

Safflower Yellow a Attenuates Osteoarthritis via Regulating Inflammation and Cholesterol Metabolism

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Abstract

Background

Osteoarthritis (OA) is known to be associated with inflammation and cholesterol metabolism disorder. As a chronic and complex disease, uncovering its molecular mechanism and finding effective therapy with low side effects are urgent. Hydroxysafflor yellow A (HSYA) is extracted from *Carthamus tinctorius* L, which has extensive pharmacological effects.

Methods

In this study, Interleukin 1 β (IL-1 β) was used to establish the OA model in vitro, and the impacts of HSYA on the OA cell model were analyzed. We used CCK-8 to measure the cell viability and Flow Cytometry to test the apoptosis. ELISA was performed to calculate the release of inflammatory cytokines. And WB was carried out to measure the expression of collagen and cholesterol relevant proteins. We also measured the protein levels in NF- κ B and PI3K/Akt/mTOR signaling pathways.

Results

The results showed that HSYA promoted cell viability and inhibited apoptosis. And it up-regulated the expression levels of collagen II (Col-II) and Sry related HMG box-9 (SOX9) while down-regulated the expression of matrix metalloproteinase-13 (MMP13). The IL-1 β induced high levels of IL-6 and TNF- α were inhibited by HSYA. Also, HSYA regulated the expression of cholesterol relevant proteins. Compared with the model group, the levels of APT-binding cassette transporter 1 (ABCA1) and cholesterol transport gene (APOA-1) were significantly elevated. However, the levels of cholesterol-processing enzymes cholesterol 25-hydroxylase (CH25H) and 25-hydroxy-cholesterol 7- α -hydroxylase (CYP7B1) were inhibited. Besides, HSYA inhibited the protein expression in NF- κ B and PI3K/Akt/mTOR signaling pathways.

Conclusions

HSYA was proved to regulate inflammatory response and cholesterol metabolism in vitro.

1. Introduction

OA is a common disease that often causes pain, stiffness, and disability among older people [1]. It causes a considerable economic burden on families and society. The main pathological feature of OA is the degeneration of articular cartilage[2], which generates changes in the bone, synovium, menisci, and ligaments[1]. As a complex disease, the risk factors for OA include age, gender, BMI, physical activity, genetics, and joint-level factors[3]. With the increasing number of obesity, the role of obesity in the development and progression of OA attracted the researcher's attention. Dozens of studies demonstrated that obesity was a risk factor of OA[4, 5]. And obesity is closely associated with the incidence of metabolic syndrome. Reduced HDL-C levels, proinflammatory, elevated serum triglyceride, rising glucose,

and insulin resistance are all characteristics of metabolism syndrome[6]. It is an independent risk factor for OA. Inflammatory processes were found in the tissue of synovium, chondrocytes, and synovial fluid in OA[7]. A study reported that inflammation in OA was chronic, low-grade, and mediated by the innate immune system[8]. This result was supported by another research. It suggested the inflammatory response in OA regulated the catabolism of chondrocyte, which caused low-grade synovitis[9]. Besides, the NF- κ B signaling pathways are activated in the early stage of OA, which regulates the release of proinflammatory cytokines and chemokines.

On the other hand, abnormal cholesterol levels were observed in the body of OA patients, and it was a risk factor of OA[10]. High levels of LDL-C sped up the development of OA in human and mouse OA models[11, 12]. The molecular mechanism of cholesterol metabolism disorder is associated with CH25H–CYP7B1–ROR α axis [12]. Up-regulated levels of cholesterol hydroxylases and an increased amount of oxysterol metabolites were found in osteoarthritic chondrocytes [13]. Besides, the PI3K/Akt/mTOR signaling pathways were activated during the development of OA[14].

Hydroxysafflor Yellow A (HSYA) is one of the main components extracted from the follower of *Carthamus tinctorius* L. It has various pharmacological activities such as anti-inflammatory, antioxidative, antitumor, immunodepressive, analgesic, and antidiabetic[15]. Xingwang Y et al[16] reported HSYA modulated the release of inflammatory cytokines via TLR4-Myd88 and MAPK-NF- κ B signaling pathways. The HSYA is also found to regulate cholesterol metabolism. With the treatment of SY, the levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) were decreased in hyperlipidemic mice[17]. However, this outcome was contrary to that of Huijun Z et al., who found SY was failed to modulate the levels of TC, TG, HDL-C, LDL-C, and insulin in obesity mice[18].

As for the therapy of OA, given the high costs of joint replacement surgery, pharmacological treatments became increasingly important. Paracetamol, NSAIDs, corticosteroid injections, and tramadol are supported by guidelines to treat OA. However, these drugs often lead to side effects[19]. Nowadays, inhibiting OA relevant signaling pathways are new therapies for OA treatment. Several reports have shown that inhibiting NF- κ B and PI3K/Akt/mTOR signaling pathways can attenuate OA[20]. Therefore, we aim to explore whether HSYA can act as the inhibitor of NF- κ B and PI3K/Akt/mTOR signaling pathways and alleviate the symptoms of OA via inhibiting inflammation and regulating abnormal cholesterol levels.

2. Materials And Methods

2.1 Regents

Hydroxysafflower yellow A was purchased from Chengdu must Biotechnology (CAS. 78281-02-4; Chengdu, China). We bought the Cell Counting Kit-8 (CCK-8) kit (Nanjing, China) from Vazyme Corporation. IL-1 β was obtained from Novoprotein Scientific Inc. (Shanghai, China). RIPA lysate was recruited from Beyotime (Shanghai, China). Primary antibodies against Col-II (Abcam, ab34712), SOX9 (Abcam, ab185230), MMP13 (Abcam, ab39012), CH25H (Abcam, ab133933), CYP7B1 (Abcam,

ab138497), ABCA1 (Abcam, ab18180), PI3K (Abcam, ab140307), phospho-Akt (Abcam ab1926623), phospho-mTOR (Abcam, ab109268), NF- κ B p65 (Abcam, ab16502), phospho-I κ B α (Abcam, ab133462), second antibody goat anti-rabbit (Abcam, ab6721), and second antibody goat anti-mouse (Abcam, ab6789) were purchased from Abcam. β -actin (Abclonal, AC026) was from Abclonal.

2.2 Cell Culture and Treatment

SW1353 cells (Chinese academy of sciences cell bank, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% FBS (Gibco, Australia), 100 units/ml penicillin and 100 μ g/ml streptomycin and incubated at 37 °C with 5% CO₂. To assess the effects of HSYA on cell viability, SW1353 cells were cultured in 96 wells overnight and then treated with increasing amounts of HSYA (0, 20, 40, 60, 80, 100, 200 μ mol/L) for 48h. Next, 10 μ l of CCK-8 reagent was added to each well for further 2 h incubation. IL-1 β was used to induce the OA cell model in vitro. After the cells were cultured in 96 wells overnight, we treated the cells with different doses of IL-1 β (0, 2, 4, 6, 8, 10 μ mol/L) to choose the optimum concentration. For the protective effect of HSYA, IL-1 β (6 μ mol/L) was added to SW1353 cells for 48h, and before this, 20, 40, and 60 μ mol/L of HSYA was pre-treated for 2h. And then treated with CCK-8 reagent incubating for 2 h. The absorbance value was assessed by a microplate reader (BioTek Epoch, USA) at 450nm. The test was repeated three times.

2.3 Flow cytometry

We measured the effects of HSYA on cell apoptosis by Flow cytometry. We divided the cells into control group, IL-1 β group, HSYA (40 μ mol/L) group, and IL-1 β +HSYA group. The cells were pre-treated as part of the protective effect of HSYA, and then cells were digested with trypsin. After centrifuging and resuspending in the buffer, we stained the cells with AnnexinV-FITC/PI kit (Invitrogen, USA). The data were collected by flow cytometer (BECKMAN, USA).

2.4 ELISA for conditioned medium

The levels of CRP, TNF- α , and IL-6 in the conditioned medium (collected from cultural supernatant) were measured with CRP ELISA kits (ZC-31853, ZCi Bio, China), TNF- α ELISA kits (ZC-35733, ZCi Bio, China), and IL-6 ELISA kits (ZC-32446, ZCi Bio, China). Experimental procedures followed the manufacturer's instructions.

2.5 Western blot analysis

RIPA buffer containing 0.1% protease inhibitor was used to homogenize cell samples, and the lysates from cells were centrifuged at 12,000 rpm for 15 min at 4 °C, and then collected supernatants for protein detection. Measuring the total protein concentrations with a BCA kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (20 μ g per lane for cell samples) were separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred onto PVDF membranes. Then blocking the membranes in TBS-T (20mM Tris-HCl pH 7.6, 150mM NaCl, 0.1% Tween-20) at room temperature for 2 h

containing 5% skimmed milk or 5% BSA. After washing in TBS-T three times, incubating the immunoblots with the following primary antibodies (3% BSA dilution) including Col-II, SOX9, MMP13, CH25H, CYP7B1, ABCA1, APOA-1, PI3K, p-Akt, p-mTOR, NF- κ B p65, p-I κ B α (Details of dilution were in the reagents part) overnight at 4°C, respectively. Then using secondary antibodies (1% BSA dilution) to incubate the membranes for 2 h at room temperature. The membranes were washed with TBST again and then detected with ECL. Quantifying the Band sizes by Scion Image 4.0 software (Scion Corporation, Frederick, MD, USA). Sample loading was normalized relative to β -actin as a reference standard. The final results are shown as "fold changes" in comparison with the control group.

2.6 Statistical analyses

We used GraphPad Prism v.6.0 (GraphPad Software, Inc.) to analyze the data and generate the charts in this experiment. All data are presented as the mean \pm standard deviation, and all tests were performed three times. Unpaired, two-tailed Student's t-test statistically analyzed differences between the two groups. Differences among the three groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. For all experiments, $P < 0.05$ was considered statistically significant.

3. Results

3.1 The effect of HSYA on SW1353 viability and the establishment of OA in vitro

SW1353 cells were treated with increasing amounts of HSYA to test the impact of HSYA on Cell viability. As shown in Figure 1A, HSYA had no cytotoxicity when the dose was below 40 μ M, while it significantly inhibited the cell viability with over 40 μ M. IL-1 β was used to induce OA cell models in vitro (Figure 1B). The results showed the inhibition ratio of SW1353 was elevated with the increasing amounts of IL-1 β . When the concentration of IL-1 β was 6 μ M, the inhibition ratio reached about 50%. Therefore, we used 6 μ M IL-1 β to establish the cell model of OA.

