α-Lipoic acid inhibits TNF-α-induced NF-κB activation and adhesion molecule expression in human aortic endothelial cells

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ABSTRACT **Endothelial activation and monocyte adhesion are initiating steps in atherogenesis thought to be caused in part by oxidative stress. The metabolic thiol antioxidant α-lipoic acid has been suggested to be of therapeutic value in pathologies associated with redox imbalances. We investigated the role of (*R*)-α-lipoic acid (LA) vs. glutathione and ascorbic acid in tumor necrosis factor α (TNF-α) -induced adhesion molecule expression and nuclear factor κB (NF-κB) signaling in human aortic endothelial cells (HAEC). Preincubation of HAEC for 48 h with LA (0.05–1 mmol/l) dose-dependently inhibited TNF-α (10 U/ml) -induced adhesion of human monocytic THP-1 cells, as well as mRNA and protein expression of E-selectin, vascular cell adhesion molecule 1 and intercellular adhesion molecule 1. LA also strongly inhibited TNF-α-induced mRNA expression of monocyte chemoattractant protein-1 but did not affect expression of TNF-α receptor 1. Furthermore, LA dose-dependently inhibited TNF-α-induced IκB kinase activation, subsequent degradation of IκB, the cytoplasmic NF-κB inhibitor, and nuclear translocation of NF-κB. In contrast, TNF-α-induced NF-κB activation and adhesion molecule expression were not affected by ascorbic acid or by manipulating cellular glutathione status with L-2-oxo-4-thiazolidinecarboxylic acid, *N*-acetyl-L-cysteine, or D,L-buthionine-S,R-sulfoximine. Our data show that clinically relevant concentrations of LA, but neither vitamin C nor glutathione, inhibit adhesion molecule expression in HAEC and monocyte adhesion by inhibiting the IκB/NF-κB signaling pathway at the level, or upstream, of IκB kinase.—Zhang, W.-J., Frei, B. α-Lipoic acid inhibits TNF-α-induced NF-κB activation and adhesion molecule expression in human aortic endothelial cells. *FASEB J.* 15, 2423–2432 (2001)**

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ARTERIAL LEUKOCYTE RECRUITMENT is an important initiating step in atherogenesis (1, 2). Leukocyte adhesion and emigration to the subendothelium, in response to chemoattractants and other activating molecules, is mediated by adhesion molecules expressed on endothelial cells including E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) (2, 3). Studies with experimental animals and humans have found increased expression of these adhesion molecules on lesion-prone arterial sites and on atherosclerotic lesions (4–7). Genetic deficiencies of adhesion molecules in mice are associated with decreased atherosclerosis (8) Therefore, modulation of monocyte–endothelial interactions may be an important target for the prevention and treatment of atherosclerosis.

Induction of endothelial adhesion molecules by inflammatory cytokines, such as tumor necrosis factor α (TNF-α), depends on activation of the transcription factor nuclear factor κB (NF-κB) (9). The promoter regions of the genes for E-selectin, VCAM-1, and ICAM-1 contain three, two, and one NF-κB binding site, respectively (10–12). In endothelial cells, the p50-p65 heterodimer of the NF-κB/Rel family is the predominant species binding to κB consensus sequences in these promoters. NF-κB is located in the cytoplasm in an inactive form associated with its inhibitors, the IκB proteins (IκBs). In response to TNF-α stimulation, IκBs are phosphorylated by IκB kinase (IKK), ubiquitinated, and proteolytically degraded, which allows NF-κB to translocate to the nucleus (9, 13). Many NF-κB-regulated genes, including adhesion molecules, monocyte chemoattractant protein-1 (MCP-1), and several cytokines, are important mediators of inflammation (14).

Agents that block NF-κB signaling and, hence, adhesion molecule expression and leukocyte–endothelial interactions in vitro (15, 16) also exert marked effects on inflammatory responses in vivo (3). Regulation of endothelial adhesion molecule expression has been related to oxidative stress through specific reduction-oxidation (redox) -sensitive transcriptional or posttranscriptional factors, including NF-κB (17, 18). Therefore, antioxidants have been suggested to be of therapeutic value. For example, the glutathione (GSH) precursor and radical scavenger *N*-acetyl-L-cysteine (NAC) and the putative antioxidant pyrrolidine dithiocarbamate have been shown to inhibit adhesion molecule expression induced by cytokines or oxidants in different cell types (17–20).

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The metabolic thiol antioxidant (*R*)-α-lipoic acid (LA, 1,2-dithiolane-3-pentanoic acid) is a compound naturally occurring in pro- and eukaryotes that may be useful in treating pathologies associated with redox imbalances (21, 22). LA has been used safely for more than 30 years in Germany to treat diabetic complications and polyneuropathies. Apart from the essential role of LA as a cofactor in oxidative metabolism, LA in its free, non-protein-bound form has potent antioxidant and metal-chelating properties (23–26). LA is readily taken up by a variety of cells and tissues and is reduced in mitochondria to the potent antioxidant dihydrolipoic acid (6,8-dithiooctanoic acid, DHLA) (24, 27–29). With a standard reduction potential of −0.32 V, the DHLA/LA couple may chemically reduce glutathione disulfide (GSSG) to GSH, which has a redox potential of −0.24 V (21).

Therefore, the goal of the present study was to investigate the role of LA vs. GSH and other cellular antioxidants in TNF-α-induced expression of adhesion molecules and monocyte adhesion to human aortic endothelial cells (HAEC). We also examined potential underlying mechanisms by studying the NF-κB/IκB signaling pathway.

