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PKA negatively regulates PP2Cβ to activate NF-κB-mediated inflammatory signaling

Hyo-Kyung Choi [a,1], Soo-Yeon Park [a,1], Hye-Jeong Oh [a], Eun-Jeong Han [a], Yoo-Hyun Lee [b], Jeongmin Lee [c], Woo Jin Jun [d], Kyung-Chul Choi [e], Ho-Geun Yoon [a,*]

[a] Department of Biochemistry and Molecular Biology, Center for Chronic Metabolic Disease Research, Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul, South Korea
[b] Department of Food Science and Nutrition, The University of Suwon, Kyunggi-do 445-743, South Korea
[c] Department of Medical Nutrition, Kyung Hee University, Yongin-si, Kyunggi-do 446-701, South Korea
[d] Department of Food and Nutrition, Chonnam National University, Gwangju, South Korea
[e] Department of Medicine, Graduate School, University of Ulsan, Seoul 138-736, South Korea

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ABSTRACT

Protein phosphatase 2Cβ (PP2Cβ) was found to act as a negative regulator of NF-κB-mediated inflammatory signaling; however, its regulatory mechanism has not been examined. Here, we show that protein kinase A (PKA) phosphorylates the PP2Cβ, which was inhibited by PKA-specific inhibitor, H89. Mutation analysis of serine residues in PP2Cβ revealed that Ser-195 in PP2Cβ is phosphorylated by PKA. Importantly, PKA inhibition by H89 abrogated the Forskolin-induced destabilization of PP2Cβ against ubiquitin-dependent proteosomal degradation pathway. Furthermore, H89 treatment efficiently reversed the negative effect of Forskolin on the anti-inflammatory function of PP2Cβ. Collectively, these data suggest that PKA destabilizes PP2Cβ upon inflammatory stimuli via phosphorylation of Ser-195 in PP2Cβ.

1. Introduction

NF-κB has been shown to play an important role in the regulation of inflammatory signaling [1]. The function of NF-κB was regulated by post-translational modifications including phosphorylation, acetylation and sumoylation [2]. Among them, phosphorylation during the activation of inflammatory signaling is well-defined event in the regulation of NF-κB activity [3–6]. In this context, multiple kinases were shown to involve in the phosphorylation by different stimuli, which leads to the nuclear import of NF-κB and subsequent activation of inflammation. For instance, PKA phosphorylates the Ser-276 of NF-κB upon lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) [6]. On the other hand, Ser-311 of NF-κB is phosphorylated by protein kinase C-ζ (PKC-ζ), which is activated by both TNF-α and interleukin (IL)-1 [7,8]. Furthermore, Ser-536 and 529 are phosphorylated by IKK and casein kinase 2, respectively [3,5]. Importantly, there are evidences indicating that RelA phosphorylation at Ser-276 and 536 is essentially prior to acetylation of RelA, demonstrating the functional importance of phosphorylation event in the activation of RelA function [9,10].

It has been well defined that phosphorylation of target protein is reversibly regulated by a different sets of phosphatase [11,12]. In regard to NF-κB-mediated inflammatory signaling, protein phosphatase 2Cβ (PP2Cβ/PPM1B) was shown to suppress the inflammatory signaling by dephosphorylation of IKKβ, which leads to stabilization of IKBβ, a well-known inhibitor of NF-κB [13]. Furthermore, PP2A was found to associate with RelA to inhibit the activation of RelA in melanoma cells [14]. Therefore, protein phosphatases are believed to act as a negative regulator of the NF-κB-mediated inflammation; however, our understanding of protein phosphatase regulation and function regard to inflammatory signaling remain incomplete.

In this study, we have found that PKA phosphorylates PP2Cβ at Ser-195 in a response to TNF-α treatment. PKA-mediated phosphorylation induced the destabilization of PP2Cβ via ubiquitin-dependent proteosomal degradation pathway. Finally, PKA activates NF-κB-mediated transcription by destabilization of PP2Cβ. Our data suggests a novel phosphorylation-dephosphorylation switch in the regulation of NF-κB-mediated inflammatory signaling.

* Corresponding author. Fax: +82 2 312 5041.
E-mail address: yhgeun@yuhs.ac (H.-G. Yoon).
[1] These authors contributed equally to the article.

