Biochemical and Biophysical Research Communications 457 (2015) 101–105

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Glatiramer acetate (GA) prevents TNF-α-induced monocyte adhesion to primary endothelial cells through interfering with the NF-κB pathway

Guoqian Wei, Xueyan Zhang, Zhendong Su, Xueqi Li *

Department of Cardiology, The Fourth Affiliated Hospital of Harbin Medical University, China

ARTICLE INFO

Article history:
Received 9 December 2014
Available online 19 December 2014

Keywords:
Glatiramer acetate
Tumor necrosis factor-alpha (TNF-α)
Endothelial dysfunction
Monocytes adhesion
NF-κB

ABSTRACT

Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) is considered to be the major one contributing to the process of development of endothelial dysfunction. Exposure to TNF-α induces the expression of a number of proinflammatory chemokines, such as monocyte chemotactic protein-1 (MCP-1), and adhesion molecules, including vascular adhesion molecule-1 (VCAM-1) and E-selectin, which mediate the interaction of invading monocytes with vascular endothelial cells. Glatiramer acetate (GA) is a licensed clinical drug for treating patients suffering from multiple sclerosis (MS). The effects of GA in vascular disease have not shown before. In this study, we found that GA significantly inhibited TNF-α-induced binding of monocytes to endothelial cells. Mechanistically, we found that GA ameliorated the upregulation of MCP-1, VCAM-1, and E-selectin induced by TNF-α. Notably, this process is mediated by inhibiting the nuclear translocation and activation of NF-κB. Our results also indicate that GA pretreatment attenuates the up-regulation of COX-2 and iNOS. These data suggest that GA might have a potential benefit in therapeutic endothelial dysfunction related diseases.

1. Introduction

The recruitment of inflammatory cells from the circulation to endothelial cells (ECs) is involved in the initiation of atherosclerosis [1]. This process is mainly mediated by the upregulation of a number of proinflammatory chemokines, such as monocyte chemotactic protein-1 (MCP-1), and adhesion molecules, including vascular adhesion molecule-1 (VCAM-1) and E-selectin. These chemokines and adhesion molecules are essential for the firm adhesion of monocytes to activated ECs [2–4]. Several studies have demonstrated that the inflammatory cytokine tumor necrosis factor-α (TNF-α), a pleiotropic proinflammatory cytokine, plays a critical role in the disruption of vascular function and the subsequent development of vascular disease. TNF-α is critically involved in the pathogenesis of atherosclerosis by mediating the interaction of invading monocytes with vascular ECs in triggering extracellular matrix deposition in aortic vessels [5]. TNF-α has been reported to trigger several intracellular signaling events that ultimately up-regulate the expression of MCP-1, VCAM-1, and E-selectin [6]. Importantly, the activation of nuclear factor (NF)-κB is essential for the transcriptional regulation of these factors induced by proinflammatory cytokines, such as TNF-α [7]. Since endothelial inflammation is essential in the development of atherosclerosis, agents that can attenuate TNF-α-induced NF-κB activation in ECs could be a novel molecular target to prevent vascular endothelial dysfunction.

Glatiramer acetate (GA) (also known as Cop-1 and Copaxone), a synthetic polymer of random sequences of four naturally occurring amino acids (L-tyrosine, L-glutamate, L-alanine, and L-lysine), is a drug approved for the treatment of multiple sclerosis (MS). It has been demonstrated that GA suppresses experimental allergic encephalomyelitis (EAE) [8,9].

GA treatment in dendritic cells and monocytes causes them to preferentially stimulate protective anti-inflammatory responses. Notably, Vieira and colleagues have demonstrated that dendritic cells from GA-treated MS patients produced less TNF-α compared to those of untreated patients [10]. In addition, GA induces a broad inhibitory effect on monocyte reactivity and promotes the development of anti-inflammatory type II monocytes characterized by increased secretion of IL-10 and TGF-β, as well as by decreased production of IL-12 and TNF-α, thereby transferring protection from EAE [11,12]. However, little information with regarding to the protective effects of GA in endothelial functions has been reported before. In this study, we report that GA inhibits the expression of endothelial adhesion molecules and attenuates the adhesion of THP-1 cells to the surface of endothelial cells.

