International Journal of Cardiology 154 (2012) 147–152

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Down-regulation of CREB-binding protein expression blocks thrombin-mediated endothelial activation by inhibiting acetylation of NF-κB

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ARTICLE INFO

Article history:
Received 30 November 2009
Received in revised form 30 June 2010
Accepted 5 September 2010
Available online 5 October 2010

Keywords:
Acetylation
CREB-binding protein
Endothelial activation
NF-κB

ABSTRACT

Objectives: CREB-binding protein (CBP) belongs to a unique class of transcription co-activators possessing histone acetyltransferase (HAT) activity. The aim of the present study was to evaluate the role of CBP in thrombin-induced endothelial activation, and also explore the underlying mechanism.
Methods: Leukocyte-endothelial adhesion was calculated as the proportion of the labeled-neutrophils that adhered to ECs relative to all neutrophils applied. Levels of adhesion molecules were analyzed by real-time RT-PCR and western blot. Electrophoretic mobility shift assay and NF-κB reporter assay were performed to evaluate NF-κB activation. Acetylation of NF-κB was measured with immunoprecipitation and western blot assay. To detect the CBP–HAT activity, acetyl residues on an acetylated histone H4 was analyzed.
Results: Leukocyte-endothelial adhesion induced by thrombin was markedly attenuated in endothelial cells with CBP knockdown. The decreased adhesion was paralleled by the reduction of vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and E-selectin. Furthermore, CBP silencing suppressed thrombin-mediated NF-κB activation, and this inhibitory effect was associated with decreased acetylation of NF-κB and CBP–HAT activity.
Conclusions: Our results indicate that CBP is involved in the regulation of endothelial activation via NF-κB-dependent pathway. Down-regulation of CBP may play a role in returning ECs from a pre-inflammatory status to a quiescent state in the pathogenesis of atherosclerosis.

1. Introduction

The endothelial injury, activation, and dysfunction caused by pro-inflammatory stimuli contribute to the pathogenesis of atherosclerosis [1–3]. One of the early detectable cellular responses in the formation of atherosclerotic lesions is the recruitment of leukocytes by the endothelial expression of specific adhesion molecules [4–6], such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin. Recently, there is increasing evidence that acetylation, controlled by histone acetyltransferase (HAT) or histone deacetylases, is one of the key control points for the regulation of inflammatory genes [7,8], and plays a critical role in mediating the inflammatory effect in inflammatory lung diseases [9,10]. It has been shown that the status of hyper nuclear acetylation occurred in atherosclerosis [11,12]. However, little is known about the role of the increased acetylation in the endothelial dysfunction and inflammation.

CREB-binding protein (CBP) belongs to a unique class of transcription co-activators possessing HAT activity [13]. In addition to histone acetylation, CBP is capable of acetylating certain nuclear proteins, such as nuclear factor-kappa B (NF-κB) [14], cAMP-responsive element-binding protein [15], and signal transducer and activator of transcription-3 [16]. NF-κB is essential for the induction of numerous pro-inflammatory genes in the vascular wall [17,18]. Enhanced activity of NF-κB could be achieved through acetylation at multiple lysine residues [14,19,20]. Given the importance of CBP in modulating nuclear acetylation of NF-κB, the effect of CBP–HAT activity on endothelial activation has been the subject of considerable investigation. Since the previous study has indicated that HAT activity derived from CBP causes hyper nuclear acetylation in atherosclerotic lesions [11,12], it is likely that acetylation of NF-κB via CBP–HAT activity has a significant role in the NF-κB-mediated endothelial activation for pro-inflammatory mediators.

Accordingly, the aim of the present study was to evaluate the role of CBP–HAT in thrombin-induced endothelial activation, and also explore the underlying mechanism. Here, we found that CBP knockdown impaired the expression of adhesion molecules and leukocyte-endothelial adhesion. This inhibitory effect was in part related to the inability to enhance the acetylation of NF-κB.

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doi:10.1016/j.ijcard.2010.09.003

2. Methods

2.1. Materials

Thrombin from rat serum and curcumin was obtained from Sigma-Aldrich. MG-132 was purchased from Calbiochem. BAY 11-7082 was from Beyotime.

