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Inhibition of mycophenolic acid on NF-κB activity in human endothelial cells

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KEY WORDS mycophenolic acid; endothelium; NF-kappa B; I-kappa B

ABSTRACT

AIM: To examine the effect of mycophenolic acid (MPA) on the activity of nuclear factor-κB (NF-κB) and its inhibitor (IκBα) in 4-phorbal-12-myristate-13-acetate (PMA) stimulated and non-stimulated human umbilical vein endothelial cells (HUVEC). **METHODS**: MPA at various concentrations were applied to cultured HUVEC, pre-incubated or not pre-incubated with PMA (100 μg/L for 30 min). The activity of NF-κB in endothelial cells was measured with electrophoretic mobility shift assays (EMSA), and IκBα protein levels were detected by Western blot method. **RESULTS**: Incubation with PMA was found to result in rapid increment of NF-κB activity in cultured HUVEC. MPA treatment decreased the activities of NF-κB, both in PMA treatment and un-treatment HUVEC. Protein level of IκBα was decreased in PMA-treatment HUVEC. MPA treatment increased IκBα protein levels in both PMA treatment and un-treatment HUVEC. **CONCLUSION**: MPA is a potent inhibitor of NF-κB activation in endothelial cells, which might explain its beneficial effect in endothelial cells.

INTRODUCTION

Mycophenolate mofetil (MMF) is a new immunosuppressant and has successfully used in the prevention of acute and chronic rejection after renal transplantation[1]. MMF is also effective in the treatment of lupus nephritis, especially for those with renal vascular lesions and refractory severe ones which traditional immunosuppressant has no effect[2]. MMF *in vivo* is metabolized to mycophenolic acid (MPA), a special inhibitor of inosine monophosphate dehydrogenase (IMPDH) involved in *de novo* purine synthesis and a suppressor of both T- and B-lymphocyte proliferation[3]. But besides lymphocytes, MPA has also effect on endothelial cell, fibroblast cell, mesangial cell, and vascular smooth muscle cell[4-7]. It was reported that MPA could inhibit endothelial cell activation, such as proliferation, adhesion molecules expression, and angiogenesis[8-10]. However, the molecular mechanisms of the endothelial effect of MPA are still not well understood.

Nuclear factor κB (NF-κB) is a central mediator of the immune response. It is a multisubunit transcription factor that can rapidly activate transcription of quite number genes, whose products include cytokines, adhesion molecules, and chemokines[11]. The change of NF-κB activity in endothelial cells plays an important role in endothelial cell activation[12]. NF-κB is composed of members of the Rel family of ubiquitous transcriptional activators that include NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), Rel B, and c-Rel, which can homo- and heterodimerize to form actiwe NF-κB[13]. Inactive NF-κB is localized in the cytoplasm of most unstimulated cells in complex with its inhibitor, I Kappa B (IκB). Phosphorylation of IκB, followed by its degradation, allows the translocation of NF-κB into the nucleus to activate NF-κB-dependent gene transcription[14].

Since MPA has a potent inhibitory effect in endothelial activation and NF-κB is a central mediator of endothelial activation, we hypothesize that NF-κB might be a target for MPA-inhibiting endothelial cell activation. Therefore, the effects of MPA on the activity of NF-κB in human endothelial cells were observed in the present study.

MATERIALS AND METHODS

Reagents Mycophenolic acid and 4-phorbol-12-

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myristate-13-acetate (PMA), were purchased from Sigma (USA). IκBα antibody was from Santa Cruz (USA). Gel shift assay systems were from Promega (USA).

Cell culture and treatments Human umbilical vein endothelial cells were purchased from American Type Culture Collection (ATCC, No CRL-1998). Cells were cultured in F-12 (Gibco, USA), supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Sijiqing Co, Hangzhou, China), glutamine 2 mmol/L, 2-mercaptoethanol 10 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 kU/L. When cell was confluent, medium was replaced with F12 with 1 % FBS for 12 h. Before incubation with MPA, endothelial cells were divided into two groups at random: stimulated group and normal culture condition group, with or without PMA treatment respectively. In normal culture condition group, cells were incubated with MPA 10, 50 μmol/L for 2 h; while in the stimulated group, cells were pretreated with MPA 10, 50 μmol/L for 2 h, and then PMA 100 μg/L was added for another 30 min.