MATERIALS AND METHODS

Materials

(*R*)-α-Lipoic acid (Asta Medica, Frankfurt/Main, Germany) stock solution was prepared in dimethyl sulfoxide (DMSO) at concentrations such that the final DMSO concentration in the cell culture media did not exceed 0.1%. [Note that in the present study, (*R*)-α-lipoic acid was used in all experiments; essentially identical results were obtained with the enantiomeric form, (*S*)-α-lipoic acid (data not shown)]. L-2-oxo-4-thiazolidinecarboxylic acid (OTC), NAC, D,L-buthionine-*S,R*-sulfoximine (BSO), GSH monoethyl ester, and ascorbic acid were obtained from Sigma (St. Louis, MO) and prepared fresh in doubly distilled water. Human recombinant TNF-α was purchased from Boehringer Mannheim (Mannheim, Germany).

Cell culture

HAEC were obtained from Clonetics (San Diego, CA) and cultured with endothelial cell growth medium (Clonetics) at 37°C in a humidified 95% air-5% CO_2 atmosphere. Cells were harvested at confluence with 0.05% trypsin-0.02% EDTA (Sigma) and plated at a split ratio of 1:5. For experiments, cells were grown to confluence in 48- or 96-well plates, 75 cm^2 flasks, or 100 mm Petri dishes (Costar, Cambridge, MA) using endothelial culture medium consisting of M199 medium (Sigma) supplemented with 20% fetal calf serum (Life Technologies, Grand Island, NY), 100 ng/ml streptomycin, 100 IU/ml penicillin, 250 ng/ml fungizone, 1 mmol/l glutamine (Life Technologies), and 1 ng/ml human recombinant basic fibroblast growth factor (Boehringer Mannheim). All experiments used HAEC at passage seven or eight.

Human monocytic THP-1 cells were purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium (Sigma) containing 10% fetal calf serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, 250 ng/ml fungizone, 1 mmol/l glutamine, and 50 μmol/l 2-mercaptoethanol and subcultured at a 1:5 ratio three times per week.

Experiments

HAEC were preincubated for 48 h with different concentrations of LA (0.05–1 mmol/l). Control cells were incubated with the same concentrations of the vehicle DMSO (≤0.1%). In some experiments, cells were preincubated with 0.2 mmol/l LA for different periods (0, 1, 4, 16, and 48 h). Subsequently, the cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for various periods: 4.5 h for protein expression of adhesion molecules and measurement of THP-1 adhesion; 2 h for measurement of mRNA levels; 1 h for measurement of NF-κB activation; and 15 min for measurement of IκB proteins and IκB kinase activity. In one experiment in which TNF-α was replaced by phorbol 12-myristate 13-acetate (PMA), HAEC were preincubated for 48 h with different concentrations of LA (0.05–1 mmol/l), washed twice, and subsequently coincubated for 5 h with the same concentrations of LA plus PMA (100 nmol/l).

Assay for THP-1 monocyte adhesion to endothelial monolayers

Adhesion of THP-1 cells to HAEC was measured as described (30). HAEC were grown in 48-well plates to confluence. After preincubation for 48 h with 0, 0.05, 0.1, 0.2, or 0.5 mmol/l LA, the cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for 4.5 h. The cells were washed twice with M199 medium; THP-1 cells (2.5×10^5/ml) were layered over the HAEC monolayers and incubated for another hour at 37°C. Thereafter, the cells were washed with HBSS and fixed in 1% glutaraldehyde in PBS. The adherent THP-1 cells were counted in five high-power fields (HPF, 40×-phase contrast objective) per well under a microscope and expressed as adherent THP-1 cells per HPF.

Measurement of cell adhesion molecules by ELISA

Surface expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1) was quantified by ELISA performed on HAEC monolayers in flat-bottom 96-well plates. After treatment, the cells were fixed in 0.1% glutaraldehyde in PBS. For cell ELISA, plates were blocked at 37°C for 1 h with 5% skim milk powder in PBS before incubation with a primary antibody to either E-selection, ICAM-1 (R&D Systems, Minneapolis, MN), or VCAM-1 (Dako, Carpinteria, CA) at 4°C overnight. The plates were then incubated with a horseradish peroxidase (HRP) -conjugated goat anti-mouse IgG secondary antibody (Amersham, Piscataway, NJ) at 37°C for 1 h. The expression of E-selectin, ICAM-1, and VCAM-1 was quantitated by the addition of the peroxidase substrate *o*-phenylendiaminehydrochloride (Sigma). The absorbance of each well was measured at 492 nm with a microplate spectrophotometer (Molecular Devices, Palo Alto, CA).

Measurement of cell surface expression of TNF-α receptor 1 (TNF-R1)

Surface expression of TNF-R1 was quantified by ELISA performed on HAEC monolayers in flat-bottom 96-well plates. After incubation without or with LA for 48 h, the cells were washed twice with PBS and fixed in 0.1% glutaraldehyde in

PBS. The plates were blocked at 37°C for 1 h with 5% skim milk powder in PBS before incubation at 4°C overnight with a primary antibody to TNF-R1 (N-20; Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the plates were washed three times with 0.1% Tween 20 in PBS and incubated at 37°C for 1 h with a HRP-conjugated donkey anti-goat IgG secondary antibody (Santa Cruz Biotechnology). Surface expression of TNF-R1 was quantitated by the addition of the peroxidase substrate *o*-phenylendiaminehydrochloride (Sigma). The absorbance of each well was measured at 492 nm with a microplate spectrophotometer (Molecular Devices).

Cell viability

Cell viability was assessed by morphology and by reduction of the tetrazolium salt MTT (3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by mitochondrial dehydrogenases, according to the manufacturer's instruction (Boehringer Mannheim).