3.2 The effects of HSYA on the cell viability, apoptosis, and the levels of collagen relevant protein in OA cell model

The degradation of chondrocyte is a classical character of OA. To discover whether HSYA can inhibit this progress, the IL-1 β induced cells were treated with various (20 μ M, 40 μ M, 60 μ M) amounts of HSYA. In Figure 2A, it was apparent that IL-1 β suppressed the cell viability. However, this effect attenuated with HSYA treatment. The results showed that 40 μ M and 60 μ M of HSYA remarkably up-regulated the cell viability of SW1353. The Flow Cytometry was performed to measure the effects of HSYA on apoptosis (Figure 2B, 2C). The data proved that HSYA markedly down-regulated the IL-1 β induced apoptosis rate. Besides, the protein expression of Col-II, SOX9, and MMP-13 was analyzed (Figure 2D-G). Compared with the model group, HSYA elevated the levels of Col-II and SOX9 and also dropped the expression of MMP-13. The results indicated that HSYA promoted cell viability and inhibited apoptosis.

3.3 HSYA affected the release of inflammatory cytokines

The release of cytokines was the main reason for the development of OA. A vital therapy to control OA was to inhibit the release of inflammatory cytokines. So we also measured the expression of IL-6, TNF- α , and CRP with the treatment of HSYA (Figure 3). As shown, HSYA successfully inhibited the expression of IL-6 and TNF α but not CRP when compared with IL-1 β induced cell model group.

3.4 The effects of HSYA on cholesterol metabolism

The abnormal level of cholesterol was also observed in OA. Here, we tested the expression of the proteins such as ABCA1, APOA-1, CH25H, and CYP7B1 (Figure 4). Compared with the model group, the levels of ABCA1 and APOA-1 were significantly elevated, while the levels of CH25H and CYP7B1 were inhibited with the treatment of HSYA. The results demonstrated the effects of HSYA in regulating the level of cholesterol.

3.5 The regulation of HSYA on NF- κ B and PI3K/Akt/mTOR signaling pathways

To explore the molecular mechanism of OA, the expression of the proteins in NF- κ B and PI3K/Akt/mTOR signaling pathways were analyzed. In NF- κ B signaling pathways (Figure 5), the IL-1 β -induced expression of NF- κ B p65 was inhibited. The level of p-IKB α had a similar trend with NF- κ B p65 but with no statistic significant. As for PI3K/Akt/mTOR signaling pathways (Figure 6), the levels of PI3K, p-Akt, and p-mTOR were all decreased with the treatment of HSYA when compared with the model group. Therefore, HSYA regulated the NF- κ B and PI3K/Akt/mTOR signaling pathways.

4. Discussion

As a chronic disease, OA is the main reason which causes disability and reduced quality of life around the globe[21]. The mechanism for the generation of OA is complex, and many factors contribute to this progress. So, further understanding its pathogenesis and finding effective and low side effects medicine is still urgent. Chinese drugs HSYA which extracted from *Carthmau tinctorius* L has various pharmacological activities. So in this study, we evaluated its pharmacological function in OA cell models to find latent therapy for OA treatment.

To maintain the integrity and physiology of joints, keeping the intact structure of articular cartilage and subchondral bone is essential. However, Chondrocyte death and matrix loss are discovered in the development of OA[22]. Also, decreased expression of collagen II and SOX9 while increased expression of MMP13 in OA was reported[23]. Interestingly, several recent studies showed that herbs such as fisetin and scutellarin were found to modulate this process. Elevated expression of MMP13 and decreased levels of coll-II and SOX9 were inhibited by these herbs [24, 25]. In this study, HSYA had a similar function, which inhibited the IL-1 β induced high expression of MMP13 and increased the expression of collagen II and SOX9. And HSYA significantly increased the cell viability and decreased the apoptosis ratio of SW1353. So, the results indicated that HSYA had a protective function in deterring the degradation of cartilage cells.

In the joint and other tissues of OA, inflammatory cytokines are released, which promote the inflammatory response in chondrocytes[26]. Several cytokines such as IL-1 β , TNF α , IL-6, IL-15, IL-17, and many other cytokines are associated with the pathogenesis of OA

Meanwhile, prior studies have noted the importance of NF- κ B signaling pathways in chondrocyte-intrinsic inflammatory responses[27]. Research showed that HSYA could reduce the expression of NF- κ B and the levels of hs-CRP, IL-1 β , and IL-6 in the serum of the acute myocardial infarction[28]. These results were in agreement with Jin M's findings, which showed HSYA injection significantly inhibited the expression of TNF- α , IL-1 β , and IL-6, and also the percentage of p65-positive cells in acute lung injury mice[29]. And Xingwang Y[16] also reported the anti-inflammatory effect of HSYA on BV2 microglia through NF- κ B signaling pathways. Here, we demonstrated the inhibition effects of HSYA on the release of IL-6 and TNF- α but not CRP. Zahra measured the effects of ginger on knee osteoarthritis patients, and he found that there was no change in the levels of CRP within 3 months[30]. Other studies suggested that the levels of CRP had no association with the incidence or progression of OA[31]. The above studies may partly explain the reason why there was no change in the level of CRP in this study.

A large number of studies had reported the high levels of cholesterol in OA patients, and it had been regarded as the new risk factor for initiating OA[10]. The mRNA levels of APOA-1 and ABCA1 were reduced in OA^[32], and the expression of CH25H and CYP7B1 increased in OA^[13]. These changes promoted the development of OA. Bao et al[17] reported that the levels of TC, TG, and LDL-C were reduced with the treatment of SY in hyperlipidemic mice. In this study, we found the expression of ABCA1 and APOA-1 were increased while the expression of CH25H and CYP7B1 were decreased, indicating the cholesterol-reducing effects of HSYA.

During the development of OA, many signaling pathways are involved, except for the NF- κ B signaling pathways, the molecules in PI3K/Akt/mTOR signaling pathways also change a lot, and inhibiting this pathway has been regarded as a therapy for OA[33, 34]. HSYA was reported that can regulate PI3K/Akt/mTOR signaling pathways in THP-1 macrophages, and also exhibited anti-apoptotic effects on reperfusion injury rat[35]. Here, the results showed the levels of PI3K, p-Akt, and p-mTOR were all decreased with the treatment of HSYA. So, it suggested that HSYA modulated PI3K/Akt/mTOR signaling pathways in OA cell model.

In conclusion, this study demonstrated that HSYA could promote the viability of chondrocyte and inhibit its apoptosis, which protected chondrocyte. Besides, HSYA inhibit the release of inflammatory cytokines IL-6 and TNF α via NF- κ B signaling pathways. On the other hand, HSYA modulated the metabolism of cholesterol and PI3K/Akt/mTOR signaling pathways in OA cells model.

Abbreviations

OA, Osteoarthritis; HSYA, Hydroxysafflor yellow A; IL-1 β , Interleukin 1 β ; Col-II, collagen II; SOX9, Sry related HMG box-9; MMP13, matrix metalloproteinase-13; IL-6, Interleukin 6; ABCA1, APT-binding cassette

transporter 1; CH25H, cholesterol 25-hydroxylase; CCK-8, Cell Counting Kit-8

Declarations

Acknowledgements

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

All the authors agreed to publish the manuscript.

Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Shaohua Ju and Mingjian Wang conceived and designed the experiments. Panwang Liu, Lirong Tan, Youli Tan, and Xiaohong Li performed the experiments. Benxiang He and Yu Xia analyzed the data. Shaohua Ju and Mingjian Wang wrote the paper. All authors read and approved the final manuscript.

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Figures

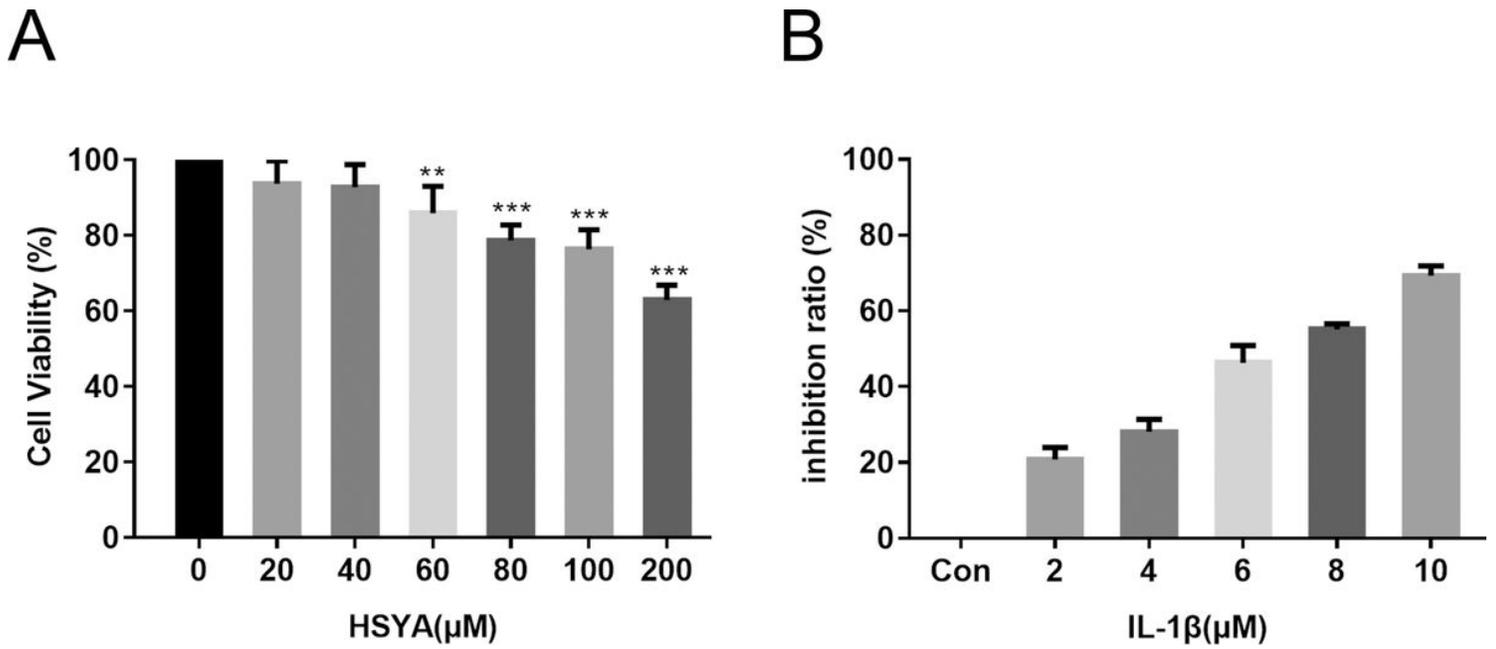


Figure 1

The effect of HSYA on SW1353 viability and the establishment of OA in vitro. A. The cell viability of SW1353 was measured by CCK-8 with an increasing amount of HSYA. B. IL-1β was used to establish the in vitro model of OA. Data are presented as means ± SD. **P < 0.01, ***P < 0.001 compared with 0μM of HSYA.

