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Original Article

Radial extracorporeal shock wave therapy alleviates acute inflammation of human primary tenocytes through the integrin-FAK-p38MAPK pathway

Ruidong Ge[1,2], Bin Ruan[1], Shengxuan Chen[3], Shuo Bai[4], Qi Gao[1], Anran Dong[1]

[1]School of Sports Medicine and Rehabilitation, Beijing Sport University, Beijing 100084, China; [2]Department of Rehabilitation Medicine, China-Japan Friendship Hospital, Beijing 100029, China; [3]Department of Rehabilitation Medicine, The Gulou Hospital of Traditional Chinese Medicine, Beijing 100009, China; [4]Department of Rehabilitation Medicine, Beijing Chaoyang District Taiyanggong Community Health Service Center, Beijing 100028, China

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Abstract: Objectives: To explore whether radial extracorporeal shock wave therapy (rESWT) can alleviate acute inflammation of human primary tenocytes by the integrin-focal adhesion kinase (FAK)-p38 mitogen-activated protein kinase (MAPK) pathway. Methods: Western blotting was used to evaluate the changes in the integrin-FAK-p38MAPK signaling pathway mediated by rESWT using specific antibodies targeting the phosphorylation sites of intracellular signal pathway proteins. Results: rESWT up-regulated FAK phosphorylation and down-regulated p38MAPK phosphorylation levels in a tumor necrosis factor (TNF)-α-induced acute inflammation model of human primary tenocytes. Pretreatment with an integrin inhibitor significantly reduced rESWT-mediated downregulation of p38MAPK phosphorylation and attenuated its reversal effect on the increased secretion of pro-inflammatory cytokines in TNF-α-induced human primary tenocytes. Conclusions: Our results imply that rESWT may partially alleviate acute inflammation in human primary tenocytes through the integrin-FAK-p38MAPK pathway.

Keywords: Inflammation, integrin, mitogen-activated protein kinases, shockwave, tenocyte

Introduction

Tendonopathy involves a complex pathologic change in the tendons, characterized primarily by pain, weakness, and decreased tolerance to exercise [1]. Approximately 30% of all consultations with a general practitioner for musculoskeletal disorders involve tendinopathies [2]. Modern molecular biology has confirmed that moderate inflammation can effectively trigger tendon repair, while excessive inflammatory cascade reactions will hinder the process and accelerate degeneration, while causing a few complications, such as adhesion, fibrosis, and calcification [3]. Therefore, an ideal intervention for tendinopathy, based on preserving inflammation-induced repair, should bidirectionally regulate inflammation to promote tendon repair stably and rapidly [3]. Radial extracorporeal shock wave therapy (rESWT) has been widely used in the clinical treatment of tendinopathy, but the molecular mechanisms of its anti-inflammatory effects have not been fully clarified.

Stress plays a vital role in the development, stability, and repair of tendons. Stress deprivation reduces the biomechanical properties of tendons [4], whereas continuous and excessive stress hinders tendon repair [5]. Tenocytes, the main matrix cells of tendons, respond widely to stress stimulation. Various approaches are available to study the effects of stress stimulation on *in vitro* tenocytes, including cyclic stretching [6], hydrostatic pressure [7], low-intensity ultrasound [8], and extracorporeal shock wave therapy (ESWT) [9]. Numerous *in vitro* studies have confirmed that ESWT can significantly promote proliferation, migration, differentiation, and secretion of type I collagen in

tenocytes [9]. Our previous experimental study also confirmed that rESWT could significantly reverse the decreased proliferation of human primary tenocytes induced by tumor necrosis factor (TNF)-α and the increased secretion of pro-inflammatory cytokines [10].

In treating of musculoskeletal diseases, ESWT, as a stress stimulus, exerts mainly a "mechanotransduction" effect. As a sound wave (mechanical wave), an extracorporeal shock wave (ESW) activates a variety of mechanotransduction receptors on the cell membrane through the shear force generated at different tissue interfaces and the cavitation effect generated in the tissue fluid, thus activating downstream signaling pathways and transcription factors in cells, regulating biological function, and ultimately affecting the physiologic and pathologic conditions of tissues and the prognosis [11].

Mechanotransduction is the process by which cells convert stress stimuli into chemical signals, affecting a series of cellular biological functions. Although the specific molecular mechanisms of mechanotransduction have not been fully clarified, many possible mechanisms have been extensively explored in recent years.

The extracellular matrix (ECM)-integrin-cytoskeleton pathway has been mostly implicated. Through integrins, cells adhere to the ECM, which provides a material basis for information exchange between the ECM and cells. During this process, the cytoskeleton, G proteins, integrins, mitogen-activated protein kinases (MAPKs), receptor tyrosine kinases (RTKs), and stretch-activated ion channels are involved [12]. As adhesion receptors and stress transducers, integrin receptors combine with ECM ligands to transmit signals across the membrane and ultimately regulate cell adhesion, proliferation, migration, and differentiation [13]. Studies have shown that integrin may be one of the mediators of ESWT at the cellular and molecular levels. ESWT can significantly up-regulate the expression of the subtypes α2, β1, and α6 [14], of which α2 and β1 integrins are widely distributed in human tendon tissues [15]. When integrins are activated, they aggregate with other integrins, adapter proteins, and kinases, such as focal adhesion kinase (FAK), to form an adhesion complex, which then activates intracellular signaling cascades to mediate cell responses [16]. Our previous quantitative proteomic analysis also showed that rESWT could significantly up-regulate the expression of integrin alpha-2 (ITGA2) in a TNF-α-induced acute inflammation model of human primary tenocytes [10], suggesting that ITGA2 may be one of the key molecular targets of rESWT on anti-inflammation, but its intracellular mechanism remains unclear. Numerous studies have confirmed that activation of the p38MAPK pathway is closely related to inflammatory development in tendinopathy [17].

Therefore, in this study, we attempted to use specific antibodies targeting the phosphorylation sites of intracellular signaling pathway proteins in western blot analysis (WB) to evaluate the changes in the rESWT-mediated integrin-FAK-p38MAPK signaling pathway. Further, pretreatment with integrin inhibitors was used to preliminarily explore whether rESWT can alleviate acute inflammation of human primary tenocytes through the integrin-FAK-p38MAPK pathway.

Materials and methods

The reagents and antibodies used in this study are listed in **Tables 1** and **2**.