Quantitation of glutathione

HAEC were incubated for 24 h with the indicated compounds in endothelial culture medium. After incubation, the cells were rinsed with HBSS and detached with a rubber policeman. Proteins were precipitated with 10% (v/v) perchloric acid and acid-soluble thiols were derivatized with iodoacetic acid (1 mmol/l), followed by conjugation to 1-fluoro-2,4-dinitrobenzene. The dinitrophenyl-derivative of GSH was separated by HPLC according to the method of Farris and Reed (31) using γ-glutamyl-glutamate as an internal standard.

Northern blot analysis

Total cellular RNA was isolated from HAEC using TRIzol Reagent (Life Technologies, Inc). Northern blot analysis was performed as described (32). RNA blots were hybridized with 10^6 cpm/ml of the [α-^{32}P]dATP-labeled oligonucleotide probes for either human E-selectin, VCAM-1, ICAM-1, or MCP-1 (R&D Systems) or cDNA for rat glyceraldehyde-3-phosphate dehydrogenase overnight at 57°C in a hybridization oven. Blots were washed, air dried, and exposed to Hyperfilm X-ray films (Amersham) at −80°C.

Western blot analysis

After treatment, cells were washed three times with ice-cold PBS containing 1 mmol/l Na_3VO_4 and 5 mmol/l EDTA, then scraped and resuspended in 0.75 ml lysis buffer as described (33). After incubation of the cell lysates on ice for 20 min and centrifugation at 10,000 *g* at 4°C for 15 min, the supernatant containing solubilized proteins was recovered, frozen in small aliquots, and stored at −80°C until use.

Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The membranes were incubated overnight with either rabbit polyclonal anti-IκBα, rabbit polyclonal anti-IκBβ, or goat polyclonal anti-TNF-R1 antibody (Santa Cruz Biotechnology) after blocking with 0.1% Tween 20 in Tris-buffered saline containing 5% skim milk powder. Thereafter, the membranes were incubated with HRP-conjugated donkey anti-rabbit antibody (Amersham) or donkey anti-goat antibody (Santa Cruz Biotechnology) for 1 h at room temperature. IκBα, IκBβ, and TNF-R1 proteins were detected by the enhanced chemiluminescence detection system (Amersham).

Immunoprecipitation and in vitro kinase assay

HAEC were treated and cellular lysates were prepared as described above. Immunoprecipitation and in vitro kinase assays were performed as described (33). Protein supernatant (100 μg) was incubated with rabbit polyclonal anti-IKKα or anti-IKKβ antibody (Santa Cruz Biotechnology) for 18 h at 4°C. The IKK–antibody complex was precipitated with protein-A agar (Santa Cruz Biotechnology) for 2 h at 4°C. The immunoprecipitate was recovered by centrifugation (1000 *g*, 5 min) and washed twice with ice-cold lysis buffer and once with kinase buffer (without ATP). The immunoprecipitated IKKα and IKKβ proteins were incubated with 500 ng IκBα GST fusion protein (Santa Cruz Biotechnology) as a substrate for 30 min at 30°C in kinase buffer as described (33). The reaction was terminated by addition of 5× SDS-PAGE sample buffer and boiling for 5 min. Proteins were separated on 10% SDS-PAGE and autoradiography of the dried gel was performed at −80°C.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and EMSA was performed as described (34). For EMSA, 5 μg of nuclear extract was incubated with ^{32}P-labeled double-stranded NF-κB oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Santa Cruz Biotechnology) at room temperature for 30 min. The incubation mixture included 1 μg of poly (dI-dC) in a binding buffer (25 mmol/l HEPES, pH 7.9, 0.5 mmol/l EDTA, 0.5 mmol/l DTT, 1% Nonidet P-40, 5% glycerol, 50 mmol/l NaCl). The DNA–protein complex was electrophoresed on 5% nondenaturing polyacrylamide gels in 0.5× Tris borate EDTA buffer. The specificity of binding was examined by incubating nuclear extracts prepared from TNF-α-treated cells with 2–4 μg of antibodies against either the p65 or p50 subunit of NF-κB (Santa Cruz Biotechnology) for 30 min at room temperature before addition of the ^{32}P-labeled oligonucleotide. Specificity was further tested by competition with a 100-fold excess of unlabeled competitor consensus oligonucleotide. Radioactive bands were detected by autoradiography at −80°C.

Data analysis

Data are reported as mean ± SD. One-way ANOVA or Student's unpaired *t* test were used for statistical analysis of the original data, and significance was accepted at the $P < 0.05$ level. Data in Figs. 2, 6, and 7 are expressed as percent of TNF-α stimulation, which was calculated as follows: (value for TNF-α plus LA-treated cells/value for TNF-α-treated cells) × 100%. Percent inhibition was calculated as follows: [1 − (value for TNF-α plus LA-treated cells − value for unstimulated cells/value for TNF-α-treated cells − value for unstimulated cells)] × 100%.

RESULTS

Lipoic acid inhibits TNF-α-induced adhesion molecule expression and monocyte adhesion to human aortic endothelial cells

Incubation of confluent HAEC with 10 U/ml TNF-α for 4.5 h caused an almost 10-fold increase in adhesion of THP-1 monocytic cells compared with adhesion of THP-1 cells to unstimulated HAEC (**Fig. 1**). This in-

