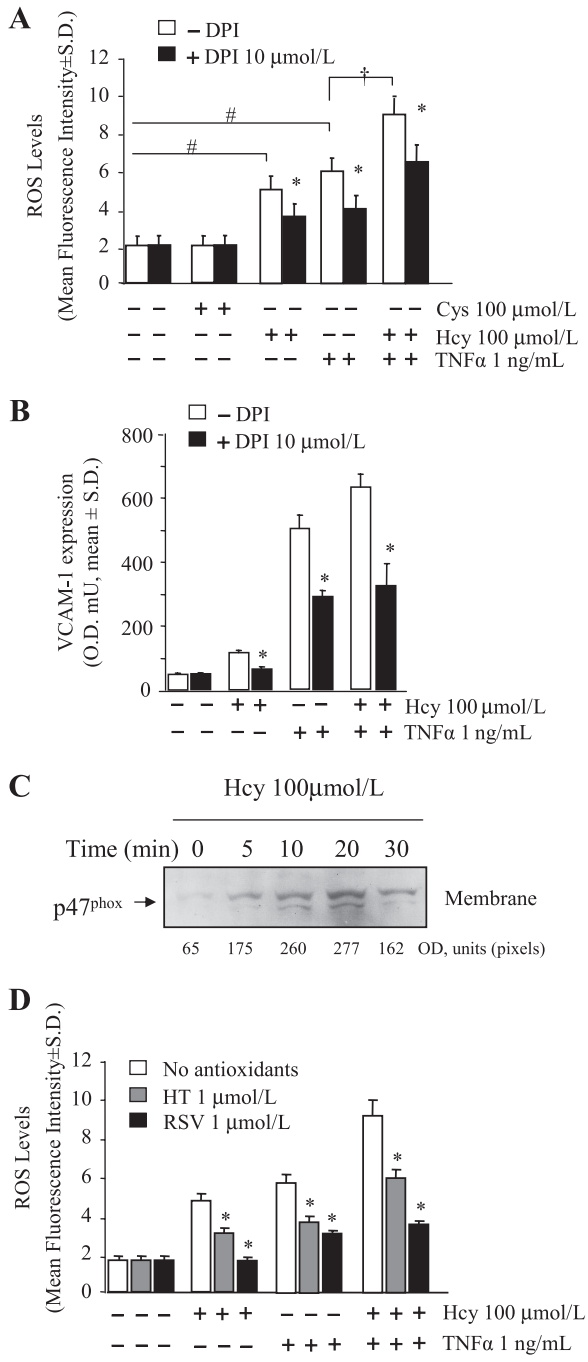

Fig. 5. Effects of Hcy on NAD(P)H oxidase activation, intracellular reactive oxygen species (ROS) production, and VCAM-1 surface expression in HUVEC. *A*: diphenyliodonium (DPI) inhibits the increase in intracellular ROS levels induced by Hcy ± TNF-α. HUVEC were treated for 50 min with Cys (100 μmol/l) or Hcy (100 μmol/l) ± TNF-α (1 ng/ml) and then incubated for 45 min with carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-H_2DCFDA, 10 μmol/l), and intracellular ROS production was monitored. Involvement of NAD(P)H oxidase was tested by addition of the NAD(P)H oxidase inhibitor DPI (10 μmol/l) to monolayers 30 min before treatment with stimuli. Fluorescence was measured by flow cytometry, and results are expressed as fluorescence intensity. Values are means ± SD of 3 independent experiments. *$P < 0.05$ vs. corresponding samples without DPI. †$P < 0.05$ vs. TNF-α (1 ng/ml). #$P < 0.05$ vs. unstimulated control. *B*: DPI reduces VCAM-1 expression induced by Hcy ± TNF-α. HUVEC were treated for 30 min with DPI (10 μmol/l) and then stimulated for 20 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml). VCAM-1 expression was assessed by cell surface EIA. Values are means ± SD, with 8 replicates per condition in each of 3 separate experiments. *$P < 0.05$ vs. corresponding samples without DPI. *C*: effects of Hcy on translocation of NAD(P)H oxidase p47^{phox} subunit to plasma membrane. HUVEC were treated with Hcy (100 μmol/l) for 0–30 min, and plasma membrane fractions were prepared and used for detection of p47^{phox} subunit translocation after protein separation by SDS-PAGE and subsequent immunoblotting. The blot is representative of 3 similar blots. *D*: dietary antioxidants *trans*-resveratrol (RSV) and hydroxytyrosol (HT) reduce Hcy-induced increase in intracellular ROS levels. HUVEC at confluence were pretreated for 60 min with RSV or HT (1 μmol/l) and stimulated with Hcy (100 μmol/l) ± TNF-α (1 ng/ml) for 50 min, and intracellular ROS production was monitored by incubation for 45 min with carboxy-H_2DCFDA (10 μmol/l). Fluorescence was measured by flow cytometry. Values are means ± SD of 3 independent experiments. *$P < 0.05$ vs. control without antioxidants.

