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Attenuation of acute pancreatitis by PPAR- α in rats: the effect on Toll-like receptor signaling pathways

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Running title: PPAR- α protect AP in the regulation of TLR pathways

Abstract

Objectives: The peroxisome proliferator-activated receptor-alpha (PPAR- α) has attracted considerable attention for its anti-inflammatory properties; however, Toll-like receptor (TLR) pathways have an essential pro-inflammatory role in acute pancreatitis (AP). This study aimed to evaluate the attenuation of inflammation by PPAR- α and to investigate the interaction between PPAR- α and TLR pathways in AP.

Methods: AP was induced in rats by administration of caerulein. The PPAR- α agonist WY14643 and/or antagonist MK886 was administered. The severity of AP was determined by measuring serum amylase, lipase, Ca^{2+} , pathological changes, myeloperoxidase (MPO) activity, serum levels of interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1). TLR2 and TLR4 mRNA and proteins were determined by real-time RT-PCR and western-blotting respectively. mRNA expressions of target molecules of TLR pathways, including IL-6, IL-10, ICAM-1 and Tumor necrosis factor-alpha (TNF- α) were also measured.

Results: Treatment with WY14643 significantly decreased amylase, lipase, MPO activity, pathological scores, IL-6 and ICAM-1 levels. TLR2 and TLR4 mRNA and proteins were markedly decreased after treatment with WY14643, along with IL-6, ICAM-1 and TNF- α mRNA levels. However, these effects were completely reversed by the co-administration of MK886.

Conclusions: Activation of PPAR- α played a protective role in AP, partially mediated by modulation of TLR pathways.

Introduction

Acute pancreatitis (AP) is a common but potentially lethal disease that has an increasing incidence. Essentially, AP is an inflammatory disease, the progress and prognosis of which are determined by local and systemic inflammatory responses. Therefore, exploring the intrinsic anti-inflammatory mechanisms is important for controlling the development of pancreatitis.

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors that are members of the class II nuclear hormone receptor superfamily that regulate cellular energy and lipid metabolism. Three subtypes have been identified: PPAR- α , - γ , and - β/δ . Recently, PPARs have attracted wide attention as essential negative regulators in inflammatory responses¹. PPAR- γ is the most frequently studied protective molecule in several inflammatory responses, such as ischemia/reperfusion, shock and AP². PPAR- α as a general modulator of the inflammatory response was first reported by Devchand et al³, who observed that inflammation is prolonged in PPAR- α -deficient mice. Subsequently, it was demonstrated that PPAR- α -deficient mice have abnormally prolonged responses in inflammatory bowel disease⁴. Recent studies have clearly demonstrated that PPAR- α activation protects brain capillary endothelial cells from oxygen-glucose deprivation-induced hyperpermeability in the blood-brain barrier⁵. In AP, PPAR- α -deficient mice show increased pancreatic inflammation and tissue injury⁶. In contrast, it was reported that PPAR- α negatively regulates the vascular inflammatory gene response by negative ‘cross-talk’ with the transcription factors, NF- κ B and AP-1⁷. Based on these results, it was hypothesized that the inflammatory progression in AP can be controlled by modulation of the activity of

PPAR- α . This study was designed to investigate the effects of administration of a PPAR- α agonist or/and antagonist on regulation of the pancreatic inflammatory response in caerulein-induced AP in rats.

Toll-like receptors (TLRs) are a group of pattern recognition receptors upstream of NF- κ B and AP-1. These molecules, including TLR2, TLR4 and TLR9, have a critical role in the pathogenesis of AP⁸⁻¹⁰. Ligand binding by TLRs results in activation of a cascade of kinases, leading to nuclear translocation of NF- κ B and AP-1, followed by release of cytokines, chemokines and adhesion molecules¹¹. Little is known about the possible interaction between the pro-inflammatory TLR pathways and the anti-inflammatory PPAR- α pathway in AP. This study aimed to evaluate attenuation of inflammation by activation of PPAR- α in AP and furthermore, to investigate the role of the TLR signaling pathways in this attenuation.