* Corresponding author at: Department of Cardiology, The Fourth Affiliated Hospital of Harbin Medical University, No. 37 Yi Yuan Street, Nan Gang District, Harbin, Hei Long Jiang Province, China. Fax: +86 045183180162.
E-mail address: xueqili075@yeah.net (X. Li).

http://dx.doi.org/10.1016/j.bbrc.2014.12.070
0006-291X/

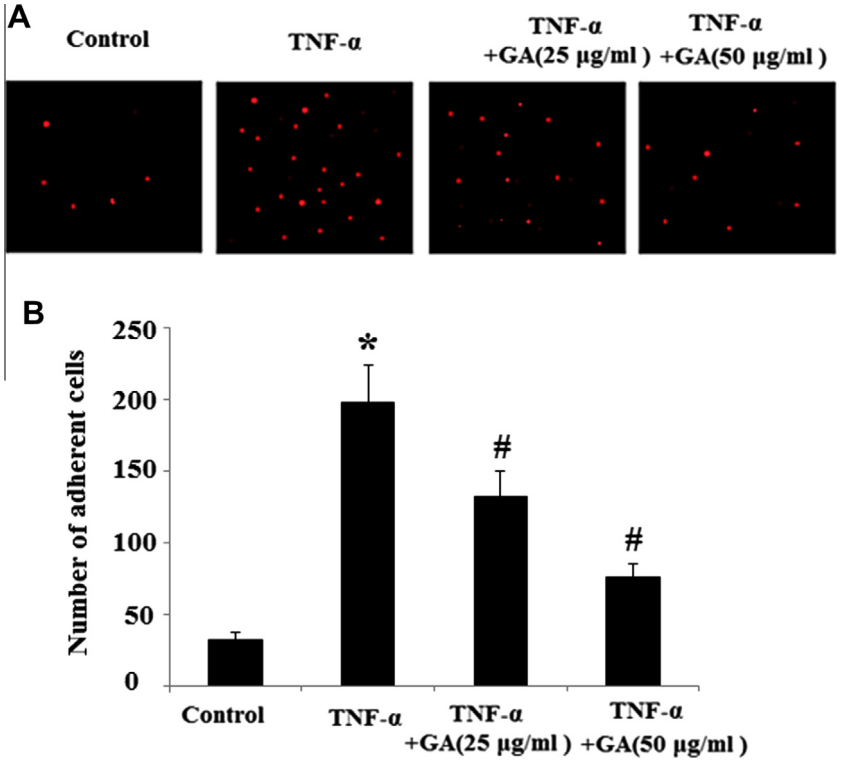

Fig. 1. Glatiramer acetate (GA) inhibited TNF-α-stimulated monocyte adhesion to HUVECs. (A) GA inhibited TNF-α-induced monocyte adhesion to ECs. HUVECs were pretreated with various concentrations of GA for 12 h before the addition of TNF-α (5 ng/ml) for 6 h. THP-1 cells were labeled with a fluorescence probe. Images were captured using a florescence microscope. (B) Quantitative analysis ($^{*}P < 0.01$; $^{\#}P < 0.01$).

2. Materials and methods

2.1. Cell culture and treatment

Primary human umbilical vein endothelial cells (HUVECs), obtained from Lonza, were maintained in endothelial growth supplement EGM-2 Single Quot Kit endothelial growth supplements EGM2 medium (Lonza, MD, USA) at 37 °C in a 5% CO_2/95% air environment. THP-1 cells were cultured in RPMI-1640 medium containing 10% FBS. HUVECs were treated with various concentrations of GA for 12 h, followed by incubated with 5 ng/ml TNF-α for another 6 h.