Hcy (100 μmol/l) induced translocation of the NAD(P)H oxidase $p47^{phox}$ subunit to the plasma membrane in HUVEC in a time-dependent fashion (Fig. 5*C*), confirming that the Hcy-induced increase of ROS production in endothelial cells occurs, at least in part, through NADPH oxidase activation.

As shown in Fig. 5*D,* the dietary phenolic antioxidants HT and RSV (1 μmol/l) quench the Hcy-induced increase in intracellular oxidative stress in HUVEC, suggesting their potential involvement in reducing ROS-mediated Hcy proinflammatory effects.

Olive oil and red wine phenolic antioxidants affect prooxidant Hcy-induced endothelial activation and monocyte adhesion. Phenolic antioxidants from typical Mediterranean foods, such as RSV from red wine and HT from extra-virgin olive oil, are considered partly responsible for the decreased risk of cardiovascular diseases in Mediterranean countries (58, 62). RSV and HT exhibit antioxidant and anti-inflammatory activities in vitro (17, 25) and in vivo (22, 62). We and others previously showed that RSV and HT, at micromolar concentrations, inhibit endothelial adhesion molecule expression by reducing cytokine-induced NF-κB activation (12, 17). To test whether these compounds can also reduce Hcy-induced endothelial activation, we treated HUVEC for 60 min with 0.1–10 μmol/l RSV or HT before stimulation with Hcy (0–500 μmol/l) for 20 h and then evaluated VCAM-1 expression by cell surface EIA. As shown in Fig. 6*A*, the antioxidant HT reduces Hcy-induced VCAM-1 expression in a concentration-dependent manner, with an almost total abolition of the Hcy effect at 1 μmol/l HT. Similar results were obtained with RVS (not shown). Both polyphenolic antioxidants tested at 1 μmol/l significantly reduced the expression of VCAM-1 induced by Hcy ± TNF-α (Fig. 6*B*), confirming a role for ROS in VCAM-1 induction by Hcy and also indicating that natural antioxidants may counterbalance Hcy prooxidant, proinflammatory effects in endothelial cells. We obtained similar results with other antioxidants such as *N*-acetylcysteine (5 mmol/l) and pyrrolidindithiocarbamate (5 μmol/l), but not with ascorbic acid (5–500 μmol/l), vitamin B6 (100 μmol/l), or folic acid

