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α-Lipoic acid inhibits matrix metalloproteinase-9 expression by inhibiting NF-κB transcriptional activity

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Abbreviations: ALA, α-lipoic acid; AP-1, activator protein-1; MMP-9, matrix metalloproteinase-9; VSMC, vascular smooth muscle cell

Abstract

The migration of vascular smooth muscle cells (VSMCs) into the intima, an important step in injury-induced neointimal hyperplasia, requires the activation of nuclear factor-κB (NF-κB) and the consequent up-regulation of matrix metalloproteinase-9 (MMP-9). This study was undertaken to test for a possible effect of α-lipoic acid (ALA), a potent inhibitor of NF-κB, on MMP-9 expression. ALA inhibited high-glucose- and TNF-α-stimulated VSMC migrations *in vitro*. It also inhibited high-glucose- and TNF-α-induced increases in MMP-9 expression. The activity of MMP-9-promoter constructs with mutations in the NF-κB binding site was not inhibited by ALA, indicating an involvement of the NF-κB signaling pathway in the ALA-specific inhibition of MMP-9. These data suggest the possibility that ALA may be useful for the prevention of neointimal hyperplasia after angioplasty, by inhibiting the NF-κB/MMP-9 pathway, especially with hyperglycemia.

Keywords: hyperplasia; matrix metalloproteinase-9; muscle, smooth, vascular; NF-κB; thioctic acid; tunica intima; vascular smooth muscle cell

Introduction

The long-term success of percutaneous transluminal angioplasty has been limited by neointimal hyperplasia resulting from vascular injury. After arterial injury, vascular smooth muscle cells (VSMCs) proliferate, migrate, and form a neointima that later accumulates extracellular matrix. VSMC migration, which depends on an alteration of the proteolytic balance within the arterial wall toward matrix breakdown, is partly mediated by matrix metalloproteinases (MMPs). MMP-9 is indispensable for the degradation of type IV collagen, a major component of the basement membrane (Zucker *et al.*, 1993; Cho and Reidy, 2002). Recent experimental data have shown that hyperglycemic diabetes can induce altered expression of the genes encoding MMP-9 (Death *et al.*, 2003) and plasma levels and zymographic activities of MMP-9 are increased in diabetics with peripheral arterial disease as compared with non-diabetics (Signorelli *et al.*, 2005).

It has been hypothesized that an increase in oxidative stress plays a key role in the pathogenesis of atherosclerosis and vascular restenosis (Kunsch and Medford, 1999; Griendling and Fitzgerald, 2003), although whether antioxidant supplementation can prevent the development of these processes remains controversial (Waters *et al.*, 2002; Salonen *et al.*, 2003). Oxidative stress induces various cytokines, including tumor necrosis factor-α (TNF-α) (Guha *et al.*, 2000). The stimulation of vascular cells by TNF-α initiates a signaling cascade leading to increases in nuclear factor-κB (NF-κB)-dependent expression of various genes (Bourcier *et al.*, 1997; Dhawan *et al.*, 1997), which play pivotal roles in the formation of neointimal hyperplasia leading to restenosis following angioplasty (Yoshimura *et al.*, 2001). α-Lipoic acid (ALA, 1,2-dithiolane-3-pentanoic acid) is a naturally occurring antioxidant which inhibits TNF-α-induced NF-κB activation and acts as a direct free-radical scavenger (Packer *et al.*, 1995; Zhang and Frei, 2001). The promoter region of MMP-9 contains binding sites for NF-κB (Sato and Seiki, 1993) and MMP-9 expression has been shown to be regulated by NF-κB (Rajagopalan *et al.*, 1996; Bond *et al.*, 2001). Thus, we hypothesized that ALA

may inhibit MMP-9 expression by inhibiting NF-κB activation. In the present study, we examined the effects of ALA on the high-glucose- and TNF- α-induced increase in MMP-9 expression and the migration of VSMCs. The underlying mechanism was evaluated by studying the possible involvement of NF-κB signaling pathways in the inhibition of MMP-9 by ALA.

