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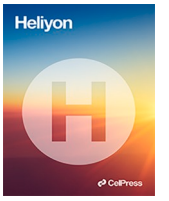

Insulin augments angiotensin II-induced myocardial fibrosis via the MEK/STAT3 pathway

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ABSTRACT

Regular insulin therapy is significantly related to worse cardiovascular outcomes in patients with type 2 diabetes and heart failure. However, the mechanisms of the causal relationship remain unclear. In this study, we observed the effect of insulin on cardiac structure and function and found that insulin dramatically augmented angiotensin II (Ang II)-induced cardiac dysfunction, as well as the proliferation and collagen production of primary cardiac fibroblasts. Total STAT3 expression, but not activation was stimulated by insulin; the effect of insulin on Ang II-induced fibrosis disappeared when STAT3 was blocked and could be entirely suppressed by the MEK inhibitor PD0325901. Our findings suggest a noninsulin-dependent glucose-lowering regimen for patients with type 2 diabetes (T2DM) and heart failure (HF).

1. Introduction

Several large-scale randomized clinical trials (RCTs) have shown that in patients with type 2 diabetes (T2DM) and heart failure (HF), insulin treatment was statistically related to worse cardiovascular outcomes [1–7]. Recently, meta-analyses obtained similar conclusions that insulin markedly increased the risk of cardiovascular death and rehospitalization for cardiac dysfunction in HF patients with reduced or preserved ejection fraction [8,9]. However, the potential mechanisms by which insulin aggravates heart failure remain unclear, which brings great challenges to improving glucose-lowering treatments.

Insulin upregulates the expression of several sodium reabsorption-related proteins, including the Na+/H+ transporter NHE3 and (Na/K) ATPase, in the proximal glomerular tubules of the opossum [10–12], and the slow release of insulin in the rat renal artery increases mean arterial pressure [13], suggesting that insulin treatment may indirectly induce or exacerbate heart failure through water and sodium retention. However, experimental studies of chronic insulin infusion in some canines have not shouwn changes in blood volume or blood pressure [14,15].

Signal transducer and activator of transcription (STAT) proteins, including STAT1-4, STAT5a, STAT5b and STAT6, are a family of cytoplasmic transcription factors, that mediate various signaling pathways [16]. Accumulating evidence indicates that STAT3 plays essential roles in various biological processes, such as cell proliferation, differentiation, survival, and angiogenesis [17]. Recently, it was reported that STAT3 plays a critical role in cardiac dysfunction by specifically mediating Ang II-induced remodeling [18,19]. Moreover, our previous study indicated that insulin could markedly increase the level of total STAT3 [20], suggesting that insulin might indirectly amplify the effect of AngII and thus contribute to worse clinical outcomes.

In this study, we observed the effect of insulin on cardiac structure and function in Ang II-induced heart failure. In addition, we

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investigated the role of insulin in Ang II-induced interstitial fibrosis and its molecular mechanism in primary rat cardiac fibroblasts. Our findings might improve the understanding of the prognostic effect of insulin on patients with T2DM and HF, provide new evidence to optimize glucose-lowering therapy and thus improve clinical outcomes.

2. Materials and methods

2.1. Insulin-treated rats with or without Ang II-induced heart failure

The animal experiments in this study were approved by the Animal Ethics Committee of the Second Affiliated Hospital of the Medicine School of Zhejiang University in Hangzhou, China. Twenty-four male Sprague–Dawley rats (160–180 g) were equally divided into four groups: normal control (control), heart failure (Ang II), regular insulin administration (Ins), and heart failure with insulin treatment (Ang II + Ins). Heart failure was induced by chronic Ang II infusion. An Alzet minipump (Alzet, No 2ML4, Palo Alto, CA, USA) was implanted into the subcutaneous tissue on the back of each rat in the Ang II and Ang II + Ins groups to deliver Ang II (1.0 mg/kg/day); rats in the other groups were administered buffer. A group of sham-operated rats without pump implantation was used as a blank control group. These animals did not differ significantly from the animals in the control group, and the data obtained from this group were grouped with the data from the control rats. Neutral insulin (16 units/kg; Novo Nordisk, Copenhagen, Denmark) was administered via subcutaneous injection twice per day at 8:00–10:00 a.m. and P.M.; non-fasting glucose was monitored right before the insulin injection, and the average level was determined for further statistical analysis. To keep the insulin efficacy stable during Ang II infusion, Alzet minipump implantation was performed 5 days after insulin treatment, and the rats were weighed and sacrificed by 5 weeks after the beginning of the experiment.

2.2. Blood pressure, glucose, IL-6 and IL-1β analysis

Systolic blood pressure (SBP) was measured by a noninvasive tail cuff blood pressure system (IITC Life Science, CA, USA) at the indicated times. Blood glucose in the tail was measured by Glucometer Gluco Touch (Roche). Radioimmunoassay kits (North Biotechnology, Beijing, China) were used to detect serum levels of IL-6 and IL-1β.

2.3. Cardiac structure and function assessment by echocardiography

One day before the samples were harvested, noninvasive echocardiographic examination was performed with a Vevo®2100 Imaging System (VisualSonics, USA) and a 21 MHz linear array ultrasound transducer under anesthesia with isoflurane. Left ventricular posterior wall thickness (LVPW), interventricular septal thickness (IVS), left ventricular end-diastolic diameter (LVDd), left ventricular end-systolic diameters (LVDs), and left ventricular ejection fraction (LVEF) were obtained by two-dimensional guided M-mode tracing measurements. The echocardiographic measurements were repeated at least 3 times during different cardiac cycles and averaged.

2.4. Histology

Histologic analysis was as performed described previously [21]. Briefly, rat hearts were collected 5 weeks after AngII administration, fixed in 4 % paraformaldehyde, sectioned longitudinally and embedded in paraffin. The samples were cut into 5-μm-thick sections according to standard procedures and stained with hematoxylin and eosin (HE, Servicebio, China). To measure the fibrotic areas, the sectioned hearts were stained with Masson trichrome. Pictures were taken with a fluorescence microscope (Axio Imager M2; Carl Zeiss, Oberkochen, Germany). The area of interstitial fibrosis was calculated as the ratio of the total area of interstitial fibrosis to the total area of the section. Quantification was performed with ImageJ software.

2.5. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from heart tissue or cells using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA with an RT Reagent Kit (Primerscript; Takara Bio Inc., Shiga, Japan). RT–qPCR was performed using a SYBR Green Kit (TAKARA). The mRNA levels of the indicated genes were normalized to β-actin. The corresponding primers are shown below:

COL1A1: F: CGATGGATTCCAGTTCGAGT,
R: TTTTGAGGGGGTTCAGTTTG;
COL3A1: F: GCCCACAGCCTTCTACACCT,
R: GCCAGGGTCACCATTTCTC; and
β-actin: F: AGCGAGCATCCCCCAAAGTT,
R: GGGCACGAAGGCTCATCATT.