Materials and Methods

Animals

Male Wistar rats (200-250 g) were used in this study (Experimental Animal Center of Sichuan University, Chengdu, China). All animals were bred and housed in standard shoebox cages in a climate-controlled room with an ambient temperature of $23 \pm 2^{\circ}\text{C}$ and a 12:12-h light-dark cycle. Animals were fed standard laboratory chow and given water *ad libitum* except for the 12 hours prior to experimentation during which animals were starved and allowed free access to water alone. All animal experiments were conducted according to the guidelines of the local Animal Use and Care Committees and according to the National Animal Welfare Law.

Model preparation and experimental animal grouping

Acute pancreatitis (AP) was induced by two subcutaneous injections of caerulein (Sigma, St. Louis, MO) at a total dose of 20 µg/kg administered at 1-hour intervals. The PPAR- α agonist, WY14643 and the antagonist, MK886, were also purchased from Sigma. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1 h and/or 30 min before the first caerulein injection. The vehicle for caerulein was saline and 10% dimethylsulfoxide (DMSO) for both WY14643 and MK886. Rats were sacrificed 6 h after the first injection of caerulein or saline. All animals were randomly assigned to eight groups: DMSO plus saline group (control group), WY14643 plus saline group, MK886 plus WY14643 plus saline group, MK886 plus saline group, DMSO plus caerulein group, WY14643 plus caerulein group, MK886 plus WY14643 plus caerulein group, and MK886 plus caerulein group.

Sampling and pretreatment

Laparotomy was performed under 1% pentobarbital anesthesia (50 mg/kg body weight). Fresh pancreatic tissue was removed, rinsed in TRIzol (Gibco, Carlsbad, CA) for RNA isolation, or frozen in liquid nitrogen overnight, then stored at -80°C or fixed with 10% formaldehyde. Portal vein blood was collected, centrifuged at 4°C and the serum was stored at -80°C for further studies.

Serum amylase, lipase and Ca^{2+} determination

Serum amylase, lipase and Ca^{2+} levels were determined by using an automatic biochemical analyzer (Olympus AU5400, Japan) in the Biochemical Center of West

China Hospital. Serum amylase and lipase levels are expressed in terms of IU/l. Ca^{2+} levels are expressed in terms of mg/dl.

Histological examination

For routine histology, 5- μm sections of 10% formalin-fixed, paraffin-embedded tissue were prepared and stained with hematoxylin and eosin (H&E). All microscopic sections were examined by two independent pathologists who were blinded to the experimental protocol. Pancreatitis severity was scored as previously described¹². Briefly, necrosis was scored on a 0-3 scale, with 0, normal; 1, periductal necrosis (<5%); 2, focal necrosis (5-20%); 3, diffuse necrosis (>50%). Inflammatory cell infiltration was scored on a 0-3 scale, as follows: 0, normal; 1, inflammatory infiltration in duct; 2, infiltration in the parenchyma (<50%); 3, infiltration in the parenchyma (>50%). Edema was also scored on a 0-3 scale, with 0, normal; 1, focal increase between lobules; 2, diffuse increase between lobules; 3, acini disrupted and separated. The total severity score was the sum of all three scores.

Myeloperoxidase (MPO) measurement

Neutrophil infiltration in the pancreas was quantified by measuring tissue MPO activity using a chromatometric kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions provided by the manufacturer. Briefly, after weighing, a segment of pancreatic tissue was suspended in 0.5% hexadecyltrimethylammonium bromide (pH 6.5, 50 mg of tissue per ml) and was then homogenized. After freezing and thawing the homogenate three times, the tissue levels of MPO were detected by using

0.0005% hydrogen peroxide as a substrate for the enzyme. One unit of MPO activity is defined as that degrading 1 μ mol of peroxide per minute at 25°C and is expressed in units per gram weight (U/g) of wet tissue.