Cytoplasmic and nuclear protein extraction At the appropriate time, endothelial cells were collected and washed twice with cold phosphate-buffered saline (PBS), resuspended in 400 μL of cold buffer A [HEPES 10 mmol/L, pH 7.9, KCl 10 mmol/L, edetic acid 0.1 mmol/L, 0.2 % NP-40, dithiothreitol (DTT) 1 mmol/L, phenylmethyl sulfonyl fluoride (PMSF) 0.1 mmol/L]. After incubated on ice for 15 min and vortexed vigorously, the lysate was centrifuged at 10 000 × *g* (Eppendof, model 5417R) at 4 ℃ for 10 min. Supernatant was recovered, placed into microcentrifuge tubes, and frozen by immersion in liquid nitrogen. The resulting nuclear pellet was resupended in 50 μL buffer B (HEPES 20 mmol/L, pH 7.9, NaCl 420 mmol/L, edetic acid 0.1 mmol/L, $MgCl_2$ 1.5 mmol/L, 25 % glycerol, DTT 1 mmol/L, PMSF 0.1 mmol/L), then incubated on ice for another 20 min and vortexed occasionally. Cell debris was pelleted by centrifugation at 4 ℃ for 15 min, the supernatant used for electrophoretic mobility shift assay (EMSA) was stored at −80 ℃ until used. The protein content of the extracts was determined by the Bradford protein assay with bovine serum albumin as the standard (Bio-rad).

Electrophoretic mobility shift assay EMSA was performed with a commercial kit (Promega, USA). Briefly, 3.5 pmol of the appropriate consensus oligonucleotide was ^{32}P end-labeled by incubation at 37 ℃ for 10 min with 10 U of T4 polynucleotide kinase in a reaction containing 0.37 MBq [γ-^{32}P]-ATP. The reaction was stopped by edetic acid. Identified quantity of nuclear protein extracts (6 − 8 μg) was equilibrated for 10 min at room temperature in a buffer [20 % glycol, $MgCl_2$ 5 mmol/L, edetic acid 2.5 mmol/L, DTT 2.5 mmol/L, NaCl 250 mmol/L, Tris-HCl 50 mmol/L, pH 7.5, 2 μL of poly (di-dc) 0.25 g/L]. When a competition assay was performed, a 100-fold molar excess of the cold oligonucleotide (unlabelled probe) was added to the reaction 10 min prior to the addition of the labeled probe. Subsequently, 35 fmol of the labeled probe were added to the reaction and incubated for another 20 min at room temperature. The reactions were run on a non-denaturing, 4 % acrylamide (80∶1 acrylamide to bisacrylamide) gel. The gel was dried and exposed to an X-ray film for about 12 − 14 h.

The following oligonucleotides were used: 1) NF-κB: 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ and 5′-AGT TGA GGC GAC TTT CCC AGG C-3′; 2) SP-1: 5′-ATT CGA TCG GGG CGG GGC GAG C-3′ and 3′-TAA GCT AGC CCC GCC CCG CTC G-5′.

Western blotting of IκBα Cytoplasmic extracts (25 μg) were electrophoresed on 10 % SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore). IκBα protein was detected according to the enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) protocol using a 1∶1000 dilution of anti-IκBα IgG.

RESULTS

Effect of PMA on NF-κB activity We initially investigated the effect of PMA on the activity of NF-κB in endothelial cells. Baseline activity of NF-κB was detected in endothelial cells cultured in F12 without FBS. In response to PMA treatment, a rapid induction was observed at 30 min. The specificity of the shift bands was verified by competition assays: all the shift bands were suppressed by incubation with a 100-fold excess of unlabelled NF-κB probe and unchanged by competition with a 100-fold of labeled SP1 probe (Fig 1).

Effect of MPA on NF-κB activity The effect of MPA on NF-κB activation was assessed in endothelial cells. In PMA stimulated group, endothelial cells were pretreated with different concentrations of MPA for 2 h. It was found that in the presence of MPA, the PMA induced NF-κB activity was markedly inhibited, and MPA 50 μmol/L was more effective than 10 μmol/L

(Fig 2).