Tissue samples and primary culture of human tenocytes

Three male patients (32, 33, and 36 years old) who were hospitalized at the China-Japan Friendship Hospital (Beijing, China) underwent anterior cruciate ligament reconstruction. After signing informed consent and agreeing to donate, the residual tissue of the semitendinosus tendon grafts was collected, and the tendon tissues were wrapped with bacteria-free gauze containing normal saline in a sterile pack. The tendon tissues and culture scheme were isolated as described in our previous study, and we completed immunofluorescence identification [10]. This study was approved by the Ethics Committee of Sports Science Experiment of Beijing Sport University (Beijing, China; 2020075H).

Stimulation with TNF-α

Twelve hours before TNF-α stimulation, the culture medium of human primary tenocytes was changed from 10% to 0.1% fetal bovine serum

Table 1. Reagents

Reagents	Manufacturer	City/state and country
Recombinant tumor necrosis factor-α	Bioworld	MN, USA
Multiplex bead-based flow fluorescent immunoassay kit	Raisecare	Qingdao, China
GRGDSP	MedChemExpress	NJ, USA
SB203580	MedChemExpress	NJ, USA

GRGDSP: glycine-arginine-glycine-aspartic acidserine-proline peptide.

Table 2. Antibodies

Antibody	Code No.	Lot No.	Manufacturer	City/state and country
Anti-FAK	#71433	1	Cell Signaling Technology	MA, USA
Anti-phospho-FAK (Tyr397)	#8556	5	Cell Signaling Technology	MA, USA
Anti-p38MAPK	#8690	9	Cell Signaling Technology	MA, USA
Anti-phospho-p38MAPK (Thr180/Tyr182)	#4511	13	Cell Signaling Technology	MA, USA
Anti-GAPDH	Ab1029t	0528	Boao Ruijing	Beijing, China
Goat anti-rabbit immunoglobulin G (H+L)	S8002	6	Jackson ImmunoResearch	West Grove, PA, USA

FAK: focal adhesion kinase; MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

(FBS), and the tenocytes were subjected to serum starvation. In accordance with our previous study [10], recombinant TNF-α dry powder was dissolved to 10 μg/mL in sterile 1 × phosphate buffer solution (PBS). When stimulating cells, 10 μg/mL TNF-α solution was diluted to 1:1000 in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 0.1% FBS, and the final concentration was 10 ng/mL. Sterile 1 × PBS was added to the other groups at the same concentration as the control.

Stimulation with inhibitor

Glycine-arginine-glycine-aspartic acidserine-proline peptide (GRGDSP) 50 μg/mL dissolved in enzyme-free sterile water and 30 μM SB203580 dissolved in dimethylsulfoxide (DMSO) were added to the medium of human primary tenocytes in two 60 mm culture plates 4 and 1 h before rESWT, respectively [18]. They were pre-incubated for 4 and 1 h at 37°C in a 5% CO_2 cell incubator. Similarly, the medium of human primary tenocytes was changed from 10% to 0.1% FBS 12 h before inhibitor pre-incubation, and cells were starved with serum. The other groups were treated with the same concentrations of enzyme-free sterile water and DMSO as controls.

In vitro rESWT for tenocytes

Referring to our previous study [10], we selected a pneumatic ballistic shock wave instrument equipped with a standard applicator of 15 mm (Gymna ShockMaster 300, GymnaUniphy NV, Bilzen, Belgium). To determine the appropriate time point for rESWT in FAK and p38MAPK phosphorylation, four primary human tenocytes (P2-P5, 80%-90% confluence) in 100 mm culture plates were starved for serum 12 h before intervention and detached using 0.25% trypsin. Approximately 1/9 of the cells were resuspended in a medium containing 0.1% FBS after centrifugation (178 × *g* for 5 min) and inoculated into a 60 mm cell culture plate as a control. The remaining 8/9 of the cell solution was centrifuged (178 × *g* for 5 min), resuspended in a medium containing 10 ng/mL TNF-α and 0.1% FBS, and transferred to two 5 mL polypropelene tubes (Taizhou, China). Each tube contained a 4×10^5 cells/mL cell suspension. One tube was treated with rESWT, 0.29 mJ/mm^2 × 1200 impulses. After the rESWT intervention, the two tubes were inoculated separately into four 60 mm cell culture plates. These nine plates were designated as control 30 min, rESWT+TNF-α 30 min, rESWT+TNF-α 1 h, rESWT+TNF-α 2 h, rESWT+TNF-α 3 h, TNF-α 30 min, TNF-α 1 h, TNF-α 2 h, and TNF-α 3 h. Cultured tenocytes were harvested at 30 min and 1, 2, and 3 h after the rESWT intervention for WB.

To detect the effects of GRGDSP on rESWT-mediated phosphorylation of p38MAPK and secretion of pro-inflammatory cytokines in

human primary tenocytes induced by TNF-α, we divided five 60 mm culture plates containing tenocytes P2 to P5 tenocytes (80%-90% confluence) into five groups: control, TNF-α, TNF-α+rESWT, GRGDSP+TNF-α+rESWT, and SB203580+TNF-α (positive control). These tenocytes were also subjected to serum starvation for 12 h prior to the intervention. After 4 h of pre-incubation with GRGDSP, human tenocytes were removed from the TNF-α+rESWT and GRGDSP+TNF-α+rESWT groups using 0.25% trypsin. These cells were resuspended in a medium containing 10 ng/mL TNF-α and 0.1% FBS and transferred to two 5 mL polypropelene tubes (Taizhou, China). Each tube contained 1×10^5 cells/mL cell suspension and was fixed on the surface of the applicator in a perpendicular manner; between them, the couplant was stuffed. Subsequently, the preparation was treated with rESWT, 0.29 mJ/mm^2 × 1200 impulses. After the rESWT intervention, the two tubes were inoculated separately in two 60 mm culture plates. In the TNF-α group, cells were separated using 0.25% trypsin, and resuspended in a medium containing 10 ng/mL TNF-α and 0.1% FBS. In the SB203580+TNF-α group, SB203580 was pretreated for 1 h, removed using 0.25% trypsin, and finally resuspended in a medium containing 10 ng/mL TNF-α and 0.1% FBS. In the control group, cells were detached using 0.25% trypsin and resuspended in a medium containing 0.1% FBS. Cells from the next three groups were inoculated into three 60-mm culture plates. The cells mentioned above were harvested for WB analysis 1 h after the rESWT intervention.