Enzyme-linked immunosorbent assay

Rat serum cytokines including interleukin-6 (IL-6) and soluble intercellular adhesion molecule-1(sICAM-1) were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN) and performed following the instructions provided by the manufacturer. The result was read by a microplate reader (Bio-Rad, M550, Hercules, CA). IL-6 and sICAM-1 concentrations are expressed in terms of pg/ml and ng/ml, respectively.

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

Fresh pancreatic tissue (50-80 mg/mouse) was collected and total RNA was extracted with TRIzol according to the instructions provided by the manufacturer. Integrity was confirmed by agarose gel electrophoresis. Concentration and purity were determined by ultraviolet spectrophotometry, and the total RNA concentration was calculated. Reverse transcription was performed in 20 μ l with 5 μ g of total RNA and random hexamers (TaKaRa Biotechnology Co., Ltd, Dalian, China). PCR amplification was performed on 1 μ l of the resulting cDNA. Specific primers were designed and synthesized by Invitrogen (Invitrogen Biotechnology Co., Ltd, Shanghai, China). The sequences of primers are shown in Table1. All the samples were normalized to β -actin gene. Conditions for all PCRs were optimized using an iCycler iQ (Bio-Rad, Hercules, CA) in a 25 μ l reaction

system for 40 cycles. Each cycle consisted of denaturation for 20 s at 94°C, annealing for 30 s at 55°C (TLR2, TLR4, and TLR9) or 52°C (ICAM-1, IL-6, IL-10 and TNF- α) and extension for 30 s at 72°C. A no-template, ddH₂O control was included for each template. All samples were amplified simultaneously in triplicate in a single run. Relative quantitative gene expression was calculated as previously described¹³ and expressed as a percentage of the control level.

Western-blotting

For total protein extracts, pancreas samples were homogenized with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 1% sodium deoxycholate, 1 mM PMSF), and centrifuged at 15,000×g for 10 min at 4°C. The supernatant was retained for the subsequent protein assays. Protein concentration was determined with a BCA kit (Pierce Biotechnology, Rockford, IL, USA). Extracts were treated with 2 × SDS-polyacrylamide loading buffer at 95°C for 10 min, and was separated by electrophoresis on 10% Tris–HCl polyacrylamide gels at room temperature. Proteins were transferred to 0.45 μ m polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA) for 90 min at 300 mA, 4°C (Bio-Rad Lab, Hercules, CA, USA). Membranes were blocked with 5% non-fat dry milk in TBS-0.05% Tween 20 (TBST) for 1 h at room temperature, washed three times for 10 min each in TBST, and incubated at 4°C with gentle shaking overnight with primary antibody rabbit anti-rat TLR2 polyclonal antibody (Abcam, Cambridge, UK) at a 1:500 dilution and mouse anti-rat TLR4 monoclonal antibody (Abcam) at a 1:500, in TBST containing 5% non-fat dry milk. After three 10-min TBST washes, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG at

1:5,000 (Bio-Rad) and goat anti-rat IgG at 1:5,000 (Bio-Rad) for 1 h at room temperature. After washing, membranes were analyzed by enhanced chemiluminescence (Pierce Biotechnology).

Statistical analysis

The data were expressed as the mean $\pm$ SE. Differences between three or more groups were evaluated by one-way ANOVA. A Student's t-test was used for comparisons of two groups. A *P*-value of less than 0.05 (2-tailed) was considered statistically significant. All tests were performed using the statistical package SPSS software 11.5 (SPSS, Chicago, IL).

