GSK-3β activates *NF-κB* to aggravate caerulein-induced early acute pancreatitis in mice

Xiaoying Fu[1,2#], Xi Zhong[3#], Xudong Chen[2], Dujiang Yang[1], Zongguang Zhou[1], Yong Liu[1]

[1]Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu, China; [2]West China School of Medicine, Sichuan University, Chengdu, China; [3]Department of Critical Care Medicine, West China Hospital, Sichuan University, Chengdu, China

Contributions: (I) Conception and design: Y Liu, X Fu, X Zhong; (II) Administrative support: Z Zhou; (III) Provision of study materials or patients: Y Liu, Z Zhou; (IV) Collection and assembly of data: X Chen, D Yang; (V) Data analysis and interpretation: X Fu, X Zhong; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Yong Liu. Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu, China. Email: beckmanliuyong@163.com.

Background: Acute pancreatitis is a life-threatening disease which causes considerable morbidity and mortality. However, no specific and effective treatments are currently available for this critical condition, which is mainly due to the insufficient understanding of the early cellular events in the initial phase of acute pancreatitis. Previous researchers have reported that two independent events, intra-acinar trypsinogen and *NF-κB* activation, are of equal importance in the early development of acute pancreatitis. *GSK-3β*, an essential molecule in multiple physiopathological processes including inflammation, is associated with the expression of the *NF-κB* pathway.

Methods: We investigated whether *GSK-3β* affected the expression of cytokines produced by intra-acinar cells and aimed to determine the probable regulatory mechanism by using single allele *GSK-3β*-deficient mice.

Results: Our data showed that *IL-6* and *TNF-α* mRNA expression in pancreatic tissue and serum *IL-6* and *TNF-α* were significantly decreased. Meanwhile, pancreatic phospho-*NF-κB* p65 (ser536) protein expression in *GSK-3β^{+/−}* mice was lower than that in wild type (WT) mice.

Conclusions: *GSK-3β* may activate intra-acinar *NF-κB* signaling to promote the production of proinflammatory cytokines, which then induces the recruitment of inflammatory cells and activation of the cytokine cascade, further promoting local and systemic inflammation and ultimately aggravating acute pancreatitis. These findings strongly indicate that *GSK-3β* may be a potential treatment target for acute pancreatitis.

Keywords: Acute pancreatitis; caerulein-induced model; *GSK-3β*; *NF-κB*; inflammatory response

Introduction

Acute pancreatitis, usually caused by gallstones and ethanol consumption (1), is an acute inflammatory disease of the exocrine pancreas, characterized by acute abdominal pain and a concomitant rise in serum amylase and lipase concentration (2). Although the majority of cases are mild and self-limited, the overall mortality of acute pancreatitis still remains at 4–8% (2), and can even reach 15% when patients with pancreatitis develop pancreatic or extrapancreatic necrosis and systemic inflammatory response syndrome (SIRS) (3). While the rate of hospitalization for acute pancreatitis has been increasing in the past decades (4), this disease lacks specific and effective interventions because of the little understanding of the early cellular events in the pathophysiology of the initiation of acute pancreatitis, posing a significant challenge to health

providers worldwide.

Over the past century, it has been presumed that intra-acinar trypsinogen activation is the key event for the onset of pancreatitis (5,6). However, some researchers found that the progression of local and systemic inflammation could not be relieved when pathological activation of trypsinogen was completely removed (7,8), indicating that there might be another parallel mechanism that mediates the inflammatory response in the early stage of acute pancreatitis. It was then found that intra-acinar *NF-κB* activation during the early development of acute pancreatitis was independent from trypsinogen activation. Thus, researchers proposed that *NF-κB* activation might be responsible for local inflammation and the development of the systemic inflammatory response (7,9-12). Furthermore, Huang *et al.* proved that *NF-κB*, which was activated early in acinar cells during acute pancreatitis, provoked the expression of multiple proinflammatory genes, and ultimately increased the severity of acute pancreatitis when transgenes that encoded the *NF-κB* p65 subunit or the inhibitor of κB kinase 2 (*IKK2*) were introduced to mice (13-18).