Cytokine detection

The level of interleukin (IL)-1β in the culture medium of the above five groups of human primary tenocytes was determined using multiplex bead-based flow fluorescent immunoassay. The specific performance of the kit was determined according to the manufacturer's instructions. Finally, the data were analyzed using flow cytometry (NAVIOS; Beckman Coulter, CA, USA).

WB analysis

Tenocytes were collected for WB analysis. A culture medium was collected to determine cytokine levels. Culture plates containing suspension tenocytes were first collected for centrifugation (178 × *g* for 5 min) and then resuspended in 45 μL cell lysate; for adherent growth tenocytes, after removing the culture medium, cells were rinsed twice with precooled PBS, and 45 μL of cell lysate was added (radioimmunoprecipitation assay lysis buffer:protein inhibitor mixture:protein phosphate inhibitor mixture:phenylmethylsulfonyl fluoride = 100:2:2:1) to each 60 mm culture plate. The protein extraction process was as follows: lyse on ice for 5 min, gently scrape the cells with a cell scraper (culture plates containing suspension tenocytes are not necessary), and transfer them to a 1.5 ml Eppendorf tube. Cell extracts were lysed on ice for 15 min and then centrifuged at 19801 × *g* at 4°C for 15 min. The supernatant was aspirated, loading buffer was added, and the mixture was heated at 100°C for 5 min.

Tenocyte protein samples (30 μg) were added to each well. A 4%-20% Tris-glycine gel was used for electrophoresis and transferred to nitrocellulose membranes. The nonspecific antibody was blocked at 20-24°C with 5% fat-free milk for 1.5 h. Primary antibodies anti-FAK (1:1000), anti-phospho-FAK (Tyr397) (1:1000), anti-p38MAPK (1:1000), anti-phospho-p38MAPK (Thr180/Tyr182) (1:1000) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000) were incubated at 4°C overnight. Goat anti-rabbit IgG (H+L) (1:10000) was incubated at 20-24°C for 30 min. Enhanced chemiluminescence reagent (Biosharp, Hefei, China) was used to visualize the reaction. Protein bands were detected using an electrochemiluminescence detection system (Thermo Fisher Scientific, MA, USA), and the gray value was analyzed using Image Lab 5.0 software (Bio Rad, CA, USA).

Statistical analysis

The Statistical Product and Service Solutions software (version 22.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. All data are expressed as mean ± standard deviation. Significant differences were assessed using Welch's t-test and one-way analysis of variance. The least significant difference test was used for multiple comparisons. $P < 0.05$ was considered significantly different.

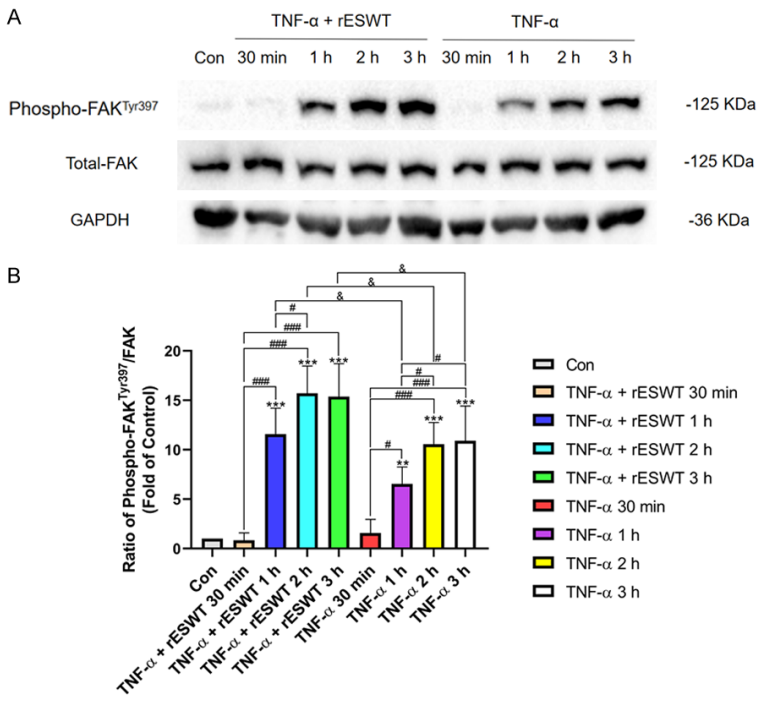

Figure 1. Influence of FAK phosphorylation at the Tyr397 residue at different time points. A. Representative graph of the protein band of phospho-FAK^{Tyr397} and FAK. GAPDH was used as an internal reference. B. Quantitative results of phospho-FAK^{Tyr397} and FAK. Data are expressed as means ± SD (n = 3). Significant differences were assessed using Welch's t-test and ANOVA. The LSD test was used for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. the control. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ represent intragroup comparisons at different time points. $^{\&}P < 0.05$ represents an intergroup comparison at the same time point. The uncropped images of this figure are shown in Supplementary Figure 1. TNF: tumor necrosis factor; rESWT: radial extracorporeal shock wave therapy; FAK: focal adhesion kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation; ANOVA: one-way analysis of variance; LSD: least significant difference.

Results

rESWT-induced FAK phosphorylation

After 30 min of rESWT intervention, FAK phosphorylation in Tyr397 was low in the control, TNF-α, and TNF-α+rESWT groups. After 1, 2, and 3 h of rESWT intervention, tenocytes showed adherent growth, and the FAK phosphorylation level at the Tyr397 residue gradually increased, reaching a peak at 2 h. Compared to the TNF-α group, the level of FAK phosphorylation at the Tyr397 residue in the TNF-α+rESWT group was significantly higher at 1, 2, and 3 h of the rESWT intervention, while no significant differences were observed in FAK phosphorylation levels between the two groups 30 min after rESWT intervention (**Figure 1**).

rESWT-reduced phosphorylation of p38MAPK

Phosphorylation of p38MAPK at the Thr180 and Tyr182 residues in the control group was low. After 30 min of the rESWT intervention, the phosphorylation of p38MAPK at the Thr180 and Tyr182 residues in groups TNF-α and TNF-α+rESWT groups all showed higher levels but did not differ statistically between comparisons between groups. Within 3 h of rESWT intervention, the levels of p38MAPK phosphorylation in Thr180 and Tyr182 gradually decreased in both TNF-α and TNF-α+rESWT groups. In the TNF-α group, after 1 h of intervention, the phosphorylation at Thr180 and Tyr182 residues was still higher than that of the control group, and at 2 h of intervention, this level returned to that of the control group. In the TNF-α+rESWT group, after 1 h of intervention, the phosphorylation of p38MAPK at the Thr180 and Tyr182 residues was much lower than that in the TNF-α group (**Figure 2**). Based on the results mentioned above, we preliminarily determined that the integrin inhibitor reversed rESWT-reduced p38MAPK phosphorylation 1 h after the intervention of rESWT.