Results

Serum amylase, lipase and Ca^{2+} determination

Levels of serum amylase, lipase and Ca^{2+} were determined (Figure 1). Compared with the DMSO plus saline (control) group, both serum amylase and lipase levels were significantly increased after caerulein treatment (14,925.59 [24.41] vs. 2,253.64 [58.12], $P < 0.05$; 781.32 [24.82] vs. 49.59 [2], $P < 0.05$, respectively, Figures 1A and 1B). Compared with the DMSO plus caerulein group, WY14643 treatment significantly inhibited the increase in serum amylase and lipase levels (12,038 [446.83]; 573.57 [29.65], $P < 0.05$, respectively). The inhibitory effects of WY14643 was completely reversed by the administration of MK886 (15,042.33 [404.61]; 782.56 [28.1], $P > 0.05$, respectively). Moreover, treatment of rats with MK886 alone profoundly increased serum amylase and lipase levels (18,876.46 [477.59]; 988.08 [29.82], $P < 0.05$, respectively).

The serum Ca^{2+} level serves as a marker for the severity of AP. After caerulein administration, serum Ca^{2+} levels were significantly reduced (6.53 [0.33] vs. 11.22 [0.54], $P < 0.05$, respectively). Compared with the DMSO plus caerulein group, treatment of WY14643 significantly attenuated this reduction (10.06 [0.42], $P < 0.05$). Co-administration of MK886 blocked the effect of the PPAR- α agonist (7.35 [0.36], $P > 0.05$), and furthermore, MK886 alone induced a more profound reduction in serum Ca^{2+} levels (3.97 [0.21], $P < 0.05$, Figure 1C).

Histological assessment of inflammation

Rat pancreatic tissues were sampled from all groups and stained by H&E (Fig. 2A, A1-5). No interstitial edema, cellular swelling, inflammatory cells infiltration and sporadic necrosis were detected in the DMSO plus saline (control) group, (Fig. 2A, A1), with a pathological score of 0.50 [0.21] (Fig. 2B). After caerulein administration, the pathological score was significantly increased (5.71 [0.29], $P < 0.05$), showing interstitial edema, cellular swelling, inflammatory cells infiltration and sporadic necrosis (Fig. 2A, A2). Compared with the DMSO plus caerulein group, the administration of WY14643 significantly attenuated pathological damage (3.29 [0.28], $P < 0.05$), (Fig. 2A, A3). However, the protective effect of WY14643 was significantly reversed by the administration of MK886 (5.73 [0.4], $P > 0.05$), (Fig. 2A, A4). Pretreatment with MK886 profoundly increased pancreatic damage (6.86 [0.41], $P < 0.05$) (Fig. 2A, A5).

Neutrophil infiltration by MPO assay

Neutrophil infiltration was assessed by the activity of MPO, an enzyme specifically

expressed by polymorphonuclear leukocytes. Compared with DMSO plus saline (control) group, caerulein treatment resulted in a marked increase in MPO activity (1,239.41 [93.9] vs. 207.9 [12.84], $P<0.05$, Figure 3). Compared with the DMSO plus caerulein group, WY14643 treatment significantly reduced the level of MPO activity (792.48 [49.08], $P<0.05$). The inhibitory effect of WY14643 was completely blocked by the administration of MK886 (1,204.42 [96.63], $P>0.05$). Furthermore, MK886 alone significantly increased MPO activity (1,685.1 [56.6], $P<0.05$).

Measurements of serum IL-6 and ICAM-1 concentrations

Serum IL-6 and ICAM-1 concentrations were assessed as a further measure of pancreatic inflammation. Compared with the DMSO plus saline (control) group, both IL-6 and ICAM-1 levels were significantly increased following caerulein administration (1,459.92 [74.05] vs. 752.82 [36.44]; 951.72 [39.76] vs. 403.47 [25.88], $P<0.05$, respectively, Figures 4A and 4B). Compared with the DMSO plus caerulein group, WY14643 treatment markedly decreased these levels (981.49 [48]; 724.78 [35.69], $P<0.05$, respectively). Co-administration of MK886 completely inhibited the effects of WY14643 (1,370.73 [65.27]; 945.47 [34], $P>0.05$, respectively). Administration of MK886 alone prior to caerulein treatment resulted in profoundly increased levels of IL-6 and ICAM-1 (1,978.12 [73.11]; 1,189.28 [38.87], $P<0.05$, respectively).