In contrast to the observations regarding *NF-κB* described above, some controversial results have also been reported in a mouse model with constitutive modifications of the *NF-κB* gene. A study reported that *NF-κB* signaling-deficient mice developed more severe pancreatitis when induced by caerulein (19). In another study, in which IkBα was specifically deleted in mouse pancreatic tissue, *NF-κB* was constitutively activated and acute pancreatitis was ameliorated when mice were administered caerulein and L-arginine (20). These conflicting outcomes highly suggest that there are signaling pathways or proteins that engage in *NF-κB* regulation and further interfere with the development of acute pancreatitis.

Glycogen synthase kinase-3β (*GSK-3β*), an isoform of glycogen synthase kinase 3 (*GSK-3*), is a serine/threonine protein kinase which plays critical roles in multiple pathophysiological processes including cell differentiation, cell cycle control, cell motility, microtubule function, tumorigenesis, and apoptosis (21-24). Recently, it was also found to be involved in inflammation processes (25-28). More importantly, *GSK-3β*-deficient mice (29) showed a similar phenotype to those that lacked the p65 subunit of *NF-κB* or *IKKβ* (30,31). Further investigations revealed that the p65 subunit of *NF-κB* was a direct phosphorylation target of *GSK-3β*, which consequently affected *NF-κB*-dependent gene expression in various cells and tissues (32-34). Moreover, other reports also demonstrated that

GSK-3β targeted *NF-κB* signaling in different pathological processes (35-38). As *NF-κB* activation provoked the expression of multiple inflammatory mediators and cytokines in pancreatic acinar cells and promoted the development of acute pancreatitis, this research used heterozygous *GSK-3β* deficient mice(*GSK-3β⁺/⁻* mice) to prove the hypothesis that *GSK-3β* is involved in caerulein-induced early acute pancreatitis through activating the *NF-κB* signaling pathway.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5701).

Methods

Generation of transgenic mice

Paired *GSK-3β⁺/⁻* mice, purchased from Jackson laboratory (strain name: B6.129P2-Gsk3β^tm1Dgen/J), were housed in a 12-h light and dark cycle in a specific pathogen-free animal facility with a constant temperature (22±1 ℃) and free access to tap water and pelleted chow. Male and female *GSK-3β⁺/⁻* mice were mated to obtain single allele *GSK-3β* deficiency (*GSK-3β⁺/⁻*) offspring because *GSK-3β* homozygous knockout (*GSK-3β⁻/⁻*) embryos would die at day E13.5 attributed to *TNF-α*-induced hepatocyte apoptosis. The genotypes of all descendants, which were consistently maintained in a C57BL/6 background, were determined by RT-PCR according to the protocol of the Jackson laboratory, and wild type (WT) littermates were assigned to the control group randomly. Mice allocation, experiment conduction, and data analysis were completed by 3 investigators independently.

A protocol was prepared before the study without registration. The study was approved by the Animal Ethics Committee of Sichuan University West China Medical School *(*No. 2021737A*)*, and all procedures with animals were conducted according to the guidelines of the local Animal Use and Care Committees of Chengdu and executed according to the National Animal Welfare Law of China.

Caerulein pancreatitis induction

To induce pancreatitis with caerulein, WT and heterozygote *GSK-3β* (*GSK-3β⁺/⁻*) littermate mice (n=8), which were 8 to 10 weeks old and weighed approximately 20 g, were fasted for 12 h with water ad libitum. The mice then received

an intraperitoneal injection of 50 µg/kg caerulein (Sigma-Aldrich, St. Louis, MO, USA) in saline. WT and *GSK-3β⁺/⁻* control mice (n=6) received an equal amount of saline. All mice were sacrificed by CO_2 asphyxiation 30 min after caerulein injection, and blood serum and tissue samples were collected for further research.

Preparation of serum and tissue samples

Whole blood samples were placed at room temperature for 2 h and then centrifuged for 15 min at 3,000 rpm. The serum was collected and stored at –80 ℃ for measurement of amylase, lipase, and cytokines. Tissues from the pancreas were removed and placed on ice, immediately frozen in liquid nitrogen, and then stored at –80 ℃ for PCR and western blot analysis.