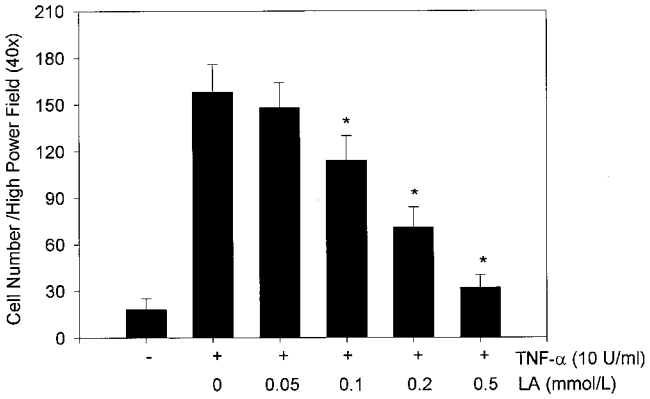

Figure 1. Lipoic acid dose-dependently inhibits adhesion of THP-1 cells to TNF-α-stimulated human aortic endothelial cells. Confluent HAEC were preincubated for 48 h with the indicated concentrations of LA. The cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for 4.5 h. THP-1 cells (2.5×10^5/ml) were layered over HAEC and incubated at 37°C for 60 min. Thereafter, the cells were washed three times with HBSS and fixed with 1% glutaraldehyde in PBS. The adherent THP-1 cells in 5 high-power fields (40× phase contrast objective) were counted under a microscope and calculated as adherent THP-1 cells per high-power field. Data shown are mean values ± SD of four separate incubations each and are representative of three independent experiments. *$P < 0.05$ compared with TNF-α alone.

crease in HAEC adhesiveness was dose-dependently reduced by preincubation of the cells for 48 h with increasing concentrations of LA (0.05–0.5 mmol/l) (Fig. 1).

Since adhesion molecules are essential for the interaction of endothelial cells with monocytes, we determined whether LA affects expression of E-selectin, VCAM-1, and ICAM-1. HAEC did not constitutively express E-selectin, but treatment with 10 U/ml TNF-α caused a transient increase in E-selectin protein levels, with a maximum level reached after 4 h of incubation. VCAM-1 was not constitutively expressed, but was maximally induced after 6 h of incubation with TNF-α. In contrast, ICAM-1 was expressed in untreated HAEC and strongly up-regulated by TNF-α, with maximum levels reached after 12 h of incubation (data not shown).

HAEC were pretreated with 0.2 mmol/l LA for up to 48 h before incubation with 10 U/ml TNF-α for 4.5 h. As shown in **Fig. 2*A***, a time-dependent inhibitory effect on protein expression of E-selectin and VCAM-1 was observed, whereas ICAM-1 expression was not affected. The maximum inhibitory effect on E-selectin and VCAM-1 expression occurred when LA was preincubated with the cells for 48 h, but no inhibition was observed when LA was added simultaneously with TNF-α. Therefore, in all subsequent experiments (as well as the experiment in Fig. 1), HAEC were pretreated with LA for 48 h.

Increasing concentrations of LA (0.05–1 mmol/l) dose-dependently inhibited TNF-α-induced expression of all three adhesion molecules (Fig. 2*B*). At 0.5 mmol/l LA, E-selectin, VCAM-1, and ICAM-1 expression was inhibited by 68 ± 2.1%, 95 ± 2.8%, and 27 ± 3.4%, respectively ($P<0.01$ vs. TNF-α-stimulated cells pretreated without LA; $n=3$). Lipoic acid (0.05–1 mmol/l) exerted similar dose-dependent inhibitory effects on PMA (100 nmol/l) -induced E-selectin and VCAM-1 expression, whereas PMA-induced ICAM-1 expression was not inhibited by LA (data not shown). The highest LA concentration used in these experiments (1 mmol/l) did not affect cell viability as assessed by cell number, cellular morphology, and MTT reduction (data not shown).

To further investigate the mechanism by which LA inhibited adhesion molecule expression, Northern blot analysis was performed. Incubation of HAEC for 2 h with 10 U/ml TNF-α resulted in strong induction of

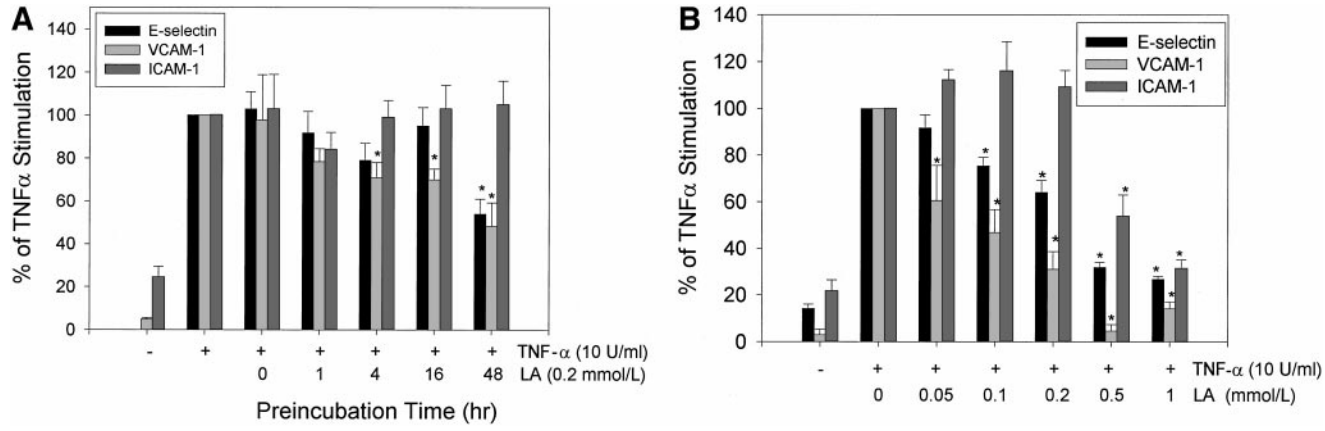

Figure 2. Lipoic acid time-dependently (*A*) and dose-dependently (*B*) inhibits TNF-α-induced cell surface protein expression of E-selectin, VCAM-1 and ICAM-1 in human aortic endothelial cells. Confluent HAEC were preincubated for the indicated times with 0.2 mmol/l of LA (*A*), or for 48 h with the indicated concentrations of LA (*B*). The cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for 4.5 h. Surface expression of adhesion molecules was measured by ELISA as described in Materials and Methods. Data are expressed as percentage of TNF-α-induced adhesion molecule expression in non-preincubated HAEC. Data shown are mean values ± SD of four separate incubations, and are representative of three independent experiments. *$P < 0.05$ compared with TNF-α alone.

mRNA levels of all three adhesion molecules. MCP-1 message was also strongly up-regulated. Pretreatment of the cells with LA (0.05–1 mmol/l) significantly inhibited induction of mRNA expression in a dose-dependent manner (**Fig. 3**). As determined by densitometry scanning, E-selectin, VCAM-1, ICAM-1, and MCP-1 message levels decreased by 93%, 77%, 67%, and 100%, respectively, when HAEC were pretreated with 0.5 mmol/l LA.