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2. Materials and methods

2.1. Cell culture and plasmids

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin (Gibco BRL), and 0.1 mg/ml streptomycin (Gibco BRL) at 37 °C under 5% CO_2. MG-132 was purchased from Calbiochem (Bad Soden, Germany), and a 10 mM stock was prepared in dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO, USA). H89, Forskolin and MG132 were from Sigma Aldrich.

The PP2Cβ construct was generated by PCR and cloned into the pSG5-KF2M1 and pSG5-KM2M1 (Sigma–Aldrich) plasmid vectors. Various PP2Cβ mutants were generated by site-directed mutagenesis and cloned into pGEX4T1 (GE Healthcare Life Science, UK). All DNA constructs were verified by sequencing.

2.2. Immunoprecipitation and western blotting

Cells were plated at 60–70% confluency and transfected using Polyexpress reagent (Excel gene, Gaithersburg, MD, USA) following the manufacturer's protocol. For IPs, cells were lysed in IP buffer [0.5% Triton X-100, 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] containing a protease inhibitor cocktail (Roche). A total of 500 μg of whole cell lysate was immunoprecipitated using protein A/G agarose (Santacruz Biotechnology, Santa Cruz, CA) and anti-HA (Sigma–Aldrich, New York, NY, USA) or anti-FLAG (Sigma–Aldrich). Western analyses were performed using commercial antibodies including anti-β-actin (Sigma–Aldrich), anti-GAPDH (Millipore Co., Bedford, MA, USA), anti-PP2Cβ (ATGEN, Seongnam, Gyeonggi-do, Korea), and anti-Myc (Cell Signaling, Danvers, MA, USA).

2.3. Real-time PCR analysis

RT-PCR analysis and quantification were performed with SYBR Green PCR Master Mix reagents on an ABI Prism 7300 Sequence Detection System. The singularity and specificity of amplification were verified using the Dissociation Analysis Software. All samples were normalized to human GAPDH. Primer sequences for amplification of IL-6 RNA were 5′-CCCCCAGGAGAAGATTCCAA-3′ (F) and 5′-GCTGCTTTCACACATGTTACTCTTG-3′ (R). Primer sequences for amplification of IL-1β RNA were 5′-ACCTGAGCTCGCCAGTGAA-3′ (F) and 5′-TCGGAGATTCGTAGCTGGAT-3′ (R). All samples were normalized to human GAPDH. All reactions were performed in triplicate. Relative expression levels and SDs were calculated using the comparative method.

2.4. In vitro kinase assay

GST-fusion proteins were incubated with 10 ng of recombinant PKA (EMD Millipore, Billerica, MA, USA) in the presence of kinase reaction buffer (10 μl 5× kinase buffer, 10 μl magnesium/ATP cocktail solution 90 μl 75 mM $MgCl_2$/500 mM ATP plus 10 μl [100 μCi] of [γ-^{32}P]-ATP [3000 Ci/mmol]) in a total volume of 50 μl for 30 min at 30 °C. Reactions were terminated by washing twice with 1× kinase buffer. Samples were resuspended in 15 μl 5× SDS sample loading buffer and boiled for 5 min. After electrophoresis, SDS polyacrylamide gels were stained with Coomassie blue and dried, and the phosphorylated products were visualized by autoradiography or quantified by PhosphorImager analysis.

2.5. Statistical analysis

Statistical analyses were done using Student's *t*-test with Bonferroni for multiple comparisons. $P < 0.05$ was considered as significant.

3. Results and discussions

3.1. PKA phosphorylates PP2Cβ at Ser-195

To uncover the regulatory mechanism of the function of PP2Cβ, we first examined the possibility whether the function of PP2Cβ is modulated by kinases involved in the activation of NF-kB-mediated inflammation. By using computational NetPhosK 1.0 prediction program, we found that PKA displays the highest probability in the phosphorylation of PP2Cβ (data not shown). To verify this, we next performed the in vitro kinase assay with recombinant PKA protein. As shown in Fig. 1A, PKA efficiently phosphorylated GST-PP2Cβ but not GST alone. Importantly, PKA-mediated phosphorylation of PP2Cβ was inhibited by PKA-specific inhibitor, H89 treatment. Sequence analysis predicted six phosphorylation sites in the PP2Cβ protein (Fig. 1B). For accurate determination of the phosphorylation site in PP2Cβ, we generated GST-tagged mutant plasmids in which serine was substituted for alanine and mutant plasmids were screened using in vitro kinase assays. Mutation of Ser-195 resulted in complete loss of phosphorylation; however, replacement of residual serine residues with alanine had a negligible effect on phosphorylation of PP2Cβ (Fig.1C). Finally, we confirmed in vivo PKA-mediated phosphorylation of PP2Cβ at Ser-195 residue after transfection of either wild-type PP2Cβ^{WT} or mutant PP2Cβ^{S195A} into HEK293 cells. PKA activator, Forskolin treatment efficiently increased the phosphorylation of PP2Cβ^{WT} but not mutant PP2Cβ^{S195A}, providing evidence that the Ser-195 residue of PP2Cβ is critical for PKA-dependent phosphorylation (Fig. 1D).