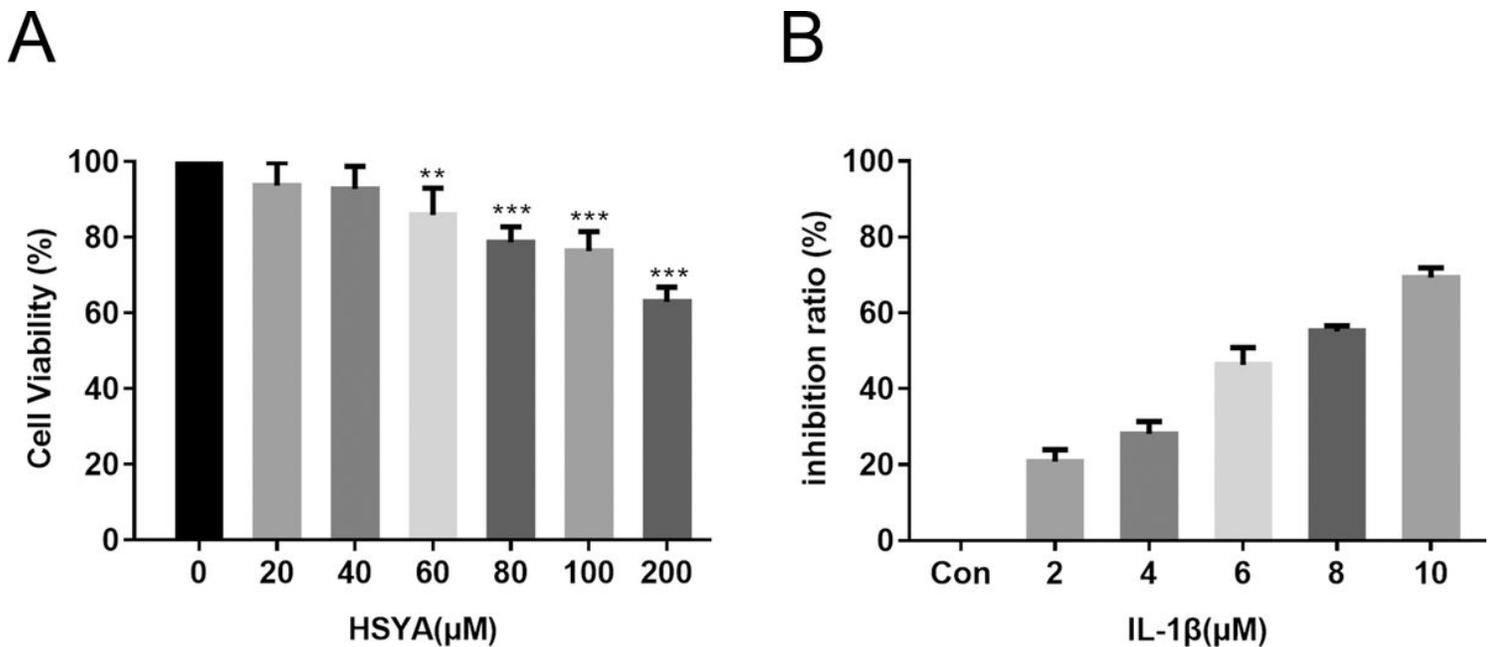


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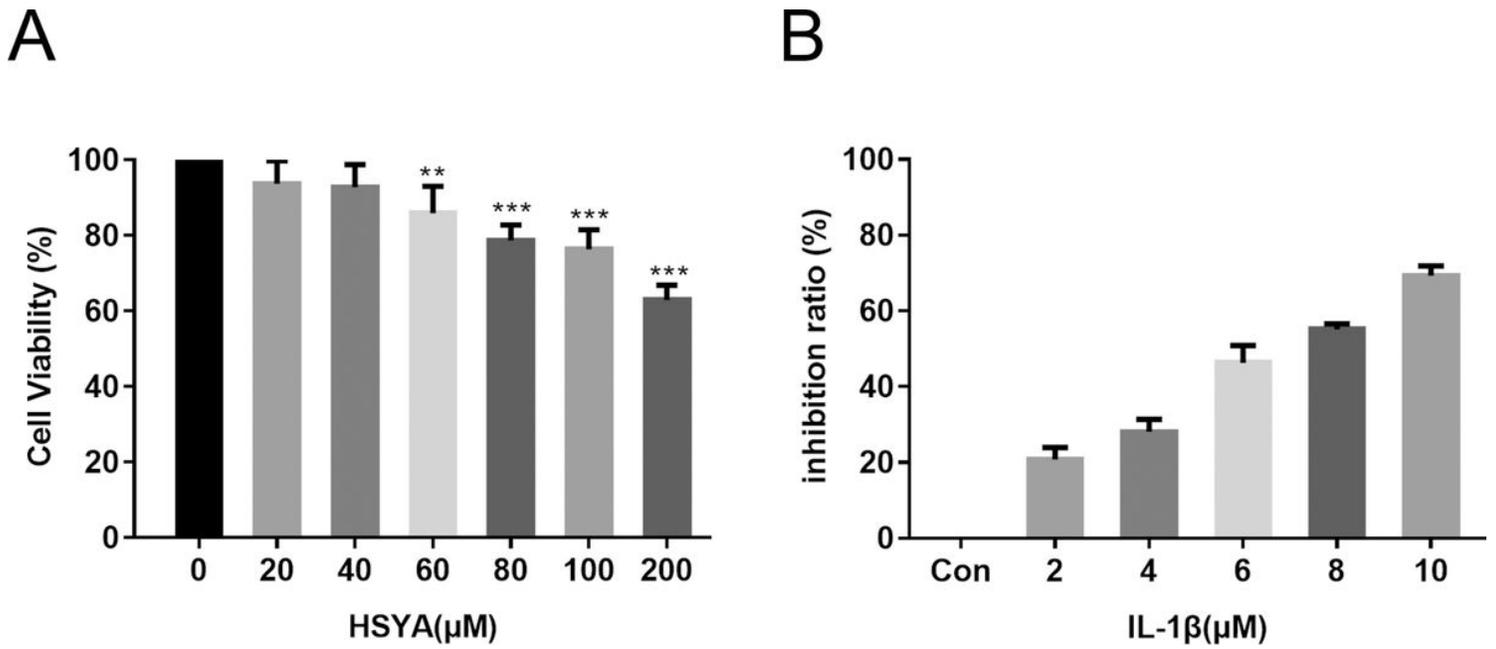


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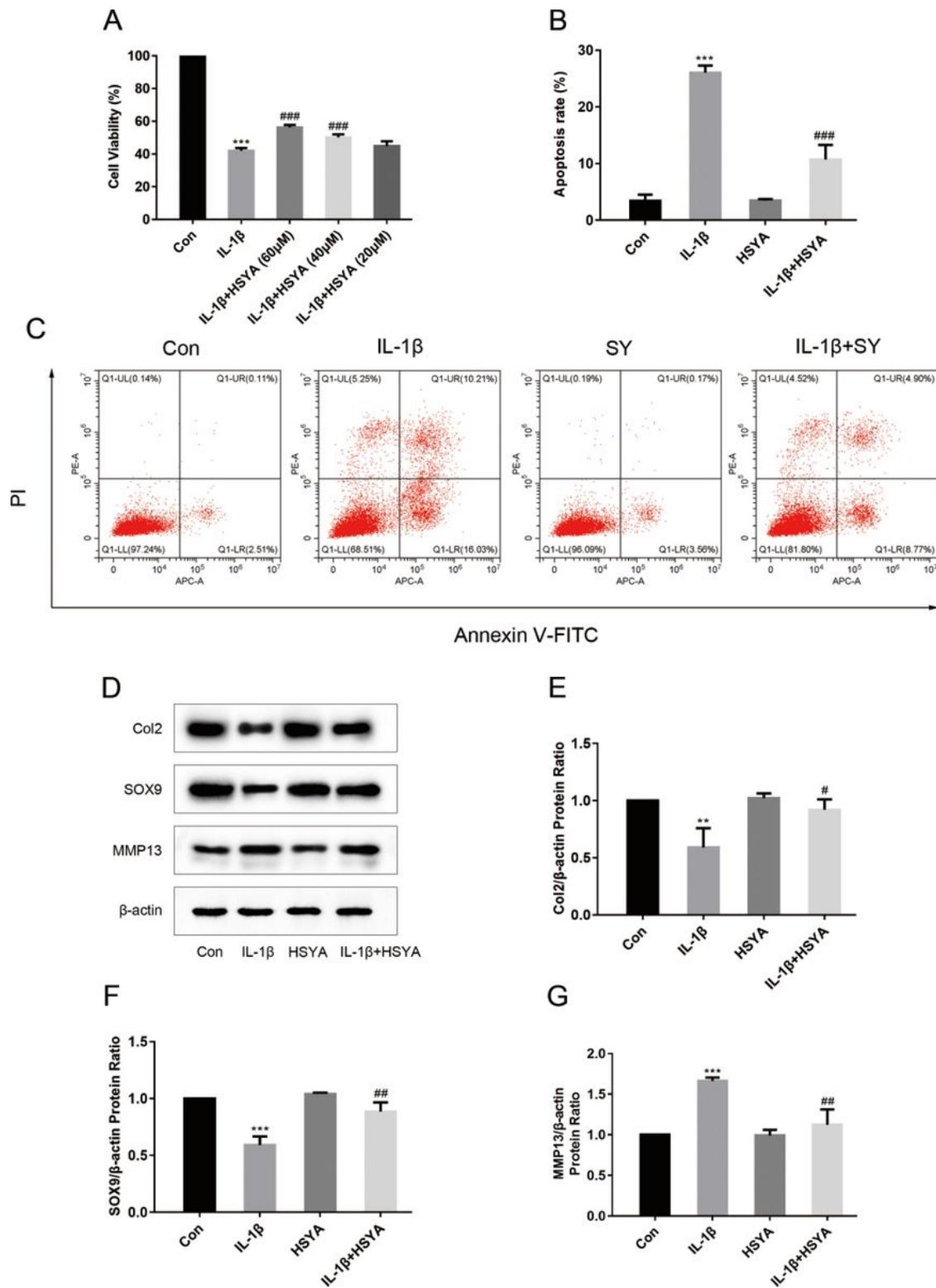


