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Activation of mTOR is involved in anti-β_2GPI/β_2GPI-induced expression of tissue factor and IL-8 in monocytes

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ABSTRACT

Previous study has demonstrated that activation of the mammalian target of rapamycin (mTOR) pathway in endothelial cells (ECs) results in the formation of chronic vascular lesions associated with antiphospholipid syndrome (APS). In addition, it has been shown that stimulation of monocytes and ECs by antiphospholipid antibodies (aPL) leads to a prothrombotic and proinflammatory state and up-regulated expression of tissue factor (TF) and inflammatory cytokines. However, the role of mTOR in pathogenic mechanisms of APS remains largely unexplored. In the present study, we aimed to investigate whether mTOR was involved in anti-β_2GPI/β_2GPI complex-induced expression of TF and interleukin-8 (IL-8/CXCL8) in monocytes and explore the relationship among TLR4, mTOR, MAPKs and NF-κB in such a process. The results showed that treatment of anti-β_2GPI/β_2GPI or APS-IgG/β_2GPI complex could markedly induce mTOR activation as well as expression of TF and IL-8 in THP-1 cells or primary monocytes. The mTOR inhibitor rapamycin (100 nM) could attenuate the elevated expression of TF and IL-8. In addition, rapamycin could also decrease the phosphorylation of p38, ERK1/2 and NF-κB p65 stimulated by anti-β_2GPI/β_2GPI or APS-IgG/β_2GPI complex, but it had no effect on JNK. Moreover, the anti-β_2GPI/β_2GPI or APS-IgG/β_2GPI complex-induced phosphorylation of mTOR in THP-1 cells was down-regulated through inhibition of p38 (10 μM, SB203580) or ERK (5 μM, U0126) rather than inhibition of JNK (90 nM, SP600125) or NF-κB (20 μM, PDTC). Finally, the mTOR activation could also be affected by exposure to TLR4 inhibitor TAK-242 (5 μM). Taken together, our results indicated that mTOR was involved in regulating anti-β_2GPI/β_2GPI-induced expression of TF and IL-8 in monocytes. In addition, the inhibition of mTOR pathway might be beneficial for the prevention and treatment of aPL-mediated thrombosis and inflammation in APS patients.

1. Introduction

As an acquired autoimmune disorder, antiphospholipid syndrome (APS) is associated with thrombotic events, pregnancy morbidity and elevated levels of antiphospholipid antibodies (aPL), especially anti-β_2-glycoprotein I (anti-β_2GPI) antibodies [1,2]. The prothrombotic status has been related to a proinflammatory and procoagulant phenotype induced by aPL that react with phospholipid binding proteins, such as β_2GPI, protein C, protein S or prothrombin [3,4]. The majority of studies have suggested that anti-β_2GPI antibodies can activate monocytes and endothelial cells (ECs), showing increased intercellular adhesion and elevated expression of tissue factor (TF), adhesion molecules (E-selectin, VCAM-1, ICAM) and inflammatory cytokines (TNF-α, IL-1β, IL-8), which are beneficial to the thrombus formation of the syndrome [5–7].

Increasing evidence has indicated that activation of ECs or monocytes by aPL is mediated by intracellular signal pathways via its specific binding molecules/receptors, such as annexin A2 (ANX2), toll-like receptor 4 (TLR4) and apolipoprotein E receptor 2′ (apoER2′) [6,8]. Although the association between monocytes and the development of thrombotic complications in APS patients has been well established, it remains largely unexplored how anti-β_2GPI/β_2GPI induces expression of TF and cytokines in monocytes. Studies from our and other laboratories have suggested that β_2GPI co-localizes with ANX2 and TLR4 on the

Abbreviations: mTOR, mammalian target of rapamycin; ECs, endothelial cells; APS, antiphospholipid syndrome; aPL, antiphospholipid antibodies; TF, tissue factor; β_2GPI, β_2-glycoprotein I; IL-8, interleukin-8; TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa B; APS-IgG, IgG from patients of antiphospholipid syndrome; p38, p38-mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; ANX2, annexin A2; apoER2′, apolipoprotein E receptor 2′.