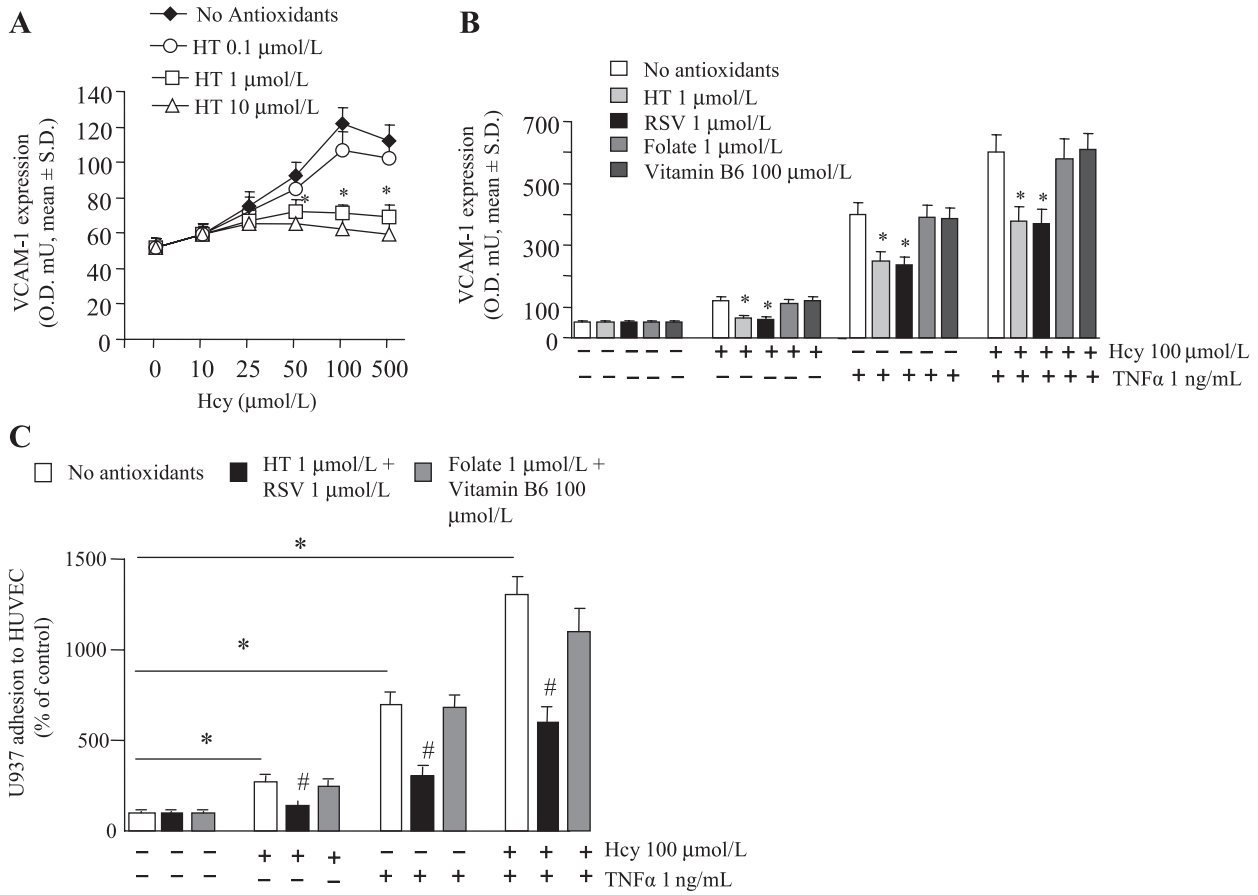

Fig. 6. Effects of phenolic antioxidants, folic acid, and vitamin B6 on Hcy-induced VCAM-1 expression and monocytoid cell adhesion to HUVEC. *A*: dietary antioxidant HT reduces Hcy-induced VCAM-1 expression in a concentration-dependent manner. HUVEC at confluence were pretreated for 60 min with HT (0.1–10 μmol/l) and then stimulated for 20 min with Hcy (0–500 μmol/l), and VCAM-1 expression was assessed by cell surface EIA. Data are based on 2 different experiments, each consisting of ≥8 replicates per condition. *$P < 0.05$ vs. control without antioxidants. *B*: antioxidants RSV and HT, but not folic acid or vitamin B6, reduce Hcy-induced VCAM-1 expression. HUVEC at confluence were pretreated for 60 min with RSV or HT (both at 1 μmol/l) and stimulated for 20 h with Hcy (100 μmol/l) ± TNF-α (1 ng/ml). VCAM-1 expression was assessed by cell surface EIA. Data are based on 3 different experiments, each consisting of ≥8 replicates per condition. *$P < 0.05$ vs. control without antioxidants. *C*: RSV and HT, but not folic acid or vitamin B6, reduce Hcy-induced U937 monocytoid cell adhesion to HUVEC. HUVEC were pretreated simultaneously with RSV and HT (1 μmol/l) or folic acid (1 μmol/l) and vitamin B6 (100 μmol/l) before 20 h of stimulation with Hcy (100 μmol/l) ± TNF-α (1 ng/ml), and U937 cell adhesion to the endothelium was evaluated. Monocyte adhesion was measured as the number of U937 cells adhering within a high-power (0.09-mm^2) field and expressed as percentage of unstimulated control. Values are means ± SD of adhering cells from 3 experiments, each consisting of 8 counts per condition. *$P < 0.05$ vs. unstimulated control. #$P < 0.05$ vs. stimulated conditions without antioxidants.

(1 μmol/l; Fig. 6*B*). Ascorbic acid, vitamin B6, and folic acid were devoid of any effect on adhesion molecule expression, even with Hcy concentrations of 25 and 50 μmol/l (data not shown). Similar to VCAM-1, RSV and HT also inhibited E-selectin expression induced by Hcy (100 μmol/l) ± TNF-α (1 ng/ml) by ~50 ± 5 and 45 ± 4%, respectively, as well as the expression of ICAM-1 induced by Hcy (100 μmol/l) + TNF-α (1 ng/ml) by ~37 ± 4 and 30 ± 3%, respectively, indicating a general quenching of endothelial activation by these phenolic antioxidants, consistent with previous results with other stimuli (12, 17, 22).