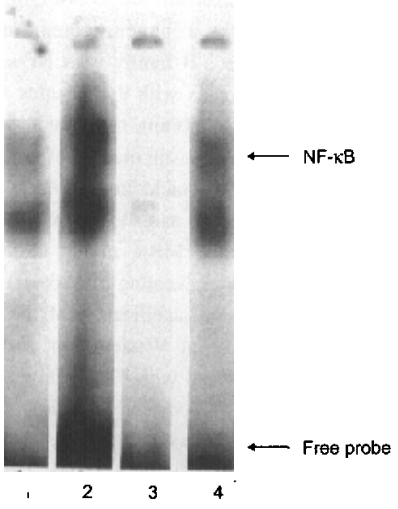

Fig 1. Effect of PMA on NF-κB activity in endothelial cell. Lane 1: control; Lane 2: treated with PMA 100 μg/L for 30 min; Lane 3: treated with PMA 100 μg/L for 30 min + 100-fold unlabeled NF-κB probe; Lane 4: treated with PMA 100 μg/L for 30 min + 100-fold unlabeled SP1 probe. Results are representative of three separate experiments.

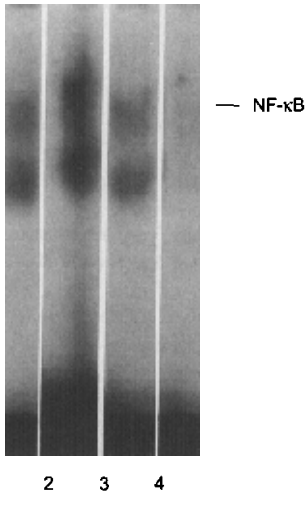

Fig 2. Effect of MPA on PMA induced-NF-κB activity in endothelial cells. Lane 1: control; Lane 2: treated with PMA 100 μg/L for 30 min; Lane 3: treated with MPA 10 μmol/L for 2 h + PMA 100 μg/L for 30 min; Lane 4: treated with MPA 50 μmol/L for 2 h + PMA 100 μg/L for 30 min. Results are representative of three separate experiments.

In the normal culture condition group, endothelial cells were only incubated with different doses MPA for 2 h. Similarly, the activity of NF-κB in endothelial cells was decreased by MPA (Fig 3).

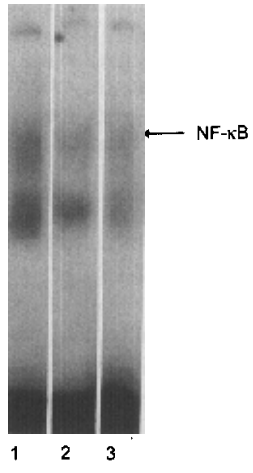

Fig 3. Effect of MPA on NF-κB activity in normal culture condition of endothelial cells. Lane 1: control; Lane 2: treated with MPA 10 μmol/L for 2 h; Lane 3: treated with MPA 50 μmol/L for 2 h; Results are representative of three separate experiments.

MPA interfering with IκBα degradation In the normal culture condition group, the protein level of IκBα in endothelial cells treated with different concentrations of MPA was increased a little (Fig 4).

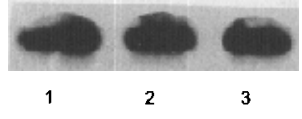

Fig 4. Effect of MPA on IκBα protein level in normal culture condition of endothelial cell. Lane 1: control; Lane 2: treated with MPA 10 μmol/L for 2 h; Lane 3: treated with MPA 50 μmol/L for 2 h; Results are representative of three separate experiments.

In the stimulated group, it was founded that IκBα protein level was markedly decreased with PMA treatment for 30 min and pretreated with MPA the decreased IκBα protein level returned to the original level (Fig 5).