2.2. Real-time polymerase chain reaction (PCR)

After indicated treatments, total RNA from HUVECs was isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. 2 μg of cellular total RNA was then used as template for reverse transcription PCR to synthesize cDNA. The synthesized cDNA was used in quantitative real-time PCR analysis using SYBR Green qPCR Master Mix (Roche). The target mRNA level was normalized to the level of GAPDH and compared with the control.

2.3. Monocyte adhesion assay

The determination of monocyte adhesion to ECs was conducted using THP-1 cells. Briefly, HUVECs were grown to confluence in 24-well plates and treated with 25 μg/ml or 50 μg/ml GA for 12 h before the addition of 5 ng/ml human recombinant TNF-α for another 6 h. HUVECs were then gently washed with serum-free medium, and calcein-AM labeled THP-1 cells (1×10^6/ml RPMI-1640 medium containing 1% FBS) were then added to HUVECs. The ratio of the labeled THP-1 cells and the HUVEC monolayer was 4:1 (monocytes to ECs). After 1 h incubation, unbound monocytes were washed away by using EC medium. The adhered monocytes were determined by using a fluorescence microscope.

2.4. Western blot analysis

HUVECs were harvested with cell lysis buffer (Cell signaling, USA). The protein lysates were separated by SDS-polyacrylamide gel electrophoresis using 10% gels and transferred to PVDF membranes [13]. Transferred membranes were blocked with 5% fat free milk in tris buffered saline with tween 20 (TBST) for 1 h then incubated with primary antibody against MCP-1, VCAM-1, E-selectin, iNOS, and IκBα (1:1000; Santa Cruz Biotechnology, USA) at 4 °C overnight. Horseradish peroxidase-conjugated secondary antibody was then applied and the blots were developed using enhanced chemiluminescence according to the manufacturer's protocol (Santa Cruz Biotechnology, USA). Images were scanned and band intensities were analyzed by using the Image J software (National Institute of Health, USA).

2.5. Luciferase reporter assays

HUVECs were transfected with pNF-κB-Luc using Lipofectamine 2000 (Invitrogen). After treated with GA and TNF-α, cells were lysed and luciferase activity was determined by use of a dual luciferase kit (Promega, USA) and TD-20/20 luminometer (TurnerDesigns, USA). Luciferase activity was normalized to the Renilla luciferase activity, which served as an internal control for transfection efficiency.

2.6. Determination of NF-κB p65 nuclear translocation

After indicated treatment, cells were washed for 3 times with PBS and fixed with 4% paraformaldehyde for 10 min at room tem-

perature (RT). After permeabilized with 0.4% Triton X-100 for 15 min on ice, cells were then blocked with 10% normal goat serum (Sigma) for 30 min at room temperature. The cells were then incubated with rabbit anti-NF-κB p65 primary antibody for 2 h at 4 °C and washed with PBS three times followed by incubation with Alexa-594 conjugated secondary antibodies for 1 h at RT. The chamber slides were then washed with PBS for 3 times and mounted with DAPI mounting medium (Sigma, USA). NF-κB p65 was visualized with fluorescence microscopy (Waltham, USA).

2.7. Boyden chamber assay

The effects of GA on THP-1 cell migration were analyzed with a modified Boyden chamber assay. Briefly, HUVECs (1×10^5 cells/ml) were seeded on a 24-transwell plate (Costar, USA) and allowed to adhere to the plate for 24 h at 37 °C. The upper chamber contains a polycarbonate filter with 8 μm pores (Nucleopore, USA). HUVECs were exposed to TNF-α (10 ng/ml) in the presence or absence of GA (25 or 50 μg/ml) for 16 h, centrifuged at 10,000×*g* for 10 min and the supernatants were harvested and used as the chemoattractant in the lower chamber. The upper wells were filled with THP-1 cells at a concentration of 1×10^6 cells/ml and incubated for 2 h at 37 °C. Cells that had actively migrated through the membrane were fixed by using 100% ethanol for 5 min and stained with crystal violet (Sigma Aldrich). Transmigration was counted in three random fields at a magnification of ×200.