Materials and Methods

Cell culture

VSMCs were harvested from the thoracic aorta of adult male Sprague-Dawley (SD) rats using the explant method. All procedures complied with the institutional guidelines for animal research. Cells were cultured in DMEM (Gibco BRL, Grand Island, NY) containing 20% FBS (Gibco BRL). VSMC purity was determined by staining with smooth-muscle-specific α-actin monoclonal antibodies (Sigma, St Louis, MO). Cells from the third to fifth passage were used.

Cell migration assay

Confluent VSMCs were subjected to injury by preincubation in serum-free medium for 24 h and scraping the cell layer with a sterile single-edged razor blade. The dishes were washed twice with PBS and the cells were incubated in conditioned medium for 60 h and fixed. The distance that the cells had migrated across the edge of the wound was measured with a digitizer (WACOM. Co., Japan).

Gelatin substrate gel zymography

Zymography was carried out as described by Overall *et al.* (1989) with minor modifications. VSMCs were plated at 8×10^5 cells/35 mm^2 dish and incubated until they reached 80% confluence. The medium was then aspirated, the cells were serum-starved for 24 h, and fresh serum-free medium was added to each dish with or without ALA. Supernatants were collected after 24 h and proteins were subjected to SDS-PAGE in 8% polyacrylamide gels copolymerized with 1 mg/ml gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h at room temperature and then incubated for 24 h at 37°C in buffer containing 1 mmol/l$CaCl_2$ and 50 mmol/l Tris-HCl (pH 7.6). The gels were stained with Coomassie blue (0.25%) and proteolytic activity was detected as a clear zone.

Western blot analysis

VSMCs were plated at 8×10^5 cells/35 mm^2 dish and incubated until they reached 80% confluence. The cells were serum-starved for 24 h and incubated in the conditioned media. Supernatants were collected after 24 h and subjected to SDS-PAGE in 8% polyacrylamide gels and electrotransferred to PVDF membranes (Millipore Corporation, Bedford, MA). Detection was performed using an enhanced chemiluminescence Western blotting kit, following the manufacturer's instructions. Densitometric measurement was performed using the digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT).

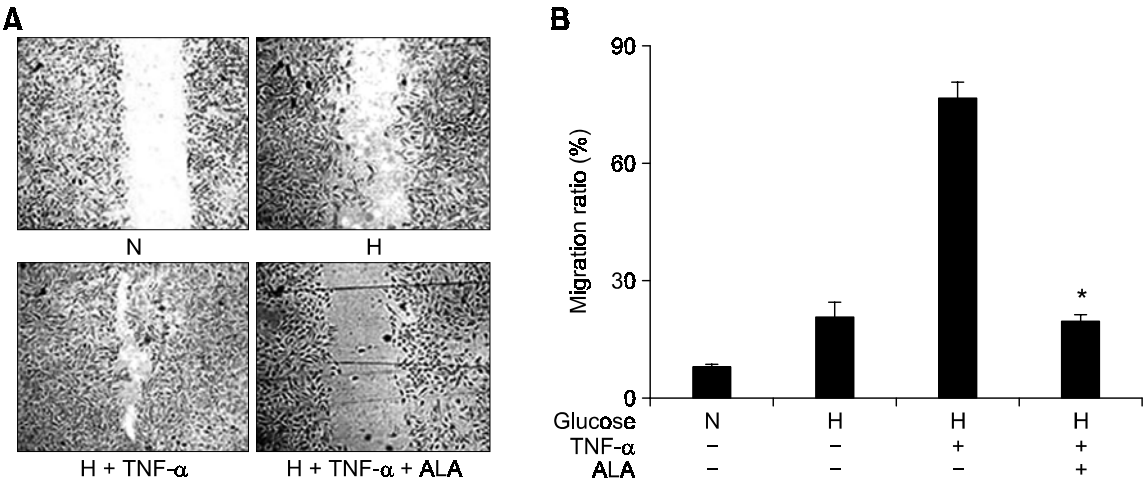

Figure 1. Inhibitory effect of ALA on migration of VSMCs stimulated by high glucose and TNF-α (10 ng/ml). (A) The effect of ALA on cell migration was assessed by the wounding migration assay. (B) The extent of cell migration was determined with a digitizer. N, normal D-glucose (5 mmol/l); H, high D-glucose (20 mmol/l). Data are expressed as the means ± SEMs of three independent experiments. *$P < 0.01$ *vs.* H+TNF-α.