Inhibitory effect of SB203580 on p38MAPK phosphorylation

TNF-α (10 ng/mL) significantly induced the phosphorylation level of p38MAPK in Thr180 and Tyr182 in human primary tenocytes. SB203580 (30 μM) significantly inhibited the level of phosphorylation of p38MAPK at the Thr180 and Tyr182 residues in TNF-α-induced human tenocytes, while 1 μM and 10 μM SB203580 did not show a significant inhibitory effect (**Figure 3**). Therefore, we initially selected 30 μM SB203580 as a positive control.

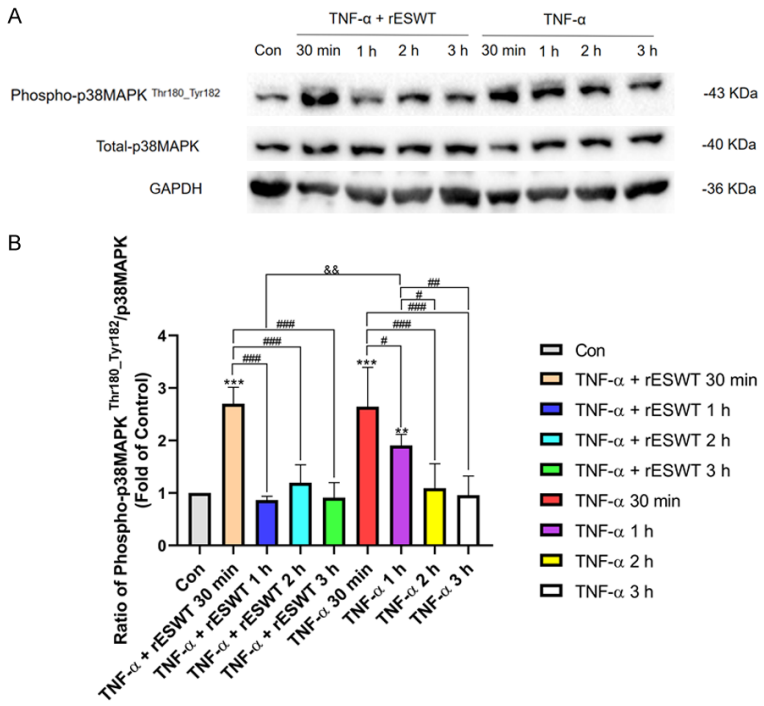

Figure 2. Influence of p38MAPK phosphorylation at the Thr180 and Tyr182 residues at different time points. A. Representative graph of the protein band of phospho-p38MAPK^{Thr180_Tyr182} and p38MAPK. GAPDH was used as an internal reference. B. Quantitative results of phospho-p38MAPK^{Thr180_Tyr182} and p38MAPK. Data are expressed as means ± SD (n = 3). Significant differences were assessed using Welch's t-test and ANOVA. The LSD test was used for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. the control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ represent intragroup comparisons at different time points. $^{\&\&}P < 0.01$ represents the intergroup comparison at the same time point. The uncropped images of this figure are shown in Supplementary Figure 1. TNF: tumor necrosis factor; rESWT: radial extracorporeal shock wave therapy; MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation; ANOVA: one-way analysis of variance; LSD: least significant difference.

The integrin inhibitor reversed rESWT-reduced p38MAPK phosphorylation

After 1 h of rESWT intervention, the phosphorylation of p38MAPK at residues Thr180 and Tyr182 in the TNF-α+rESWT group was significantly lower than in the TNF-α group, while in the GRGDSP+TNF-α+rESWT group, the phosphorylation of p38MAPK at these two residues was higher than in the TNF-α+rESWT group. There were no significant differences in the phosphorylation of p38MAPK at these two residues between the TNF-α and GRGDSP+TNF-α+rESWT groups. SB203580+TNF-α as a positive control group, compared to the TNF-α and GRGDSP+TNF-α+rESWT groups, the level of phosphorylation of p38MAPK at the Thr180 and Tyr182 residues decreased significantly, and there was no significant difference between the SB203580+TNF-α group and the control group (**Figure 4**).

Inhibitor of integrin reversed rESWT-induced reduction in IL-1β levels

After stimulation with 10 ng/mL TNF-α, IL-1β levels increased significantly in the TNF-α group compared to those in the control group. However, 1 h after the rESWT intervention, IL-1β levels in the TNF-α+rESWT group were still significantly higher than those in the control group but were much lower than those in the TNF-α group. GRGDSP was pre-incubated for 4 h, and rESWT was administered for 1 h. IL-1β levels in the GRGDSP+TNF-α+rESWT group increased slightly compared to those of the TNF-α+rESWT group but were still slightly lower than those of the TNF-α group (P = 0.036). IL-1β levels in the SB203580+TNF-α group were significantly lower than those in the TNF-α group but still higher than those in the control group and slightly higher than those in the TNF-α+rESWT group (P = 0.020) (**Figure 5**).

Discussion

rESWT has been used successfully to treat chronic tenopathy. Mechanotransduction is one of the core pathways that exert its biological effects. Integrins play an important role as classical receptors of mechanotransduction. Previous studies have confirmed that one of the possible molecular mechanisms of anti-inflammatory ESWT is the rapid inducement of tissue to release nitric oxide, thus inhibiting Nuclear Factor kappa-B (NF-κB) activity [19]. Currently, no study has explored the anti-inflammatory molecular mechanism of rESWT throu-