Lipoic acid inhibits TNF-α-induced activation of NF-κB and degradation of IκBs

Because activation of NF-κB is required for the transcriptional induction of adhesion molecules, we examined the effect of LA pretreatment on TNF-α-induced NF-κB activation. EMSA was used to determine NF-κB binding activity of nuclear extracts. As shown in **Fig. 4**, preincubation of HAEC with LA (0.1–0.5 mmol/l) inhibited TNF-α-induced NF-κB binding activity in a dose-dependent manner. At 0.5 mmol/l LA, TNF-α-induced NF-κB activation was inhibited by 81%, as determined by densitometry scanning. The specificity of the protein–DNA complex for the NF-κB sequence was demonstrated by competition with excess unlabeled NF-κB oligonucleotide and by supershift of the bands after incubation of the nuclear extracts with antibodies to the p65 and p50 subunits of NF-κB (Fig. 4). A complete supershift was observed with the p65 antibody and a partial supershift with the p50 antibody. These observations agree with previous data and suggest that in endothelial cells, the TNF-α-activated NF-κB complex is composed predominantly of the p65 subunit (35, 36).

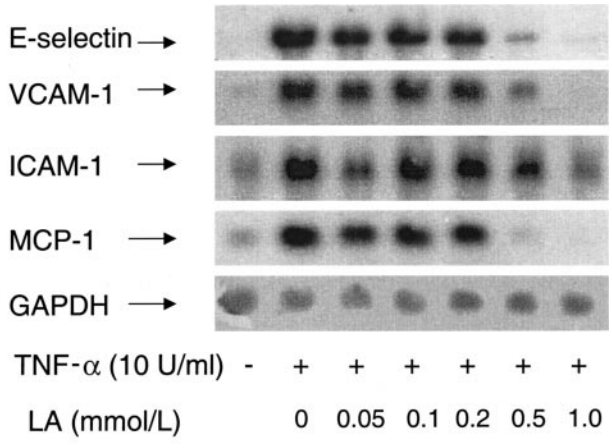

Figure 3. Lipoic acid dose-dependently inhibits TNF-α-induced up-regulation of E-selectin, VCAM-1, ICAM-1, and MCP-1 mRNA levels in human aortic endothelial cells. Confluent HAEC were preincubated for 48 h with the indicated concentrations of LA. The cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for 2 h. Northern blot analysis of total RNA extracts was performed as described in Materials and Methods. Data shown are representative of two independent experiments.

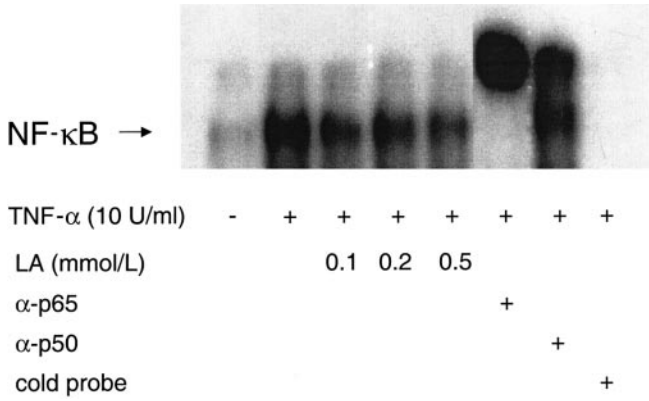

Figure 4. Lipoic acid dose-dependently inhibits TNF-α-induced NF-κB activation in human aortic endothelial cells. Confluent HAEC were preincubated for 48 h with the indicated concentrations of LA. The cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for 1 h. Isolation of nuclear extracts and EMSA were performed as described in Materials and Methods. Data shown are representative of two independent experiments. α-p65 and α-p50, antibodies to the p65 and p50 subunits of NF-κB, respectively.

To determine whether LA affects TNF-α-induced phosphorylation and degradation of IκBs, immunoblotting studies were performed. Analysis of HAEC extracts using IκB-specific antibodies showed that stimulation of the cells with TNF-α caused rapid degradation of both IκBα and IκBβ. Protein levels of IκBα and IκBβ dropped by 58% and 67%, respectively, after incubation of HAEC with TNF-α for 15 min and by $> 80\%$ after 30 min; thereafter, IκB levels increased gradually up to 120 min of incubation due to NF-κB-induced de novo synthesis of IκBs (data not shown).

When HAEC were treated with increasing concentrations of LA (0.1–1.0 mmol/l) before incubation with TNF-α for 15 min, the degradation of both IκBs was dose-dependently inhibited (**Fig. 5**). IκBα protein levels dropped to 32.0% ± 16.8% of controls after a 15 min incubation with TNF-α, but only to 44.3 ± 17.2% and 86.3 ± 1.5% (n=3) after pretreatment of HAEC with 0.1 and 0.5 mmol/l LA, respectively. Similar results were obtained for IκBβ (Fig. 5).