Measurement of serum amylase and lipase

Serum amylase and lipase were detected using commercially available amylase and lipase assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

LiquiChip multiple cytokine assay

Serum cytokines, *IL-6* and *TNF-α*, were simultaneously measured on the LiquiChip 100 workstation (Luminex, Austin, TX, USA) using a commercially available luminex assay kit (R&D, USA) according to the manufacturer's protocol.

Quantitative real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)

Snap frozen pancreatic tissue was pulverized in liquid nitrogen and total RNA was extracted by the Trizol method with RNAiso Plus (TaKaRa, DaLian, China). First-strand cDNA was synthesized from 5 µg total RNA using oligo-dT primers (18mer) and M-MLV reverse transcriptase (TaKaRa, Dalian, China). For real-time PCR amplification, 1-µL cDNA was used for each PCR amplification in a 25-µL reaction system in triplicate with SYBR Green chemistry using the Icycler IQ Multicolor Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Gene-specific, intron-spanning primers were designed with primer premier 5.0 software and synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The sequences of the primers are provided as follows: *IL-6* (forward 5'-AGT CAC AGA AGG AGT GGC TAA-3', reverse 5'-CAC TAG GTT TGC CGA GTA GA-3'), *TNF-α* (forward 5'-ACG TCG TAG CAA ACC ACC AA-3', reverse 5'-CTG GGA GTA GAT AAG GTA CA-3'), and GAPDH (forward 5'-CCT CAA GAT TGT CAG CAA T-3', reverse 5'-CCA TCC ACA GTC TTC TGA GT-3'). A standard program of real-time PCR was performed with the first denaturation step at 94 ℃ for 5 min, 40 cycles of denaturation at 94 ℃ for 20 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s. Relative quantitation of the mRNA level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method as described previously (39) and was expressed as fold change compared with WT control.

Western blot assay

For the western blot assay, snap frozen mouse pancreatic tissue was rapidly pulverized in liquid nitrogen and the resulting tissue powder was resuspended in ice-cold RIPA buffer containing 1 mM phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China). Samples were centrifuged at 4 ℃ for 10 min at 12,000 rpm. Supernatants were collected and the concentration of each sample was determined using the Pierce(R) BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA). Then, 30 µg samples of protein were subjected to 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Non-specific binding to the membrane was blocked by 5% (w/v) dry non-fat milk dissolved in Tris-buffer saline/0.05% Tween-20 (TBST) at room temperature for 1 h. Membranes were then incubated with rabbit monoclonal anti-phospho-*NF-κB* p65 (ser536) antibody (1:500 dilution, Cell Signaling Technology, Beverly, MA, USA) and anti-β-actin antibody (1:1,000 dilution, Cell Signaling Technology, Beverly, MA, USA), which was used as the internal reference, overnight at 4 ℃. Membranes were washed for 10 min 3 times with TBST and then incubated with goat polyclonal anti-rabbit IgG (H+L) antibody conjugated to horseradish peroxidase (1:5,000 dilution, KPL, Maryland, USA) diluted in 5% (w/v) dry non-fat milk in TBST for 1 h at room temperature. Finally, after washing for 10 min 3 times with TBST, membranes were developed using the Alpha Innotech FluorChem imaging system (USA) to visualize the target bands with the enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA), and the semi-quantitative grayscale intensity was measured with Quantity
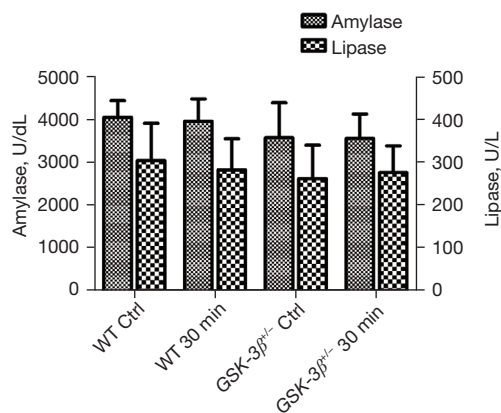
Figure 1 The levels of serum amylase and lipase. The levels of serum amylase and lipase in *GSK-3β*^{+/–} mice were lower than in WT mice, but there was no significant difference between *GSK-3β*^{+/–} mice and WT mice 30 min after caerulein challenge, or compared to respective controls, P>0.05.