Finally, to examine the functional consequences of HUVEC treatment with polyphenolic antioxidants, we evaluated their effect on endothelial-monocyte interactions (adhesion) induced by Hcy by documenting the increased monocytoid cell adhesion after treatment of HUVEC with Hcy ± TNF-α and the inhibition of endothelial adhesive properties with RSV and HT (Fig. 6*C*). Monocytoid cells did not adhere to unstimulated HUVEC but adhered to Hcy-stimulated HUVEC and, to a greater extent, to HUVEC treated with Hcy (100 μmol/l) + TNF-α (1 ng/ml). When HUVEC were pretreated with RSV or HT (1 μmol/l) for 60 min before Hcy stimulation, monocytoid cell adhesion was reduced to a degree comparable with the inhibition of adhesion molecule expression (Fig. 6*C*). The combination of the two polyphenolic antioxidants at micromolar concentration resulted in an increased inhibition of monocyte adhesion to the endothelium, suggesting that concentrations of these polyphenols attainable with Mediterranean diets can quench inflammation caused by HHcy. On the contrary, folic acid and vitamin B6, alone or in combination, did not affect monocyte adhesion to the endothelium (Fig. 6*C*).

DISCUSSION

Even modestly elevated Hcy concentrations are recognized as an independent risk factor for atherosclerotic disease, but the mechanisms linking HHcy to the development of vascular disease, and even the causal role of Hcy, have not been fully clarified. The possibility that Hcy might affect lesion progression stimulating VCAM-1 expression was first raised by in vivo findings that HHcy induced by methionine loading in healthy subjects modifies the adhesive properties of the endothelium as reflected by circulating levels of soluble VCAM-1 and ICAM-1 (44), as well as E-selectin (37). Such results were later confirmed by Silverman et al. (51), who demonstrated increased VCAM-1-dependent monocyte adhesion to endothelial cells after treatment of human aortic endothelial cells with Hcy, without, however, an assessment of underlying mechanisms.

Since it is known that Hcy induces the release of soluble cytokines, such as TNF-α (19), thus possibly causing endothelial activation indirectly (29), we first investigated the ability of Hcy to induce the endothelial release of TNF-α. We observed that Hcy does not affect the release of TNF-α in the endothelium, in contrast to monocytoid cells (19) and smooth muscle cells from coronary arteries (59). Rather, we report that Hcy directly affects the expression of adhesion molecules in endothelial cells and, in particular, VCAM-1 by increasing its gene expression. This occurs at ≥50 μmol/l Hcy, which is compatible with the in vivo definition of moderate HHcy (31–100 μmol/l) (54). We also demonstrate a global state of endothelial activation by Hcy, with the increased expression, by Hcy alone or in combination with inflammatory cytokines, of all the main endothelial adhesion molecules, including E-selectin and ICAM-1, the latter only in combination of Hcy with near-threshold classical proinflammatory stimuli.

To address the mechanisms by which Hcy induces endothelial activation, we show that, in HUVEC, Hcy (100 μmol/l), but not Cys, induces NF-κB activation and the nuclear translocation of its p65 (RelA) subunit, as well as the degradation of IκB-α and IκB-β, confirming and extending previous data (3). It is known that vascular endothelial cells in a quiescent state express little VCAM-1 and other endothelial activation markers. This normal quiescent status is maintained by a complex balance of interconnected activators or repressors of endothelial inflammatory genes (21, 55). Thus the mere presence of activated NF-κB in the nucleus of endothelial cells challenged with Hcy is necessary, but not sufficient, for VCAM-1 induction by Hcy. Using transient transfection assays with several deletional mutants of the VCAM-1 promoter linked to the CAT reporter gene (45), we report, for the first time, that Hcy induces the expression of the reporter gene driven by the VCAM-1 promoter and that the two tandem NF-κB sites are necessary for VCAM-1 promoter activity, since reporter activity is virtually abolished in the absence of such regions in the VCAM-1 promoter.

We also found that Hcy induces the activation of the redox-sensitive transcription factors NF-κB and AP-1, as assessed by EMSA. We show that Hcy alone induces increased DNA binding of NF-κB and, in combination with low concentrations of proinflammatory stimuli, also AP-1. Hcy activation of AP-1, in a proinflammatory milieu containing low concentrations of activating cytokines, can explain the more-than-additive effects of Hcy and inflammatory cytokines on the expression of VCAM-1, E-selectin, and ICAM-1. Indeed, it is known that AP-1 can synergize with the NF-κB transactivation of several inflammation-inducible genes (1).