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lipid rafts of monocytes and anti-β_2GPI antibodies stimulate expression of TF and cytokines in monocytes [8,9].

As a conserved protein kinase in a signaling pathway downstream from PI3K [10], the mammalian target of rapamycin (mTOR) exists in at least two functionally distinct complexes, mTOR complex 1 (mTORC1) and mTORC2 [11]. The mTORC1 is exquisitely sensitive to inhibition by rapamycin, whereas mTORC2 is resistant to rapamycin for an unknown reason [11]. The mTOR signaling pathway executes various biological functions, such as differentiation, proliferation and survival of cells. Studies in vivo and in vitro have indicated that mTOR plays a crucial role in the vascular stenosis caused by mechanical endothelial injury [12,13]. A recent study has revealed that the activation of mTOR can stimulate intimal hyperplasia, leading to the formation of chronic vascular lesions as seen in APS [14]. Consequently, the inhibition of mTOR through sirolimus or rapamycin prevents or minimizes the neointimal formation after injury [15]. Taken together, we hypothesized that the activation of mTORC pathway played a role in aPL/anti-β_2GPI-induced thrombosis and activation of ECs in APS.

Our previous study has demonstrated that anti-β_2GPI/β_2GPI complex can induce expression of TF and inflammatory cytokines (such as IL-8) in monocytes [6,8]. However, it remained unknown whether mTOR was involved in anti-β_2GPI/β_2GPI-induced expression of TF and inflammatory cytokines (such as IL-8) in monocytes. In the present study, we mainly aimed to investigate the roles of mTOR in anti-β_2GPI/β_2GPI complex-induced expression of TF and inflammatory cytokines/chemokines (such as IL-8) in THP-1 cells and primary monocytes, and explore whether mTOR inhibitor rapamycin could effectively reverse the effects of anti-β_2GPI/β_2GPI complex in vitro.

2. Materials and methods

2.1. Cell culture

2.1.1. THP-1 cells

Human monocytic-derived THP-1 cell line was purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified incubator with 5% CO_2. The THP-1 cells were seeded in 6-well plates at a density of 2×10^6 cells/well and incubated for 24 h. All experimental data were obtained from cells between passage 3 and 10.

2.1.2. Monocytes

The peripheral blood mononuclear cells (PBMCs) were collected from blood buffy coats of healthy volunteers without personal/family history of allergies as previously described [16,17]. Each subject gave written informed consent according to the Institutional Review Board of Jiangsu University. After washed two times, the PBMCs were resuspended in RPMI-1640 medium and incubated at 37 °C in a humidified incubator with 5% CO_2 for 1 h. Non-adherent cells were removed by washing three times with RPMI-1640 medium. The purity of primary monocytes was evaluated by flow cytometry, and cells consisting of >90% $CD14^+$ cells, <1% $CD3^+$ cells, and <1% $CD19^+$ cells were utilized in the following experiments. The adherent cells were seeded in 24-well plates at a density of 1×10^6 cells/well and incubated for 2 h prior to administration of different stimuli.