Lipoic acid inhibits TNF-α-induced IκB kinase activation

Because TNF-α-induced NF-κB activation and IκB degradation were inhibited by LA and are known to be initiated by phosphorylation of IκB by IKK, we investigated whether LA affects IKK activity. The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ. We found that LA inhibits TNF-α-induced activation of both IKKα and IKKβ in a dose-dependent manner (**Fig. 6**). IKKα activity was inhibited by 23% and 89% (n=2) and IKKβ by 26% and 60% (n=2) in cells pretreated with 0.1 and 0.5 mmol/l LA, respectively.

It is noteworthy that there was a close correlation

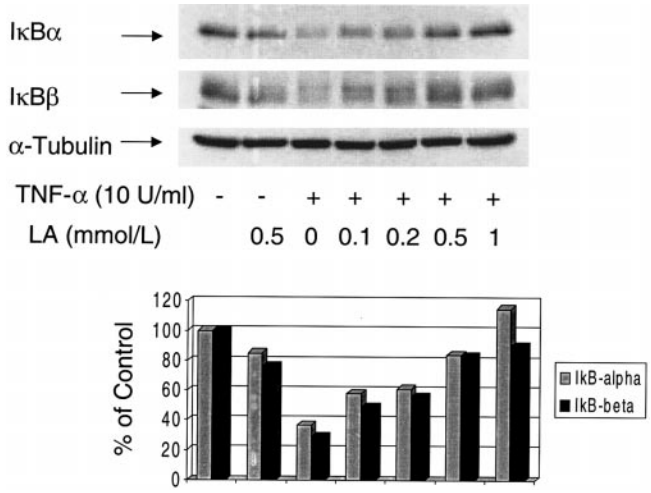

Figure 5. Lipoic acid dose-dependently inhibits TNF-α-induced degradation of IκBα and IκBβ proteins in human aortic endothelial cells. Confluent HAEC were preincubated for 48 h with the indicated concentrations of LA. The cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for 15 min. Total protein extracts of the cells were prepared and analyzed by Western blotting as described in Materials and Methods (top panel). The intensity of the bands was quantitated by densitometry and, after normalization to α-tubulin protein, expressed as percentage of IκB levels in unstimulated HAEC (bottom panel). Data shown are representative of three independent experiments.

between the dose-dependent effects of LA pretreatment on IKK activity, IκB degradation, NF-κB activation, E-selectin and VCAM-1 expression, and THP-1 adhesion (Figs. 1–6). Each of these processes was 50% inhibited by LA concentrations of 0.1–0.2 mmol/l. In contrast, LA (0.05–1 mmol/l) did not inhibit cell surface or total cellular protein expression of TNF-R1, the main receptor through which TNF-α activates the IκB/NF-κB signaling pathway (data not shown). Our data, therefore, strongly suggest that LA inhibits TNF-α-induced IKK activation in HAEC and subsequent proteolytic degradation of IκBs, thereby preventing NF-κB nuclear translocation and NF-κB-mediated inflammatory gene transcription.

TNF-α-induced adhesion molecule expression is not affected by glutathione or ascorbic acid

Ascorbic acid and GSH are present in human cells in millimolar concentrations and play a pivotal role in cellular antioxidant defenses and redox status. Furthermore, they have been implicated in regulation of NF-κB activation (9, 37), and are affected by treatment of cells with LA (21, 22, 28) Therefore, we investigated the role of cellular GSH levels and redox status (GSH:GSSG ratio) and ascorbic acid in TNF-α-induced adhesion molecule expression. Glutathione levels in HAEC were either decreased with BSO, an irreversible inhibitor of γ-glutamyl-cysteine synthetase, or increased with the cellular cysteine delivery agents OTC or NAC. Treatment of cells with 1 mmol/l BSO for 24 h resulted in a decrease in GSH levels from 6.54 to 0.76 nmol/10^6 cells and in GSSG from 0.68 nmol/10^6 cells to undetectable levels (<0.25 nmol/10^6 cells). In contrast, treatment of HAEC with 2 mmol/l OTC for 24 h resulted in an increase in GSH levels from 6.54 to 10.19 nmol/10^6 cells and in the GSH:GSSG ratio from 10.1 to 98.4. However, BSO did not enhance and OTC did not inhibit TNF-α-induced adhesion molecule expression in HAEC. The same observation was made for NAC and ascorbic acid (**Fig. 7**). Moreover, OTC, ascorbic acid and the cellular GSH delivery agent GSH monoethyl ester failed to exert an inhibitory effect on TNF-α-induced NF-κB activation (**Fig. 8**). These results indicate that neither cellular GSH levels, the GSH:GSSG redox ratio, nor vitamin C plays a significant role in TNF-α-induced adhesion molecule expression in HAEC.

DISCUSSION

Recruitment of leukocytes from the circulation to the extravascular space is a critical initiating event in atherogenesis and vascular inflammation (1–4). The process of leukocyte emigration involves several steps (38, 39). The leukocytes initially roll along the vessel