We have confirmed that Hcy stimulation of endothelial cells induces phosphorylation of the main endothelial IκB isoforms (3, 29), which are ubiquitinated and degraded via the proteasome-dependent pathway. The resulting nuclear translocation of active forms of NF-κB leads to transcription, translation, and expression of the VCAM-1 gene, which is abolished by the proteasome inhibitor lactacystin. Our results thus unequivocally support the hypothesis that proteasome-dependent processes, including NF-κB activation, are necessary for Hcy-elicited increases in VCAM-1 expression and suggest a likely effect of Hcy on IκB phosphorylation and, therefore, on activation of the IκB kinase (IKK) signalosome (39).

VCAM-1 expression is regulated by redox-sensitive steps, and antioxidants are known to quench VCAM-1 induction (12, 17, 38). On the other hand, Hcy-induced endothelial dysfunction was previously suggested to be mediated by intracellular oxidative stress (60). The mechanisms through which Hcy induces endothelial activation were not, however, clear from previous literature. We show here that VCAM-1 induction by Hcy is mediated by an increase of ROS, mainly through activation of NAD(P)H oxidase, as shown by the increased membrane translocation of the cytosolic $p47^{phox}$ subunit of NAD(P)H oxidase and by the reduced ROS production and VCAM-1 expression in the presence of the NAD(P)H oxidase inhibitors DPI and apocynin. Such results are consistent with

those obtained in monocytoid cell culture (52) and in the coronary arteries of mice with HHcy (59). Hcy activation of NADPH oxidase, as well as $p47^{phox}$ membrane translocation, is abolished by PKC inhibitors (experiments not shown), suggesting some involvement of PKC in Hcy endothelial effects, in agreement with reports of other studies using monocytoid cells (52, 69).

Increased oxidative stress is often quoted as a mechanism of Hcy-induced endothelial dysfunction, and antioxidants have been examined to prevent Hcy-induced endothelial dysfunction (5, 33). Having highlighted a critical role of ROS overproduction in VCAM-1 induction by Hcy, we tested the possibility that the prooxidant and proinflammatory effects of Hcy could be reduced by quantitatively minor Mediterranean dietary components, the antioxidant and anti-inflammatory properties of which have been long recognized (25, 61, 63). We previously showed that the olive oil and red wine phenolic antioxidants HT and RSV reduce VCAM-1 expression and endothelial activation induced by LPS or cytokines by inhibiting NF-κB and AP-1 activation (12). Here we show that they also reduce Hcy-induced VCAM-1 induction and monocytoid cell adhesion, suggesting a novel possible tool to ameliorate Hcy proinflammatory effects mediated by ROS overproduction and NF-κB activation. Further studies are warranted to explain the mechanisms by which these antioxidant polyphenols may counterbalance enhanced ROS production and expression of endothelial adhesion molecules by Hcy and whether such effects also occur in vivo. Such phenolic antioxidants are known to be highly bioavailable and to reach plasma levels in the micromolar range when they are included in the diet (43). They may therefore represent an alternative dietary approach to prevent prooxidant and proinflammatory effects of Hcy.

Although the association of HHcy and vascular disease is no longer a matter of discussion, the causal role of Hcy, supported by our findings, still needs to be definitely accepted through intervention trials in humans showing clinical benefits of lowering Hcy. In three recently reported trials (7, 35, 56), no effects of folic acid and vitamin B6/B12 supplementation on clinical end points were found. Although there are multiple explanations for the apparent lack of efficacy of Hcy-lowering strategies in these trials, including, as a main explanation, minor Hcy lowering against the background of very minor HHcy, strategies to counteract vascular effects of Hcy might be alternative or complementary to those of Hcy lowering. Natural antioxidants of Mediterranean diets might represent one such therapeutic strategy in vascular disease.

ACKNOWLEDGMENTS

We are grateful to the Division of Obstetrics and Gynecology, Vito Fazzi Hospital (Lecce, Italy), for providing umbilical cords and to Drs. F. Sanapo and S. Scolozzi [Laboratory of Clinical Pathology, "Cardinale G. Panico" Hospital, Tricase (Lecce)] for use of the flow cytometry apparatus. We also gratefully acknowledge the kind donations of monoclonal antibody-producing hybridomas by Michael A. Gimbrone, Jr., and of the VCAM-1 promoter constructs by Andrew S. Neish.