3.2. PKA destabilizes PP2Cβ by phosphorylation at Ser-195

To examine the effect of PKA-mediated phosphorylation on the function of PP2Cβ, we assessed the level of PP2Cβ protein in the presence or absence of Forskolin. As shown in Fig. 2A, Forskolin treatment significantly reduced the protein level of PP2Cβ^{WT}, whereas PKA activation had no effect on the stability of PP2Cβ^{S195A} mutant. Intriguingly, a well-known PKA inhibitor, H89 treatment abrogated the Forskolin-induced reduction of PP2Cβ stability. Furthermore, MG132 treatment also restored the protein level of PP2Cβ (Fig. 2B). Additionally, a time-course experiment with cycloheximide treatment again verified that PKA-dependent phosphorylation decreases PP2Cβ stability (Fig. 2C). Finally, we verified above results in the context of endogenous PP2Cβ protein (Fig. 2D). Collectively, these demonstrated that PKA destabilizes the PP2Cβ protein via phosphorylation of Ser-195 in PP2Cβ.

3.3. PKA-dependent destabilization of PP2Cβ is mediated by ubiquitin-dependent proteosmal degradation

Since MG132 treatment reversed the PKA-mediated destabilization of PP2Cβ, we next examined whether PKA activation induced the ubiquitination of PP2Cβ. Forskolin treatment increased the ubiquitination of the PP2Cβ^{WT} and MG132 treatment further enhanced the effect by PKA (Fig. 3A). Importantly, ubiquitination of PP2Cβ^{S2436A} was not observed by either Forskolin or MG132 treatment, indicating that PKA promotes the ubiquitination of PP2Cβ via phosphorylation of Ser-195 in PP2Cβ. Consistently, H89 treatment efficiently reduced Forskolin-induced the ubiquitination of