2.8. Statistical analysis

All data were subjected to analysis of variance using SPSS software. Experimental data are expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to determine statistical significance. $P < 0.05$ was considered as the minimum level of statistical significance.

3. Results

Since cytokines-induced mononuclear cell adhesion to ECs is an important step in the development of atherosclerosis, we determined if GA has a regulatory effect on the adhesion of monocytes to ECs. Exposure of ECs to 5 ng/ml TNF-α for 6 h significantly increased adhesion of monocytes to EC under static condition. Pretreatment with GA at a concentration of 25 μg/ml and 50 μg/ml significantly inhibited TNF-α-induced binding of monocytes to ECs (Fig. 1A and B). The supernatant of HUVECs exposed to 10 μg/ml of TNF-α significantly increased the number of THP-1 monocytes that passed through the transwell microporous membrane, compared with the control (Fig. Suppl. 1). However, cotreatment with GA significantly inhibits the migration of THP-1 in a dose dependent manner from 25 μg/ml to 50 μg/ml.

Adhesion of monocytes to ECs is critically mediated by both chemotactic cytokines and vascular adhesion molecules. Therefore, we assessed mRNA levels and protein abundance of MCP-1, VCAM-1 and E-selectin. As shown in Fig. 2A, treatment of HUVECs with 5 ng/ml TNF-α strongly induced MCP-1, VCAM-1 and E-selectin. In contrast, pretreatment with GA strongly inhibited the induction of MCP-1, VCAM-1, and E-selectin. Consistent with these observations, western blot analysis revealed that pretreatment with GA inhibited MCP-1, VCAM-1 and E-selectin protein expression induced by TNF-α (Fig. 2B).

Multiple lines of evidence have demonstrated that the activation of NF-κB plays a critical role in the transcriptional regulation of chemotactic cytokines and vascular adhesion molecules that are involved in leukocyte adhesion to endothelium [14]. NF-κB exists in the cytoplasm in the forms of heterodimer containing p50 and p65 subunits. NF-κB proteins are retained in the cytoplasm by a family of inhibitory proteins referred to as IκBα. Stress stimulation such as inflammatory cytokines induces phosphorylation and subsequent degradation of IκBα, thereby leading to the liberation of NF-κB heterodimers, which can then translocate to the nucleus, bind specific DNA sequences, and affect target gene expression. The inhibitory effect of GA was verified by confocal microscopic examination of NF-κB p65 nuclear translocation, which showed that TNF-α induced p65 nuclear translocation was blocked by pretreatment with GA (Fig. 3A). Notably, NF-κB-Luc reporter experiment displayed that TNF-α drastically induced NF-κB luciferase activity. This induction was markedly suppressed by pretreatment with GA, thereby confirming the inhibition effect of GA in NF-κB activation (Fig. 3B). NF-κB activation is controlled by its specific inhibitor protein family IκB. IκB phosphorylation and subsequent degradation result in nuclear translocation and activation of NF-κB. Here, our results indicate that the phosphorylation level of