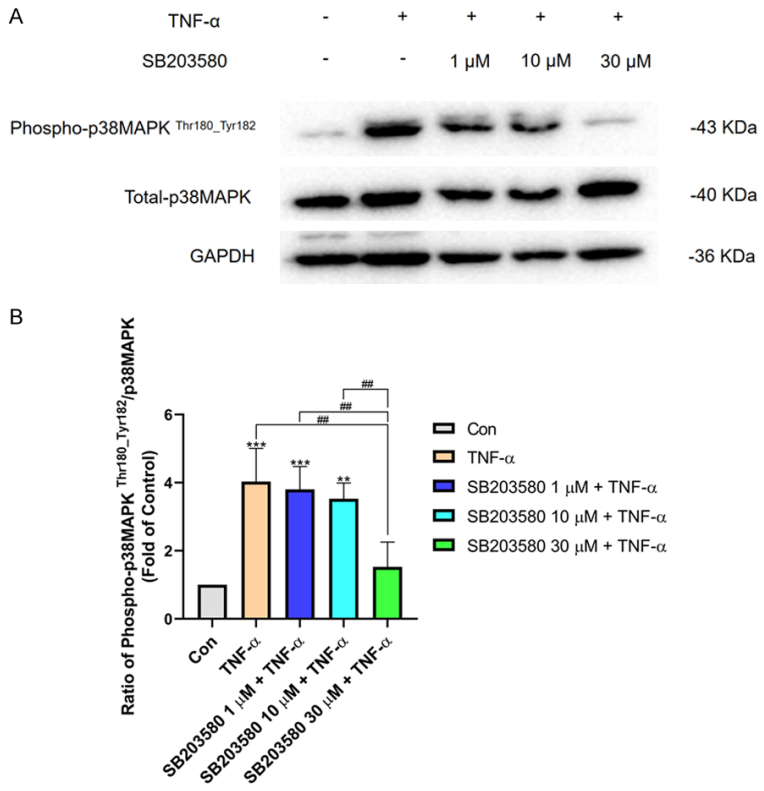

Figure 3. Influence of p38MAPK phosphorylation levels at the Thr180 and Tyr182 residues treated with different concentrations of SB203580. A. Representative graph of the protein band of phospho-p38MAPK^{Thr180_Tyr182} and p38MAPK. GAPDH was used as an internal reference. B. Quantitative results of phospho-p38MAPK^{Thr180_Tyr182} and p38MAPK. Data are expressed as means ± SD (n = 3). Significant differences were assessed using Welch's t-test and ANOVA. The LSD test was used for multiple comparisons. $^{**}P$ < 0.01, $^{***}P$ < 0.001 *vs.* control. $^{\#\#}P$ < 0.01 represent intergroup comparisons (except the control). The uncropped images of this figure are available in Supplementary Figure 2. TNF: tumor necrosis factor; MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation; ANOVA: one-way analysis of variance; LSD: least significant difference.

gh the integrin-mediated mechanotransduction pathway.

Integrins are transmembrane heterodimers that consist of two subunits: α and β. They are made up of extracellular, transmembrane, and cytoplasmic domains [13]. Integrin mediates cell adhesion, migration, and ECM synthesis through the transmission of inside-out signals and biological functions such as inflammation, immunity, and development through the transmission of outside-in signals [13]. Numerous studies have confirmed that MAPK phosphorylation is significantly regulated by integrin inhibitors (GRGDSP) or specific integrin-blocking antibodies [20]. Previous studies have confirmed that ESW can regulate cell biological functions by activating integrin [14]. Our previous study also showed that rESWT could significantly up-regulate ITGA2 expression in primary human tenocytes induced by TNF-α in an acute inflammation model [10]. This study found that GRGDSP significantly attenuated the alleviative effect of IL-1β secreted from human primary tenocytes induced by TNF-α after rESWT (**Figure 5**). In summary, rESWT partially alleviated TNF-α-induced acute inflammation in human primary tenocytes by activating integrins.

The main mechanism of integrin activation involves conformational changes. Due to its lack of internal catalytic activity, the activated integrin is mainly based on its intracellular domain to bind to FAK and induce phosphorylation, further regulating downstream signaling pathways [21]. When the integrin is activated, FAK automatically phosphorylates the Tyr397 residue [22]. Numerous studies have confirmed that FAK phosphorylated at Tyr397 can target the regulation of MAPK phosphorylation [23], thus confirming that FAK phosphorylated at Tyr397 plays an important role in integrin-mediated mechanotransduction. This study showed that rESWT could significantly induce FAK phosphorylation at the Tyr397 residue (**Figure 1**). Due to the radial shape and limited applicator diameter of the pneumatic ballistic extracorporeal shock wave therapeutic apparatus, the suspended cell model by rESWT intervention was selected in this study to perform even shock wave radiation for all human primary tenocytes. However, rESWT in suspended human primary tenocytes has limitations [24]. **Figure 1** shows that after 30 min of rESWT intervention, FAK of human primary tenocytes in each group did not show signifi-

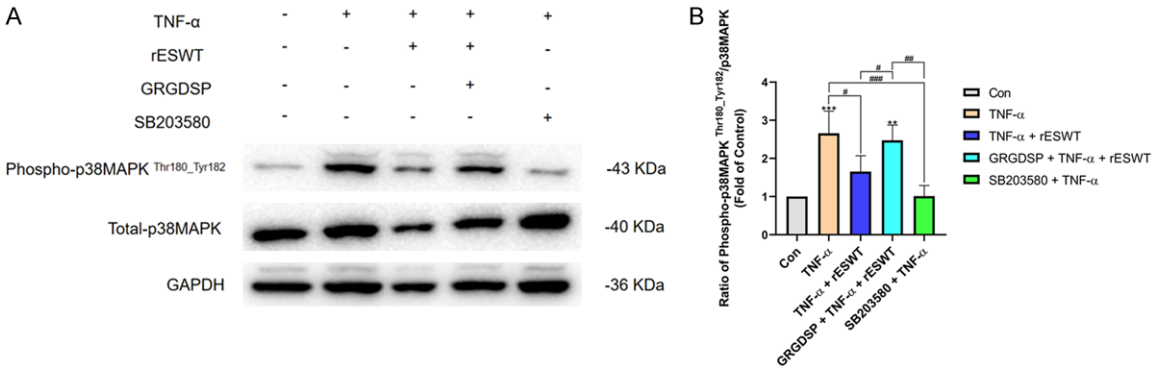

Figure 4. Influence of p38MAPK phosphorylation levels at the Thr180 and Tyr182 residues treated with GRGDSP. A. Representative graph of the protein band of phospho-p38MAPK^{Thr180_Tyr182} and p38MAPK. GAPDH was used as an internal reference. B. Quantitative results of phospho-p38MAPK^{Thr180_Tyr182} and p38MAPK. Data are expressed as means ± SD (n = 3). Significant differences were assessed using Welch's t-test and ANOVA. The LSD test was used for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. the control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ represents comparisons between groups (except the control). The uncropped images of this figure are available in Supplementary Figure 3. TNF: tumor necrosis factor; rESWT: radial extracorporeal shock wave therapy; GRGDSP: glycine-arginine-glycine-aspartic acidserine-proline peptide; MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation; ANOVA: one-way analysis of variance; LSD: least significant difference.