REFERENCES

1. **Ahmad M, Theofanidis P, Medford RM.** Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-α. *J Biol Chem* 273: 4616–4621, 1998.
2. **Austin RC, Lentz SR, Werstuck GH.** Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease. *Cell Death Differ* 11 *Suppl* 1: S56–S64, 2004.
3. **Au-Yeung KK, Woo CW, Sung FL, Yip JC, Siow YL, O K.** Hyperhomocysteinemia activates nuclear factor-κB in endothelial cells via oxidative stress. *Circ Res* 94: 28–36, 2004.
4. **Baeuerle PA.** IκB-NF-κB structures: at the interface of inflammation control. *Cell* 95: 729–731, 1998.
5. **Bagi Z, Cseko C, Toth E, Koller A.** Oxidative stress-induced dysregulation of arteriolar wall shear stress and blood pressure in hyperhomocysteinemia is prevented by chronic vitamin C treatment. *Am J Physiol Heart Circ Physiol* 285: H2277–H2283, 2003.
6. **Basta G, Lazzerini G, Del Turco S, Ratto GM, Schmidt AM, De Caterina R.** At least 2 distinct pathways generating reactive oxygen species mediate vascular cell adhesion molecule-1 induction by advanced glycation end products. *Arterioscler Thromb Vasc Biol* 25: 1401–1407, 2005.
7. **Bønaa KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, Wang H, Nordrehaug JE, Arnesen E, Rasmussen K.** Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N Engl J Med* 354: 1578–1588, 2006.
8. **Bowie A, O'Neill LA.** Oxidative stress and nuclear factor-κB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* 59: 13–23, 2000.
9. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976.
10. **Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knuechel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D.** Activated transcription factor nuclear factor-κB is present in the atherosclerotic lesion. *J Clin Invest* 97: 1715–1722, 1996.
11. **Carluccio MA, Massaro M, Bonfrate C, Siculella L, Maffia M, Nicolardi G, Distante A, Storelli C, De Caterina R.** Oleic acid inhibits endothelial activation: a direct vascular antiatherogenic mechanism of a nutritional component in the Mediterranean diet. *Arterioscler Thromb Vasc Biol* 19: 220–228, 1999.
12. **Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, Visioli F, Distante A, De Caterina R.** Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler Thromb Vasc Biol* 23: 622–629, 2003.
13. **Chomczynski P, Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
14. **Collins T.** Endothelial nuclear factor-κB and the initiation of the atherosclerotic lesion. *Lab Invest* 68: 499–508, 1993.
15. **Collins T, Cybulsky MI.** NF-κB: pivotal mediator or innocent bystander in atherogenesis? *J Clin Invest* 107: 255–264, 2001.
16. **Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T.** Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J* 9: 899–909, 1995.
17. **Csiszar A, Smith K, Labinskyy N, Orosz Z, Rivera A, Ungvari Z.** Resveratrol attenuates TNF-α-induced activation of coronary arterial endothelial cells: role of NF-κB inhibition. *Am J Physiol Heart Circ Physiol* 291: H1694–H1699, 2006.
18. **Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS.** A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest* 107: 1255–1262, 2001.
19. **Dalal S, Parkin SM, Homer-Vanniasinkam S, Nicolaou A.** Effect of homocysteine on cytokine production by human endothelial cells and monocytes. *Ann Clin Biochem* 40: 534–541, 2003.
20. **De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, Shin WS, Liao JK.** Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 96: 60–68, 1995.
21. **Dela Paz NG, Simeonidis S, Leo C, Rose DW, Collins T.** Regulation of NF-κB-dependent gene expression by the POU domain transcription factor Oct-1. *J Biol Chem* 282: 8424–8434, 2007.
22. **Dell'Agli M, Fagnani R, Mitro N, Scurati S, Masciadri M, Mussoni L, Galli GV, Bosisio E, Crestani M, De Fabiani E, Tremoli E, Caruso D.** Minor components of olive oil modulate proatherogenic adhesion mole-