2.6. Western blot (WB) analysis

Homogenates of rat hearts or primary cardiac fibroblasts were prepared for WB analysis. In this study, the following antibodies were used: anti-β-actin, anti–phospho-STAT3 (p-STAT3), anti-STAT3, anti-p-STAT1, anti-STAT1, anti-p-STAT5, and anti-STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA). Signals of the indicated proteins were quantified by densitometry and were normalized to β-actin

levels.

2.7. Isolation, culture and treatment of primary rat cardiac fibroblasts (CFs)

Rat CFs were isolated from neonatal rat hearts as described previously with minor modifications [21]. Briefly, the hearts were collected from 1- to 2-day-old rats, rinsed with PBS, and minced into 1- to 3-cubic-millimeter pieces. The samples were enzymatically lysed, and the cells were collected and incubated for 60 min at 37 °C in a humidified incubator containing 5 % CO_2-95 % air. Non-cardiomyocytes adhered more readily to the dishes, and the medium containing cardiomyocytes was aspirated and discarded. Cardiac noncardiomyocytes were prepared by passaging the cells twice, and the nature of the cells was determined by immunofluorescence staining with anti-retinoic acid to identify fibroblasts; more than 95 % of the cells were stained positively. Cells at passages 3 and 4 were used for the experiments and were serum-deprived for 24 h before being treated with 100 nM Ang II (Aladdin, Shanghai, China) and/or 100 nM insulin (Sigma, USA). STAT3 and STAT5 silencing was performed by transfection with small interfering RNAs (siRNAs) against STAT3 and STAT5 (sc-29494 and sc-29496; Santa Cruz Biotechnology, USA) as described in our previous study [20]. To examine the insulin pathway, cardiac fibroblasts were pretreated with the PI3K inhibitor LY294002 (10 μmol/L) or the mitogen-activated protein kinase (MEK) inhibitor PD0325901 (1 μmol/L) for 30 min and then treated with insulin (100 nmol/L) for 24 h.

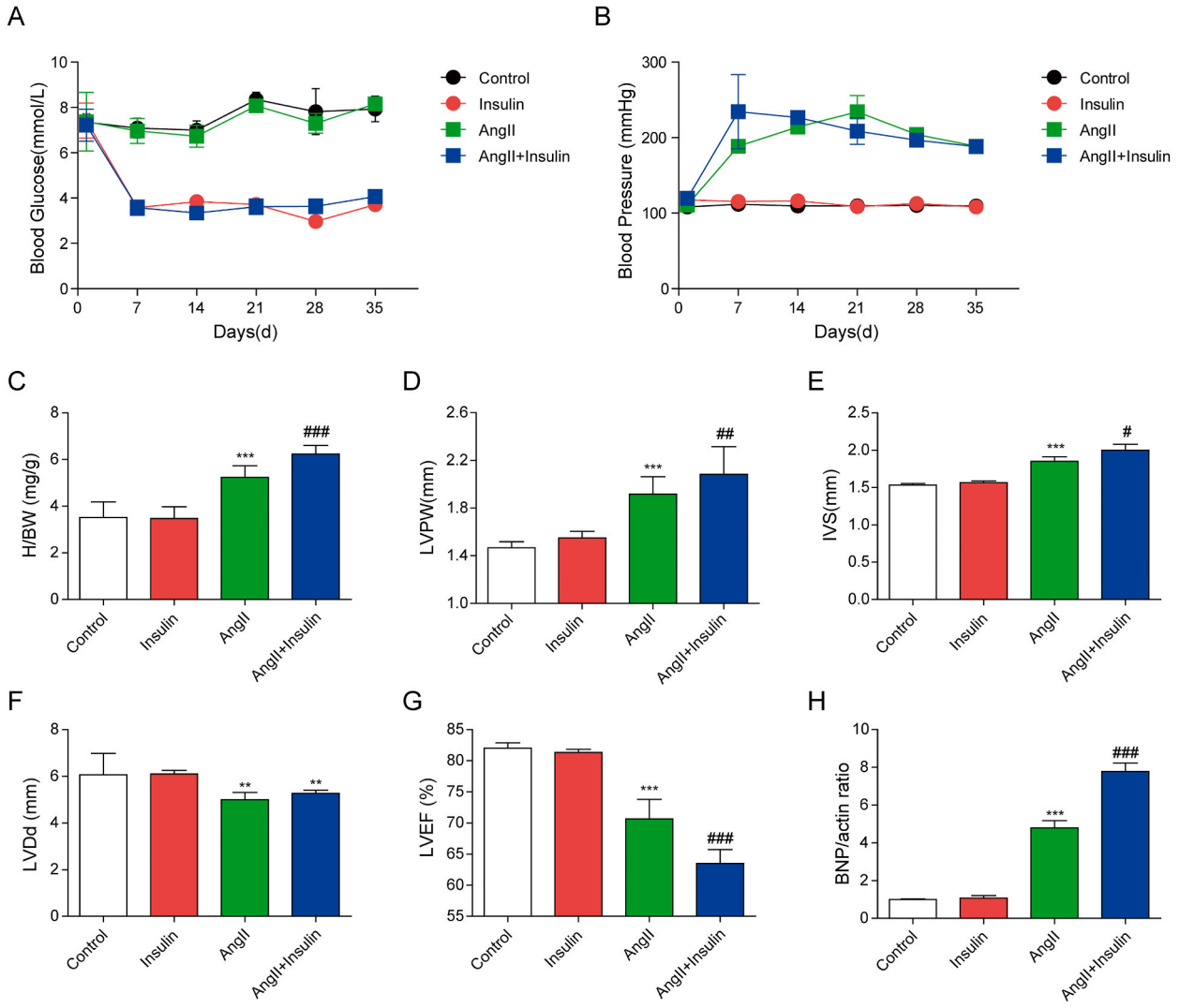

Fig. 1. Insulin aggravated Ang II-induced heart failure. A and B: Sprague−Dawley rats (n = 24) received regular insulin administration with or without chronic subcutaneous Ang II infusion. Glucose and blood pressure were monitored throughout the experiment. C: Statistical analysis of the heart weight/body weight ratio. D–G: Cardiac ultrasonography was performed using a small animal ultrasound imager (Vevo 2100) one day before harvest. LVPW, IVS, LVDd, and LVEF were measured. H: qPCR analysis of the mRNA expression of BNP in rat heart tissue. **, P < 0.01 compared with the control group; ***, P < 0.001. #, P < 0.05 compared to the Ang II group, ##, P < 0.01; ###, P < 0.001.