TLR2, TLR4 and TLR9 gene expression

Pancreatic gene expression of TLR2, TLR4 and TLR9 was simultaneously examined by quantitative RT-PCR (Figures 5A, 5B, 5C). Compared with the DMSO plus saline

(control) group, TLR2, TLR4 and TLR9 mRNA increased significantly following caerulein administration (9.11; 10.49; 10.80 times higher than the control, $P<0.05$, respectively). Compared with the DMSO plus caerulein group, WY14643 treatment significantly inhibited this increase in TLR2, TLR4 mRNA (5.03; 6.05 times higher than the control, $P<0.05$, respectively), but not TLR9 mRNA (10.85 times higher than the control, $P>0.05$). The inhibitory effect of WY14643 on TLR2 and TLR4 mRNA expression was significantly antagonized by MK886 (9.03; 9.18 times higher than the control, $P<0.05$, respectively). Furthermore, MK886 treatment alone resulted in significantly higher expression of TLR2 and TLR4 mRNA (23.28; 14.61 times higher than the control, $P<0.05$, respectively), but not TLR9 mRNA (10.37 times higher than the control, $P>0.05$).

TLR2 and TLR4 protein levels by western-blotting

Western-blotting was used to see whether pancreatic TLR2 and TLR4 protein levels correlated to changes in their mRNA levels (Figures 6A and 6B). Compared with the DMSO plus saline (control) group, TLR2 and TLR4 protein levels increased significantly following caerulein administration. Compared with the DMSO plus caerulein group, WY14643 treatment significantly decreased protein levels of TLR2 and TLR4. The inhibitory effect of WY14643 was completely blocked by the co-administration of MK886. Furthermore, MK886 treatment alone resulted in significantly higher protein levels of TLR2 and TLR4.

IL-6, TNF- α , IL-10, and ICAM-1 gene expression

Pancreatic gene expression of IL-6, TNF- α , IL-10, and ICAM-1 was also examined (Figures 7A, 7B, 7C and 7D). Compared with the DMSO plus saline (control) group, IL-6, TNF- α , IL-10, and ICAM-1 mRNA increased significantly following caerulein treatment (8.49; 13.83; 6.05; 14.70 times higher than the control, $P < 0.05$, respectively). Compared with the DMSO plus caerulein group, WY14643 treatment significantly decreased levels of IL-6, TNF- α and ICAM-1 mRNA (4.09; 5.05; 7.02 times higher than the control, $P < 0.05$, respectively), although the level of IL-10 mRNA was not decreased (6.76 times higher than the control, $P > 0.05$). The inhibitory effect of WY14643 on IL-6, TNF- α and ICAM-1 mRNA expression was significantly reversed by MK886 (7.07; 12.99; 6.70 times higher than the control, $P > 0.05$, respectively). Furthermore, MK886 treatment alone resulted in significantly higher expressions of IL-6, TNF- α and ICAM-1 mRNA (16.27; 20.94; 26.57 times higher than the control, $P < 0.05$, respectively), but not IL-10 (7.17 times higher than the control, $P > 0.05$).

Discussion

Peroxisome proliferator-activated receptor- α (PPAR- α) is one of the three subtypes of the nuclear receptor PPAR family. In rats, PPAR- α is most highly expressed in brown adipose tissue and in the pancreas¹⁴. Activation of PPAR- α , by either natural ligands, such as polyunsaturated fatty acids and eicosanoids, or synthetic ligands, such as fibrates and WY14643, stimulates target-gene transcription via the formation of heterodimeric transcription factor complexes with the retinoid X receptor¹⁵. PPAR- α has a wide range of effects on metabolism, cellular proliferation and the immune response¹⁶. A growing body of evidence suggests that PPAR- α activation induces anti-inflammatory effects in a

number of organs. Several studies have indicated that WY14643 inhibits the expression of many pro-inflammatory molecules in organs such as the heart and brain against ischemia/reperfusion injury^{17, 18}, although its role in acute pancreatitis (AP) is not fully understood. This study investigated the protective effects of PPAR- α activation by the selective agonist WY14643 on rat pancreatitis injury in the caerulein-induced model of AP. MK886 is a high-affinity and specific blocker for PPAR- α , inhibiting the conformational change or preventing other ligands from accessing the conformational-sensitive site of PPAR- α ¹⁹. In the present study, the potential of MK886 to attenuate these protective effects was also examined.