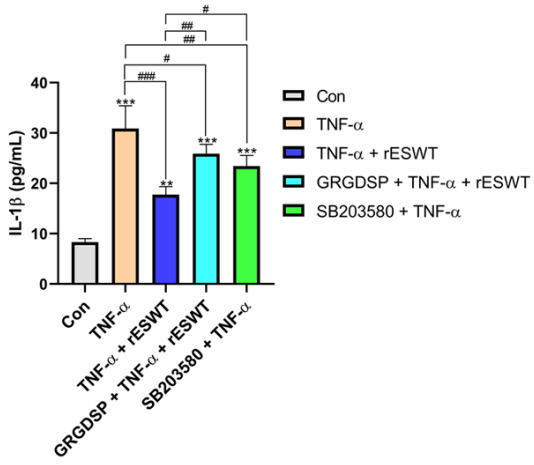

Figure 5. Influence of GRGDSP on IL-1β levels in the culture medium of TNF-α induced human primary tenocytes in an acute inflammation model intervened by rESWT. Data are expressed as means ± SD (n = 3). Significant differences were assessed using Welch's t-test and ANOVA. The LSD test was used for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. the control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ represent comparisons between groups (except the control). IL: interleukin; TNF: tumor necrosis factor; rESWT: radial extracorporeal shock wave therapy; GRGDSP: glycine-arginine-glycine-aspartic acidserine-proline peptide; SD: standard deviation; ANOVA: one-way analysis of variance; LSD: least significant difference.

cant phosphorylation at the Tyr397 residue, which may be related to the fact that cells did not adhere to the culture plate. However, 1 h after the rESWT intervention, with gradual cell adhesion, FAK phosphorylation at the Tyr397 residue of human primary tenocytes in each group was significantly enhanced, and rESWT further increased FAK phosphorylation at the Tyr397 in a TNF-α-induced acute inflammation model of human primary tenocytes. In summary, FAK phosphorylation at Tyr397 may play a vital role in rESWT-regulated integrin mechanotransduction.

The P38MAPK pathway may play a key role in mechanotransduction. Low-intensity pulsed ultrasound may regulate the activity of the integrin-p38MAPK pathway to affect chondrocyte ECM synthesis, and p38MAPK may play different roles when low-intensity pulsed ultrasound interferes with chondrocytes in physiologic or pathologic states [25]. Furthermore, low-intensity pulsed ultrasound could also regulate the integrin-FAK-MAPK pathway to alleviate early inflammation in knee osteoarthritis in a rabbit model [26]. Tenocytes are also typical mechanically sensitive fibroblasts, and rESWT is also a classical mechanical intervention method, but there are no reports that rESWT may regulate the Integrin-FAK-MAPK pathway to affect the phenotype of tenocytes. The results of this study show that TNF-α significantly regulates the phosphorylation level of p38MAPK at Thr180 and Tyr182 in primary tenocytes, and 1

h after stimulation, the phosphorylation of p38MAPK at the residues Thr180 and Tyr182 was still slightly higher than the baseline level and then gradually returned to the baseline level. rESWT significantly inhibited the phosphorylation level of p38MAPK 1 h after the intervention; that is, rESWT reduced the phosphorylation of p38MAPK at the Thr180 and Tyr182 residues in the TNF-α induced acute inflammation model of human primary tenocytes and returned it to the baseline level more rapidly (**Figure 2**). The GRGDSP integrin inhibitor greatly weakened rESWT inhibition at the level of phosphorylation of p38MAPK at Thr180 and Tyr182 in human primary tenocytes (**Figure 4**). However, **Figures 4** and **5** show that rESWT can significantly regulate p38MAPK dephosphorylation at Thr180 and Tyr182 residues in the TNF-α induced acute inflammation model of human primary tenocytes. However, after rESWT intervention, IL-1β levels secreted by the TNF-α induced acute inflammation model of human primary tenocytes did not completely return to that of the control group. Therefore, we speculate that rESWT alleviation of IL-1β levels secreted in an acute inflammation model through the integrin-FAK-p38MAPK pathway was not the only pathway involved. In this study, FAK phosphorylation at the Tyr397 residue also exerted a specific protein interaction targeting the induction of p38MAPK dephosphorylation at the Thr180 and Tyr182 residues. However, all these mechanisms require further exploration. In summary, rESWT can partially regulate the integrin-FAK-p38MAPK pathway to alleviate acute inflammation in human primary tenocytes.

This study had some limitations. First, the tenocytes adhered to the ECM *in vivo*. Due to the limitations of the rESW waveform and equipment, suspended human primary tenocytes were preliminarily selected by rESWT intervention, and further *in vivo* validation is warranted. Second, if human primary tenocytes were used in the pathological state, this study would be more convincing. However, because few patients with acute tendon injury are subjected to surgery, it is difficult to obtain sufficient tendon tissues in the acute inflammatory stage clinically. Third, the formation of FAK will ultimately affect the expression of related genes, affecting the inflammatory phenotype of human primary tenocytes. Therefore, in our next phase of investigations, we will research the regulatory effect of rESWT on gene expression through the integrin-FAK-p38MAPK pathway, using reverse transcription-polymerase chain reaction or immunofluorescence. In this study, we conducted a preliminary evaluation of the protein expression regulated in the integrin-FAK-p38MAPK pathway at the protein phosphorylation level mediated by rESWT. Further, we applied key protein molecular inhibitors in this pathway, which suggested that rESWT may partially play its anti-inflammatory role on the acute inflammation model of human primary tenocytes through this pathway. This experimental design is basically consistent with a previous study [20].

Conclusions

This study preliminarily confirmed that rESWT could up-regulate FAK phosphorylation and down-regulate p38MAPK phosphorylation in a TNF-α-induced acute inflammation model of human primary tenocytes. Pretreatment with an integrin inhibitor markedly attenuated rESWT-mediated downregulation of p38MAPK phosphorylation and its reversal effect on the increased secretion of pro-inflammatory cytokines in TNF-α-induced human primary tenocytes. These results indicate that rESWT may partially regulate the integrin-FAK-p38MAPK pathway to alleviate acute inflammation in human primary tenocytes (**Figure 6**). However, FAK and p38MAPK phosphorylation sites require dominant-negative expression or site-specific mutations to verify further the signaling pathway proposed in this study. Furthermore, the experimental results must be validated using an *in vivo* model of acute tendinitis.