2.2. Preparation of β_2GPI, IgGs and chemicals

The β_2GPI (US Biological, Swampscott, MC, USA) was treated as described by Agar C et al. [18]. Conversion from the closed circular conformation to the open conformation β_2GPI was performed in a 14 kD MWCO dialysis film by dialysis against 20 mM HEPES containing 1.15 M NaCl, pH 11.5, for 48 h at 4 °C followed by dialysis with 20 mM HEPES containing 50 mM NaCl, pH 7.4, for 3 h at 4 °C. The total serum IgG containing aPL antibodies (APS-IgG) was purified using Protein G Sepharose columns (GE Healthcare, Buckinghamshire, UK) from APS patient with high-titer of anti-β_2GPI antibodies (≥66.9 SGU/mL) [16]. The total IgG fractions from five APS patients were finally pooled and stored at −80 °C for the experiments. Similarly, each subject gave written informed consent according to the Institutional Review Board of Jiangsu University. The anti-β_2GPI antibody used in this study was purchased from a commercial institution (Chemicon, Temecula, CA, USA). It was demonstrated that anti-β_2GPI antibody (purchased above) could activate monocytes and induce TF overexpression [8,19]. Rapamycin (Sigma, Saint Louis, MO, USA) was dissolved in DMSO, aliquoted and stored at −20 °C, and this solution was further diluted with RPMI-1640 medium for the in vitro experiments until the DMSO content was <0.1%. Endotoxin contamination (<0.03 EU/mL) was removed from all β_2GPI, IgG samples and reagents (in particular anti-β_2GPI/β_2GPI or APS-IgG/β_2GPI complex) with Detoxi-GelTM (Pierce, Rockford, IL, USA) by the Limulus amebocyte lysate assay (ACC, Falmouth, MA, USA).

2.3. Cell viability assay

THP-1 cells or primary monocytes were seeded in 96-well plates at a density of approximate 2×10^4 cells/well and incubated at 37 °C in a humidified incubator with 5% CO_2 for 24 h. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 5 mg dissolved in 1 mL of PBS, Sigma) assay was used to determine cell viability at the different time points after the cells were treated with rapamycin at indicated concentrations. The absorbance of cells at a wavelength of 490 nm was determined using the ELISA plate reader (Microplate Reader, Bio-Rad, Hercules, CA, USA).

2.4. Real-time quantitative reverse transcription–polymerase chain reaction (RT–qPCR) analysis

THP-1 cells or primary monocytes were seeded in 24-well plates at a density of 1×10^6 cells/mL and serum-starved overnight prior to stimulation with monoclonal anti-β_2GPI (10 μg/mL)/β_2GPI (100 μg/mL); APS-IgG (250 μg/mL)/β_2GPI (100 μg/mL); isotype control rabbit immunoglobulin G (R-IgG) (10 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA)/β_2GPI (100 μg/mL); anti-β_2GPI (10 μg/mL)/bovine serum albumin (BSA) (100 μg/mL; Sigma); or 500 ng/mL LPS (Sigma) for 2 h. In some experiments, cells were pretreated with 100 nM rapamycin for 20 h before administration of above-mentioned stimuli. Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Subsequently, 1 μg purified total RNA was reversely transcribed into cDNA using oligo-dT primers in a 10-μL reaction system (Applied Biosystems, Foster City, CA, USA; 2720 Thermal Cycler). The expression of target genes at the mRNA level were determined by RT-qPCR using SYBR Green I dye (Takara Biotec, Kyoto, Japan) with primer pairs as follows: 5′-CACGAAACTACCTTCAACTCC-3′ and 5′-CATACTCCTGCTTGCTGATC-3′ for β-actin (amplicon size of 262 bp), 5′-GCATCTGGCAACCCTACAAC-3′ and 5′-TCTGGACCCCAAGGAAAACT-3′ for IL-8 (amplicon size of 184 bp), 5′-TCAGGTGATCCACCCACCTT-3′ and 5′-GCACCCAATTTCCTTC CATTT-3′ for TF (amplicon size of 132 bp). The β-actin was used as the control housekeeping gene. Briefly, amplifications were carried out on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with 38 cycles at a melting temperature of 95 °C for 30 s, an annealing temperature of 60–61 °C (60 °C for TF and β-actin; 61 °C for IL-8) for 30 s, and an extension temperature of 72 °C for 30 s. Each experiment was performed in triplicate, and relative quantification of mRNA expression was analyzed by the comparative threshold cycle (Ct) method.