Luciferase assay

Point mutations in the activator protein-1 (AP-1)- and NF-κB-binding sites in the MMP-9 promoter were generated as described previously (Woo *et al.*, 2003). NF-κB-luciferase (pNF-κB-Luc) and AP-1-luciferase (pAP1(PMA)-TA-Luc) constructs were purchased from Clontech (Palo Alto, CA). To analyze luciferase expression, cells were washed twice with PBS and lysed with 100 μl 1 × Reporter Lysis Buffer (Promega, Madison, WI). Each lysate (20 μl) was assayed for luciferase activity.

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts from VSMCs and EMSA were performed as described previously (Ahn *et al.*, 2001).

Statistical analysis

All results are expressed as means ± SEMs. Statistical comparisons were done with a subsequent Duncan's test for multiple comparisons. A probability value of < 0.05 was taken as statistically significant. All experiments were performed at least three times.

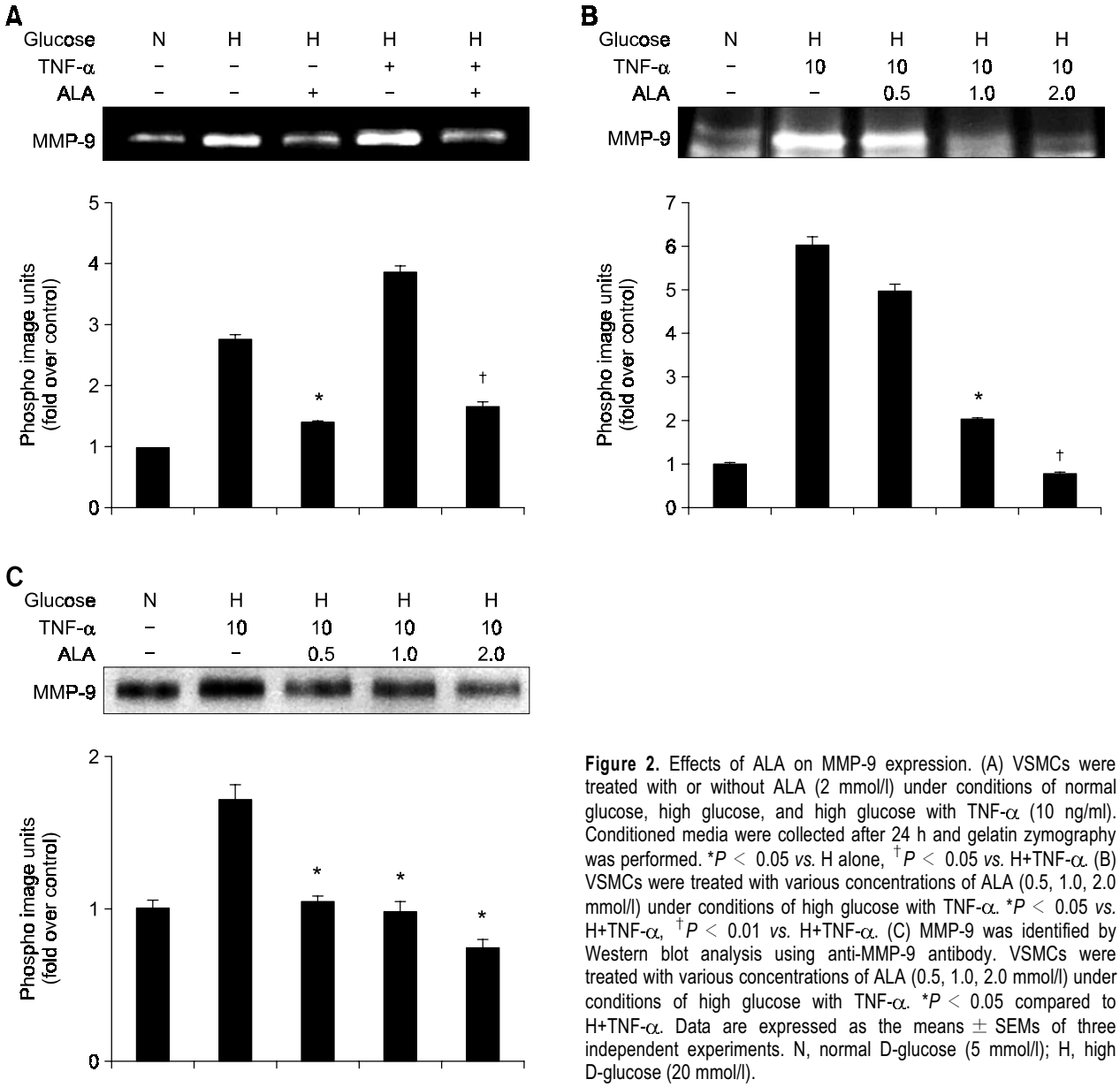

Figure 2. Effects of ALA on MMP-9 expression. (A) VSMCs were treated with or without ALA (2 mmol/l) under conditions of normal glucose, high glucose, and high glucose with TNF-α (10 ng/ml). Conditioned media were collected after 24 h and gelatin zymography was performed. $^{*}P < 0.05$ *vs.* H alone, $^{\dagger}P < 0.05$ *vs.* H+TNF-α. (B) VSMCs were treated with various concentrations of ALA (0.5, 1.0, 2.0 mmol/l) under conditions of high glucose with TNF-α. $^{*}P < 0.05$ *vs.* H+TNF-α, $^{\dagger}P < 0.01$ *vs.* H+TNF-α. (C) MMP-9 was identified by Western blot analysis using anti-MMP-9 antibody. VSMCs were treated with various concentrations of ALA (0.5, 1.0, 2.0 mmol/l) under conditions of high glucose with TNF-α. $^{*}P < 0.05$ compared to H+TNF-α. Data are expressed as the means ± SEMs of three independent experiments. N, normal D-glucose (5 mmol/l); H, high D-glucose (20 mmol/l).