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Disclosure of conflict of interest

None.

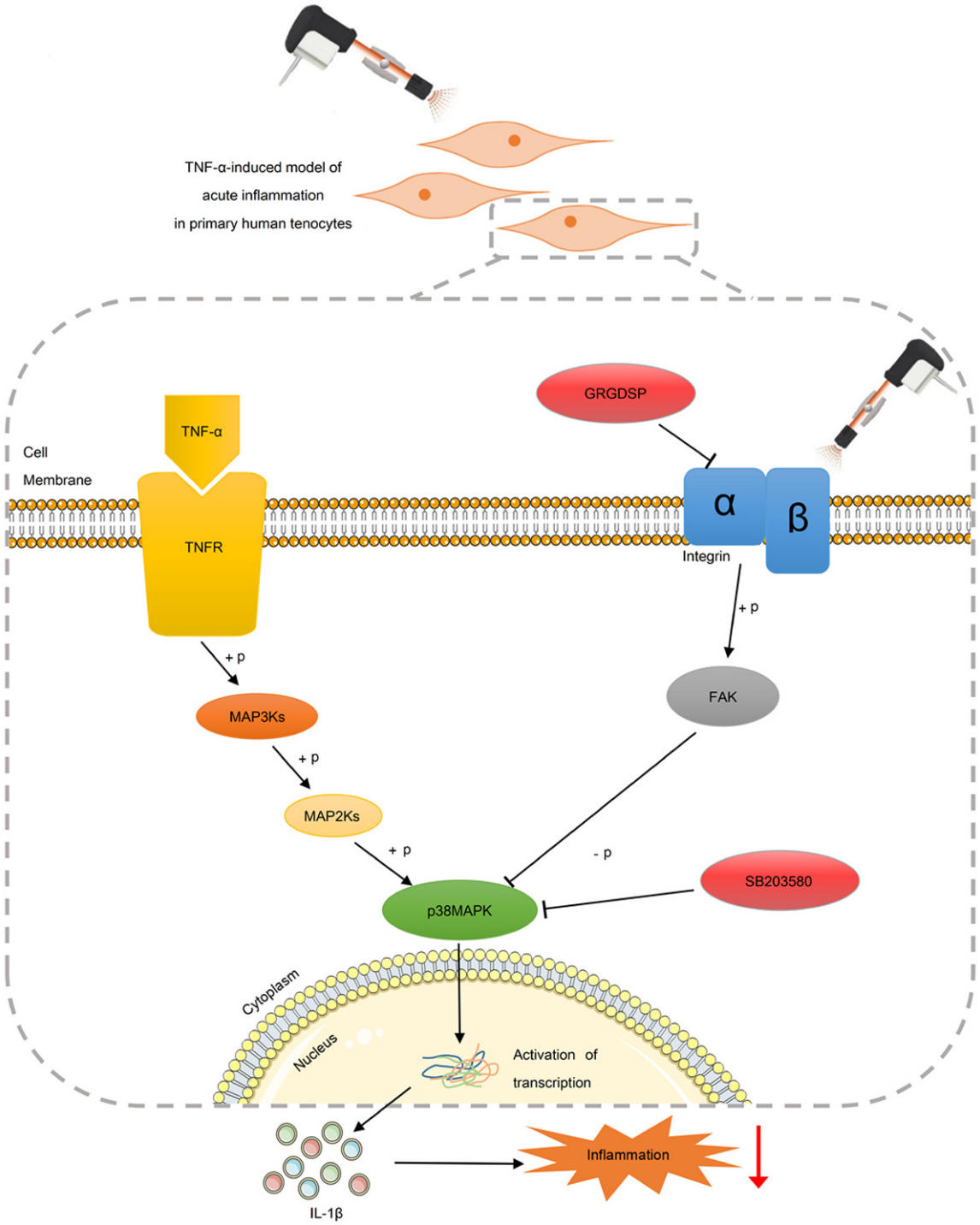

Figure 6. rESWT may partially regulate the Integrin-FAK-p38MAPK pathway to alleviate acute inflammation of human primary tenocytes. TNF: tumor necrosis factor; TNFR: tumor necrosis factor receptor; MAPK: mitogen-activated protein kinase; GRGDSP: glycine-arginine-glycine-aspartic acidserine-proline peptide; FAK: focal adhesion kinase; IL: interleukin; rESWT: radial extracorporeal shock wave therapy.

Address correspondence to: Qi Gao, School of Sports Medicine and Rehabilitation, Beijing Sport University, Xinxi Road, Haidian District, Beijing 100084, China. Tel: +86-18611918556; E-mail: bsugaoqi@126.com