The results obtained revealed that treatment with WY14643 and/or MK886 had no effect on serum amylase, lipase, Ca^{2+} , pancreatic MPO activity, pathological scores and pancreatic physiological functions. Following caerulein administration, the levels of serum amylase, lipase, pathological scores and MPO activity significantly increased. In contrast, caerulein treatment resulted in significantly decreased serum Ca^{2+} level, indicative of the severe damage associated with high dose caerulein-induced pancreatitis. Pretreatment with WY14643 significantly decreased the levels of serum amylase, lipase, pathological injury and MPO activity measured 6 h after the induction of pancreatitis. A simultaneous increase in the level of serum Ca^{2+} was detected following administration of WY14643. Interestingly, this protective effect was significantly blocked by co-administration of WY14643 and MK886. Furthermore, MK886 alone resulted in profoundly increased inflammatory responses. Together, these data demonstrate that activation of PPAR- α by WY14643 attenuated caerulein-induced pancreatitis.

To further characterize the effects of activation of PPAR- α in AP, changes in

inflammatory mediators were also examined. The results showed that administration of WY14643 and/or MK886 had no effect on baseline levels of IL-6 and ICAM-1 while levels markedly increased following caerulein treatment. Pretreatment with WY14643 significantly inhibited the increase in levels of IL-6 and ICAM-1 associated with caerulein treatment although co-administration of WY14643 and MK886 significantly diminished the effects of WY14643. Furthermore, MK886 alone resulted in more profoundly increased levels of serum IL-6 and ICAM-1. These data indicate that PPAR- α activity is centrally involved in mediation of the effects of WY14643 and MK886 on AP and that PPAR- α activation attenuates the severity of the inflammatory responses responsible for pancreatic damage in AP. However, the mechanism by which this effect is mediated remains to be elucidated.

Over the past five years, the involvement of Toll-like receptors (TLRs) in the pathophysiology of AP has been recognized²⁰. Sharif et al. reported that deletion of TLR4 resulted in significantly less severe AP and associated lung injury assessed by hyperamylasemia, edema, MPO activity and pancreatic necrosis²¹. Therefore, it can be speculated that there is 'cross-talk' between these two counteractive pathways. The results revealed that treatment with WY14643 and/or MK886 had no effect on baseline levels of TLR2, TLR4 and TLR9 mRNA while levels significantly increased following caerulein treatment. The observed increase in TLR2 and TLR4 mRNA expression was markedly downregulated by WY14643 treatment, while this effect was abolished by co-administration of WY14643 (PPAR- α agonist) and MK886 (PPAR- α antagonist). Furthermore, MK886 alone resulted in profoundly increased expression of TLR2 and TLR4 mRNA. However, in contrast to these effects, TLR9 mRNA expression was not

affected by WY14643 and/or MK886. Subsequently, the protein levels of TLR2 and TLR4 were also measured. As expected, the protein levels of TLR2 and TLR4 correlated to changes in their mRNA levels. TLRs are widely expressed in pancreas and are profoundly involved in acute pancreatitis²⁰; PPAR, repressing transcriptional responses to inflammatory signaling and is an essential component of its homeostatic functions. In the present study, activation of PPAR- α markedly attenuated the inflammatory response, simultaneously decreased the expressions of TLR2 and TLR4 mRNA and proteins, together with the target molecules of TLR activation, revealed a protective role for PPAR- α in AP, partially by modulating TLR signaling pathways.