One software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Experimental data were presented as mean ± SD. Statistical analysis was performed using a non-paired Student's *t*-test or one way analysis of variance (ANOVA) followed by Scheffe's post-hoc test when a significant difference was found among groups (SPSS statistical software 16.0; SPSS Inc., Chicago, IL, USA). P values that were less than 0.05 were considered to indicate a significant statistical difference.

Results

Effect of GSK-3β^{+/–} *on the level of serum amylase and lipase*

Commercially available amylase and lipase assay kits were used to determine the level of serum amylase and lipase. Although the levels of serum amylase and lipase were decreased in *GSK-3β*^{+/–} mice compared to WT mice, statistical analysis indicated that there was no significant difference between *GSK-3β*^{+/–} and WT mice at 30 min after caerulein challenge (P>0.05, *Figure 1*).

Effect of GSK-3β^{+/–} *on the level of serum IL-6 and TNF-α*

To determine the level of serum cytokines, *IL-6* and *TNF-α*

were simultaneously detected by the LiqiChip multiple cytokine assay system. Compared to the WT control, the levels of serum *IL-6* and *TNF-α* were significantly increased at 30 min after caerulein challenge in WT mice (P<0.01). Moreover, the levels of serum *IL-6* and *TNF-α* in *GSK-3β*^{+/–} mice were markedly reduced compared with WT mice at 30 min after caerulein challenge (P<0.01). While serum *IL-6* and *TNF-α* were modestly elevated in *GSK-3β*^{+/–} mice at 30 min after caerulein challenge, only *TNF-α* was significantly increased compared to the *GSK-3β*^{+/–} control group (P<0.05, *Figure 2A,2B*).

Effect of GSK-3β^{+/–} *on the expression of IL-6 and TNF-α mRNA in pancreatic tissue*

The expression levels of *IL-6* and *TNF-α* mRNA in pancreatic tissue were detected by real-time RT-PCR. Consistent with the expression trend of serum *IL-6* and *TNF-α* protein, *IL-6* and *TNF-α* mRNA were markedly increased in WT mice at 30 min after caerulein challenge compared to *GSK-3β*^{+/–} mice and the WT control group (P<0.01). The expression levels of *IL-6* and *TNF-α* mRNA in *GSK-3β*^{+/–} mice were also moderately increased at 30 min after caerulein challenge compared to the *GSK-3β*^{+/–} control group, but only the expression of *TNF-α* mRNA showed a statistically significant difference (P<0.05). Both *IL-6* and *TNF-α* mRNA were significantly decreased compared with WT when challenged by caerulein (P<0.01, *Figure 3A,3B*).

Effect of GSK-3β^{+/–} *on the expression of phospho-NF-κB p65 protein*

As *NF-κB* modulates multiple inflammatory mediators and cytokine expression, and the p65 subunit has a phosphorylation site of *GSK-3β*, we detected phospho-*NF-κB* p65 (ser536) protein expression in pancreatic tissue by western blot analysis. Phospho-*NF-κB* p65 (ser536) protein was significantly elevated in WT mice at 30 min after caerulein challenge compared to the WT control group. However, it was slightly elevated in *GSK-3β*^{+/–} mice, which was striking decreased compared with WT mice, at 30 min after caerulein challenge (*Figure 4*).