Results

Effect of ALA on VSMC migration and MMP-9 expression *in vitro*

We first examined whether ALA could affect VSMC migration and MMP-9 expression induced by high-glucose and TNF-α stimulation. Figure 1 shows that high glucose (20 mmol/l) stimulated VSMC migration after linear wounding. Simultaneous treatment with high glucose and TNF-α (10 ng/ml) exacerbated VSMC migration. ALA (2 mmol/l) effectively inhibited these responses ($P < 0.01$). Similarly, high glucose and TNF-α significantly increased MMP-9 expression, especially in co-stimulated cells, and ALA decreased MMP-9 expression effectively (Figure 2A). High glucose- and TNF-α-induced MMP-9 expression was inhibited by ALA in a concentration-dependent manner (Figure 2B). Western blot analysis showed that the level of MMP-9 protein decreased after treatment with ALA (Figure 2C). These results suggest that vascular injury, which can induce TNF-α release, especially under high-blood-glucose conditions such as diabetes, may stimulate MMP-9 expression and that ALA can effectively suppress these responses.

Effect of ALA on MMP-9 transcription

MMP-9 promoter activity was examined to determine whether ALA regulates MMP-9 expression at the transcriptional level, using a luciferase reporter gene linked to the MMP-9 promoter sequence. As shown in Figure 3A, luciferase gene expression was activated by stimulation with high glucose and TNF-α, in a pattern similar to that obtained by zymography, and decreased in response to ALA. ALA decreased high-glucose- and TNF-α-induced promoter activity in a concentration-dependent manner (Figure 3B).