TNF- α , IL-6 and ICAM-1 are important pro-inflammatory mediators influenced by the TLR signaling pathways, whereas IL-10 plays an anti-inflammatory role. The results demonstrated that the expression of TNF- α , IL-6, ICAM-1 and IL-10 mRNA were markedly increased by caerulein administration. In rats treated with WY14643, pancreatic expression of TNF- α , IL-6 and ICAM-1 mRNA significantly decreased while co-administration of WY14643 and MK886 abolished the effects of the PPAR- α agonist. Furthermore, MK886 alone resulted in profoundly increased expression of TNF- α , IL-6 and ICAM-1 mRNA, suggesting that blockade of PPAR- α activation exacerbated the severity of pancreatitis. In contrast, IL-10 mRNA expression was not influenced by either the PPAR- α agonist or antagonist, although levels were increased following caerulein administration. These results indicate that the protective effects of PPAR- α are partially mediated by suppression of TNF- α , IL-6 and ICAM-1, but not by enhanced IL-10 expression.

In conclusion, this study clearly demonstrates that activation of PPAR- α

significantly reduces pancreatic injury in caerulein-induced pancreatitis. Furthermore, these protective effects are associated with the modulation of TLR signaling pathways, thus indicating the potential of regulation of the PPAR- α pathway as an approach to therapeutic intervention in acute pancreatitis.

Disclosure. The authors have no conflicts of interest to disclose.

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Table 1. Specific primers for the TLR2, TLR4, TLR9, IL-6, ICAM-1, TNF- α , IL-10 and β -actin genes.

Gene	Primer	Length(bp)
TLR2		
Forward	5'-GGTCTCCAGGTCAAATCTCA-3'	129
Reverse	5'-CACACCAGCAGCATCACA-3'	
TLR4		
Forward	5'-ATCTCAGCAAAATCCCTCAT-3'	123
Reverse	5'-AATCCAGCCACTGAAGTTGT-3'	
TLR9		
Forward	5'-ATGGTTCTCTGTAGCAGGAC-3'	113
Reverse	5'-TTCAGTTCACAGGGTAGGAA3-3'	
β -actin		
Forward	5'-CGTGAAAAGATGACCCAGAT-3'	158
Reverse	5'-ACCCTCATAGATGGGCACA-3'	
IL-6		
Forward	5'-AGTCACAGAAGGAGTGGCTAA-3'	100
Reverse	5'-CACTAGGTTTGCCGAGTAGA-3'	
ICAM-1		
Forward	5'-TGCTATATGGTCCTCACCTG-3'	123
Reverse	5'-GATCATGGTACAGCACTGTCA-3'	
TNF- α		
Forward	5'-AGACCCTCACACTCAGATCAT-3'	162
Reverse	5'-GTACCACCAGTTGGTTGTCTT-3'	
IL-10		
Forward	5'-TGCAGGACTTTAAGGGTTACT-3'	156
Reverse	5'-GCTGTATCCAGAGGGTCTTC-3'	

Figure Legends

Fig. 1 Serum amylase, lipase, and Ca^{2+} determination. Acute pancreatitis was induced by two subcutaneous injection of caerulein at a total dose of 20 $\mu\text{g/kg}$ given at 1-hour intervals. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1h and/or 30 min before the first caerulein injection. Serum was harvested 6h after the first injection of caerulein or saline.

A: serum amylase.

B: serum lipase.

C: serum Ca²⁺.

Results are means $\pm$ SE for eight animals per group; * $p < 0.05$ versus DMSO + sal (control) group, # $p < 0.05$ versus DMSO + cae group. sal, saline; cae, caerulein.

Fig. 2 Histological examination and pathological scores of pancreas. Acute pancreatitis was induced by two subcutaneous injection of caerulein at a total dose of 20 μ g/kg given at 1-hour intervals. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1h and/or 30 min before the first caerulein injection. Pancreas was harvested 6h after the first injection of caerulein or saline.