Discussion

As a common clinical emergency, acute pancreatitis has considerable morbidity and mortality. However, specific and effective treatments to ameliorate acute
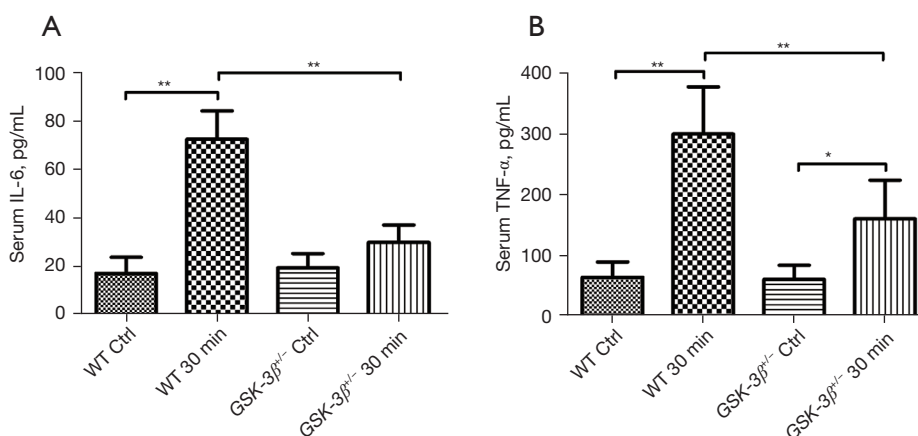
Figure 2 The levels of *IL-6* and *TNF-α* in serum. The levels of serum *IL-6* (A) and *TNF-α* (B) in WT mice were significantly elevated at 30 min after caerulein challenge. Moreover, in *GSK-3β+/−* mice, the levels of serum *IL-6* and *TNF-α* were observed to be moderately elevated at 30 min after caerulein challenge, though were significantly reduced compared with WT mice. **, $P<0.01$; *, $P<0.05$.
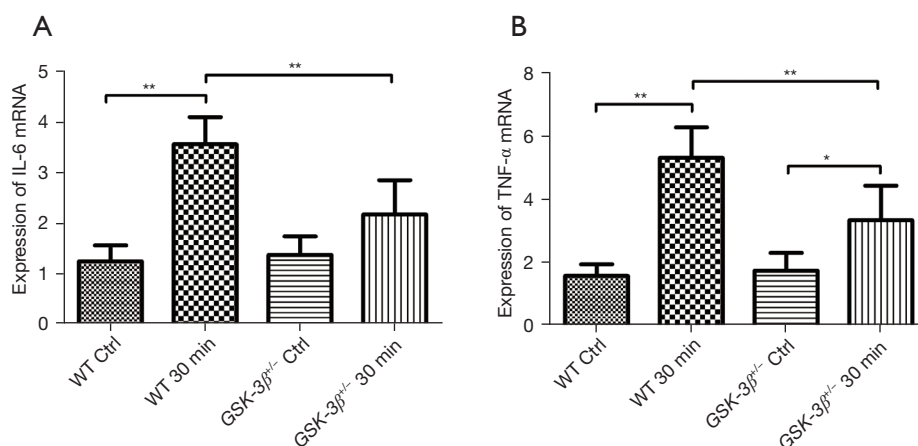

Figure 3 The expression of *IL-6* and *TNF-α* mRNA in pancreatic tissue. Pancreatic tissue *IL-6* (A) and *TNF-α* mRNA (B) levels in WT mice were significantly increased at 30 min after caerulein challenge compared to *GSK-3β+/−* mice and the WT control. Tissue mRNA expression levels of *IL-6* and *TNF-α* were found to be modestly increased, but only *TNF-α* mRNA showed a significant difference compared to *GSK-3β+/−* control. **, $P<0.01$; *, $P<0.05$.

pancreatitis are lacking, which can be attributed to the insufficient understanding of the etiopathogenesis and pathophysiological progression of pancreatitis. Recent research has indicated that intra-acinar trypsinogen and *NF-κB* activation during the early development of acute pancreatitis might be two key independent factors that result in acinar insult and the progression of local and systemic inflammatory responses (7-12), respectively. However, paradoxical results have been demonstrated from different reports (19,20), implying that the underlying

mechanisms of the development of acute pancreatitis, remains to be further investigated.

GSK-3β, a multifunctional phosphorylase kinase, was discovered to be involved in multiple pathophysiological processes (21-24). Recent research revealed that *GSK-3β* played various but critical roles in inflammatory reactions (25-28). Our former research about the expression of *GSK-3β* in the acute edematous pancreatitis shows that both *GSK-3β* mRNA and protein expression level were associated with the severity of the disease (40). Also, Jin
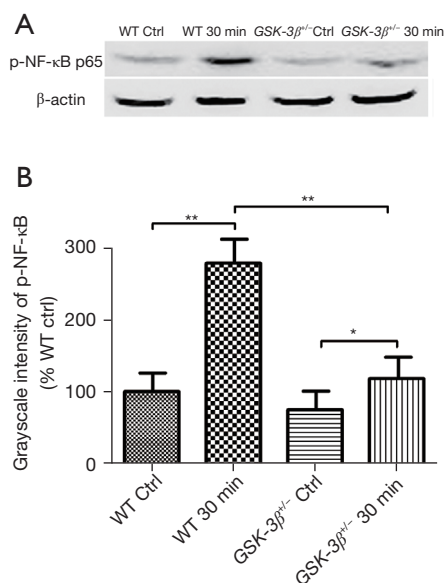
Figure 4 The expression of phospho-*NF-κB* p65 (ser536) protein in pancreatic tissue. (A) The protein expression of phospho-*NF-κB* p65 was measured by western blot analysis; (B) the grayscale intensity of *NF-κB* was presented as a percentage compared to WT control, which showed that the phospho-*NF-κB* p65 protein was significantly elevated in WT mice at 30 min after caerulein challenge compared to *GSK-3β+/−* mice and WT control, while a moderate change was detected in *GSK-3β+/−* mice. **, P<0.01; *, P<0.05.