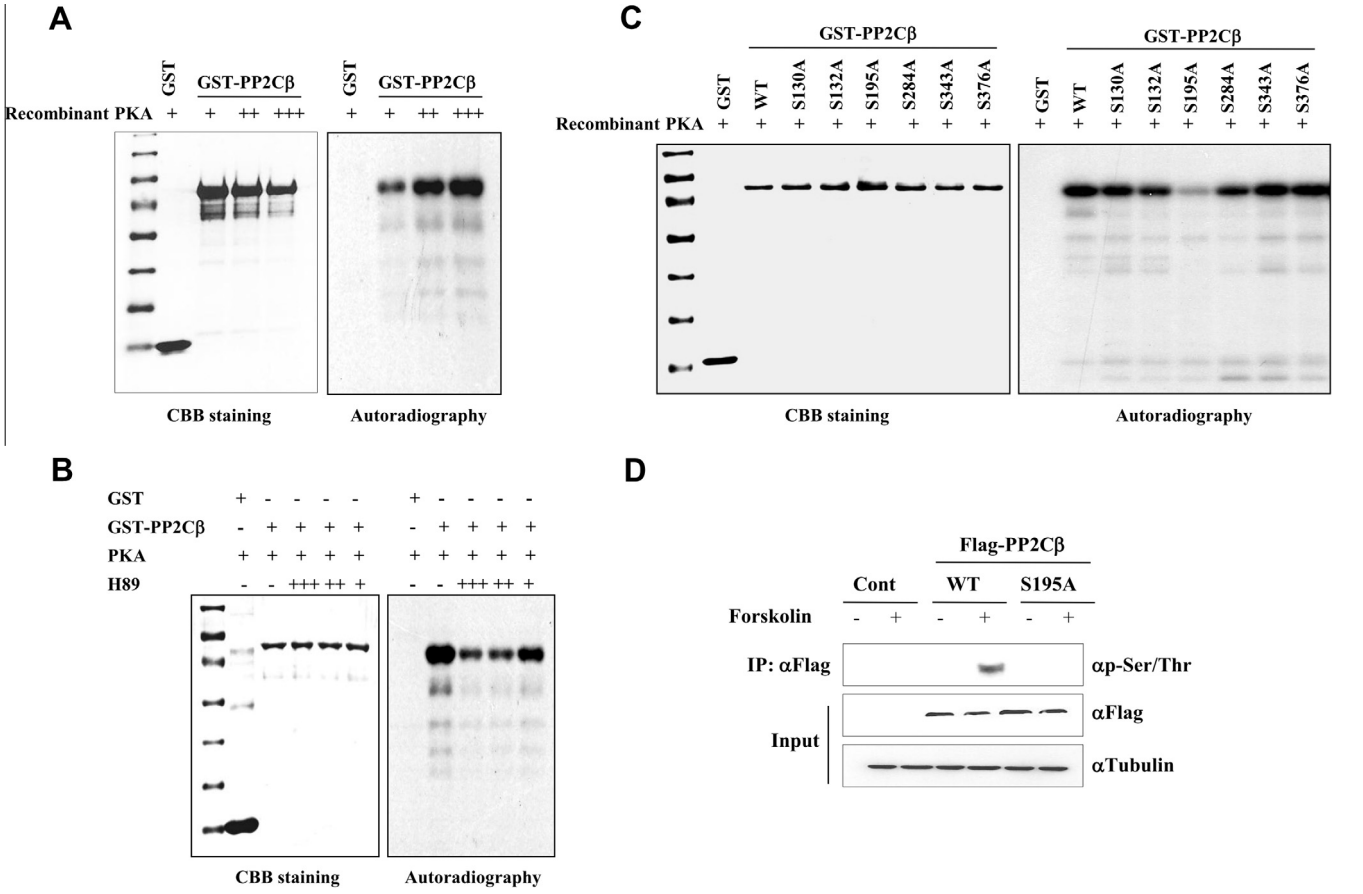

Fig. 1. PKA phosphorylates the Ser-195 of PP2Cβ. (A) PP2Cβ is phosphorylated by PKA. In vitro kinase assays were performed with recombinant PKA and the indicated GST-fused PP2Cβ proteins. The samples were processed for SDS–PAGE and subsequently visualized by autoradiography. *CBB*, Coomasie blue staining. (B) Validation of PKA-mediated PP2Cβ phosphorylation. In vitro kinase assays were performed with recombinant PKA and GST-fused PP2Cβ proteins in the presence of PKA inhibitor, H89 (50, 100, 150 μM). (C) PKA phosphorylates the Ser-195 of PP2Cβ in vitro. In vitro kinase assays were performed with recombinant PKA enzyme and the indicated GST-fused PP2Cβ proteins. (D) PKA phosphorylates PP2Cβ at Ser-195 in vivo. Either Flag-PP2Cβ^{WT} or Flag-PP2Cβ^{S195A} plasmid was transfected into HEK293 cells, and treated with Forskolin (10 μM) for 30 min. Cell lysates were analyzed by Western blotting with indicated antibodies.