To further demonstrate a possible signaling pathway for the inhibition of MMP-9 by ALA, we performed assays using MMP-9-promoter constructs with mutations in the NF-κB- and AP-1-binding sites (Figure 4A). MMP-9 promoters with point mutations in the NF-κB- and AP-1-binding sites showed a diminished response to treatment with high glucose and TNF-α compared with the wild-type MMP-9 promoter (Figure 4B). The ALA-induced inhibition of high-glucose- and TNF-α-stimulated MMP-9 promoter activity was not observed with the NF-κB-mutated MMP-9 promoter (Figure 4B).

Effects of ALA on NF-κB activity

Experiments with reporter-gene constructs containing the NF-κB-binding site showed that treatment with ALA markedly attenuated luciferase gene expression activated by high glucose and TNF-α (Figure 5A). To determine whether ALA inhibited activation by NF-κB by preventing it from binding to DNA, the effect of ALA on NF-κB-DNA-binding activity was examined by EMSA. The amount of the NF-κB-DNA complex increased in conditioned media containing high glucose and TNF-α and ALA

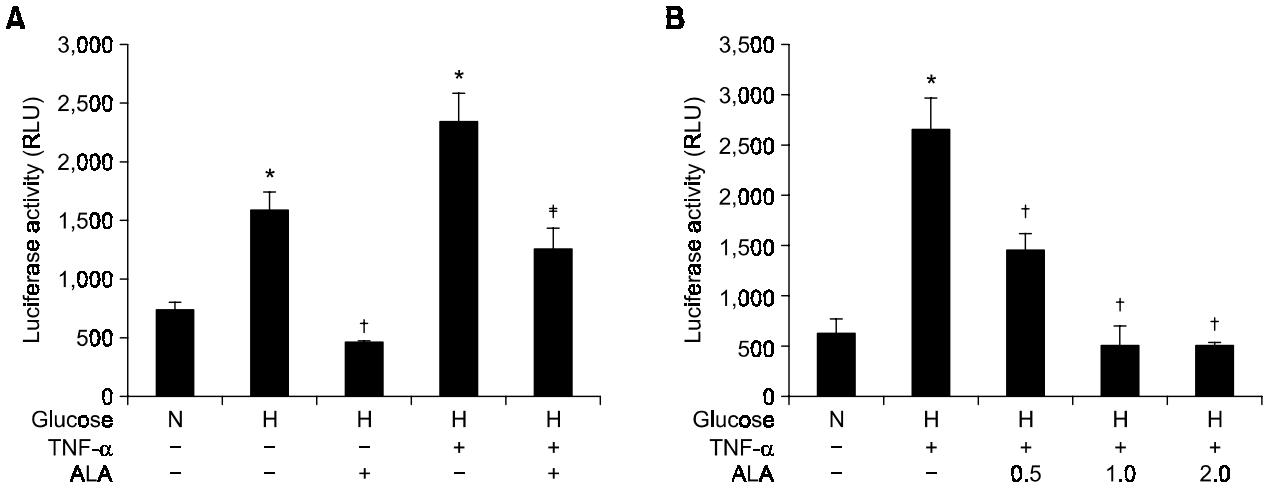

Figure 3. Effect of ALA on MMP-9 promotor activity. (A) Cells were transfected with a MMP-9-promotor-luciferase-reporter vector and treated with ALA (2 mmol/l) under conditions of normal glucose, high glucose, and high glucose with TNF-α (10 ng/ml). Luciferase activity was measured in cell lysates. *$P < 0.05$ *vs.* N, †$P < 0.05$ *vs.* H alone, ‡$P < 0.05$ *vs.* H+TNF-α. (B) Cells were transfected with a MMP-9-promotor-reporter vector and treated with various concentrations of ALA (0.5, 1.0, 2.0 mmol/l) in the absence or presence of high glucose and TNF-α. Luciferase activity was measured in cell lysates. *$P < 0.05$ *vs.* N, †$P < 0.05$ *vs.* H+TNF-α. Data are expressed as the means ± SEMs of three independent experiments. N, normal D-glucose (5 mmol/l); H, high D-glucose (20 mmol/l).