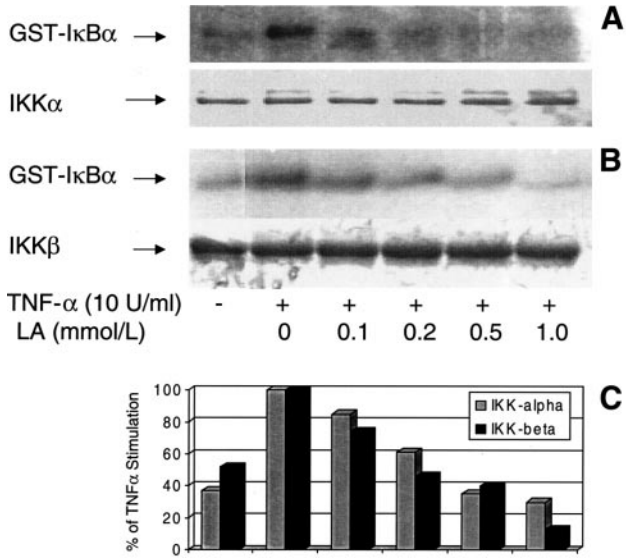

Figure 6. Lipoic-acid dose-dependently inhibits TNF-α-induced IKKα (*A*) and IKKβ (*B*) activity in human aortic endothelial cells. Confluent HAEC were preincubated for 48 h with the indicated concentrations of LA. The cells were washed twice with M199 medium and coincubated with the same concentrations of LA plus TNF-α (10 U/ml) for 15 min. Total protein extracts of the cells were prepared and analyzed by immunoprecipitation and protein kinase assays as described in Materials and Methods (*A*, IKKα; *B*, IKKβ). The intensity of the bands was quantitated by densitometry and, after normalization to IKKα or IKKβ proteins, expressed as percentage of TNF-α-induced IKK activation in HAEC preincubated without LA (*C*). Data shown are representative of two independent experiments.

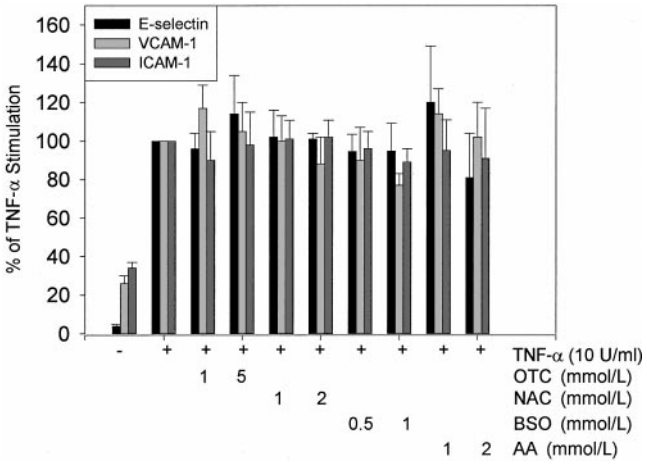

Figure 7. Ascorbic acid and manipulation of cellular glutathione status do not affect TNF-α-induced adhesion molecule expression in human aortic endothelial cells. Confluent HAEC were preincubated with the indicated concentrations of OTC, NAC or BSO for 24 h, or ascorbic acid for 4 h. The cells were washed twice with M199 medium and coincubated with same concentrations of OTC, NAC, BSO or ascorbic acid plus TNF-α (10 U/ml) for 4.5 h. Surface expression of adhesion molecules was measured by ELISA as described in Materials and Methods. Data are expressed as percentage of TNF-α-induced adhesion molecule expression in non-preincubated HAEC. Data shown are mean values ± SD of four separate incubations, and are representative of three independent experiments.

wall, then become activated by local factors generated by the endothelium (leading to their firm adhesion), and finally transmigrate across the endothelium. These complex processes are regulated in part by specific adhesion molecules and their counter-receptors. The initial rolling interactions are mediated by the selectins whereas firm adhesion and diapedesis are mediated by the interaction of integrins on the surface of leukocytes with endothelial adhesion molecules (38, 39). Factors affecting the expression of endothelial adhesion molecules, therefore, are important in regulating vascular inflammatory processes.

Activation of the transcription factor NF-κB, e.g., by inflammatory cytokines, phorbol esters, or lipopolysaccharides, is required for the transcriptional activation of endothelial cell adhesion molecules and MCP-1. Therefore, NF-κB is believed to play a pivotal role in atherosclerosis (9). An important component of the NF-κB/IκB pathway is IKK (40). IKKα and IKKβ both contribute to the activity of the IKK complex and are able to induce IκB phosphorylation and degradation, and thus NF-κB activation. Recent studies of IKKα and IKKβ knockout mice indicate that only IKKβ is required for cytokine-induced IκB degradation whereas IKKα appears to be important for epidermal differentiation and skeletal morphogenesis (41).

In the present study, we found that LA inhibits NF-κB activation and adhesion molecule expression in human aortic endothelial cells. The effective LA concentrations (≥50 μmol/l) were within the clinically relevant range. Pharmacokinetic studies of LA have shown that after a single oral dose of 10 mg/kg body weight, plasma concentrations can reach 60–70 μmol/l; higher concentrations can be achieved by intravenous administration (42, 43). Our data demonstrate that LA effectively inhibits TNF-α-stimulated mRNA and protein synthesis of cellular adhesion molecules (particularly E-selectin and VCAM-1) and consequent monocyte adhesion. LA inhibited IKK activity and nuclear translocation of NF-κB. The latter finding confirms earlier studies using Jurkat T cells and endothelial cells (42, 44).

Roy et al. (42) reported that LA inhibits VCAM-1 and ICAM-1 protein expression in human endothelial cells by a mechanism that appears to be largely independent of gene transcription, although NF-κB activation was inhibited by LA (42). These results are difficult to explain and differ from ours with respect to adhesion molecule gene transcription, which was inhibited by LA (see Fig. 3). These apparent discrepancies may be explained in part by the use of different cell types, i.e., primary human aortic endothelial cells (our study) vs. the transformed human endothelial cell line ECV304 (42).