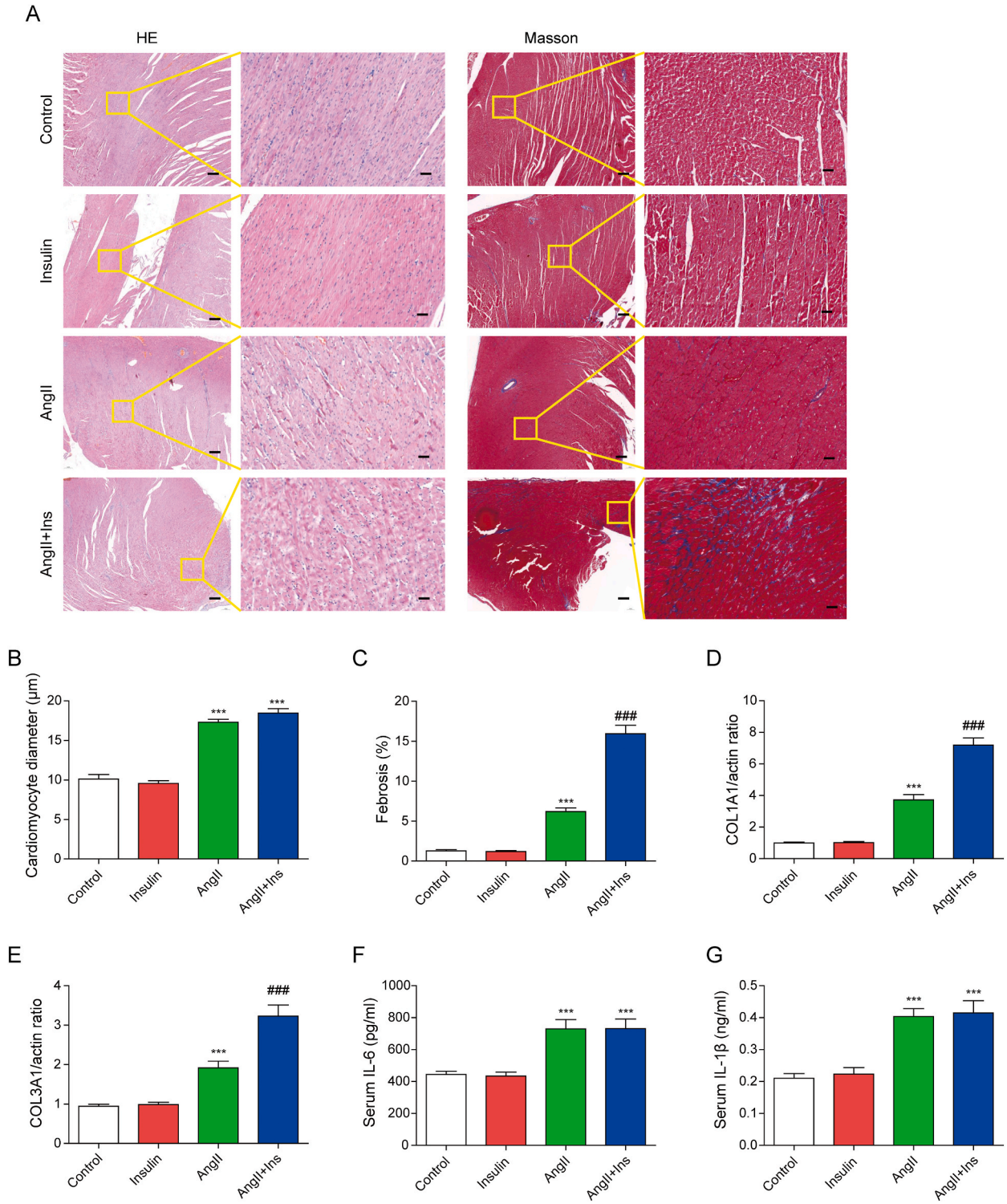

Fig. 2. Insulin aggravated Ang II-induced myocardial fibrosis. A: Hearts of twenty-four rats were fixed in formalin solution for 48 h and embedded in paraffin, the left ventricle was serially sectioned (thickness 3–4 μm), and histological analysis of heart tissues was performed with HE staining and Masson's staining (Scale bar = 50 μm). B: Quantification of cardiomyocyte diameter in HE-stained sections by ImageJ software. C: Quantification of the fibrotic area, as measured by Masson staining. D–E: Real-time PCR analysis of the mRNA expression of the fibrotic genes collagen *COL1A1* and *COL3A1*. F–G: Radioimmunoassay of IL-6 and IL-1b levels in serum. ***, $P < 0.001$ compared to the control group; ###, $P < 0.001$ compared to the Ang II group.

2.8. Cell proliferation and collagen synthesis

To measure the synthesis of new DNA during proliferation, cardiac fibroblasts were incubated for 24 h in media containing 1 μCi/mL [3H]-thymidine or 2 μCi/mL [3H]-proline. The cells were then harvested with 5 % trichloroacetic acid and solubilized in 0.1 M NaOH, and radioactivity was detected by a liquid scintillation counter (LS6500, Beckman, US).

2.9. Statistical analysis

The data are presented as the mean ± SEM. Statistical analysis was performed using StatView software (SAS Institute Inc., Cary, NC). Statistical differences between two groups were assessed by the independent *t*-test. One-way ANOVA followed by the least significant difference (LSD) t and Student-Newman–Keuls (SNK) post hoc test was performed to analyze the differences between three or more groups. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Insulin-aggravated Ang II-induced heart failure

No previous study has focused on the direct or indirect effect of insulin on cardiac remodeling or heart failure. To better clarify the mechanisms underlying the relationship between insulin and adverse cardiovascular events, this study performed regular insulin administration in normal rats but in those undergoing cardiac remodeling induced by angiotensin II. The results showed that rats that received insulin alone exhibited continuous hypoglycemia (Fig. 1A), but there were no significant changes in echocardiographic parameters including ejection fraction (Fig. 1C–H). And Ang II did not affect the level of blood glucose compared with that in the control group (Fig. 1A). Chronic Ang II infusion caused significant hypertension (Fig. 1B), which led to cardiac remodeling, as