also found that the expression of *GSK-3β* is elevated in acute necrotizing pancreatitis) (41). It could be assumed that *GSK-3β* was activated in acute pancreatitis and was associated with the severity of the pancreatitis. The latest data showed that *GSK-3β* could directly phosphorylate the *NF-κB* p65 subunit and consequently regulate the transcription of *NF-κB*-dependent gene transcription (32-34). Moreover, *GSK-3β*-deficient mice presented a phenotype similar to that of p65 subunit or *IKKβ* knock-out mice (29-31). As *GSK-3β* knockout homozygous mice will die mid gestation or as newborns due to severe liver degeneration (29), in this research, we utilized single allele *GSK-3β*-deficient (*GSK-3β+/−*) mice to investigate whether *GSK-3β* interferes with the early development of acute pancreatitis *in vivo* through the *NF-κB* signaling pathway. In the western blot assay, pancreatic *GSK-3β* protein in *GSK-3β+/−* mice decreased more than 60% compared to WT mice (data not shown), so this research based on heterozygous transgenic mice is reliable. Although serum amylase and lipase levels slightly declined in *GSK-3β+/−* mice compared

to the respective control, we did not detect a significant difference between *GSK-3β+/−* and WT mice challenged by caerulein. We propose that *GSK-3β* may not induce intra-acinar trypsinogen activation, which is responsible for acinar injury and consequently increases serum amylase and lipase during early acute pancreatitis (7-9). Meanwhile, we did not observe obvious morphological changes in the pancreatic tissues of all groups by hematoxylin and eosin (HE) staining (data not shown), such as pancreatic acinar necrosis and inflammatory cell infiltration, which to some extent supports the results that came from the amylase and lipase data.

Several lines of evidence revealed that acute pancreatitis was initiated in acinar cells and that SIRS and multiple organ failure were consequences of the synthesis and release of proinflammatory cytokines and chemokines in injured acinar cells (42-45). Therefore, we detected the expression of *IL-6* and *TNF-α* mRNA in pancreatic tissue and measured the levels of serum *IL-6* and *TNF-α*. Consistent with previous research, when using small interfering RNA-mediated knockdown of *GSK-3β* or a panel of different *GSK-3*-selective pharmacological inhibitors (46), our data showed that expression levels of *IL-6* and *TNF-α* mRNA in pancreatic tissue were significantly elevated in WT mice at 30 min after caerulein challenge compared with *GSK-3β+/−* mice and the WT control, which was consistent with the trend of the serum level of *IL-6* and *TNF-α*. The expression levels of *IL-6* and *TNF-α* mRNA in WT mice were increased 2.8 and 3.4 folds, respectively, after caerulein challenge, but in *GSK-3β+/−* mice the expression of *IL-6* and *TNF-α* mRNA both declined approximately 40% compared with the WT mice. However, serum *IL-6* and *TNF-α* were increased 4.2 and 4.6 folds, respectively, after caerulein challenge, while *IL-6* was decreased by approximately 60% and *TNF-α* was decreased by 50% in *GSK-3β+/−* mice compared with the WT mice. Despite overwhelming evidence suggesting that the recruitment of inflammatory cells into the pancreas is the predominant source of cytokines (10,47,48), the synthesis and release of cytokines in pancreatic acinar cells often occur within 30 min after caerulein challenge and prior to recognizable pathohistological changes (14,47,49). According to our observation, when increasing cytokines were detected the inflammatory cells have not been recruited. Our pathomorphological results found that no infiltrating inflammatory cells and histological injury were observed by HE staining after 30 min challenge by caerulein. Thus, we proposed that the marked increase of serum *IL-6* and