cules involved in endothelial activation. *J Agric Food Chem* 54: 3259–3264, 2006.
23. **De Winther MP, Kanters E, Kraal G, Hofker MH.** Nuclear factor κB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol* 25: 904–914, 2005.
24. **Finkelstein JD.** Methionine metabolism in mammals. *J Nutr Biochem* 1: 228–237, 1990.
25. **Frankel EN, Kanner J, German JB, Parks E, Kinsella JE.** Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* 341: 454–457, 1993.
26. **Hajra L, Evans AI, Chen M, Hyduk SJ, Collins T, Cybulsky MI.** The NF-κB signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc Natl Acad Sci USA* 97: 9052–9057, 2000.
27. **Handy DE, Zhang Y, Loscalzo J.** Homocysteine down-regulates cellular glutathione peroxidase (GPx1) by decreasing translation. *J Biol Chem* 280: 15518–15525, 2005.
28. **Hankey GJ, Eikelboom JW.** Homocysteine and vascular disease. *Lancet* 354: 407–413, 1999.
29. **Hofmann MA, Lalla E, Lu Y, Gleason MR, Wolf BM, Tanji N, Ferran LJ Jr, Kohl B, Rao V, Kisiel W, Stern DM, Schmidt AM.** Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. *J Clin Invest* 107: 675–683, 2001.
30. **Inanami O, Johnson JL, McAdara JK, Benna JE, Faust LR, Newburger PE, Babior BM.** Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of $p47^{PHOX}$ on serine 303 or 304. *J Biol Chem* 273: 9539–9543, 1998.
31. **Johnson DR, Douglas I, Jahnke A, Ghosh S, Pober JS.** A sustained reduction in IκB-β may contribute to persistent NF-κB activation in human endothelial cells. *J Biol Chem* 271: 16317–16322, 1996.
32. **Kalogeris TJ, Laroux FS, Cockrell A, Ichikawa H, Okayama N, Phifer TJ, Alexander JS, Grisham MB.** Effect of selective proteasome inhibitors on TNF-induced activation of primary and transformed endothelial cells. *Am J Physiol Cell Physiol* 276: C856–C864, 1999.
33. **Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR, Haynes WG.** Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. *Circulation* 100: 1161–1168, 1999.
34. **Li N, Karin M.** Is NF-κB the sensor of oxidative stress? *FASEB J* 13: 1137–1143, 1999.
35. **Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, McQueen MJ, Probstfield J, Fodor G, Held C, Genest J Jr.** Homocysteine lowering with folic acid and B vitamins in vascular disease. *N Engl J Med* 354: 1567–1577, 2006.
36. **Lusis AJ.** Atherosclerosis. *Nature* 407: 233–241, 2000.
37. **Mansoor MA, Seljeflot I, Arnesen H, Knudsen A, Bates CJ, Mishra G, Larsen TW.** Endothelial cell adhesion molecules in healthy adults during acute hyperhomocysteinemia and mild hypertriglyceridemia. *Clin Biochem* 37: 408–414, 2004.
38. **Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM.** Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 92: 1866–1874, 1993.
39. **Massa PE, Li X, Hanidu A, Siamas J, Pariali M, Pareja J, Savitt AG, Catron KM, Li J, Marcu KB.** Gene expression profiling in conjunction with physiological rescues of IKKα-null cells with wild type or mutant IKKα reveals distinct classes of IKKα/NF-κB-dependent genes. *J Biol Chem* 280: 14057–14069, 2005.
40. **Massaro M, Basta G, Lazzerini G, Carluccio MA, Bosetti F, Solaini G, Visioli F, Paolicchi A, De Caterina R.** Quenching of intracellular ROS generation as a mechanism for oleate-induced reduction of endothelial activation and early atherogenesis. *Thromb Haemost* 88: 335–344, 2002.
41. **Medina M, Urdiales JL, Amores-Sanchez MI.** Roles of homocysteine in cell metabolism: old and new functions. *Eur J Biochem* 268: 3871–3882, 2001.
42. **Minami T, Abid MR, Zhang J, King G, Kodama T, Aird WC.** Thrombin stimulation of vascular adhesion molecule-1 in endothelial cells is mediated by protein kinase C (PKC)-δ-NF-κB and PKC-ξ-GATA signaling pathways. *J Biol Chem* 278: 6976–6984, 2003.
43. **Miro-Casas E, Covas MI, Farre M, Fito M, Ortuno J, Weinbrenner T, Roset P, de la Torre R.** Hydroxytyrosol disposition in humans. *Clin Chem* 49: 945–952, 2003.
44. **Nappo F, De Rosa N, Marfella R, De Lucia D, Ingrosso D, Perna AF, Farzati B, Giugliano D.** Impairment of endothelial functions by acute hyperhomocysteinemia and reversal by antioxidant vitamins. *JAMA* 281: 2113–2118, 1999.
45. **Neish AS, Williams AJ, Palmer HJ, Whitley MZ, Collins T.** Functional analysis of the human vascular cell adhesion molecule 1 promoter. *J Exp Med* 176: 1583–1593, 1992.
46. **Pueyo ME, Gonzalez W, Nicoletti A, Savoie F, Arnal JF, Michel JB.** Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor-κB activation induced by intracellular oxidative stress. *Arterioscler Thromb Vasc Biol* 20: 645–651, 2000.
47. **Ross R.** Atherosclerosis—an inflammatory disease. *N Engl J Med* 340: 115–126, 1999.
48. **Royall JA, Ischiropoulos H.** Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch Biochem Biophys* 302: 348–355, 1993.
49. **Schindler U, Baichwal VR.** Three NF-κB binding sites in the human E-selectin gene required for maximal tumor necrosis factor-α-induced expression. *Mol Cell Biol* 14: 5820–5831, 1994.
50. **Selhub J.** Homocysteine metabolism. *Annu Rev Nutr* 19: 217–246, 1999.
51. **Silverman MD, Tumuluri RJ, Davis M, Lopez G, Rosenbaum JT, Lelkes PI.** Homocysteine upregulates vascular cell adhesion molecule-1 expression in cultured human aortic endothelial cells and enhances monocyte adhesion. *Arterioscler Thromb Vasc Biol* 22: 587–592, 2002.
52. **Siow YL, Au-Yeung KK, Woo CW, O K.** Homocysteine stimulates phosphorylation of NADPH oxidase $p47^{phox}$ and $p67^{phox}$ subunits in monocytes via protein kinase Cβ activation. *Biochem J* 398: 73–82, 2006.
53. **Starkebaum G, Harlan JM.** Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J Clin Invest* 77: 1370–1376, 1986.
54. **Still R, McDowell I.** Clinical implication of plasma homocysteine measurement in cardiovascular disease. *J Clin Pathol* 51: 183–188, 1998.
55. **Tham DM, Martin-McNulty B, Wang YX, Wilson DW, Vergona R, Sullivan ME, Dole W, Rutledge JC.** Angiotensin II is associated with activation of NF-κB-mediated genes and downregulation of PPARs. *Physiol Genomics* 11: 21–30, 2002.
56. **Toole JF, Malinow MR, Chambless LE, Spence JD, Pettigrew LC, Howard VJ, Sides EG, Wang CH, Stampfer M.** Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death: the Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial. *JAMA* 291: 565–575, 2004.
57. **Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA.** Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. *EMBO J* 14: 2876–2883, 1995.
58. **Trichopoulou A, Vasilopoulou E, Lagiou A.** Mediterranean diet and coronary heart disease: are antioxidants critical? *Nutr Rev* 57: 253–255, 1999.
59. **Ungvari Z, Csiszar A, Edwards JG, Kaminski PM, Wolin MS, Kaley G, Koller A.** Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of tumor necrosis factor-α, NAD(P)H oxidase, and inducible nitric oxide synthase. *Arterioscler Thromb Vasc Biol* 23: 418–424, 2003.
60. **Upchurch GR Jr, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF Jr, Loscalzo J.** Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem* 272: 17012–17017, 1997.
61. **Visioli F, Bellomo G, Galli C.** Free radical-scavenging properties of olive oil polyphenols. *Biochem Biophys Res Commun* 247: 60–64, 1998.
62. **Visioli F, Galli C.** The role of antioxidants in the Mediterranean diet. *Lipids* 36 *Suppl*: S49–S52, 2001.
63. **Visioli F, Galli C, Plasmati E, Viappiani S, Hernandez A, Colombo C, Sala A.** Olive phenol hydroxytyrosol prevents passive smoking-induced oxidative stress. *Circulation* 102: 2169–2171, 2000.