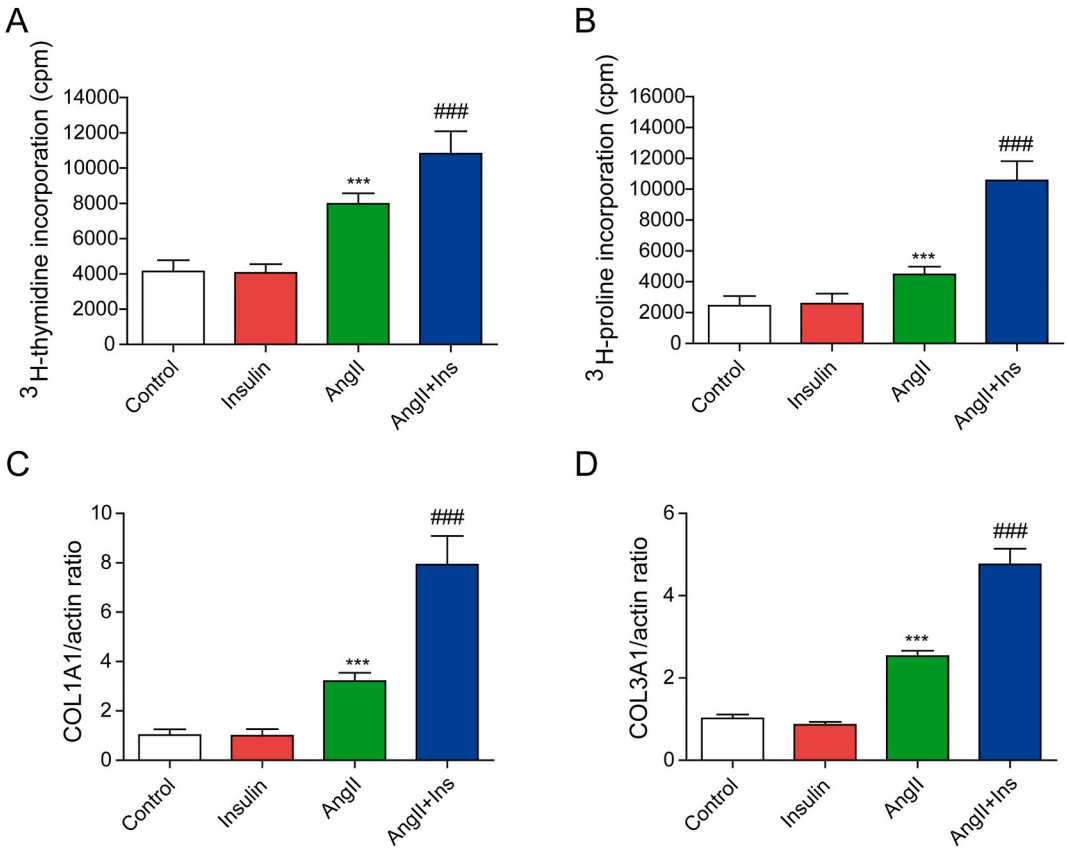

Fig. 3. Insulin promoted Ang II-induced proliferation and collagen synthesis in primary rat CFs. A, B: Primary cultured rat cardiac fibroblasts were treated with 100 nM insulin and/or 100 nM angiotensin II. The proliferation of CFs was detected via [3H]-thymidine incorporation assays (A), and [3H]-proline incorporation assays were used as indirect indicators of collagen synthesis (B). C, D: qPCR analysis of the mRNA expression of the fibrotic genes collagen COL1A1 and COL3A1. Data are expressed means ± SEM, determined in three independent experiments. Oneway ANOVA, followed by LSD t and SNK post hoc test, was performed to analyze difference between the groups. ***, $P < 0.001$ compared to the control group; ###, $P < 0.001$ compared to the Ang II group.

characterized by heavier hearts ($P < 0.001$, Fig. 1C), increased LVPW and IVS thickness ($P < 0.001$, Fig. 1D and E), and cardiac dysfunction, as represented by a reduced LVEF (vs. control, 70.67 ± 3.14 vs. 82.00 ± 0.89, $P < 0.001$, Fig. 1H). When Ang II infusion was accompanied by insulin treatment, we found a slight but notable aggravation of cardiac hypertrophy (Ang II + Ins vs. Ang II, for H/BW, 6.04 ± 0.16 vs. 5.02 ± 0.18, $P = 0.001$; for LVPW thickness, 2.12 ± 0.17 vs. 1.92 ± 0.10, $P = 0.004$; for IVS thickness, 2.00 ± 1.90 vs. 1.85 ± 0.15, $P = 0.054$; Fig. 1C–E), markedly deteriorated heart failure, as shown by lower LVEF (Ang II + Ins vs. Ang II, 63.50 ± 2.24 vs. 70.67 ± 3.14, $P < 0.001$, Fig. 1G), and increased tissue levels of B-type natriuretic peptide (BNP), a classic biomarker that was secreted in response to cardiac dysfunction (Fig. 1H).

3.2. Insulin-aggravated Ang II-induced myocardial fibrosis

To provide a preliminary explanation for how insulin aggravates AngII-induced heart failure, we performed histopathological examination of the left ventricular myocardium in rats by HE and Masson staining (Fig. 2A), with treated by insulin, AngII and the combination of insulin and AngII. Obvious cardiomyocyte hypertrophy was found in the AngII group, while insulin alone or in combination with AngII induced no changes in morphology (Fig. 2B). Almost no fibrosis was found in normal or insulin-treated rat