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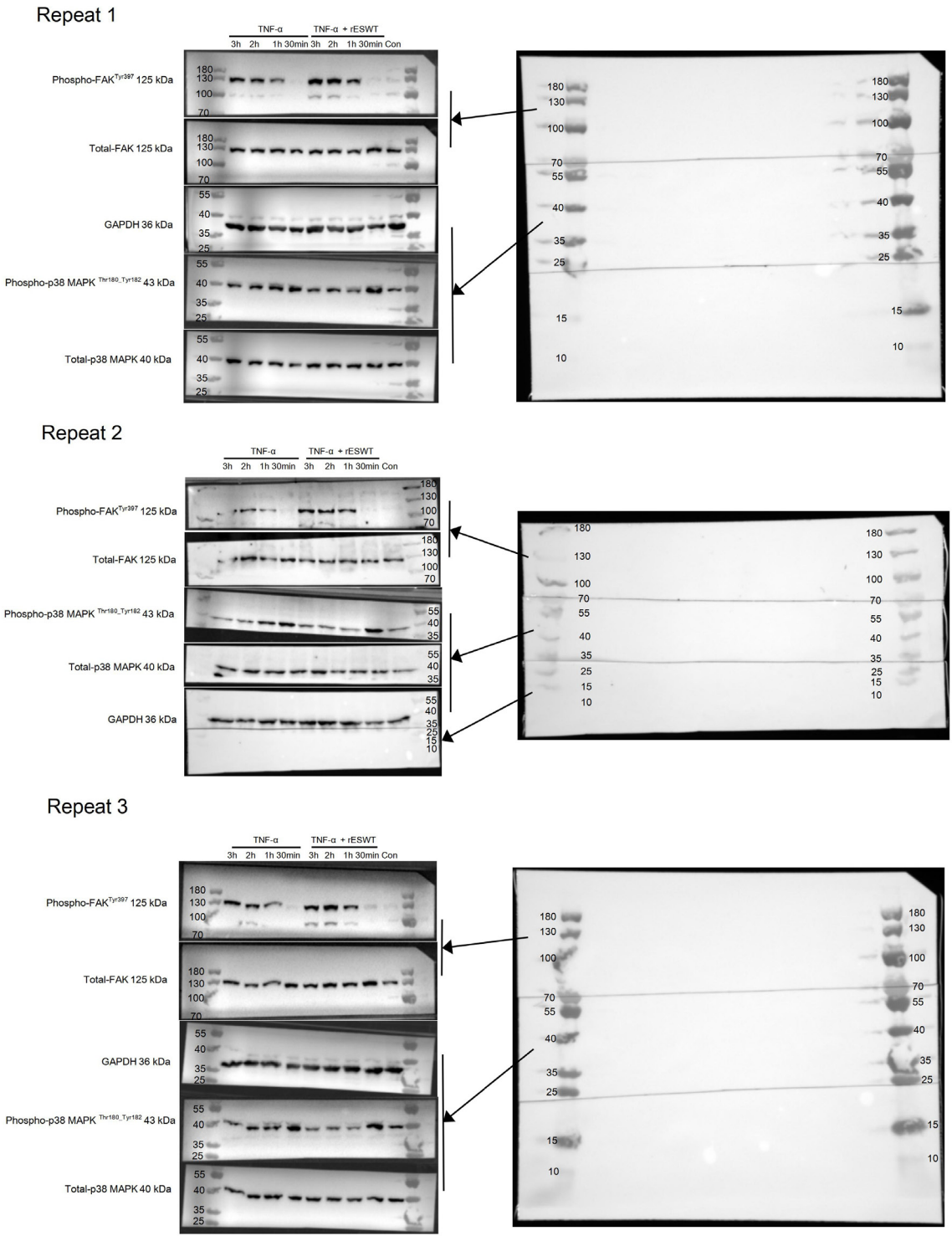
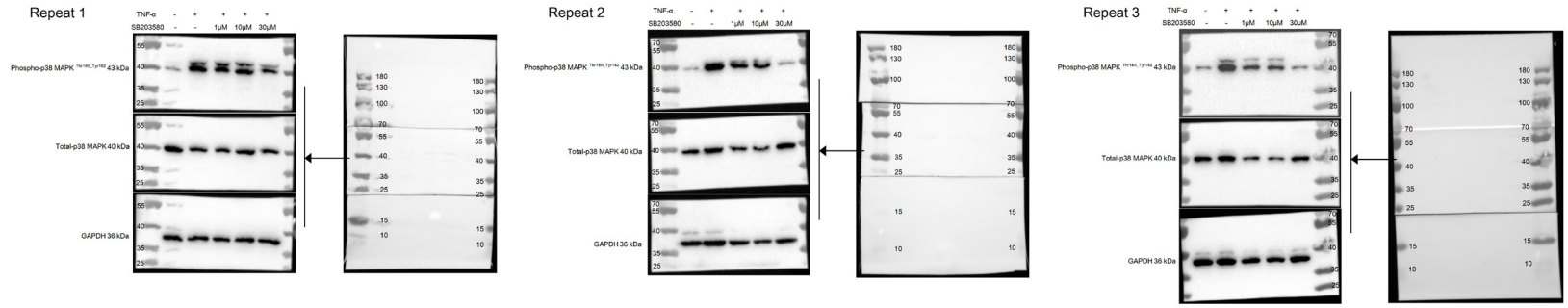
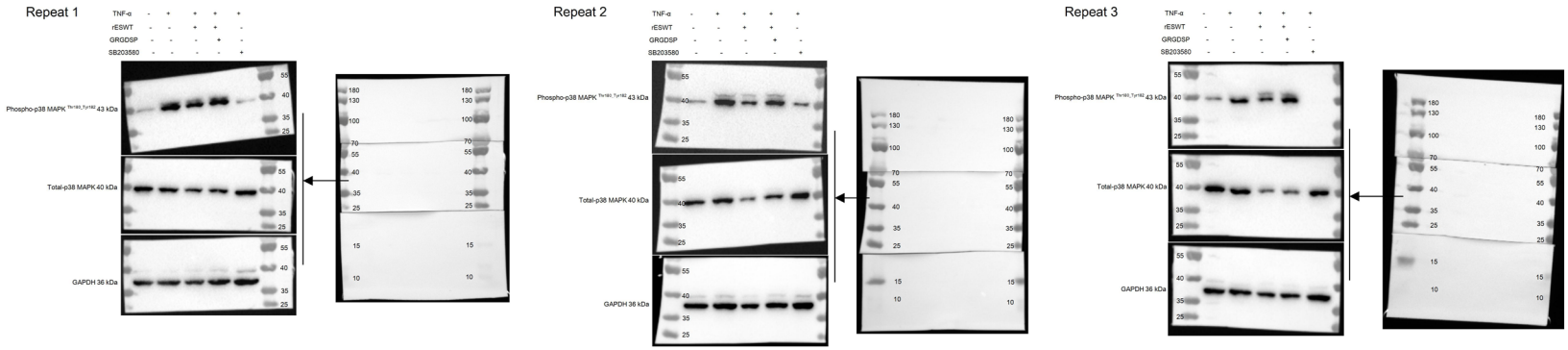


Supplementary Figure 1. Full and non-adjusted images for **Figures 1**, **2**. Western blot full images of FAK, phospho-FAK^{Tyr397}, p38MAPK, phospho-p38MAPK^{Thr180_Tyr182} and GAPDH. TNF: tumor necrosis factor; rESWT: radial extracorporeal shock wave therapy; FAK: focal adhesion kinase; MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.



Supplementary Figure 2. Full and non-adjusted images for **Figure 3**. Western blot full images of p38MAPK, phospho-p38MAPK^{Thr180_Tyr182} and GAPDH. TNF: tumor necrosis factor; MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.



Supplementary Figure 3. Full and non-adjusted images for **Figure 4**. Western blot full images of p38MAPK, phospho-p38MAPK^{Thr180_Tyr182} and GAPDH. TNF: tumor necrosis factor; rESWT: radial extracorporeal shock wave therapy; GRGDSP: glycine-arginine-glycine-aspartic acidserine-proline peptide; MAPK: mitogen-activated protein kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.