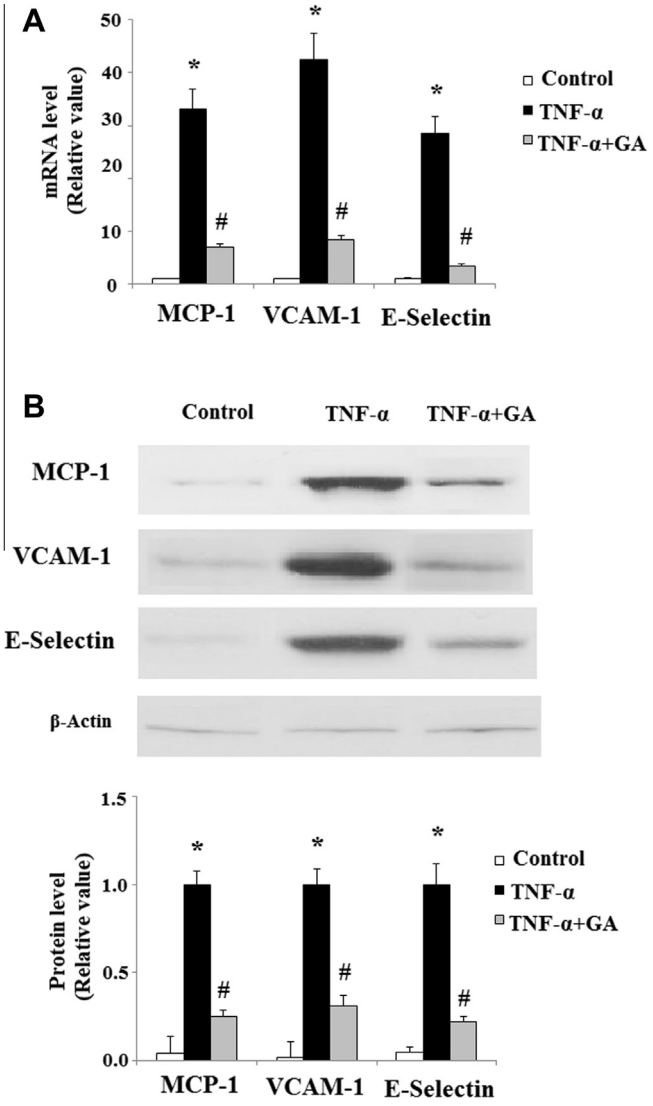

Fig. 2. Glatiramer acetate (GA) suppressed the production of MCP-1, VCAM-1, and E-selectin. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The target mRNA level was normalized to the level of GAPDH using the $_{\Delta\Delta}$Ct method. The results were compared with untreated control, which was defined as 1. (A) mRNA levels of MCP-1, VCAM-1, and E-selectin determined by real time PCR analysis. (B) Protein levels of MCP-1, VCAM-1, and E-selectin determined by western blot analysis ($^{*}P < 0.01$; $^{\#}P < 0.01$).