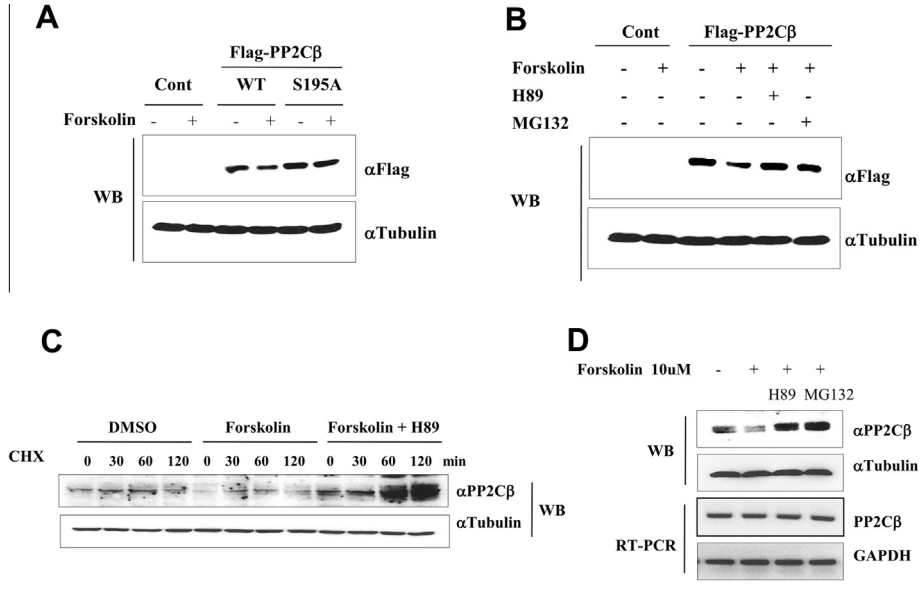

Fig. 2. PKA-dependent PP2Cβ phosphorylation decreases PP2Cβ stability. (A) Either Flag-PP2Cβ^{WT} or Flag-PP2Cβ^{S195A} plasmid was transfected into HEK293 cells, and treated with Forskolin (10 μM) for 2 h. Cell lysates were analyzed by Western blotting with indicated antibodies. (B) Flag-PP2Cβ^{WT} plasmid was transfected into HEK293 cells, and treated with H89 (10 μM) or MG132 for 6 h. Cell lysates were analyzed by Western blotting with indicated antibodies. (C) HEK293 cells were treated with cycloheximide and Forskolin or H89 for various time periods, and cell lysates were analyzed by Western blotting. (D) HEK293 cells were treated Forskolin or H89 for various time periods, and cell lysates were analyzed by Western blotting.

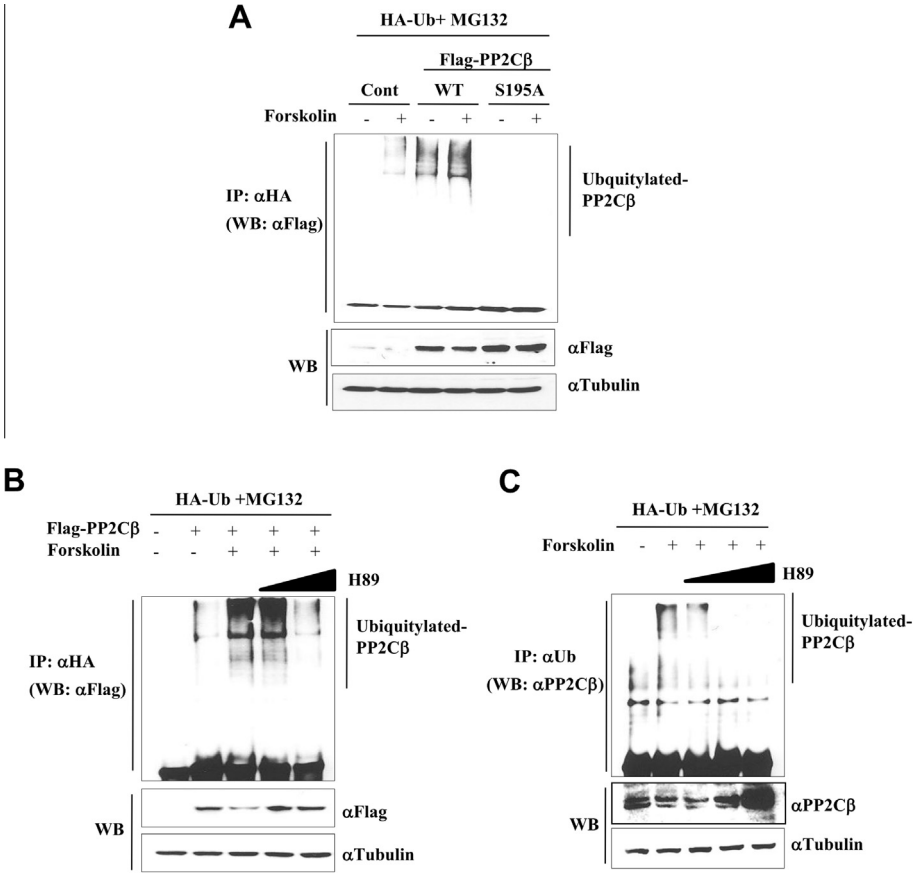

Fig. 3. PKA destabilizes PP2Cβ via enhancing of the ubiquitin-dependent proteosomal pathway. (A) Either Flag-PP2Cβ^{WT} or Flag-PP2Cβ^{S195A} plasmid was co-transfected with HA-Ub into HEK293 cells, and treated with MG132 and/or Forskolin (10 μM). Whole cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (B) Both Flag-PP2Cβ^{WT} and HA-Ub were co-transfected into HEK293 cells, and treated with MG132 and/or Forskolin (10 μM). Whole cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. (C) HA-Ub was transfected into HEK293 cells, and treated with MG132 and/or Forskolin (10 μM). Whole cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies.