TNF-α at the specific time point might not be related to the activation of inflammatory cells but was associated with other pathways. Besides proinflammatory cytokines, it is known that *IL-10*, an anti-inflammatory cytokine, plays a pivotal role in controlling excessive inflammation and maintaining immune homeostasis. In contrast to previous results which demonstrated that *GSK-3β* modulated the Toll like receptor (*TLR*)-induced inflammatory response of blood monocytes and promoted the production of proinflammatory cytokines such as *IL-1β*, *IL-6*, *TNF*, *IL-12*, and *IFN-γ* while concurrently suppressing *IL-10* secretion (46,50-52), we did not detect the promotion of serum *IL-10* in *GSK-3β⁺/⁻* mice after caerulein challenge (data not shown). Moreover, contrary to Jhun *et al.*'s results (53), we did not find that serum *IL-2* was elevated in WT mice challenged by caerulein (data not shown). It is possible that the contrasting results of the serum levels of *IL-2* and *IL-10* may be caused by virulence factors, the experimental model, and the stage of acute pancreatitis.

Previous research has indicated that *GSK-3β* participates in the regulation of the eukaryotic transcription factor *NF-κB*, which modulates many diverse cellular processes, including promoting proinflammatory cytokine synthesis and release (29,32,54-56). Several researches used *GSK-3β* inhibitors to investigate the role of it in acute pancreatitis to explore the relationship between *GSK-3β* and *NF-κB* pathway. It was found that 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), an inhibitor of *GSK-3β*, could ameliorate the severity of sodium taurocholate-induced severe acute pancreatitis (SAP) pancreatitis in the rat model and reduced the expression level of *NF-κB* (57). Similar results also have been found in cerulein-induced acute pancreatitis mice model (58). And different inhibitors showed consistent result that *GSK-3β* inhibition could alleviate acute pancreatitis through *NF-κB* pathway (41). It is well known that the *NF-κB/HIF-1α* pathway involves in intestinal barrier dysfunction. Further research showed that *NF-κB* could be activated to induce the intestinal mucosal barrier injury in mice with acute pancreatitis (59). So it could be critical to further investigate the role of *NF-κB* since it is not only function in the progression of acute pancreatitis but also involves in the systematic complications of acute pancreatitis. In this research, we utilized a phosphor-*NF-κB* p65 (ser536) monoclonal antibody to investigate whether *GSK-3β* modulated the *NF-κB* signal pathway by western blot assay. Our data showed that phospho-*NF-κB* p65 protein expression was significantly increased in WT mice, but in *GSK-3β⁺/⁻* mice

the protein expression was decreased approximately 60% compared to WT mice at 30 min after caerulein challenge, which was consistent with previous reports (52,60-62).

This study also shows some limitations. Although the animal model was highly effective for investigating the mechanisms of acute pancreatitis, it would be more convincing to establish a *GSK-3β* full deletion model. This may be achieved by using the technique of temporal and spatial control of gene expression in the mouse model, which has been established in congenital lung diseases (63).

Conclusions

In summary, our research, for the first time, proved that *GSK-3β* might activate *NF-κB* in pancreatic acinar cells and consequently promote the synthesis and release of proinflammatory cytokines by acinar cells at the early stage of the development of acute pancreatitis after caerulein challenge *in vivo* using *GSK-3β* heterozygote transgenic mice. Consequently, proinflammatory cytokines secreted by acinar cells might recruit inflammatory cells into pancreatic tissue and then promote and aggravate acute pancreatitis. Thus, we conclude that *GSK-3β* may be an ideal therapeutic target in the early stage of acute pancreatitis, and a *GSK-3β* inhibitor may provide beneficial effects in prophylaxis, surgical intervention, and endoscopic retrograde cholangiopancreatography (ERCP)-induced acute pancreatitis.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-5701

Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-5701

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Animal Ethics Committee of Sichuan University West China Medical School (No. 2021737A), and all procedures with animals were conducted according to the guidelines of the local Animal Use and Care Committees of Chengdu and executed according to the National Animal Welfare Law of China.

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