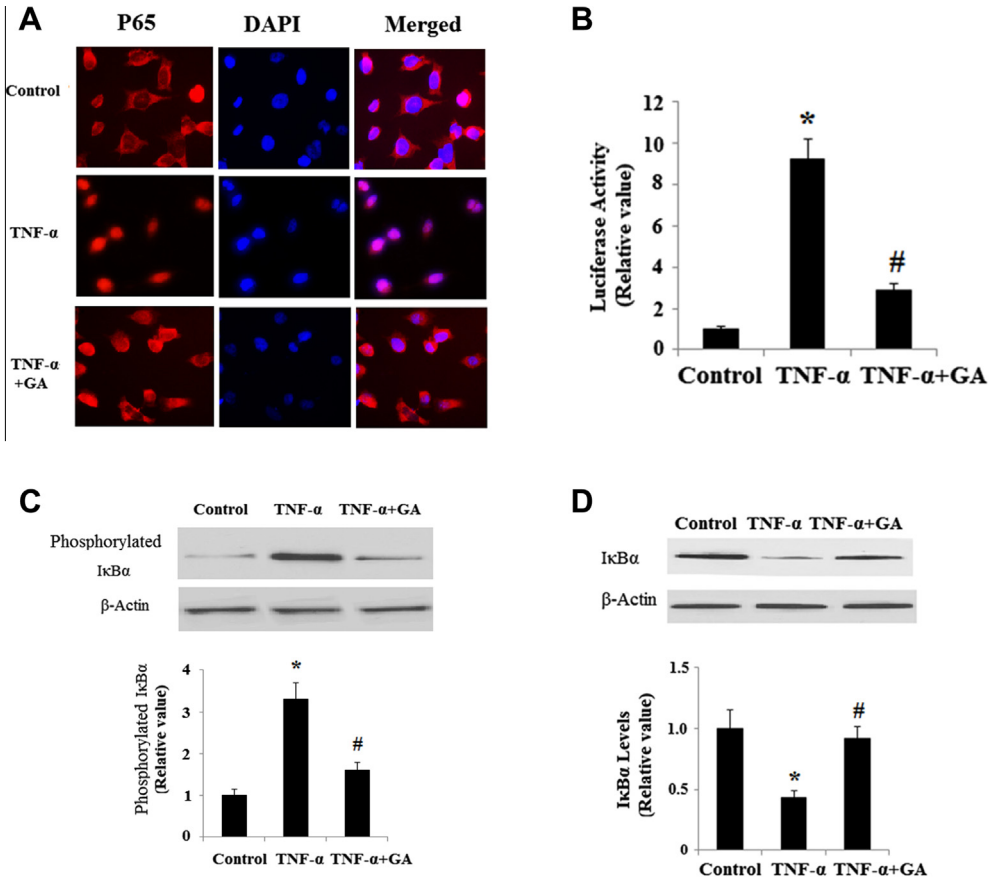

Fig. 3. Glatiramer acetate (GA) suppressed the activation of NF-κB. (A) Effects of GA on p65 nuclear translocation. HUVECs were pretreated with 25 μg/ml GA for 12 h before the addition of TNF-α (5 ng/ml) for 6 h. The cells were immunostaining with anti-p65 antibodies. (B) NF-κB luciferase reporter assays. HUVECs transfected with pNF-κB-Luc reporter were treated with 25 μg/ml GA for 12 h before the addition of TNF-α (5 ng/ml) for 6 h. luciferase activity was determined by a luminescence assay. (C) Western blot and quantification analysis revealed that the increased phosphorylation of IκBα as induced by the administration of TNF-α was attenuated by GA ($^{*}P < 0.01$; $^{\#}P < 0.01$); (D) Western blot and quantification analysis revealed that the reduction of IκBα as induced by the administration of TNF-α was attenuated by GA ($^{*}P < 0.01$; $^{\#}P < 0.01$).

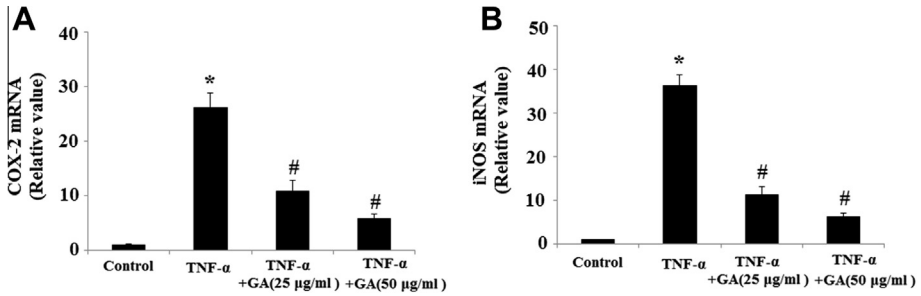

Fig. 4. Inhibitory effects of Glatiramer acetate (GA) on TNF-α-induced induction of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The target mRNA level was normalized to the level of GAPDH using the $_{\Delta\Delta}$Ct method. The results were compared with untreated control, which was defined as 1. (A) mRNA levels of COX-2 and iNOS were determined by real time PCR analysis. (B) Protein levels of COX-2 and iNOS were determined by western blot analysis ($^{*}P < 0.01$; $^{\#}P < 0.01$).

IκBα was significantly increased by TNF-α treatment (Fig. 3C). However, GA treatment significantly attenuates TNF-α-induced phosphorylation of IκBα in HUVECs. Furthermore, western blot analysis showed that TNF-α-induced IκBα degradation was inhibited in GA-treated cells (Fig. 3D).

Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are involved in the regulation of homeostatic and inflammatory processes. Both COX-2 (Fig. 4A) and iNOS (Fig. 4B) are target genes of NF-κB. And our results indicate that GA pretreatment attenuates the upregulation of COX-2 and iNOS. These results suggest that GA may inhibit inflammation through suppressing NF-κB signaling.

4. Discussion

Recruitment of immune cells to the surface of the activated endothelial cells has been found to be involved in certain chronic vascular inflammatory diseases. Inflammatory cytokines such as TNF-α and IL-1β are responsible for the activation of endothelial cells, a condition characterized by the expression of proinflammatory chemokine MCP-1 and adhesion molecules VCAM-1 and E-selectin, which promote the attachment of circulating monocytes to the endothelium [15]. In this study, we report that GA can possibly attenuate monocyte adhesion to endothelial cells caused by TNF-α. In the presence of TNF-α, there is an increase in the expres-

sion of MCP-1, VCAM-1 and E-selectin, which is prevented by pretreatment with GA. Importantly, pretreatment with GA decreases the transcriptional activity of NF-κB. These findings suggest that GA has advantages in its pleiotropic vascular protective effects.

As a clinical drug applied in the treatment in multiple sclerosis, the primary mechanism for GA activity has been attributed to its ability to skew T-cell response from the pro-inflammatory to the anti-inflammatory pathway. Several studies have demonstrated a shift from a pro-inflammatory Th1-biased cytokine profile toward anti-inflammatory Th2-biased profile in GA-treated MS patients [16,17]. Importantly, GA has been associated with the inhibition of NF-κB activation. Li and colleagues have found that GA may inhibit IL-1β-stimulated RANTES expression in human glial cells by blocking NF-κB activation, thus identifying part of the molecular basis for its anti-inflammatory and immunosuppressive effects in demyelinating diseases [18]. Furthermore, another study has reported that the increase in NF-κB binding activity induced by TNF-α is prevented by pretreatment with GA or the NF-κB inhibitors, suggesting that GA might exert its therapeutic effect in MS partially through inhibiting NF-κB activation and chemokine production [19]. An early proinflammatory event occurs in the cytoplasm which leads to IκB phosphorylation. This phosphorylation results in the degradation of IκB, thus allowing NF-κB translocation to the nucleus. NF-κB has been considered a prototypical proinflammatory signaling pathway, and the role of NF-κB in the expression of other proinflammatory genes including cytokines, chemokines, and adhesion molecules, which has been extensively studied. In this study, our results also demonstrated that GA could attenuate the expression of COX2 and iNOS, two important NF-κB downstream proteins. Importantly, a clinical study demonstrated that treatment with GA results in a decreased surface expression of the linked to the pro-migratory activity of peripheral blood mononucleated cells (PBMCs). Particularly, GA induced an overall decline of ICAM-1 and ICAM-3 expression in the majority of the immune cell subsets (B cells, CD8+ T cells and monocytes) studied [20], which is consistent with our findings in this study. Our study is also supported by studies from Kim and co-workers, which revealed that GA treatment suppresses the inflammatory potential of T cells and subsequently inflammatory responses at the blood brain barrier (BBB) [21].

Taken together, our findings suggest that GA may help protect endothelial cells against inflammatory factors. However, the underlying mechanisms associated with GA's inhibition of NF-κB activity are still unknown. Further studies will provide a complete picture of the potential benefits of GA in endothelial inflammation and vascular physiology and pathology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.070.