Figure 2

The change in the cell viability and apoptosis with the treatment of HSYA in the OA cell model and collagen relevant proteins in response to HSYA. A. The cell viability of SW1353 with different doses of HSYA. B and C represented the impact of HSYA on apoptosis in the OA cell model. The protein expression of Col-II (E), SOX9 (F), and MMP-13(G) was analyzed by WB (D). Data are presented as means \pm SD. **P <

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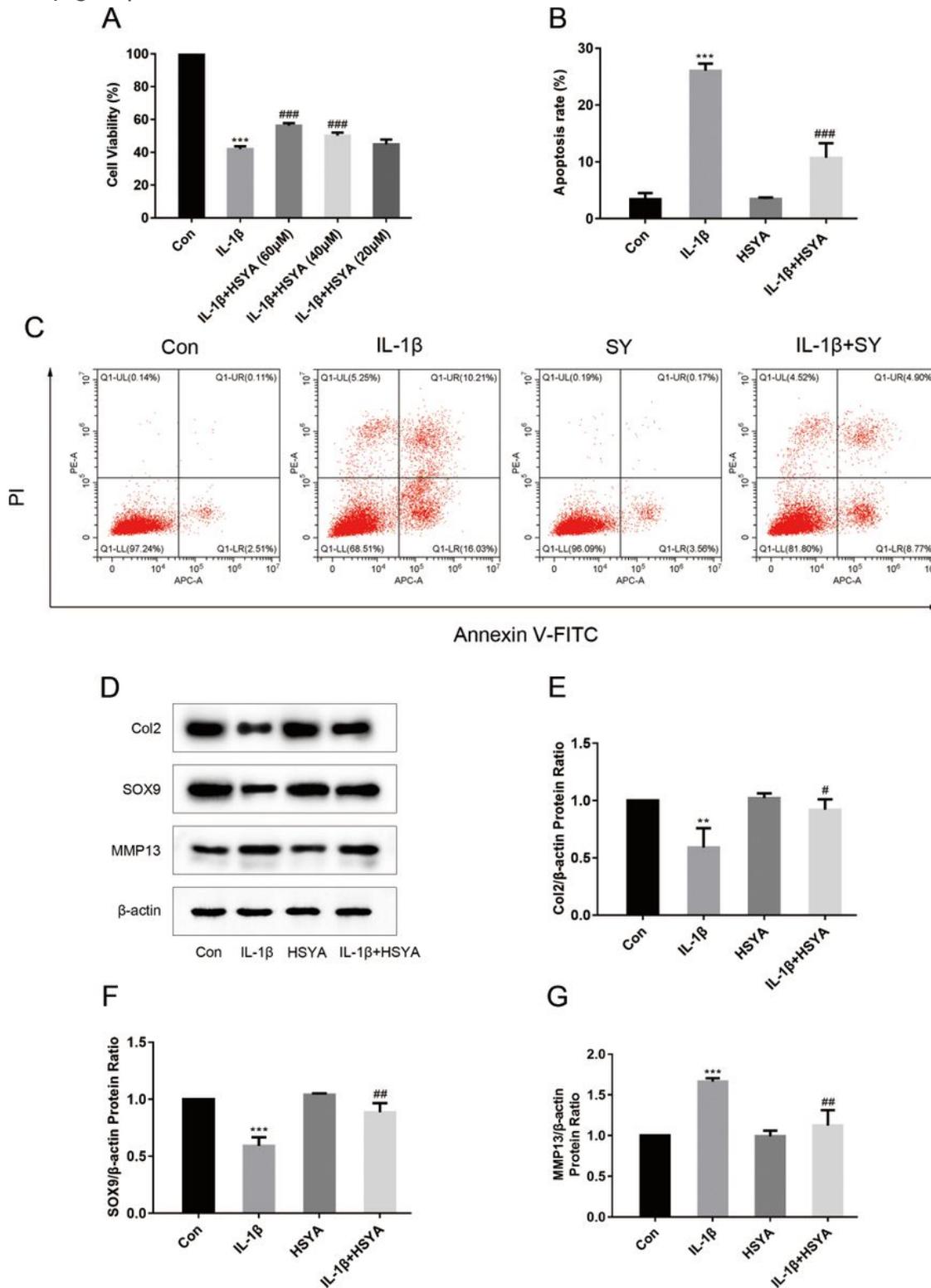


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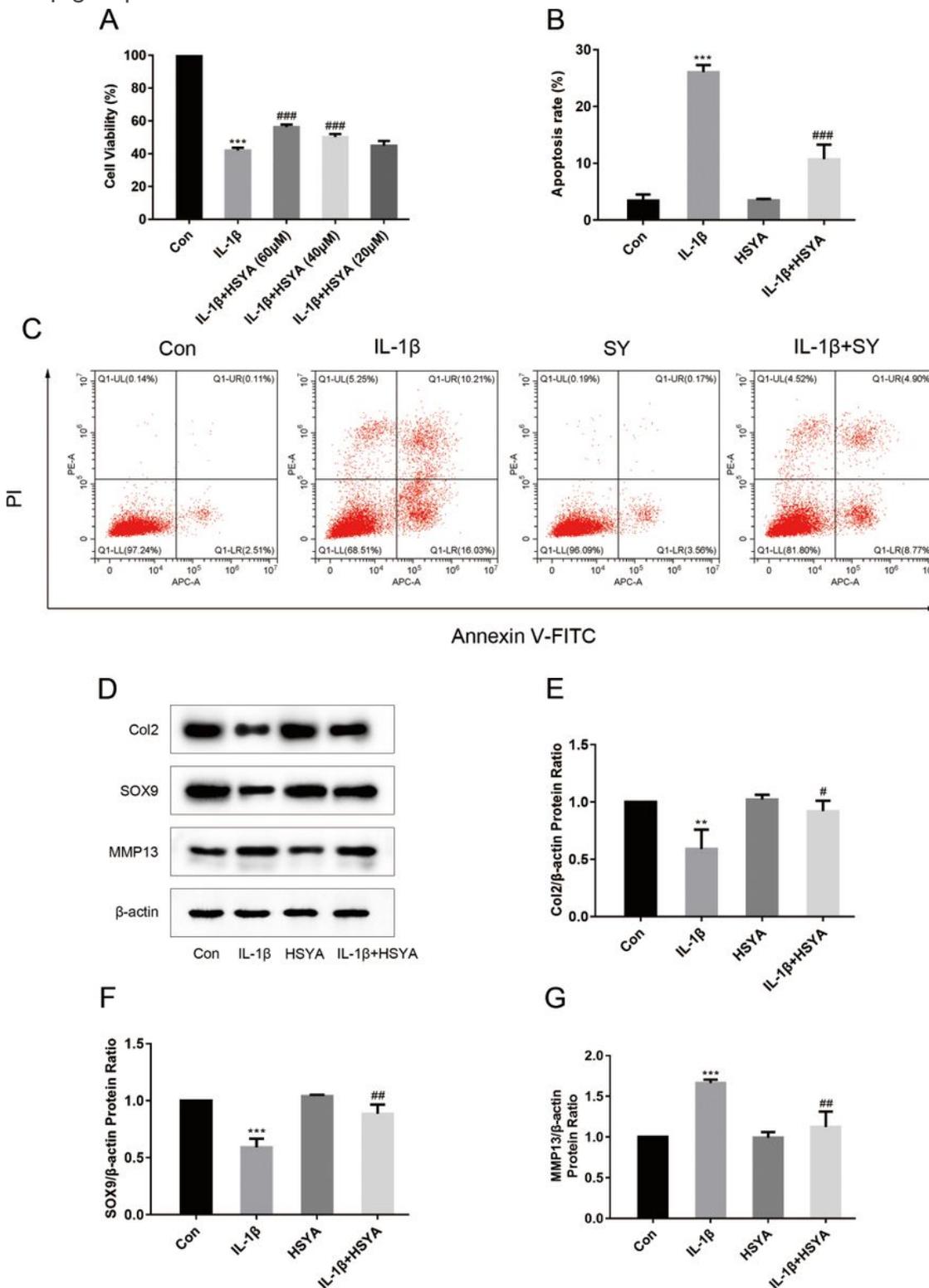


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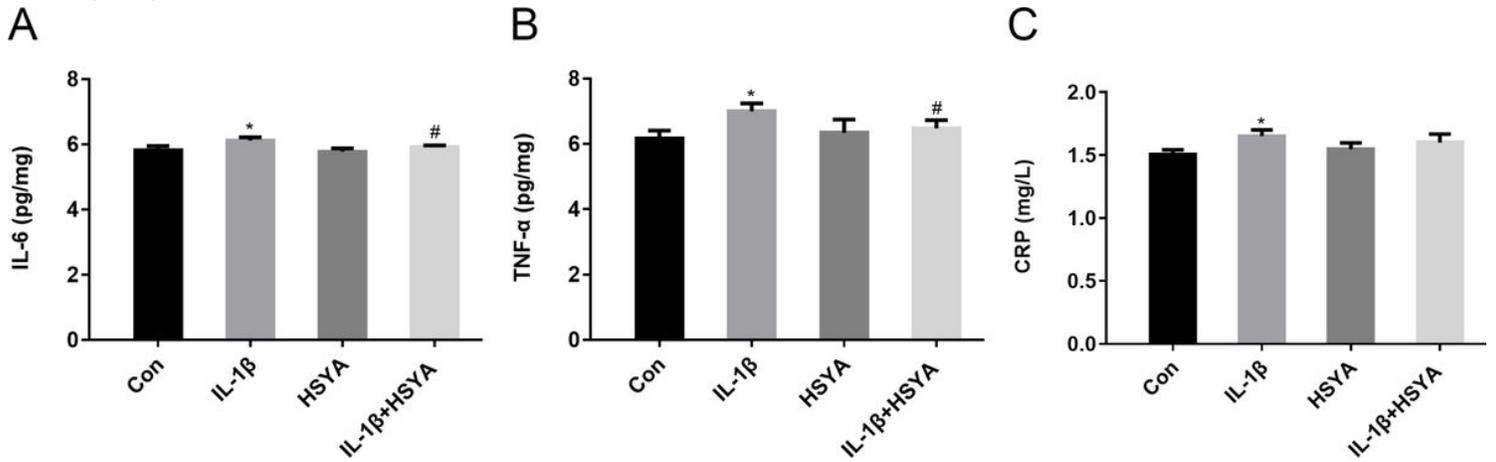


Figure 3

Inflammatory cytokines in response to HYSA. The doses of IL-6 (A), TNF- α (B), and CRP (C) were measured by ELISA. Data are presented as means \pm SD. *P < 0.05 compared with control group; #P < 0.05 compared with the IL-1 β group.