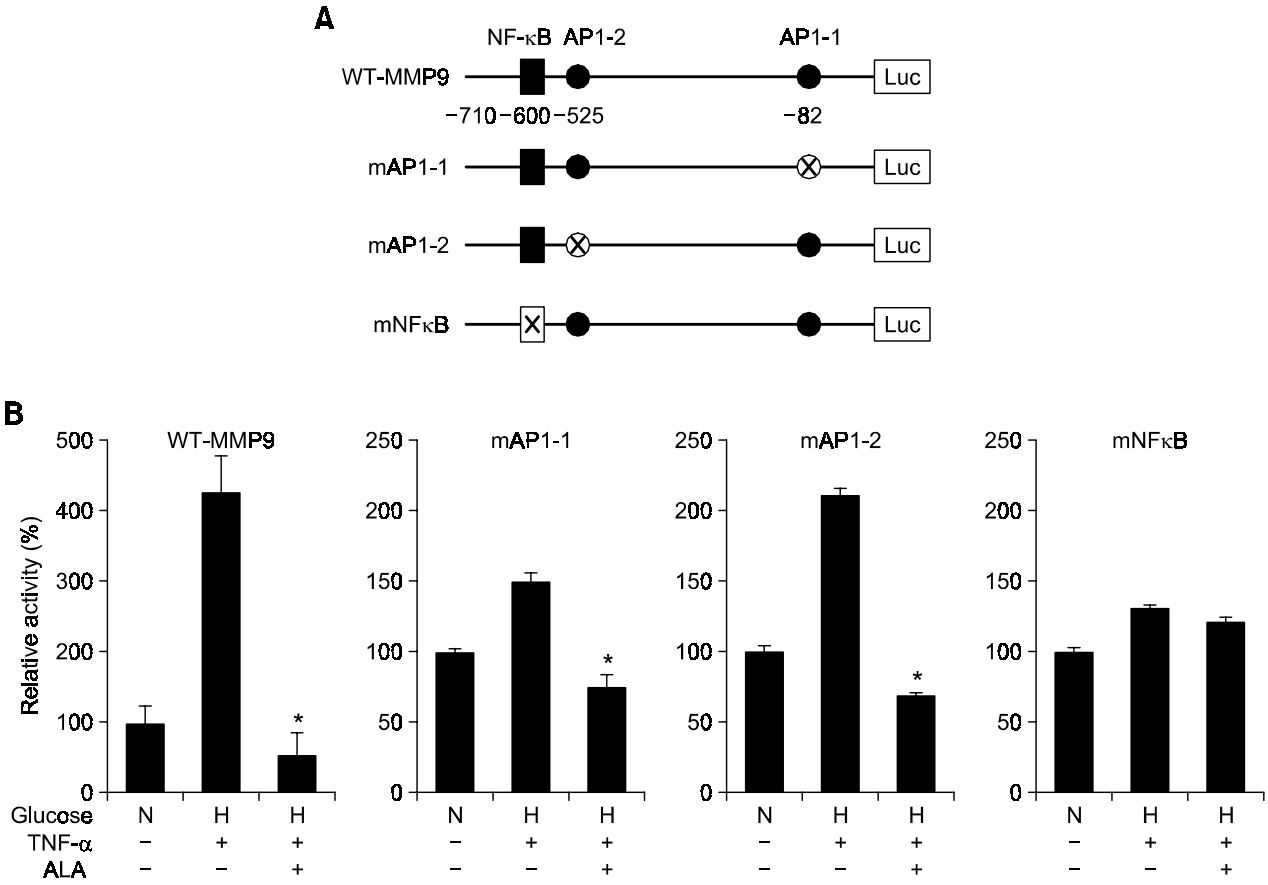

Figure 4. Effects of ALA on the activities of NF-κB and AP-1. (A) Schematic structures of the wild-type and mutant MMP-9-promoter constructs used for the promoter assays. (B) Cells were transfected with WT or mutant MMP-9 promoters and treated with ALA (2 mmol/l) in the presence of high glucose and TNF-α (10 ng/ml) for 24 h and luciferase activity was measured. Data are expressed as the means ± SEMs of three independent experiments. *$P < 0.05$ *vs.* H+TNF-α. N, normal D-glucose (5 mmol/l); H, high D-glucose (20 mmol/l); WT-MMP9, wild-type MMP-9 promoter; mAP1-1, mutant-AP1-1 MMP-9 promoter; mAP1-2, mutant-AP1-2 MMP-9 promoter; mNFκB, mutant- NF-κB MMP-9 promoter.

efficiently suppressed the increase in NF-κB-binding activity (Figure 5B).

Discussion

The principal findings of this study are: (a) ALA inhibits VSMC migration in the condition of high glucose and TNF-α; (b) ALA inhibits the high-glucose- and TNF-α-stimulated increases in MMP-9 promoter activity and expression; and (c) these effects are mediated by suppression of the NF-κB pathway.