64. **Wang G, Woo CW, Sung FL, Siow YL, O K.** Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemokine and adhesion molecules. *Arterioscler Thromb Vasc Biol* 22: 1777–1783, 2002.
65. **Wang H, Jiang X, Yang F, Gaubatz JW, Ma L, Magera MJ, Yang X, Berger PB, Durante W, Pownall HJ, Schafer AI.** Hyperhomocysteinemia accelerates atherosclerosis in cystathionine β-synthase and apolipoprotein E double knock-out mice with and without dietary perturbation. *Blood* 101: 3901–3907, 2003.
66. **Wang N, Verna L, Liao H, Ballard A, Zhu Y, Stemerman MB.** Adenovirus-mediated overexpression of dominant-negative mutant of c-Jun prevents intercellular adhesion molecule-1 induction by LDL: a critical role for activator protein-1 in endothelial activation. *Arterioscler Thromb Vasc Biol* 21: 1414–1420, 2001.
67. **Welch GN, Loscalzo J.** Homocysteine and atherothrombosis. *N Engl J Med* 338: 1042–1050, 1998.
68. **Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ.** Increase in plasma homocysteine associated with parallel increases in plasma *S*-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem* 275: 29318–29323, 2000.
69. **Zeng X, Dai J, Remick DG, Wang X.** Homocysteine mediated expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human monocytes. *Circ Res* 93: 311–320, 2003.
70. **Zhou J, Moller J, Danielsen CC, Bentzon J, Ravn HB, Austin RC, Falk E.** Dietary supplementation with methionine and homocysteine promotes early atherosclerosis but not plaque rupture in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 21: 1470–1476, 2001.