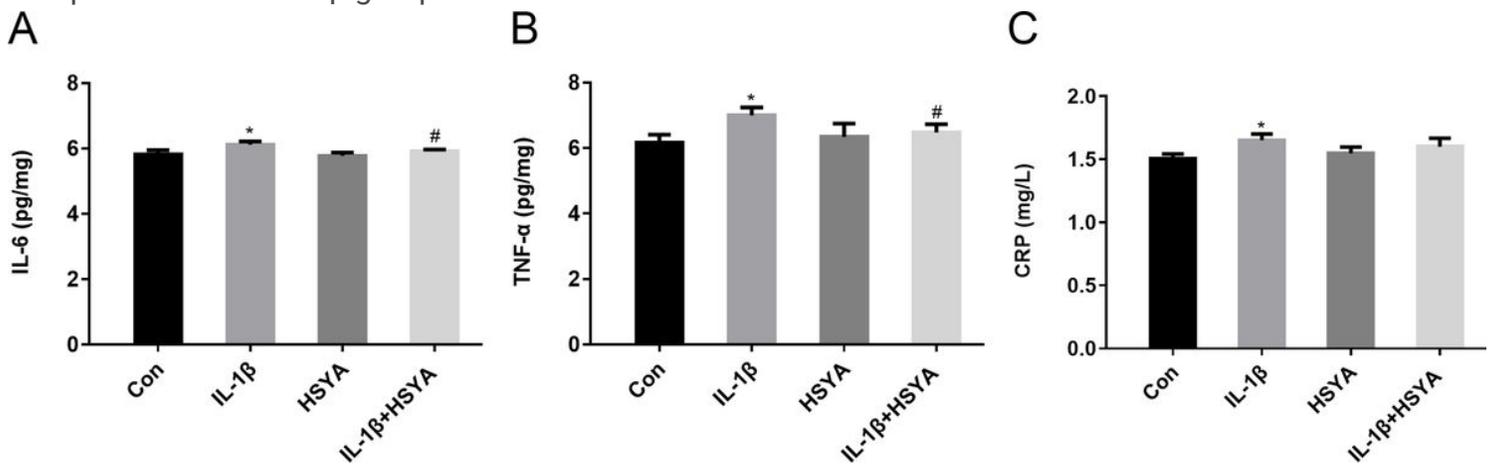


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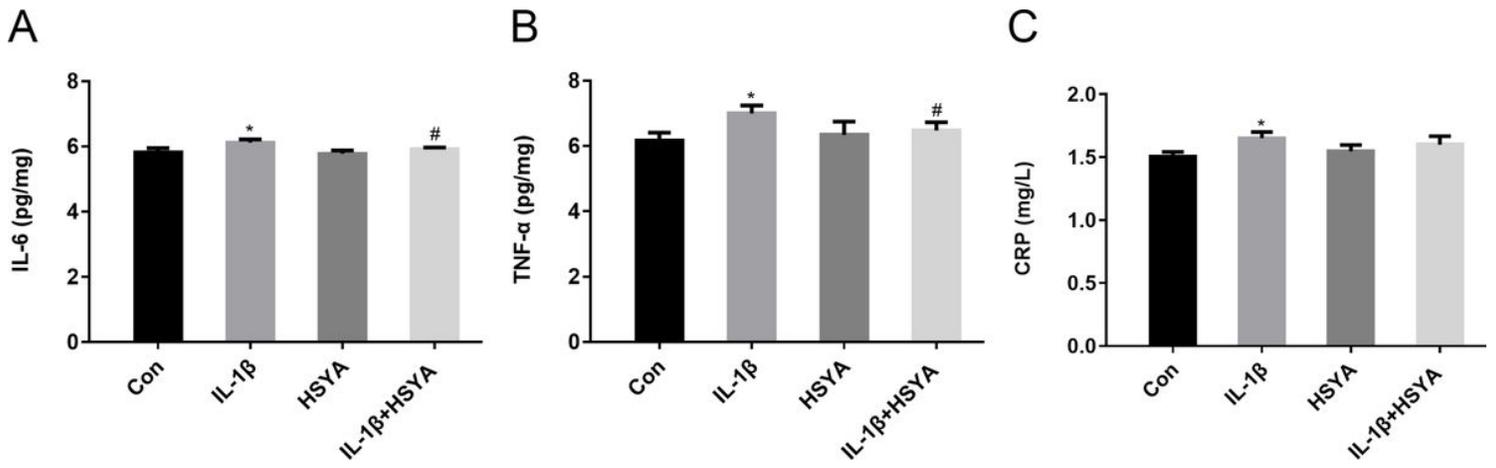


Figure 3

Inflammatory cytokines in response to HSYA. The doses of IL-6 (A), TNF- α (B), and CRP (C) were measured by ELISA. Data are presented as means \pm SD. *P < 0.05 compared with control group; #P < 0.05 compared with the IL-1 β group.

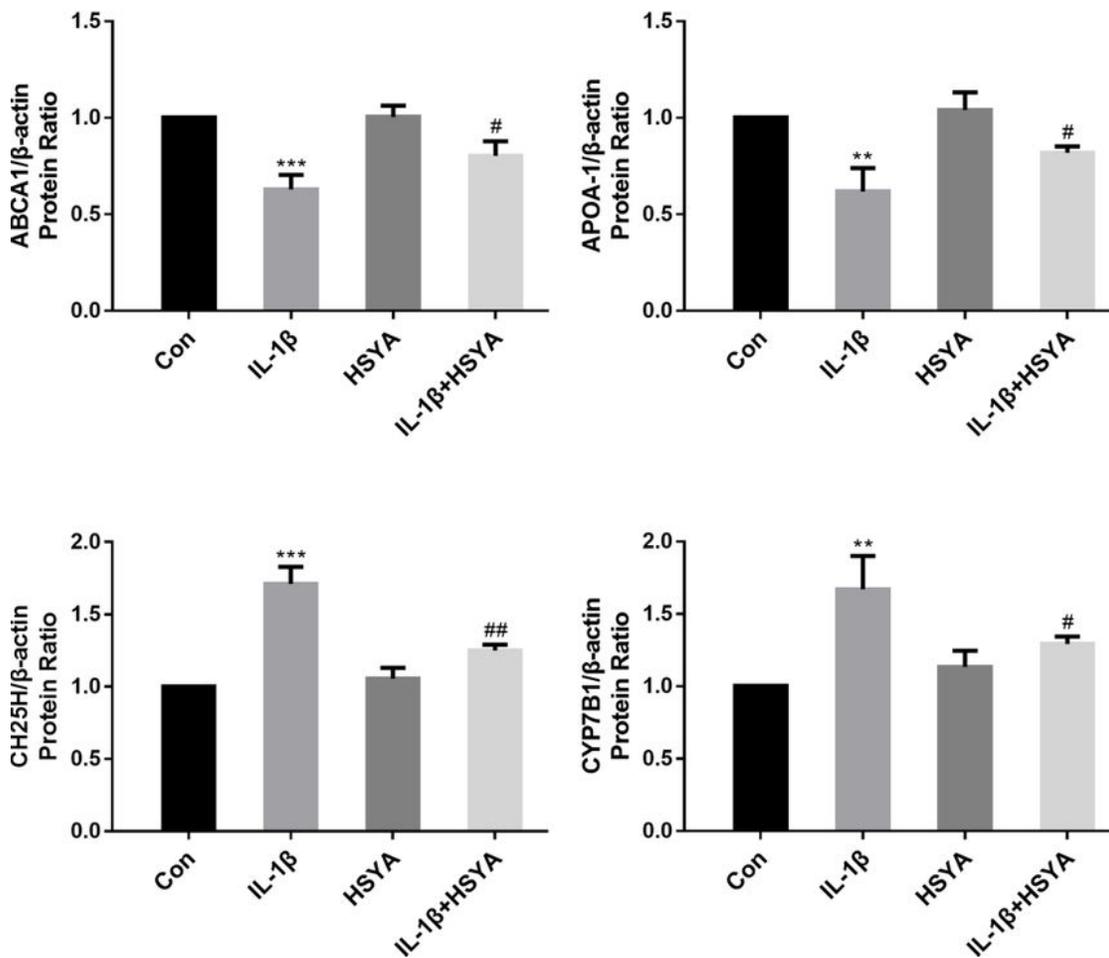
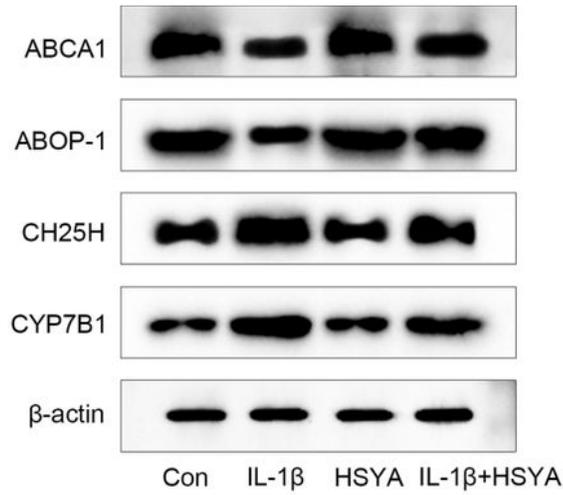


Figure 4

The regulation of cholesterol metabolism with HSYA. Data are presented as means \pm SD. **P < 0.01, ***P < 0.001 compared with control group; #P < 0.05, ##P < 0.01 compared with the IL-1 β group.