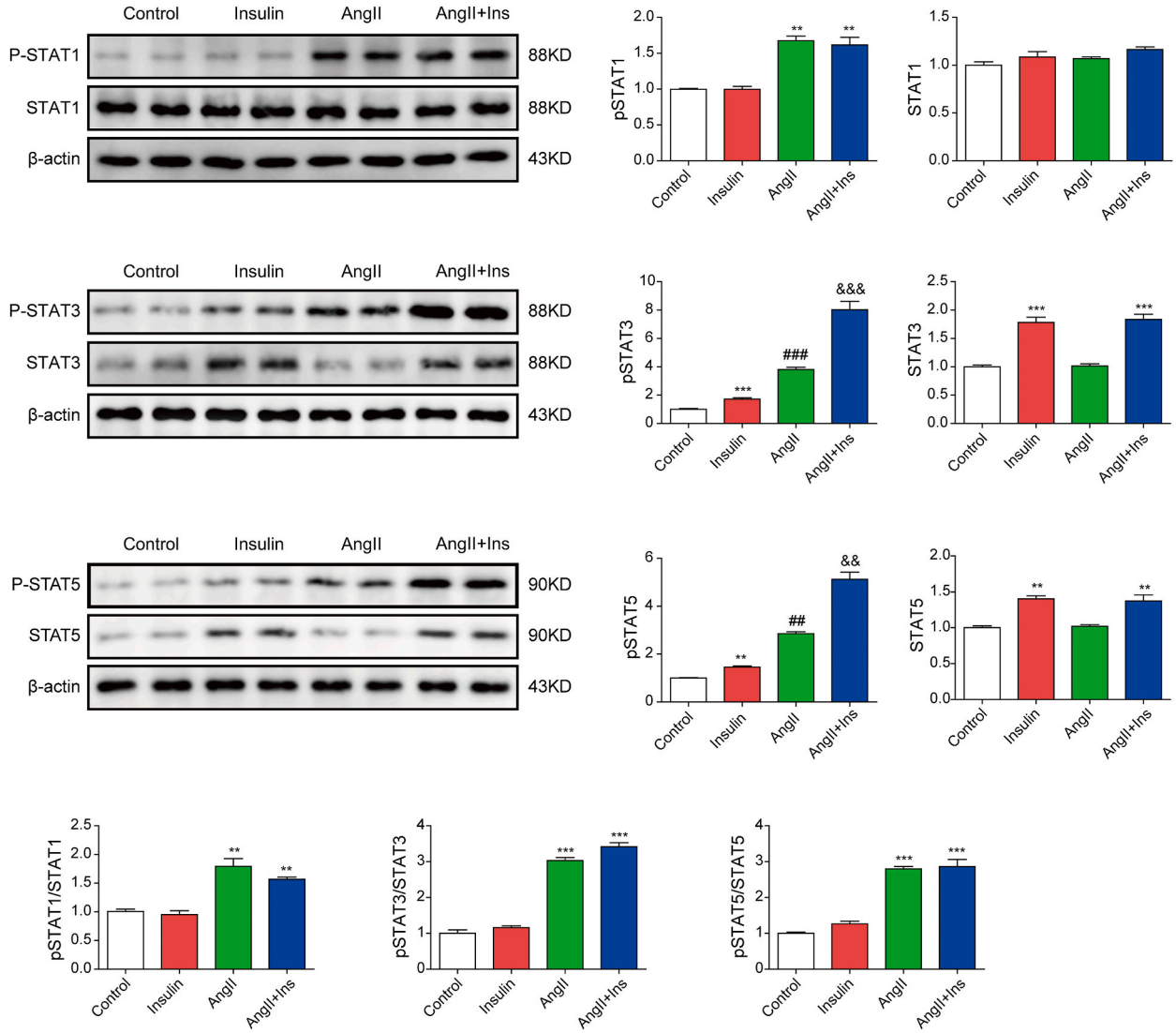

Fig. 4. Insulin facilitated Ang II-mediated STAT expression and activation in rat heart tissue. Protein levels of p-STAT1, STAT1, p-STAT3, STAT3, p-STAT5 and STAT5 in the heart tissues of insulin-treated rats with or without Ang II-induced heart failure were detected by WB analysis, and the phosphorylation of STAT1, 3, and 5 was determined by calculating the ratio of p-STATs/STATs. The original image of Western blot is in the supplementary material. *, $P < 0.05$ compared with the control group; Data are expressed means ± SEM, determined in three independent experiments. Oneway ANOVA, followed by LSD t and SNK post hoc test, was performed to analyze difference between the groups. ***, $P < 0.001$. #, $P < 0.05$ compared to the insulin group, ##, $P < 0.01$; ###, $P < 0.001$. &, $P < 0.05$ compared with the Ang II group, &&, $P < 0.01$; &&&, $P < 0.001$.

hearts, and there was obvious collagen deposition in rats after AngII infusion. Importantly, AngII-induced fibrosis was further aggravated in the presence of insulin (Fig. 2C). Consistently, the mRNA expression of the collagen genes COL1A1 and COL3A1 was significantly increased in AngII-treated rats and further stimulated when insulin was administered (Fig. 2D and E). Notably, serum IL-6 and IL-1β levels were sensitive indicators of inflammation in the AngII group, but we failed to observe further changes after insulin treatment (Fig. 2F and G).

3.3. Insulin-promoted Ang II-induced proliferation and collagen synthesis in primary rat CFs

To further explore the role of insulin in cardiac fibrosis, proliferation and collagen synthesis in primary cultured rat CFs were detected in response to insulin treatment with or without Ang II. A [3H]-thymidine incorporation assay was used to examine CF proliferation. The transcription of the collagen genes COL1A1 and COL3A1 was determined by RT−qPCR, and collagen synthesis was detected by the [3H]-proline incorporation assay. Although the proliferation and collagen synthesis of normal CFs showed no responses to insulin treatment, clear changes were observed in Ang II-treated CFs in the absence or presence of insulin (Fig. 3A and B). [3H]-thymidine incorporation, as well as collagen transcription and synthesis, were significantly stimulated by Ang II and further

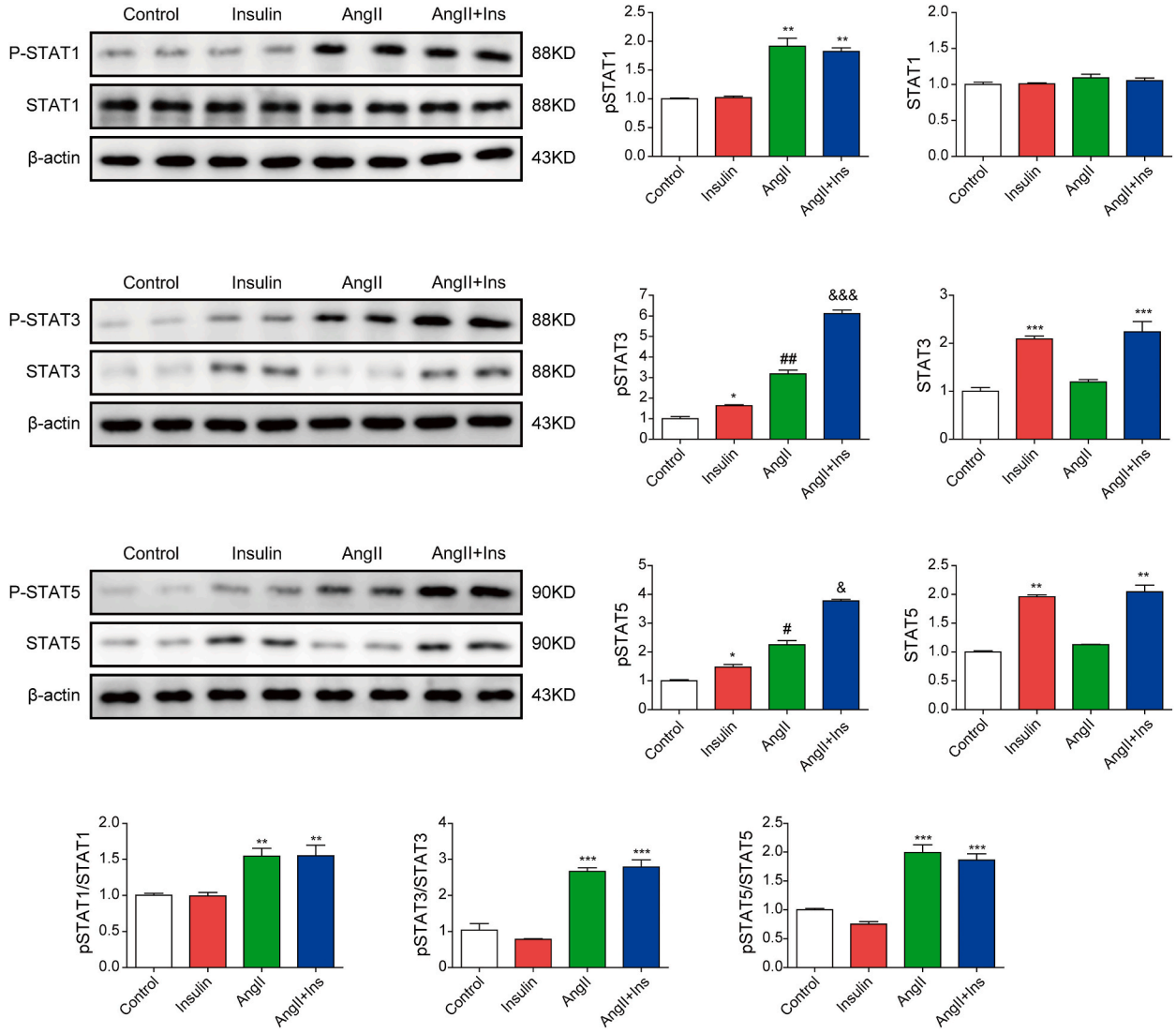

Fig. 5. Insulin facilitated Ang II-mediated STAT expression and activation in primary rat cardiac fibroblasts. Primary rat cardiac fibroblasts were treated with 100 nM insulin with or without 100 nM angiotensin II, and the phosphorylation of STAT1, 3, and 5 was calculated by determining the ratio of p-STATs/STATs. The original image of Western blot is in the supplementary material. Data are expressed means ± SEM, determined in three independent experiments. Oneway ANOVA, followed by LSD t and SNK post hoc test, was performed to analyze difference between the groups. *, $P < 0.05$ compared with the control group; **, $P < 0.01$; ***, $P < 0.001$. #, $P < 0.05$ compared to the insulin group, ##, $P < 0.01$. &, $P < 0.05$ compared with the Ang II group, &&&, $P < 0.001$.

enhanced by insulin treatment (Fig. 3A-D).