Vascular injury causes an early up-regulation of MMP-9 (Bendeck *et al.*, 1994; Wilcox *et al.*, 2001). Cho and Reidy (2002) have demonstrated in mice lacking the MMP-9 gene that MMP-9 is critical for VSMC migration and the formation of neointima. Diabetes mellitus has been identified as an independent risk factor for the development of atherosclerosis and is considered a coronary-heart-disease risk equivalent. It is also a recognized independent risk factor for poor outcome after percutaneous transluminal coronary angioplasty for symptomatic coronary disease (Kip *et al.*, 1996; Holmes *et al.*, 1997; Kastrati *et al.*, 1997; Mathew *et al.*, 2004). TNF-α is increased in arterial smooth muscle cells following injury and has multiple biological effects, such as promoting the migration of VSMCs and inflammatory cells by a mechanism dependent on the activation of NF-κB (Wang *et al.*, 2001). In this study, we verify that high glucose increases the activities of NF-κB and MMP-9 in VSMCs and stimulates their migration. Under conditions of combined high glucose and TNF-α, these responses are exacerbated. Furthermore, TNF-α- and high-glucose-induced increases in luciferase activity are reduced in MMP-9 promoters with point mutations in the NF-κB- and AP-1-binding sites, as compared with the wild-type MMP-9 promoter. These results suggest that NF-κB and AP-1 are