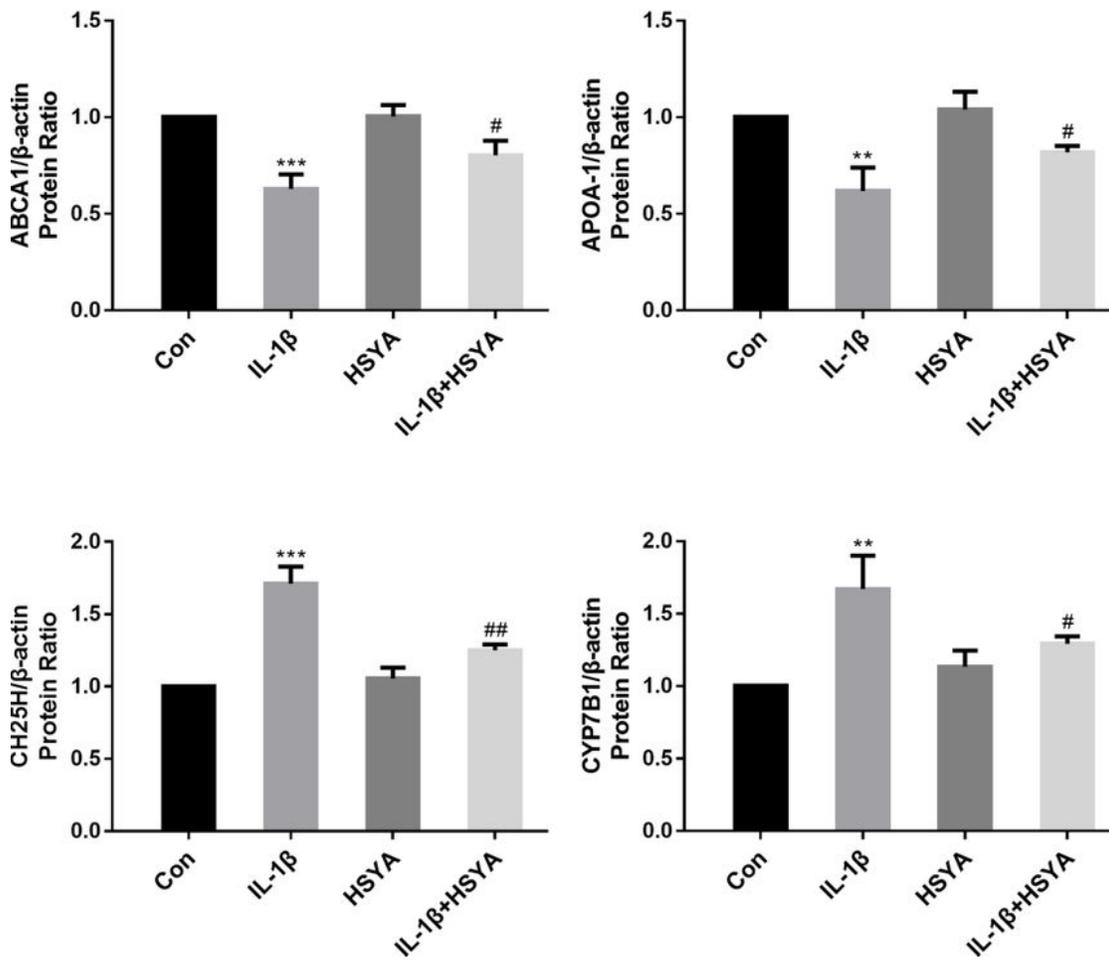
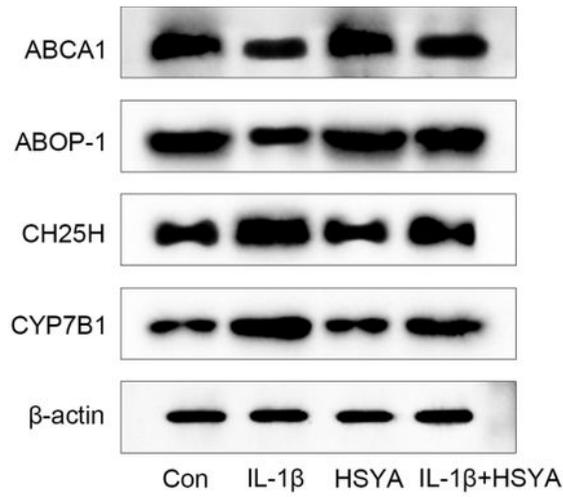


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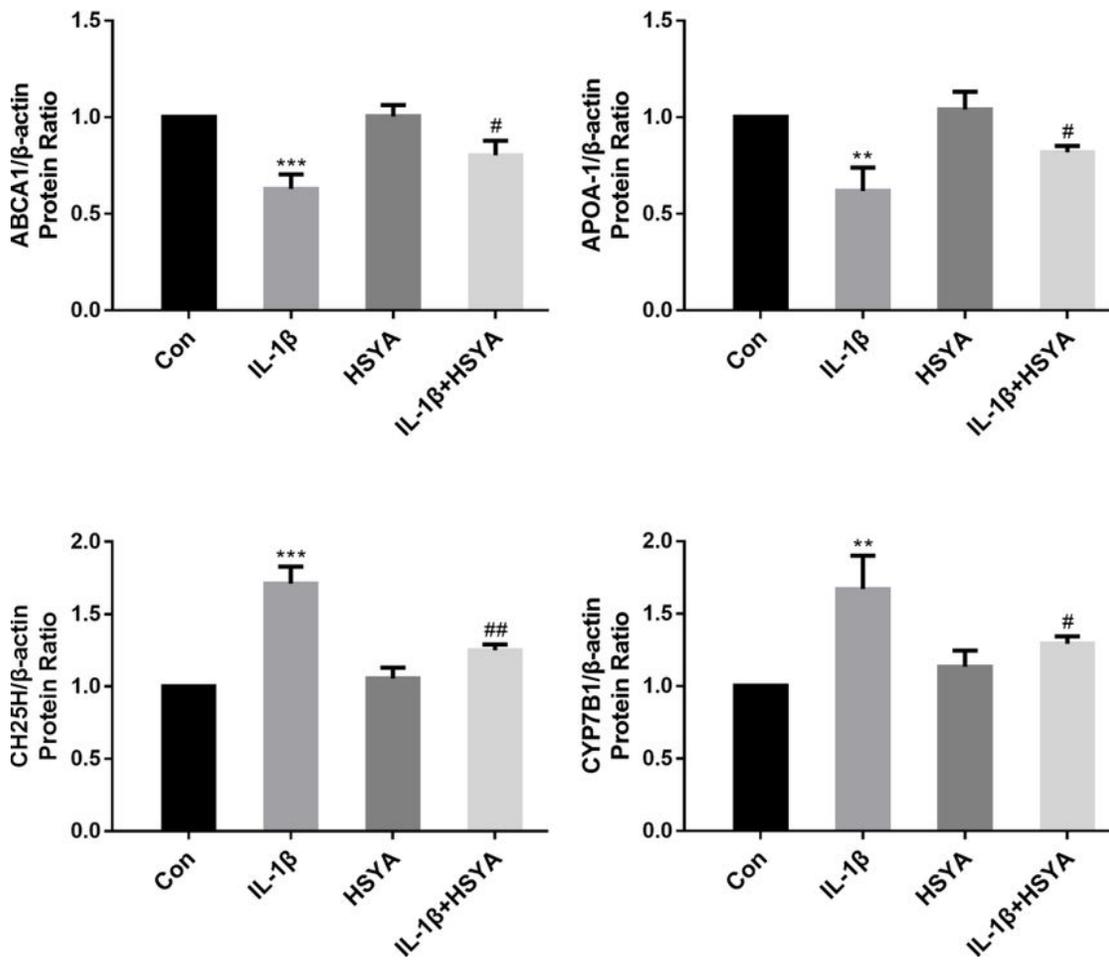
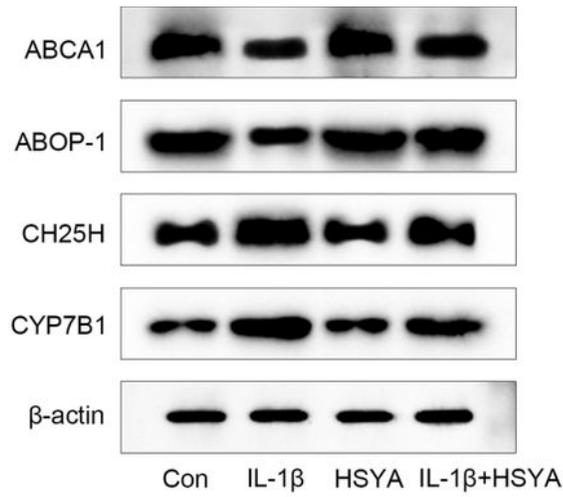


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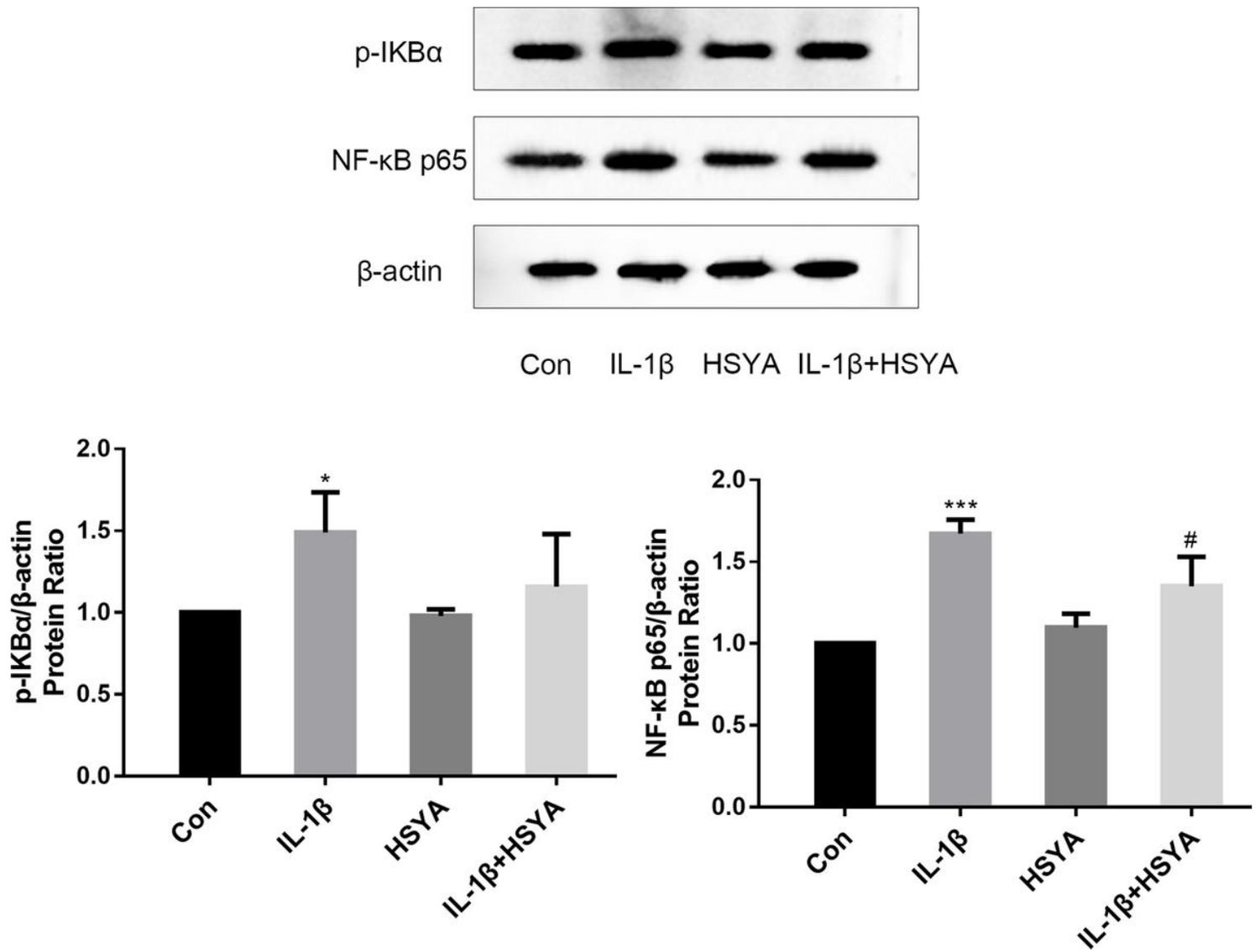