References

[1] A. Zernecke, C. Weber, Chemokines in atherosclerosis: proceedings resumed, Arterioscler. Thromb. Vasc. Biol. 34 (2014) 742–750.
[2] K. Mayer, M. Merfels, M. Muhly-Reinholz, et al., Omega-3 fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation, Am. J. Physiol. Heart Circ. Physiol. 283 (2002) H811–H818.
[3] J.W. Chen, Y.H. Chen, F.Y. Lin, et al., Ginkgo biloba extract inhibits tumor necrosis factor-alpha-induced reactive oxygen species generation, transcription factor activation, and cell adhesion molecule expression in human aortic endothelial cells, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1559–1566.
[4] M.A. Carluccio, L. Siculella, M.A. Ancora, et al., Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 622–629.
[5] W.T. Gerthoffer, Mechanisms of vascular smooth muscle cell migration, Circ. Res. 100 (2007) 607–621.
[6] T.K. Mukherjee, L. Nathan, H. Dinh, et al., 17-epiestriol, an estrogen metabolite, is more potent than estradiol in inhibiting vascular cell adhesion molecule 1 (VCAM-1) mRNA expression, J. Biol. Chem. 278 (2003) 11746–11752.
[7] E.M. Boyle Jr., J.C. Kovacich, T.G. Canty Jr., et al., Inhibition of nuclear factor-kappa B nuclear localization reduces human E-selectin expression and the systemic inflammatory response, Circulation 98 (1998) II282–II288.
[8] D. Teitelbaum, M. Fridkis-Hareli, R. Arnon, M. Sela, Copolymer 1 inhibits chronic relapsing experimental allergic encephalomyelitis induced by proteolipid protein (PLP) peptides in mice and interferes with PLP-specific T cell responses, J. Neuroimmunol. 64 (1996) 209–217.
[9] D. Teitelbaum, A. Meshorer, T. Hirshfeld, et al., Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide, Eur. J. Immunol. 1 (1971) 242–248.
[10] P.L. Vieira, H.C. Heystek, J. Wormmeester, et al., Glatiramer acetate (copolymer-1, copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells, J. Immunol. 170 (2003) 4483–4488.
[11] M.S. Weber, T. Prod'homme, S. Youssef, et al., Type II monocytes modulate T cell-mediated central nervous system autoimmune disease, Nat. Med. 13 (2007) 935–943.
[12] M.S. Weber, M. Starck, S. Wagenpfeil, et al., Multiple sclerosis: glatiramer acetate inhibits monocyte reactivity in vitro and in vivo, Brain 127 (2004) 1370–1378.
[13] F. Zhou, L. Zhang, K. Gong, et al., LEF-1 activates the transcription of E2F1, Biochem. Biophys. Res. Commun. 365 (2008) 149–153.
[14] T. Collins, M.I. Cybulsky, NF-kB: pivotal mediator or innocent bystander in atherogenesis?, J Clin. Invest. 107 (2001) 255–264.
[15] P. Libby, Inflammation in atherosclerosis, Nature 420 (2002) 868–874.
[16] O. Neuhaus, C. Farina, A. Yassouridis, et al., Multiple sclerosis: comparison of copolymer-1 reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 7452–7457.
[17] P.W. Duda, M.C. Schmied, S.L. Cook, et al., Glatiramer acetate (Copaxone) induces degenerate, TH2-polarized immune responses in patients with multiple sclerosis, J. Clin. Invest. 105 (2000) 967–976.
[18] Q.Q. Li, C.T. Bever, Glatiramer acetate blocks interleukin-1-dependent nuclear factor-kappaB activation and RANTES expression in human U-251 MG astroglial cells, Brain Res. Mol. Brain Res. 87 (2001) 48–60.
[19] Q.Q. Li, D.R. Burt, C.T. Bever, Glatiramer acetate inhibition of tumor necrosis factor-alpha-induced RANTES expression and release from U-251 MG human astrocytic cells, J. Neurochem. 77 (2001) 1208–1217.
[20] J. Sellner, W. Koczi, A. Harrer, et al., Glatiramer acetate attenuates the pro-migratory profile of adhesion molecules on various immune cell subsets in multiple sclerosis, Clin. Exp. Immunol. 173 (2013) 381–389.
[21] H.J. Kim, K. Biernacki, A. Prat, et al., Inflammatory potential and migratory capacities across human brain endothelial cells of distinct glatiramer acetate-reactive T cells generated in treated multiple sclerosis patients, Clin. Immunol. 111 (2004) 38–46.