Insulin facilitated Ang II-mediated expression and activation of STATs in rat heart tissue and primary rat CFs.

STAT3 is a key mediator of Ang II-induced myocardial fibrosis [18,19]. Moreover, our previous study confirmed in the rat liver that insulin could significantly increase the level of total STAT3 in a dose-independent manner [20]. In addition, other STAT members, such as STAT1 and STAT5, were activated by Ang II and could participate in ischemia/reperfusion injury [22–25]. To verify whether STAT3 and other STAT family members were involved in promoting Ang II-induced myocardial fibrosis, we further observed the effect of insulin on STAT1, STAT3 and STAT5 protein levels in heart tissue in the rat model (Fig. 4) and primary rat cardiac fibroblasts (Fig. 5). Consistently, insulin had no effect on the protein expression of STAT1 but markedly upregulated the total protein levels of STAT3/5 (P

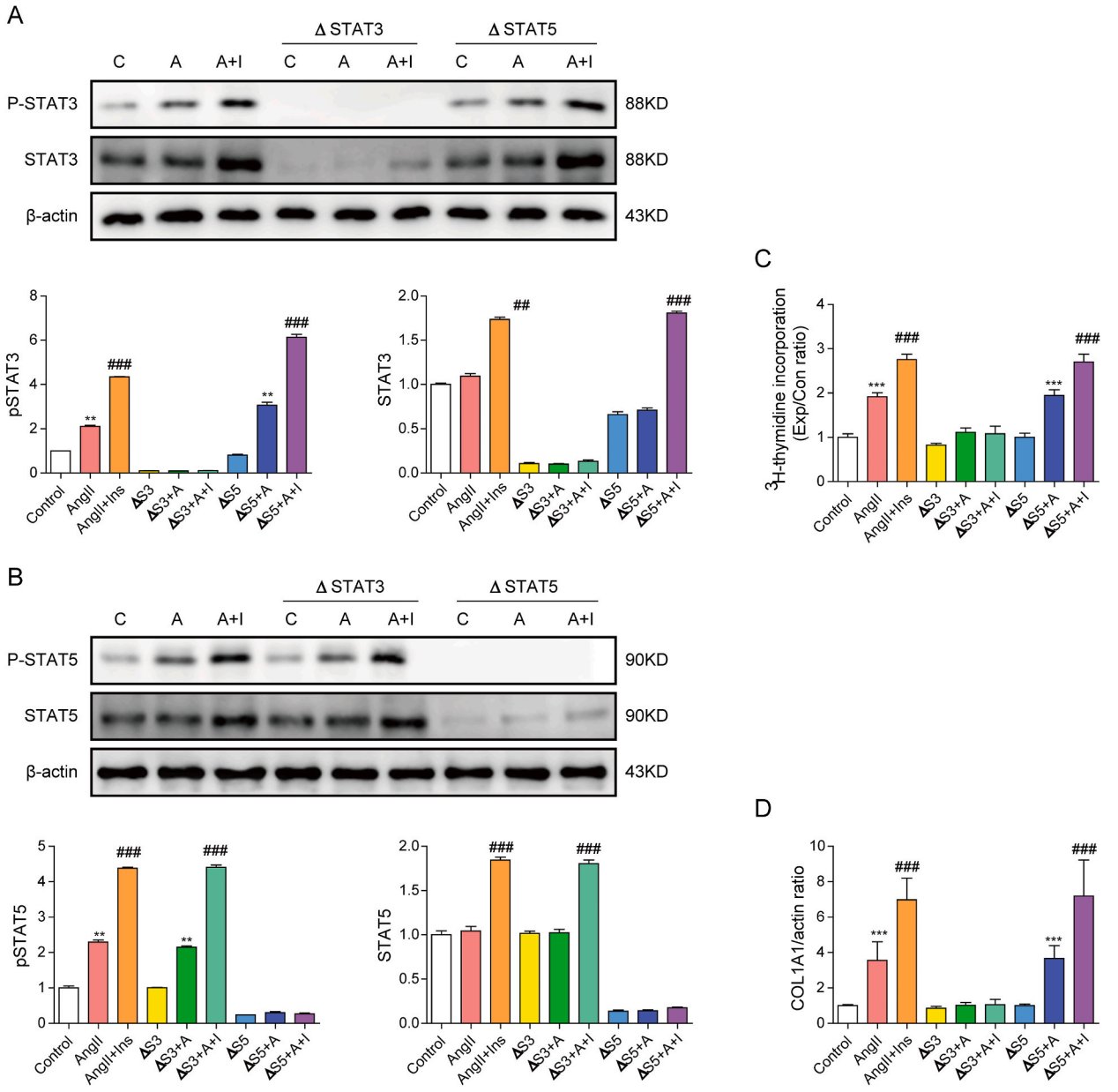

Fig. 6. Insulin-mediated promotion of AngII-induced proliferation and collagen synthesis in rat CFs is dependent on the upregulation of STAT3. A, B: Primary rat cardiac fibroblasts were transfected with invalid, STAT5, or STAT3 siRNA for 24 h and were then exposed to 100 nmol/L Ang II and/or 100 nmol/L insulin for another 24 h. The protein levels of p-STAT3, STAT3 (A), p-STAT5 and STAT5 (B) were measured by WB analysis. C: A [3H]-thymidine incorporation assay was used to measure the proliferation of CFs. D: qPCR analysis of the mRNA expression of the fibrotic gene collagen COL1A1. The original image of Western blot is in the supplementary material. Data are expressed means ± SEM, determined in three independent experiments. Oneway ANOVA, followed by LSD t and SNK post hoc test, was performed to analyze difference between the groups. ***, $P < 0.001$, compared with the control group; ###compared with the Ang II group, $P < 0.001$.

< 0.01). We also noted that insulin did not affect the phosphorylation ratio of STAT3/5 (P > 0.05) and only resulted in a limited increase in their phosphorylation (P < 0.01, Fig. 4; P < 0.05, Fig. 5). STAT1/3/5 were activated by Ang II, and the total levels of p-STAT3 and p-STAT5 were highly increased in the presence of insulin, which was consistent with the upregulation of total STAT3/5 by insulin (for p-STAT3, P < 0.001, Figs. 4 and 5; for p-STAT5, P < 0.01 in Fig. 4 and P < 0.05 in Fig. 5). Collectively, these data indicated that insulin reversed Ang II-mediated STAT expression and activation in rat heart tissue and primary rat CFs.