DISCUSSION

Mycophenolic acid (MPA) is a selective, uncom-

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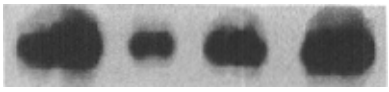

Fig 5. Effect of MPA on IκBα protein level in PMA stimulated endothelial cell. Lane 1: control; Lane 2: treated with PMA 100 μg/L for 30 min; Lane 3: treated with PMA 100 μg/L for 30 min + MPA 10 μmol/L for 2 h; Lane 4: treated with PMA 100 μg/L for 30 min + MPA 50 μmol/L for 2 h. Results are representative of three separate experiments.

petitive reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), the key enzyme of guanine nucleotide synthesis[3]. Lymphocytes are especially sensitive to GTP depletion because these cells rely on the *de novo* pathway for purine synthesis and are not able to synthesize guanosine nucleotides by salvaging from the adenine or guanine nucleotide pathway. Therefore, the immunosuppressive effect of MPA in humans, which is based on IMPDH inhibition, is associated with a marked decrease in lymphocyte proliferation. MPA can inhibit endothelial cell proliferation, migration, angiogenesis, and adhesion molecule expression. But it was shown that the effect of MPA on cell adhesion molecules in human endothelial cells was not reversed by addition of exogenous guanosine, which suggested that in endothelial cells the salvage pathway for guanosine synthesis might be more active than the *de novo* pathway[15]. There might be other mechanisms, which MPA has effected on endothelial cells.

NF-κB plays a key role in the induction of many proinflammatory genes expression in endothelial cell activation. Products among these genes are adhesion molecules (such as E-selectin, ICAM, VCAM) and cytokines (such as interleukin-1, -6, -8). The expression of the adhesion molecules and secretion of chemokines leads the attraction of host cells that can contribute to the inflammatory response. The results of EMSA showed that: (1) endothelial cell cultured in F12 with 1 % FCS showed a low baseline activity of NF-κB; while treatment with PMA for 30 min could induce NF-κB activity rapidly and markedly; (2) incubation with MPA could decrease NF-κB activity in both groups treated with or without PMA. The inhibitory effect of MPA on the activity of NF-κB was more obvious in PMA stimulated ones. These results demonstrated that MPA could inhibit the activity of NF-κB in endothelial cell, and this effect was more obvious in the activated endothelial cell.

An important mechanism in controlling NF-κB activation is provided by a protein-protein interaction involving members of the IκB family with NF-κB. IκB binds to and forms a complex with the subunits (p65 and p50) of NF-κB, thereby inhibiting transmigration of NF-κB into the nucleus. Upon stimulation of cells with a wide array of reagents including PMA, IκB is phosphorylated, ubiquitinated and subsequently degraded. We further tested whether MPA might influence the activation of NF-κB by preventing the degradation of IκB, which would result in stabilization of the IκB-NF-κB complex. The results of Western blot showed that stimulation of endothelial cell with PMA 100 μg/L led to rapid proteolysis of IκB, as virtually the IκB present in the cytoplasm was degraded within 30 min. In contrast, pre-treated with MPA 10, 50 μmol/L for 2 h prior to addition of PMA for 30 min, showed no changes in the amount of IκB present in the cytoplasm. The effect of MPA on endothelial cell IκBα protein level was consistent with the effect on NF-κB activity. It was suggested that MPA exerted either a direct effect on an IκBα kinase or on another upstream signal.

In summary, our data suggest that MPA is a strong and specific inhibitor of NF-κB activation, which might explain its beneficial effect in endothelial cells.

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霉酚酸抑制内皮细胞核因子-κB的活性

R96 A

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关键词 霉酚酸；内皮；NF-κB；IκB

目的：研究霉酚酸对内皮细胞核因子-κB (NF-κB)活力及其抑制因子 IκBα 的影响. **方法**：利用凝胶迁移率实验(EMSA)检测不同浓度的霉酚酸对一般培养状态和佛波脂(PMA)激活的内皮细胞的 NF-κB 活力影响，并用 Western blot 方法检测霉酚酸对 NF-κB 抑制因子 IκBα 的作用. **结果**：PMA 显著激活内皮细胞中的 NF-κB 活性，降低 IκBα 的蛋白水平，霉酚酸抑制 PMA 刺激的内皮细胞 NF-κB 活性升高，并抑制内皮细胞胞浆 IκBα 蛋白的降解. **结论**：霉酚酸可影响内皮细胞 NF-κB 的活性. 这可能是 MPA 影响内皮细胞功能的作用机制之一.

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