Our data that LA inhibits transcriptional activation of adhesion molecules could indicate that LA inhibits binding of NF-κB to the upstream regulatory promoter sequences of these genes. However, the effects of LA on adhesion molecule expression were closely paralleled by inhibition of IKK activity and IκB degradation, and the LA doses required for half-maximal inhibition of these processes and NF-κB activation were remarkably similar, i.e., 100–200 μmol/l LA. Therefore, our data strongly suggest that LA inhibits TNF-α-induced endothelial activation by affecting the NF-κB/IκB signaling pathway at the level (or upstream) of IKK rather than

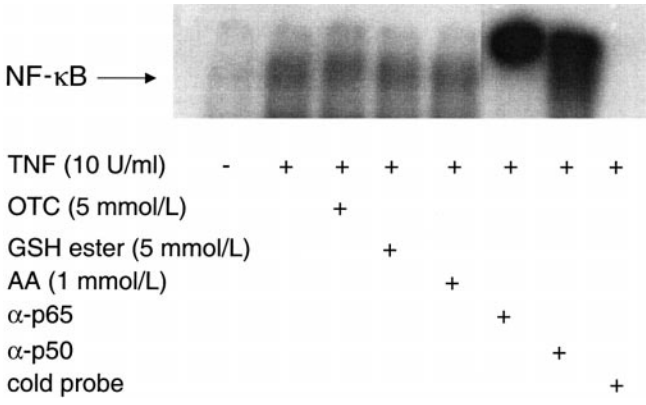

Figure 8. Ascorbic acid and manipulation of cellular glutathione status do not inhibit TNF-α-induced NF-κB activation in human aortic endothelial cells. Confluent HAEC were preincubated with the indicated concentrations of OTC or GSH monoethyl ester for 24 h, or ascorbic acid for 16 h. Subsequently, the cells were washed twice with M199 medium and coincubated with the same concentrations of OTC, GSH ester, or ascorbic acid plus TNF-α (10 U/ml) for 1 h. Isolation of nuclear extracts and EMSA were performed as described in Materials and Methods. Data shown are representative of two independent experiments. α-p65 and α-p50, antibodies to the p65 and p50 subunits of NF-κB, respectively.

by preventing DNA binding of NF-κB (45). Our conclusion is further supported by observations that LA also inhibited PMA-induced adhesion molecule expression in HAEC (this study) and NF-κB activation in other cells (42, 44) and that LA did not affect expression of TNF-R1.

NF-κB has been proposed to be a redox-sensitive transcription factor (37, 46). In most cell types, NF-κB can be activated by a diverse range of stimuli (14, 47), suggesting that several signaling pathways are involved. Some studies have suggested that reactive oxygen species play a role in the signaling events leading to NF-κB activation (46). This notion is supported by the observation that H_2O_2 is capable of activating NF-κB in some cell types, e.g., HeLa cells and subclone JR of Jurkat T cells, also known as Wurzburg cells (48). Furthermore, chemically unrelated compounds with antioxidant activity can inhibit NF-κB activation by various stimuli (17, 37, 48–52).

However, more recent data have questioned the hypothesis that reactive oxygen species are the universal second messengers for NF-κB activation, and suggest that this mechanism may be restricted to certain stimuli and certain cell types (53–59). In these studies, NF-κB could not be activated by H_2O_2 in human umbilical vein endothelial cells (HUVEC), human aortic smooth muscle cells, the human epithelial carcinoma cell line KB, or a murine thymoma line EL4.NOB; NF-κB activation by interleukin 1β (IL-1β), TNF-α, and PMA could not be inhibited by NAC or overexpression of catalase. Additional studies showed that stimulation with TNF-α or IL 1β did not increase H_2O_2 concentrations in HUVEC and bovine aortic endothelial cells (56, 60), and up to 40 mmol/l NAC was unable to inhibit cytokine-induced NF-κB activation and E-selectin expression in HUVEC (56, 61). In agreement with these observations, our data show that neither OTC, NAC, nor ascorbic acid can inhibit TNF-α-induced adhesion molecule expression and NF-κB activation in HAEC. Taken together, these results suggest that neither reactive oxygen species, cellular GSH levels, nor redox status play a significant role in cytokine-induced activation of human endothelial cells.

Lipoic acid has been shown to increase cellular GSH levels, and its reduced form, DHLA, can act as an antioxidant (21–24) However, if LA does not inhibit NF-κB activation by increasing cellular GSH levels and/or acting as an antioxidant, what could be its mechanism of action? Lipoic acid is also an effective chelator of iron and, in particular, copper (25, 26). It has been suggested that the metal chelating rather than antioxidant, properties of pyrrolidine dithiocarbamate is responsible for its inhibitory effect on NF-κB activation (56). This notion is further supported by the observation that the iron chelators desferrioxamine and 2,2,6,6-tetramethylpiperidine-1-oxyl inhibit NF-κB activation (56). In other experiments, we have observed that desferrioxamine, but not iron-loaded desferrioxamine, and the copper-specific chelator neocuproine strongly inhibit adhesion molecule and MCP-1 expression in HAEC (W. J. Zhang and B. Frei, unpublished results). Therefore, we speculate that the inhibitory effects of LA on TNF-α-induced endothelial activation are due to metal chelation rather than a general antioxidant effect. This may also explain why pretreatment of HAEC for 48 h was required for LA to exert its maximal inhibitory effect, as chelation of intracellular metal ions and extrusion from the cells is a slow process.

Since our study has shown that LA inhibits NF-κB/IκB signaling, it would be expected that LA also affects NF-κB-dependent expression of many other inflammatory genes, such as IL-1 and IL-6, tissue factor, and TNF-α, in numerous cell types, e.g., lymphoid cells, monocytes, and endothelial cells. Thus, the observed anti-inflammatory action of LA in HAEC probably extends to many other important mediators of inflammation in a variety of cells and tissues. Understanding the mechanism(s) by which LA disrupts the NF-κB/IκB regulatory pathway will be important in identifying novel anti-inflammatory agents that are both specific and effective in inhibiting the initiation and progression of atherosclerosis and other inflammatory diseases. FJ

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