A: Representative hematoxylin/eosin-stained sections of pancreas (original magnification, $\times 200$).

A1: DMSO + sal group (control group);

A2: DMSO + cae group;

A3: WY14643 + cae group;

A4: MK886 + cae + WY14643 group;

A5: MK886 + cae group.

B. Pathological scores.

Results are means $\pm$ SE for eight animals per group; * $p < 0.05$ versus DMSO + sal (control) group, # $p < 0.05$ versus DMSO + cae group. sal, saline; cae, caerulein.

Fig. 3 Pancreatic MPO activity determination. Acute pancreatitis was induced by two subcutaneous injection of caerulein at a total dose of 20 $\mu\text{g/kg}$ given at 1-hour intervals. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1h and/or 30 min before the first caerulein injection. Pancreas was harvested 6h after the first injection of caerulein or saline. Results are means $\pm$ SE for eight animals per group; * $p < 0.05$ versus DMSO + sal (control) group, # $p < 0.05$ versus DMSO + cae group. sal, saline; cae, caerulein.

Fig.4 Serum IL-6 and ICAM-1 determination. Acute pancreatitis was induced by two subcutaneous injection of caerulein at a total dose of 20 $\mu\text{g/kg}$ given at 1-hour intervals. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1h and/or 30 min before the first caerulein injection. Serum was harvested 6h after the first injection of caerulein or saline.

A: serum IL-6 concentrations.

B: serum ICAM-1 concentrations.

Results are means $\pm$ SE for eight animals per group; * $p < 0.05$ versus DMSO + sal (control) group, # $p < 0.05$ versus DMSO + cae group. sal, saline; cae, caerulein.

Fig.5 Pancreatic levels of TLR2, TLR4 and TLR9 mRNA. Acute pancreatitis was induced by two subcutaneous injection of caerulein at a total dose of 20 $\mu\text{g/kg}$ given at 1-hour intervals. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1h and/or 30 min before the first caerulein injection. Pancreas was harvested 6h after the first injection of caerulein or saline.

A: TLR2 mRNA expression.

B: TLR4 mRNA expression.

C: TLR9 mRNA expression.

Results are means $\pm$ SE for eight animals per group; * $p < 0.05$ versus DMSO + sal (control) group, # $p < 0.05$ versus DMSO + cae group. sal, saline; cae, caerulein.

Fig.6 Pancreatic levels of TLR2 and TLR4 proteins. Acute pancreatitis was induced by two subcutaneous injection of caerulein at a total dose of 20 μ g/kg given at 1-hour intervals. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1 h and/or 30 min before the first caerulein injection. Pancreas was harvested 6h after the first injection of caerulein or saline.

A: TLR2 protein expression.

B: TLR4 protein expression.

1, DMSO + sal (control) group; 2, WY14643 + sal group; 3, MK886 + WY14643 + sal; 4, MK886 + sal group; 5, DMSO + cae group; 6, WY14643 + cae group; 7, MK886 + WY14643 + cae group; 8, MK886 + cae group. sal, saline; cae, caerulein.

Fig.7 Pancreatic levels of IL-6, TNF- α , IL-10 and ICAM-1 mRNA. Acute pancreatitis was induced by two subcutaneous injection of caerulein at a total dose of 20 μ g/kg given at 1-hour intervals. Rats were treated with WY14643 and/or MK886 (6 mg/kg, i.p.) 1h and/or 30 min before the first caerulein injection. Pancreas was harvested 6h after the first injection of caerulein or saline.

A: IL-6 mRNA expression.

B: TNF- α mRNA expression.

C: IL-10 mRNA expression.

D: ICAM-1mRNA expression.

Results are means $\pm$ SE for eight animals per group; * $p < 0.05$ versus DMSO + sal (control) group, # $p < 0.05$ versus DMSO + cae group. sal, saline; cae, caerulein.