2.2. Cell culture and adenovirus infection

All procedures followed were in accordance with the Animal Ethical Commission of Wuhan University. Isolation of ECs from rat aorta was mainly performed as previously described [21,22]. Recombinant adenovirus CBP-shRNA/Ad containing small hairpin RNA (shRNA) against CBP was prepared as described previously [23]. Ad(N) which has a non-homologous shRNA sequence was used as the control adenovirus. For adenovirus infection, sub-confluent ECs were incubated with CBP-shRNA/Ad or Ad(N) at a multiplicity of infection (MOI) of 25 or 50 in serum-free media for about 4–6 h. Then the media was removed, and cells were incubated with complete medium for the indicated time courses.

2.3. Adhesion assays

Polymorphonuclear neutrophils (PMNs) were isolated from rat whole blood using the hetastarch exchange transfusion and sedimentation technique [24,25]. Freshly isolated PMNs were stained with red fluorescent dye (PKH26, Sigma-Aldrich). To investigate PMNs adherence to ECs, ECs were seeded in gelatin-coated 96-well plates at a density of 10 000 cells/well. Following treatment, ECs were washed extensively, and labeled PMNs were added to the confluent ECs monolayer at a ratio of 5:1. After incubation for 30 min, the plates were washed three times to remove nonadherent PMN. The fluorescence intensity of PMNs was measured with the fluorescence plate reader. The PMNs adhesion rate was calculated as the proportion of the fluorescence of the PMNs that adhered to ECs relative to that of all PMNs applied. Data were expressed as fold change with respect to cell adhesion in the absence of treatment.

2.4. Real-time RT-PCR

Total RNA was extracted from cells with Trizol reagent (Invitrogen). Reverse transcription was performed to produce cDNA from total RNA with oligo(dT), and then the fragments were amplified with SYBR Green-based assays kit (Invitrogen) according to manufacturer's instructions. Thermal cycling conditions comprised an initial denaturation step at 94 °C for 10 min, followed by 40 cycles (94 °C for 30 s; 60 °C for 30 s; and 72 °C for 60 s). GAPDH was used for normalization, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers were as follows:

VCAM-1, forward primer 5′-GCCACTTGATCCCTTGCTG-3′,
reverse primer 5′-AACGACCTCGCAATGACATC-3′.
ICAM-1, forward primer 5′-GCTGCCTATCGGGATGGTGAA-3′,
reverse primer 5′-ATGGACGCCACGATCACGAA-3′.
E-selectin, forward primer 5′-GCGATGCTGCCTACTTGTGA-3′,
reverse primer 5′-TGAGGTTGCTGCCACAGAGA-3′.
GAPDH, forward 5′-TCAACGGCACAGTCAAGG-3′,
reverse 5′-TGAGCCTTCCACGATG-3′.

2.5. Western blot analysis

For whole-cell protein extraction, cells were washed three times with cold PBS and subsequently lysed in cold RIPA lysis buffer (20 mM Tris Cl, pH 7.4/137 mM NaCl/2 mM EDTA/1% Triton/10% glycerol/25 mM β-glycerol phosphate /1 mM PMSF and protease inhibitor mixture). Cell lysis was performed on ice for 30 min, and clear protein extracts were obtained by centrifugation at 10 000 *g* for 20 min. Protein concentration was determined by the bicinchoninic acid protein assay (Bipec). Proteins were separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. For immunoblotting, nitrocellulose membranes were blocked and probed with antibodies against CBP, VCAM-1, ICAM-1, E-selectin or GAPDH (All from Santa Cruz) overnight at 4 °C. After three washes, the blots were incubated with peroxidase-conjugated secondary antibodies (Pierce) for 1 h at room temperature, and subsequently analyzed by an ECL detection system.

2.6. Immunoprecipitation analysis

Whole-cell protein extracts were obtained in cold PIPA lysis buffer as described before. 0.5 mg of whole-cell extracts was first incubated overnight at 4 °C with 2 μg anti-CBP or anti-p65 antibodies (Santa Cruz), and then with protein A/G-conjugated agarose (Santa Cruz) for 2 h at 4 °C to allow precipitation of target proteins. The immunoprecipitates were washed three times with cold lysis buffer. Subsequently, the immunoprecipitates were separated by SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with antibodies against CBP, p65 or acetylated-lysine antibody (cell signaling).