Insulin-enhanced cell proliferation and collagen synthesis in rat CFs induced by Ang II via MEK-mediated upregulation of STAT3.

To further investigate the mechanism by which insulin affects Ang II-induced myocardial fibrosis, we interfered with STAT3/5 protein synthesis by transfecting specific siRNAs into primary rat cardiac fibroblasts and then treated the cells with 100 nM Ang II and/or 100 nmol/L insulin. In response to siRNA treatment, p-STAT3 or p-STAT5 was almost undetectable, even after Ang II stimulation (ΔSTAT3, for STAT3 siRNA; ΔSTAT5, for STAT5 siRNA; Fig. 6A and B). We found that insulin had no effect on Ang II-induced [3H]-thymidine incorporation and COL1A1 mRNA synthesis when STAT5 was silenced but was mostly suppressed after the knockdown of STAT3 (Fig. 6C and D). It is worth noting that the effect of Ang II on the proliferation and collagen synthesis of CFs was dependent on STAT3 but not STAT5 (Fig. 6C and D). These results indicate that insulin enhanced Ang II-induced proliferation and collagen synthesis in rat CFs via STAT3 but not STAT5.

Furthermore, to explore the pathway by which insulin regulates STAT3 expression, CFs were pretreated with the insulin pathway inhibitors LY294002 (10 μmol/L) or PD0325901 (10 μmol/L) for 30 min and then treated with insulin (100 nmol/L) for 24 h. The Ang II-induced increase in p-STAT3 in response to insulin disappeared in the presence of PD0325901 (Ang + Ins vs. Ins, P > 0.05, Fig. 7A and B) but not LY294002 (Ang + Ins vs. Ins, P < 0.001, Fig. 7A and B), which was consistent with the effect of insulin on Ang II-induced proliferation and collagen synthesis in rat CFs (Fig. 7C and D).

4. Discussion

T2DM is present in 30–50 % of patients with HF and has been recognized as a strong independent predictor for poor clinical outcomes [26,27]. As a commonly used second-line treatment, insulin has been reported to be associated with poorer cardiovascular outcomes in patients with T2DM and HF by a recent meta-analysis of RCTs [8,9]. However, basic mechanisms by which insulin is involved in the development of cardiac dysfunction are still unclear. In this study, we observed the effect of insulin on cardiac structure

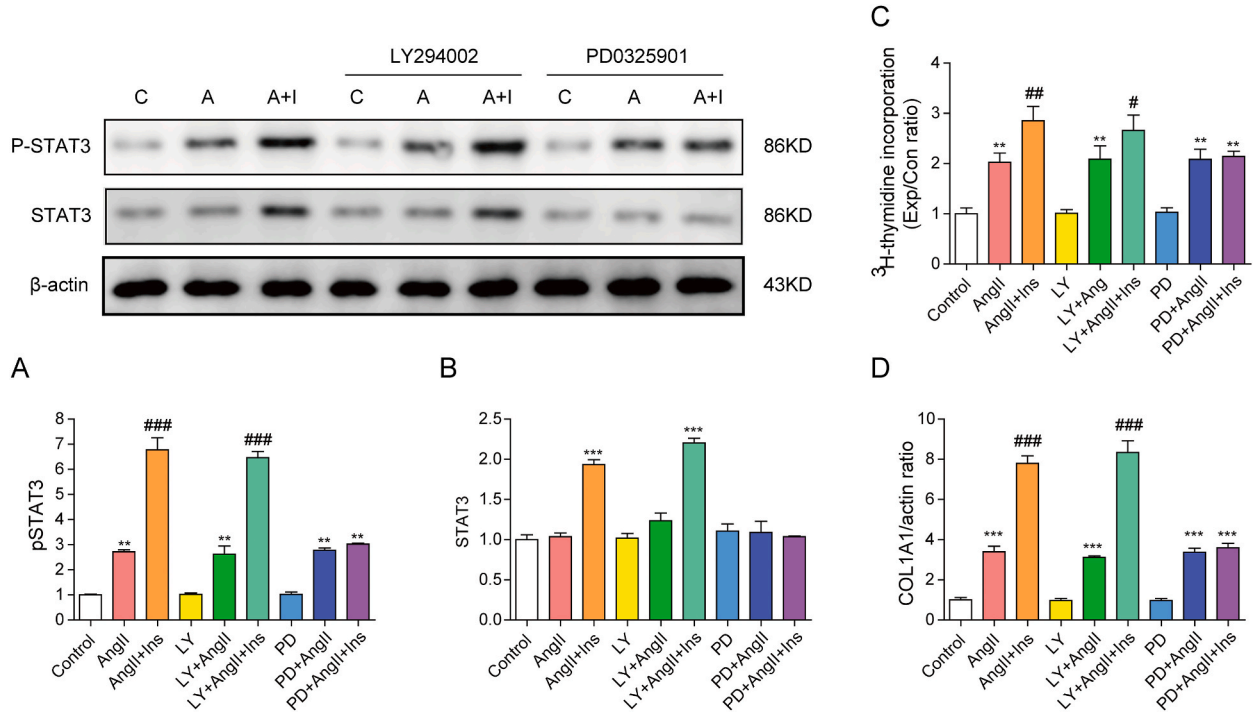

Fig. 7. The effect of insulin on STAT3 expression and AngII-induced proliferation and collagen synthesis in rat CFs was mediated by the MEK pathway. Primary rat cardiac fibroblasts were pretreated with the PI3K inhibitor LY294002 (10 μmol/L) or MEK inhibitor PD0325901 (1 μmol/L) for 30 min and then exposed to 100 nmol/L Ang II and/or 100 nmol/L insulin for 24 h. A, B. The protein levels of p-STAT3 and STAT3 were measured by WB analysis. C: A [3H]-thymidine incorporation assay was used to measure the proliferation of CFs. D: qPCR analysis of the mRNA expression of the fibrotic gene collagen COL1A1. The original image of Western blot is in the supplementary material. Data are expressed means ± SEM, determined in three independent experiments. Oneway ANOVA, followed by LSD t and SNK post hoc test, was performed to analyze difference between the groups. Significantly different from control, **, P < 0.01; ***, P < 0.001; compared with the Ang II (or Ang II + LY) group, #, P < 0.05; ##, P < 0.01; ###, P < 0.001.

and function in rats with or without Ang II-induced HF and demonstrated that insulin could markedly enhance Ang II-induced cardiac fibrosis and thus augment heart failure. Furthermore, we found that insulin could promote Ang II-induced proliferation and collagen synthesis in rat CFs by upregulating total STAT3, which was specifically mediated by the MEK pathway.