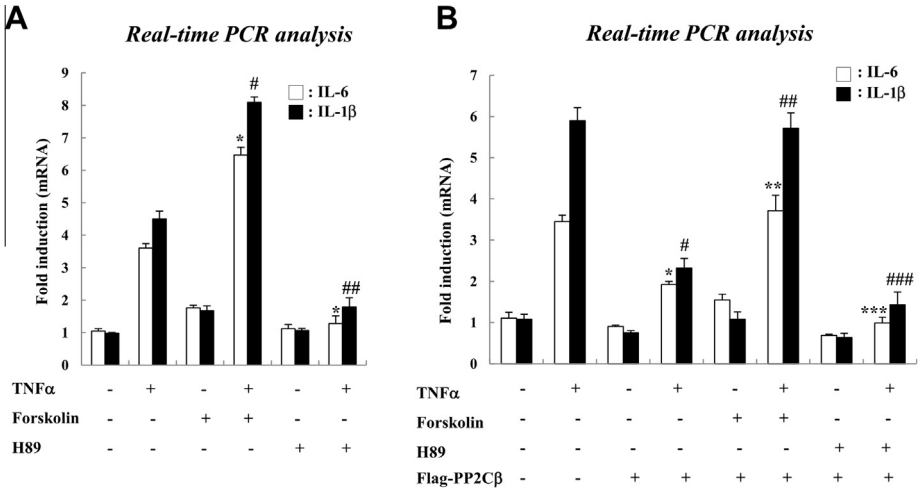

Fig. 4. PKA antagonizes PP2Cβ-mediated anti-inflammatory signals. (A) Validation of PKA action in the production of inflammatory cytokines. HEK293 cells were treated with TNFα and Forskolin or H89, and the mRNA levels of cytokines were analyzed by real-time PCR. $^{*}P < 0.05$ vs +TNFα; $^{\#}P < 0.05$ vs +TNFα; $^{\#\#}P < 0.01$ vs +TNFα. (B) PKA activation abrogated the anti-inflammatory action of PP2Cβ. Either Flag-PP2Cβ^{WT} or control plasmid was transfected into HEK293 cells, and treated with TNFα and Forskolin or H89. The mRNA levels of cytokines were analyzed by real-time PCR. $^{*}P < 0.01$ vs +TNFα; $^{\#}P < 0.05$ vs +TNFα; $^{**}P < 0.05$ vs Flag-PP2Cβ + TNFα; $^{\#\#}P < 0.05$ vs +Flag-PP2Cβ + TNFα; $^{***}P < 0.01$ vs Flag-PP2Cβ + TNFα; $^{\#\#\#}P < 0.05$ vs +Flag-PP2Cβ + TNFα.

Flag-tagged PP2Cβ as well as endogenous PP2Cβ Fig. 3B and C). Collectively, these data indicate that PKA-dependent phosphorylation is critical for PP2Cβ destabilization via the ubiquitin-dependent proteasomal degradation pathway.

3.4. PKA antagonizes the anti-inflammatory role of PP2Cβ in the expression of cytokines

We next examined the effect of PKA activation on PP2Cβ-mediated anti-inflammatory signaling. As expected, Forskolin treatment further enhanced TNFα-induced inflammatory response, whereas H89 negatively regulates cytokine production (Fig. 4A). Overexpression of $PP2C\beta^{WT}$ dramatically suppressed TNF-α-induced NF-κB activation; however, Forskolin treatment efficiently blocked the PP2Cβ-mediated inhibition of NF-κB function. Consistently, H89 treatment further potentiated the anti-inflammatory function of PP2Cβ (Fig. 4B). These data collectively demonstrated that PKA antagonizes the anti-inflammatory function of PP2Cβ in a phosphorylation-dependent way.

In this study, we for the first time identified the regulatory mechanism by which PKA activates NF-κB-mediated inflammatory signaling via antagonizing the action of PP2Cβ. Until now, there are several evidences that PP2Cβ mediates anti-inflammatory signaling by dephosphorylation of IKKβ. However, interplay between kinase and phosphatase involved in the regulation of inflammatory signaling has not been fully examined. Here, we propose a novel model that PKA phosphorylates the Ser-195 of PP2Cβ, and subsequently leads to the proteosomal degradation of PP2Cβ, which corresponds to derepression of inflammatory signaling.

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