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cells (2×10^6/ml) with a commercially available kit (Pierce). EMSA were performed with a non-radioactive EMSA kit (Viagen) according to the manufacturer's instruction. Briefly, equal amounts of nuclear protein were incubated with the biotin-labeled NF-κB oligonucleotide (5′-AGTTGAGGG-GACTTTCCCAGGC-3′) and poly dI:dC for 20 min at room temperature in binding reaction buffer. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. The DNA–protein complexes were resolved on a 6.5% polyacrylamide gel pre-electrophoresed in 0.25× Tris borate/EDTA at 120 V for 1 h. Then the gel was transferred on to positively charged nylon membrane. The transferred DNA was cross-linked to the membrane and detected with horseradish peroxidase-conjugated streptavidin.

2.8. NF-κB reporter assay

Cells were seeded at 40–60% confluence 24 h before transfection. The next day cells were transfected with a NF-κB-Luc plasmid containing the luciferase gene under the control of the NF-κB promoter (Bipec) using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24-h transfection, cells were infected with CBP-shRNA/Ad or Ad (N) as described before. To construct an endothelial cell line that stably expressed an NF-κB-luciferase reporter gene, cells were selected for resistance to neomycin. Then cells were stimulated with thrombin (0.5 U/mL) for 8 h. Cells that survived the selection were screened by a luciferase reporter assay kit (Bipec), according to the manufacturer's instructions. Luminescence was normalized to protein concentrations. Data were expressed as fold change with respect to luciferase activity in the absence of treatment.

2.9. Histone acetylation activity

CBP/HAT activity was measured using an indirect enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Upstate Biotechnology). Briefly, nuclear fractions were isolated as described before. Nuclear CBP was immunoprecipitated with 5 μg anti-CBP antibody from 500 μg of nuclear protein. The CBP/HAT activity was assayed for the detection of acetyl residues on an acetylated histone H4. HAT activity was determined on a plate reader at a wavelength of 450 and 570 nm. Data were expressed as fold change with respect to HAT activity in the absence of treatment.

2.10. Statistical analysis

Data were presented as mean ± SEM. All values were analyzed using Student's t-test for comparisons between two groups or 1-way ANOVA for multiple comparisons. A P-value <0.05 was considered statistically significant.

3. Results

3.1. Role of CBP in thrombin-stimulated PMNs adhesion

ECs were stimulated with 0.5 U/ml thrombin for 8 h, and labeled PMNs were added to ECs, then, after 30 min, adherence of PMNs was measured. As shown in Fig. 1, thrombin significantly increased the levels of PMNs adhesion to ECs. To assess whether CBP was involved in regulating PMNs adhesion to thrombin-induced ECs, ECs were pretreated with curcumin, a selective CBP inhibitor [26,27], for 1 h before thrombin stimulation. The results showed that curcumin inhibited PMNs adhesion to ECs induced by thrombin in a dose-dependent manner (Fig. 1A). Next to specifically examine the role of CBP, ECs were infected with CBP-shRNA/Ad or Ad (N) for 72 h and then subjected to 0.5 U/ml thrombin for 8 h. CBP-shRNA/Ad significantly reduced levels of CBP protein, while Ad (N) had no effect of CBP expression (Fig. 1C). These experiments showed that PMNs adhesion induced by thrombin was markedly attenuated in ECs with CBP-shRNA/Ad, but not affected by Ad (N) (Fig. 1B).

3.2. CBP knockdown inhibits thrombin-mediated induction of adhesion molecules in ECs

Because leukocyte–endothelial interactions were directly regulated by the expression of adhesion molecules on ECs [4–6], levels of VCAM-1, ICAM-1 and E-selectin were assayed. Expression levels of VCAM-1, ICAM-1 and E-selectin were significantly increased in thrombin-treated ECs. As shown in Fig. 2, down-regulation of CBP with CBP-shRNA/Ad