Figure 5

NF-κB signaling pathway in response to HSYA. A. The protein expression bands in the NF-κB signaling pathway. The levels of p-IκBα (B) and NF-κB p65 (C) were analyzed. Data are presented as means ± SD. *P < 0.05, ***P < 0.001 compared with control group; #P < 0.05 compared with the IL-1β group.

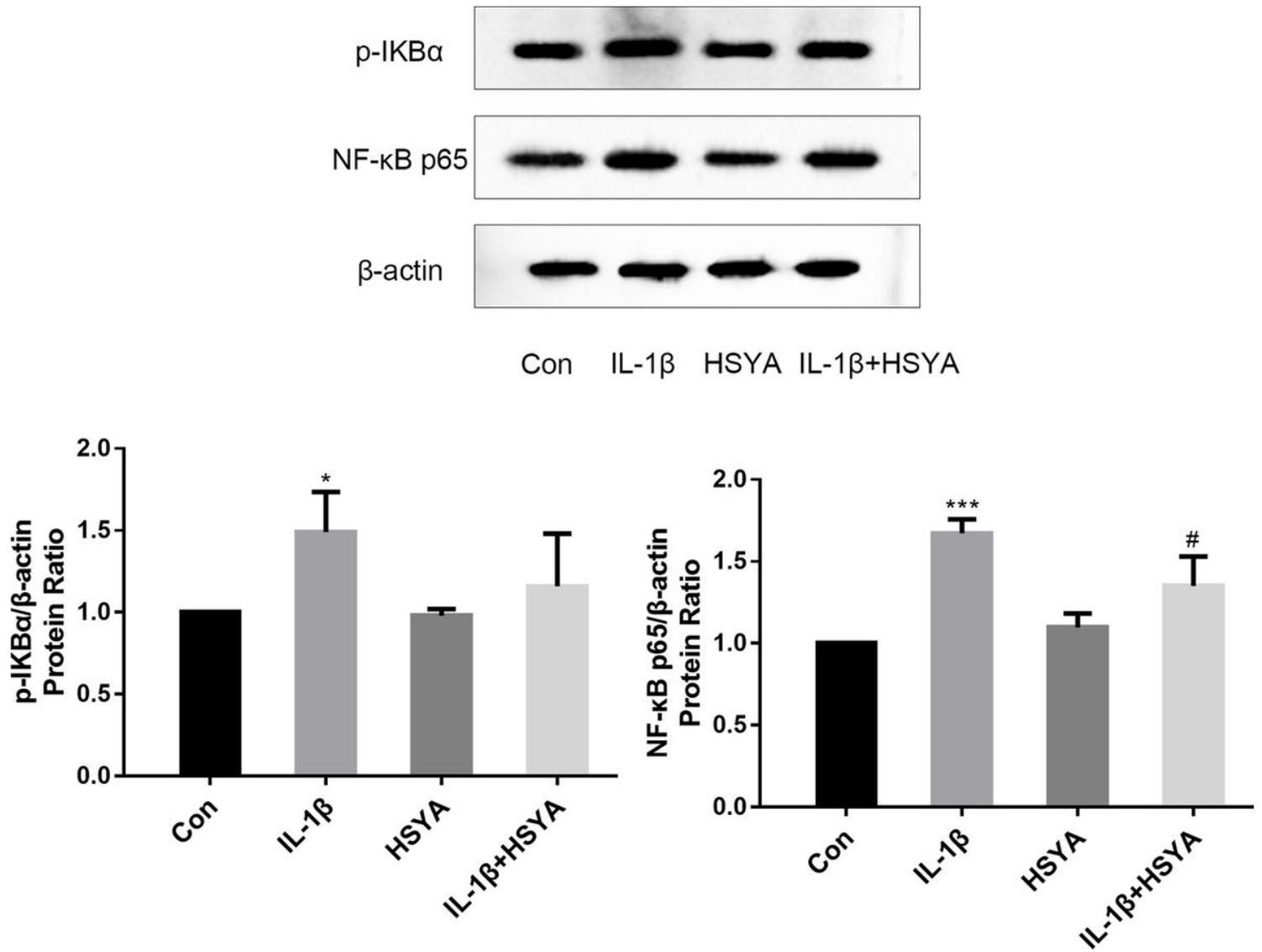


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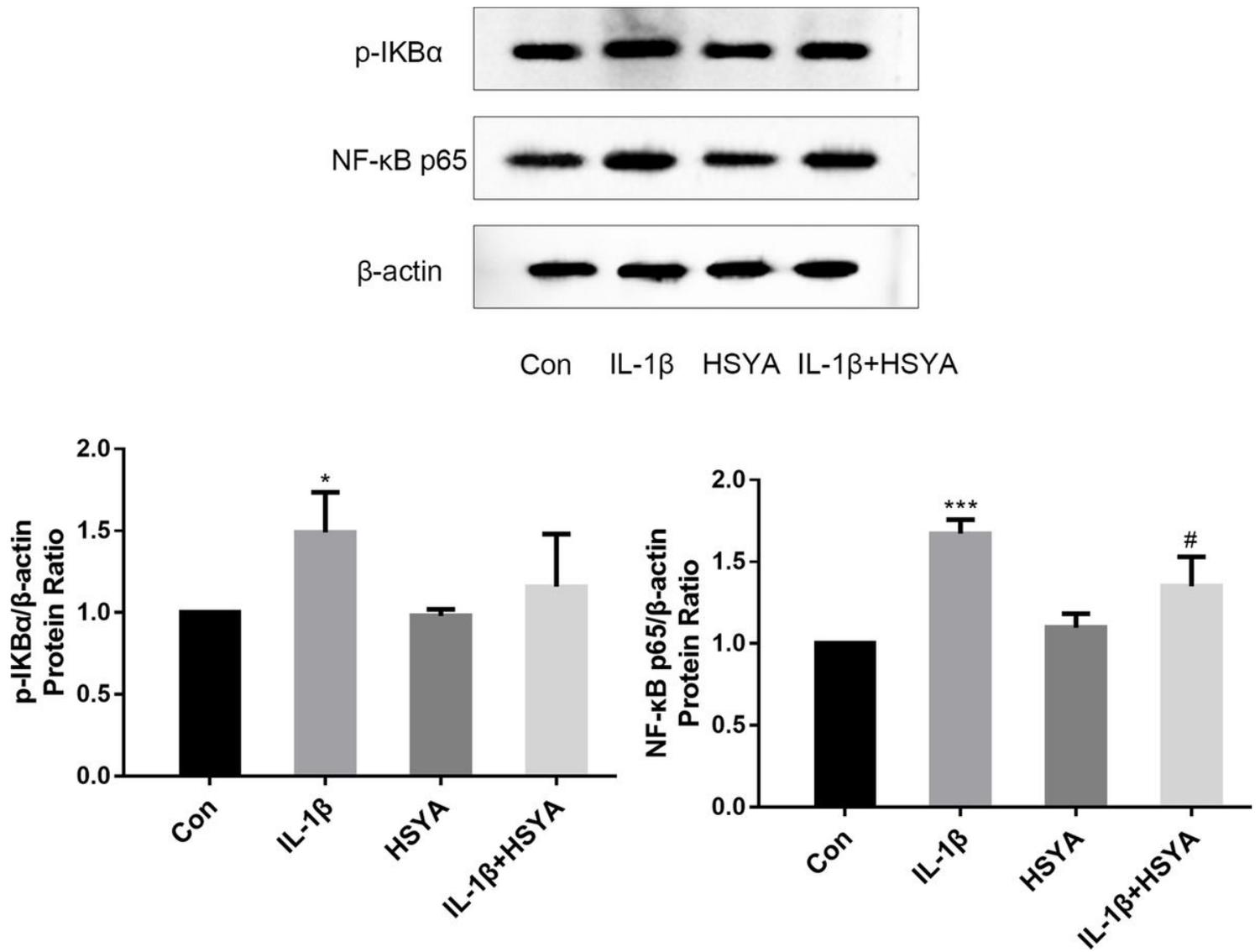