As a core mediator of cardiac remodeling, Ang II not only increases blood pressure but also directly induces cardiomyocyte hypertrophy, fibroblast proliferation and collagen production [28], and is therefore commonly used in animal models of heart failure. In this study, we found that insulin alone had no impact on cardiac structure and function in normal rats but significantly aggravated Ang II-induced systolic dysfunction in the left ventricle, which was consistent with the change in BNP, a biomarker that is increased during heart failure. Histological examination showed that insulin could further increase intercellular collagen accumulation when it was administered with Ang II, which was confirmed by the increase in collagen gene transcription in rat heart tissue. Notably, we found that neither blood pressure nor inflammation levels were significantly changed when insulin was administered to normal or Ang II-treated rats, which mainly excludes those confounding factors. Our results strongly suggest that insulin is not independently involved in the onset of heart failure but may play an important regulatory role in promoting Ang II-induced cardiac fibrosis, leading to exacerbated cardiac dysfunction.

To further determine the effect of insulin on fibrosis, we administered insulin to primary cultured rat CFs with or without Ang II. Our data indicated that insulin significantly enhanced the effect of Ang II on proliferation and collagen synthesis in CFs but had no impact when used alone, which was consistent with in the results of the animal experiments, indicating a regulatory effect of insulin that further augmented Ang II-induced cardiac fibrosis.

To explore the mechanisms by which insulin modulates this process, we next analyzed the levels of phosphorylated and total STAT/1/3/5 proteins in rat CFs treated with insulin and/or Ang II, as well as heart tissues from previous animal experiments. STAT3 acts as a transcriptional regulator of cardiac injury and is activated through phosphorylation at amino acid position 705 (Tyr705), after which it translocates into the nucleus in the form of a homo- or heterodimer of STAT proteins [29]. Recently, STAT3 was shown to play a critical role in Ang II-induced cardiac modeling. Jibo Han et al. reported that Ang II-activated STAT3 directly bound to the promoter of the collagen gene *COL1* and that suppression of STAT3 significantly alleviated Ang II-induced expression of COL1 mRNA [18]. Chia-Ti Tsai et al. showed that transfecting inactive STAT3 without phosphorylation sites effectively interfered with Ang II-induced collagen synthesis, while constitutively active STAT3 independently promoted it [19], suggesting that STAT3 phosphorylation controlled Ang II-induced fibrosis. It is worth noting that our previous study showed that insulin could significantly upregulate total STAT3 in the rat liver in a dose-dependent manner [20], which suggests the potential role of STAT3 in mediating the effect of insulin on Ang II-induced cardiac fibrosis. In addition to STAT3, STAT1/5 are activated by Ang II and participate in ischemia/reperfusion injury [22–25], indicating that they may also be involved in mediating the regulatory effect of insulin.

Our results illustrated that Ang II activated STATs through phosphorylation *in vivo* and *in vitro*, and insulin significantly increased total STAT3/5 but not STAT1, which further increased the levels of p-STAT3 and p-STAT5, suggesting that insulin might strengthen Ang II-induced STAT3/5 activation by providing more U-STATs as substrates downstream of the Ang II axis. Furthermore, to identify the specific role of STATs, we interfered with their protein synthesis by transfecting selective siRNAs. The results showed that Ang II-induced proliferation and collagen gene transcription in CFs was significantly suppressed by STAT3 knockdown and could not be restored by additional insulin treatment. However, Ang II induced CF growth and collagen production after STAT5 siRNA transfection, which could also be enhanced by insulin administration. Our data suggest that STAT3 plays a critical role in Ang II-induced cardiac remodeling and mediates the regulatory effect of insulin. It is worth mentioning that although insulin alone could increase p-STAT3 to a certain extent, our study demonstrated that the slight change in the absence of the activators might not be enough to result in significant histopathological changes or biological effects. Our previous study showed that the increase in phosphorylated and total STAT3 expression induced by insulin was significantly but partially inhibited by U0126 [20], which might be due to the participation of Janus kinase 2 (JAK2) in insulin-mediated activation of STAT3 [30]. Therefore, in this study, we chose PD0325901 as a MEK inhibitor to focus on the upstream MAPK pathway [31,32], in addition to the PI3K inhibitor LY294002 as a negative control. Our results showed that the MEK inhibitor could entirely suppress the STAT3 response to insulin, while the PI3K inhibitor LY294002 had no effect, which was consistent with the enhancement of CF growth and collagen production in response to Ang II.

5. Limitation

Limitations of the current study should also be noticed. The effect of insulin on Ang-II induced HF was observed in normal but not diabetic rats, which differed from the clinical real-world practice. So on one hand we have given new insight concerning the effect of insulin on cardiac structure and function, on the other hand further study involving a diabetic rat model and non-insulin-dependent antihyperglycemic drugs was also essential to eventually answer the clinical question. Besides, hypoglycemia might be confounding factors of adverse cardiovascular events. However, no significant change on cardiac structure and function was observed in only-insulin-treated rats. This may stem from the relatively short experiment duration, and the fact that severe hypoglycemia (<50 mg/dL), which might be risk factor of re-hospitalization due to heart failure [33,34], was rarely recorded in the current study.

6. Conclusion

Our study demonstrated that insulin could dramatically augment Ang II-induced cardiac fibrosis and therefore further aggravate cardiac dysfunction. The regulatory effect of insulin on Ang II was specifically mediated by the upregulation of total STAT3 via the MEK pathway, which increased the level of phosphorylated STAT3 in the context of Ang II stimulation. It is also important to note that the current study preliminarily demonstrated the effect of insulin on the axis of AngII-STAT3-cardiac fibrosis in non-DM rats, and it is

necessary to further observe the impact of insulin, as well as other non-insulin-dependent hypoglycemic drugs on AngII-induced cardiac remodeling in diabetic rat model, so as to better answer the clinical observation. In fact, a new diabetic drug such as SGLT-2, which increases urinary glucose excretion, has been proven to improve clinical outcomes in patients with heart failure [35,36]. Our findings provide a new theoretical basis for the use of glucose-lowering treatments in patients with T2DM and HF and suggests a noninsulin-dependent strategy.

Data accessibility statement.

Data will be made available on request.

Patient consent statement

Not applicable.

Ethics approval statement

The animal experiments in this study were approved by the Animal Ethics Committee of the Second affiliated hospital of the Medicine school of Zhejiang University in Hangzhou. All authors confirm that all methods are carried out in accordance with relevant guidelines and regulations.

Credit author statement

Nanyu Cao: Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization. Heyang Wang: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

List of abbreviations

Ang II	angiotensin II
BNP	B-type natriuretic peptide
CFs	cardiac fibroblasts
HF	heart failure
IHC	immunohistochemistry
JAK2	Janus kinase 2
IVS	interventricular septum thickness
LVPW	left ventricular posterior wall thickness
LVDd	left ventricular end-diastolic diameter
LVDs	left ventricular end-systolic diameter
LVEF	left ventricular
MEK	mitogen-activated protein kinase
RT−qPCR	real-time quantitative PCR
RCTs	randomized clinical trials
STAT	the signal transducer and activator of transcription
SBP	systolic blood pressure
T2DM	type 2 diabetes
WB	Western blot

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22860.

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