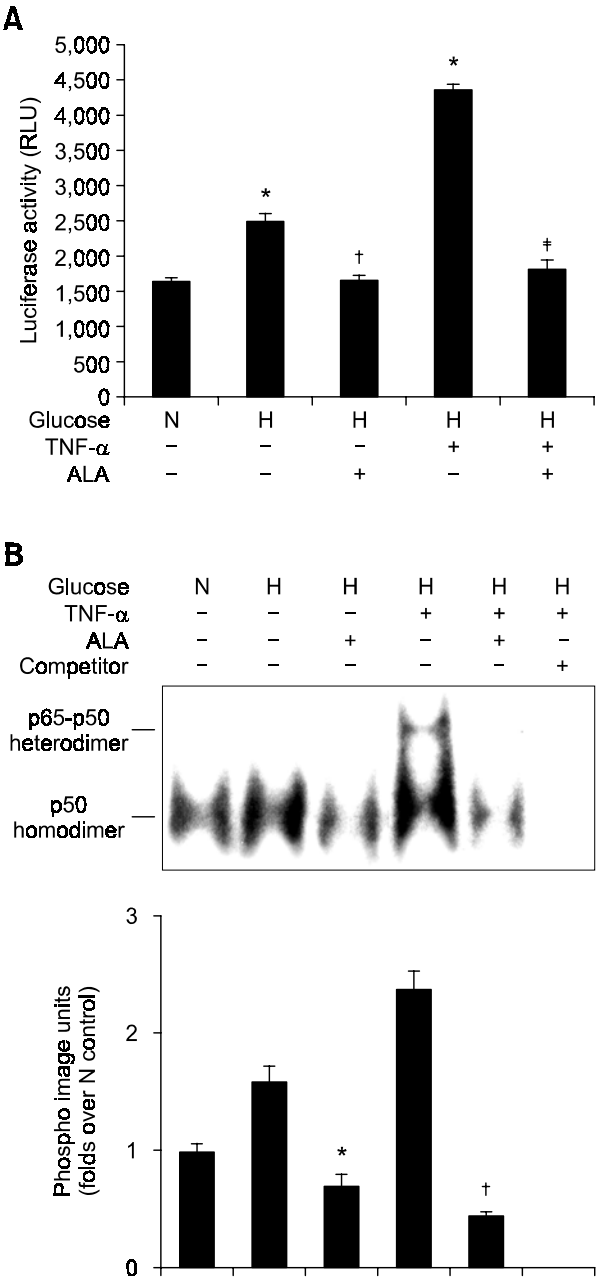

Figure 5. Effects of ALA on NF-κB activity. (A) Cells were transfected with a reporter vector containing NF-κB binding sites and treated with ALA (2 mmol/l) under conditions of high glucose and TNF-α (10 ng/ml). Luciferase activity was measured. *$P < 0.05$ *vs.* N, †$P < 0.05$ *vs.* H alone, ‡$P < 0.05$ *vs.* H+TNF-α. (B) A typical a gel-shift assay for VSMCs stimulated with high glucose and TNF-α, followed by treatment with ALA (2 mmol/l). N, normal D-glucose (5 mmol/l); H, high D-glucose (20 mmol/l).

crucial transactivators for the expression of MMP-9 induced by TNF-α and high glucose.

ALA is considered an ideal antioxidant since it possesses many beneficial characteristics, including specific free-radical quenching, metal-chelating activity, interaction with other antioxidants, and suppressive effects on redox-sensitive gene expression (Bast and Haenen, 1988; Ou *et al.*, 1995; Packer, 1998). ALA is widely used in pathological conditions associated with increased oxidative stress, including diabetic neuropathy (Ziegler *et al.*, 1995). In the present study, we have found that ALA inhibits high-glucose- and TNF-α-stimulated increases in MMP-9. It has been shown that ALA supplementation prevents angiotensin II-induced renal injury by reducing the activity of NF-κB and AP-1 (Mervaala *et al.*, 2003). Therefore, we have focused on the role of the NF-κB and AP-1 pathways in the ALA-induced decrease in MMP-9 in VSMCs. We find that ALA suppresses NF-κB promoter activity in a concentration-dependent manner, but that it has no effect on AP-1 promoter activity (data not shown). ALA also attenuates the DNA-binding activity of NF-κB. More importantly, ALA-mediated inhibition is present with the AP-1-mutated MMP-9 promoters, but not with the NF-κB-mutated MMP-9 promoter. These results suggest that ALA reduces MMP-9 expression by inhibiting the NF-κB pathway, but not the AP-1 pathway.

Further experiments were performed to clarify the mechanism by which ALA controls NF-κB transcriptional activation. It has been reported that NF-κB translocates from the cytoplasm into the nucleus and binds specifically to the NF-κB-target DNA sequence (Baldwin, 1996). After specific binding, transcriptional activation by NF-κB is regulated through phosphorylation of p65 (Wang and Baldwin, 1998). We have shown by EMSA that ALA attenuates the DNA-binding activity of NF-κB. This result indicates that ALA blocks MMP-9 expression, at least in part, by decreasing DNA-binding by NF-κB.

In summary, we demonstrate that ALA can suppress VSMC migration and inhibits MMP-9 expression by suppressing the NF-κB pathway under conditions of elevated TNF-α and glucose levels. The inhibition of MMP-9 may interfere with the ability of VSMCs to cross the endothelial basement membrane and therefore ALA may be useful in the prevention of vascular restenosis after angioplasty.

Acknowledgement

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