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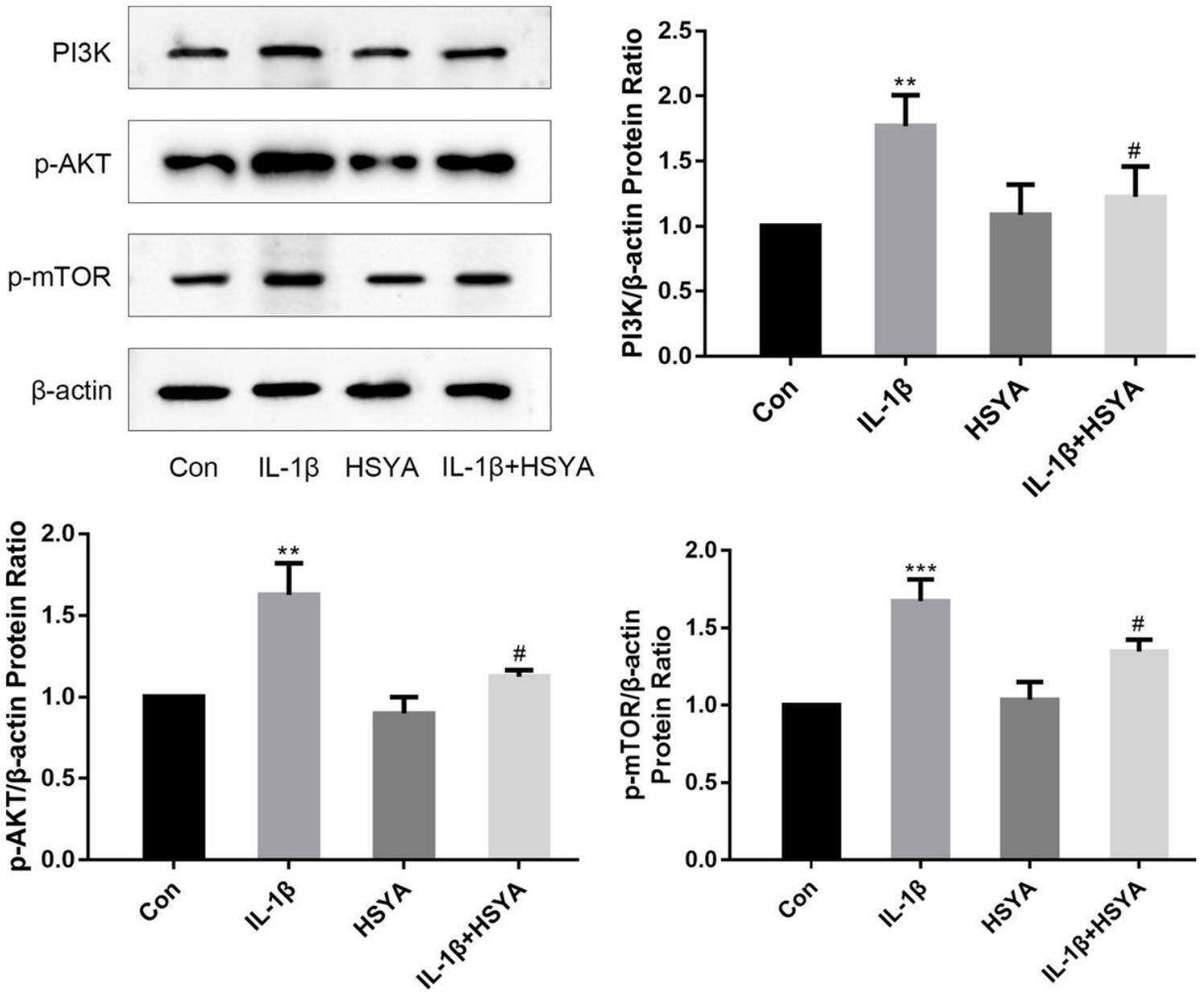


Figure 6

PI3K/Akt/mTOR signaling pathway in response to HSYA. Data are presented as means \pm SD. ** $P < 0.01$, *** $P < 0.001$ compared with control group; # $P < 0.05$ compared with the IL-1 β group.

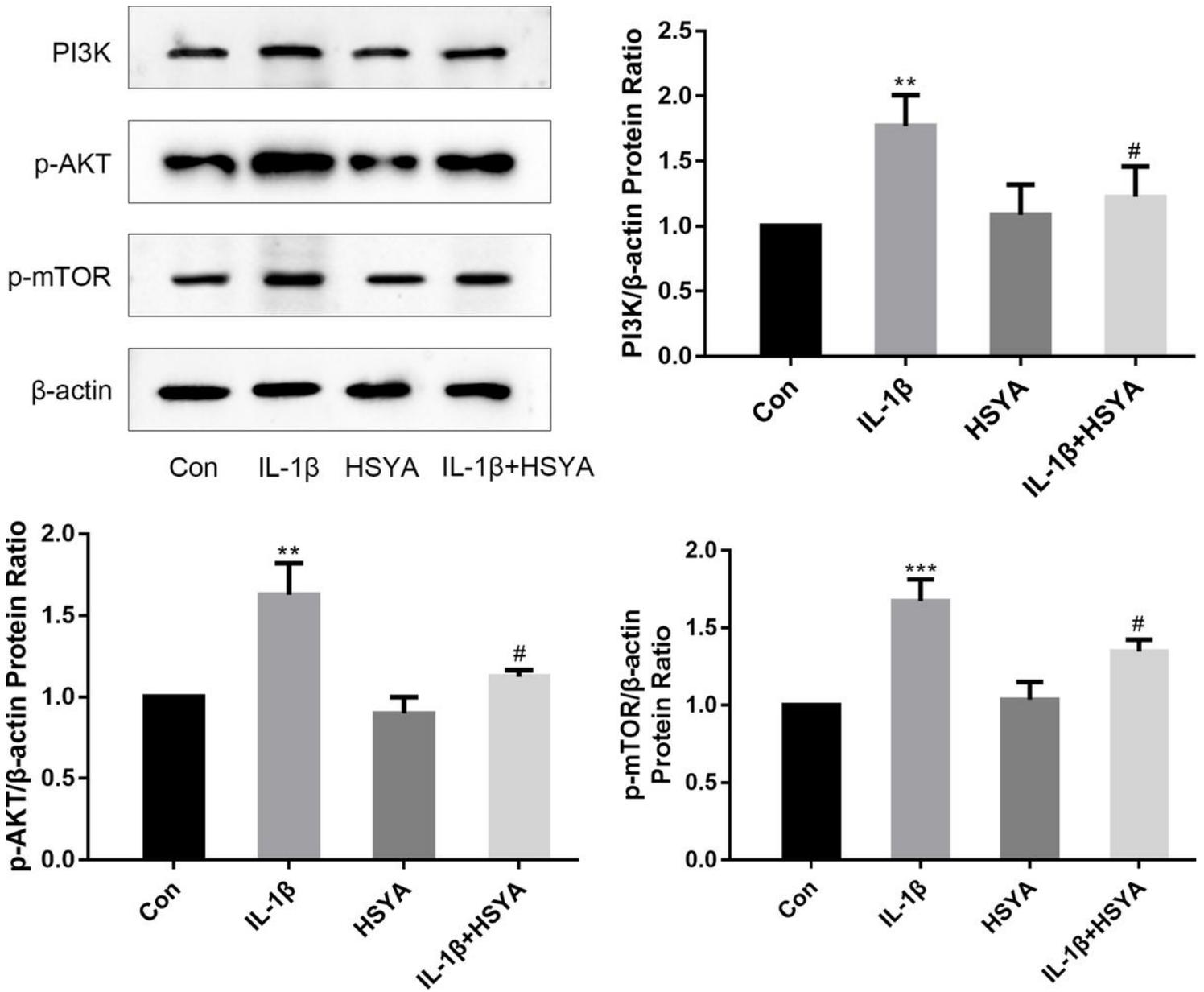


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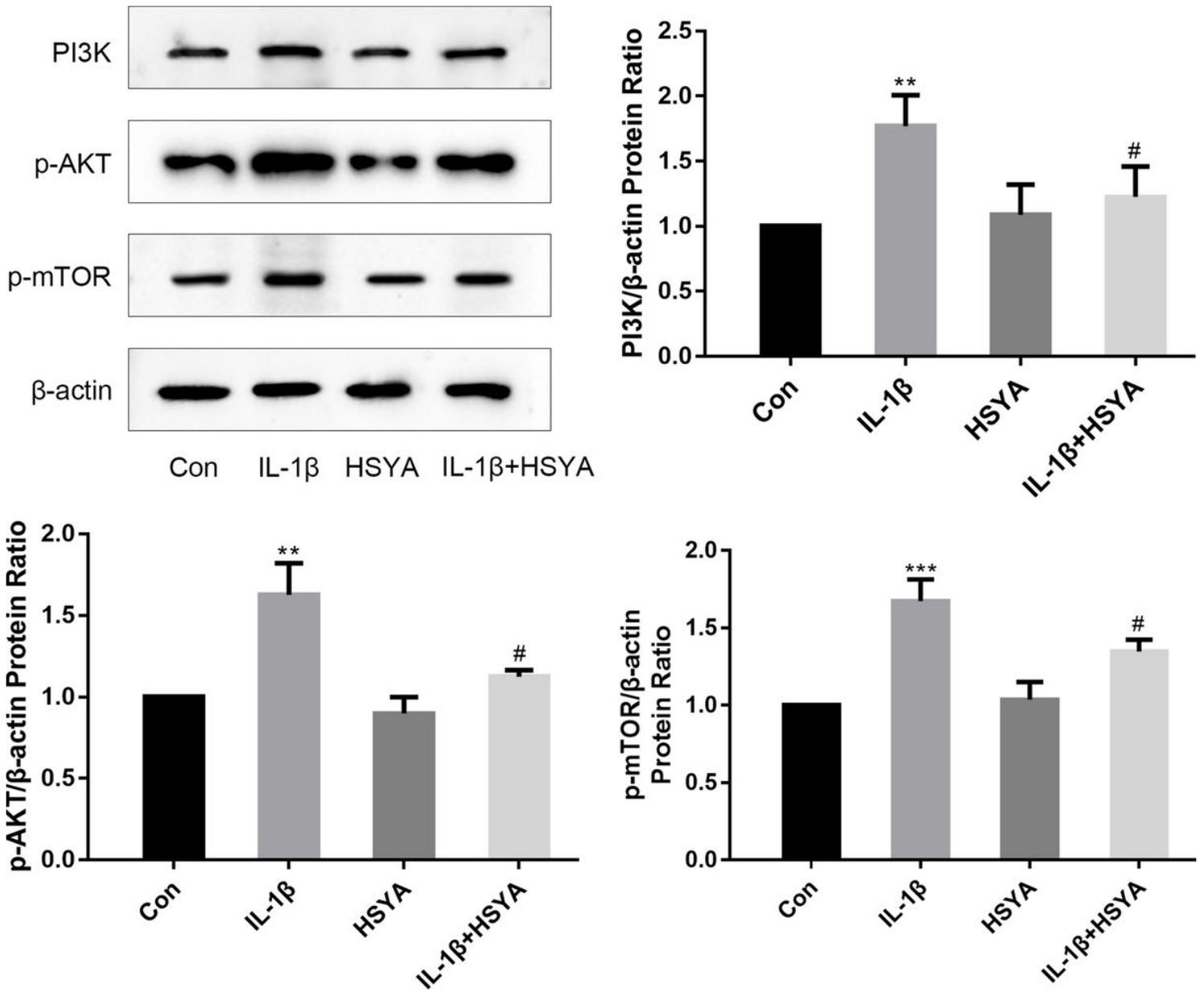


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