HIF-1α-PPARγ-mTORC1 signalling pathway mediated autophagy induces inflammatory response of pancreatic cells in rats with hyperlipidemic acute pancreatitis

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Research Article

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Abstract

Objective: The incidence of hyperlipidemic acute pancreatitis (HLAP) has been increasing rapidly in recent years in China. Autophagy has been implicated in the inflammatory response of pancreatic cells in HLAP, but the molecular mechanisms remain unclear.

Methods: In this study, the role of the HIF-1α-PPARγ-mTORC1 pathway-mediated autophagy in the inflammatory response of pancreatic cells and the molecular mechanism are investigated in a rat model of HLAP using immunohistochemistry, ELISA, electron microscope and Western blot analysis.

Results: The results reveal that autophagy is significantly increased and pancreatic injury is exacerbated in HLAP rats, and the inflammatory response is further exacerbated by treatment with rapamycin but relieved by treatment with 3-MA. Hyperlipidemia induces up regulation of HIF-1α but down regulation of PPARγ, which in turn leads to an increase in autophagy and consequently exacerbation of inflammatory response of pancreatic cells.

Conclusions: It is concluded that the HIF-1α-PPARγ- mTORC1 pathway-mediated autophagy plays a critical role in the inflammatory response of pancreatic cells in HLAP, and interference with the HIF-1α-PPARγ-mTOR pathway can provide new targets for prevention and treatment of HLAP.

Introduction

In recent years, the incidence of hyperlipidemic acute pancreatitis (HLAP) has been increasing rapidly in China as a result of bad habits such as high fat diet, smoking, drinking and lack of exercise. For instance, the incidence was increased by approximately 2.6 times in Guangdong province in eastern China during the period 1990-2005, and it was even higher than that of acute biliary pancreatitis [1]. HLAP has unique clinical characteristics that distinguish it from other types of acute pancreatitis such as high severity, recurrence rate and possibility of progression to critical illness. It should also be noted that the mortality of HLAP is high as no effective treatment is currently available for this condition. As such, a better understanding of the pathogenesis of HLAP is needed in order to develop more effective therapeutic strategies.

It is found that excessive autophagy may lead to increased cell death and consequently impaired functioning of tissues and organs [2]. Autophagy was significantly increased in pancreatic acinar cells of rats with acute pancreatitis[3-5], and pancreatic injury and inflammatory response were exacerbated by treatment with autophagy inducer but relieved by knockdown of autophagy-related genes, suggesting that autophagy could be involved in the pathophysiological process of acute pancreatitis [4]. There is also evidence that autophagy is involved in the onset and progression of HLAP and the inflammatory response of pancreatic cells, but the molecular mechanisms remain unclear. In this study, a rat model of HLAP was are established to investigate the role and molecular mechanism of the HIF-1α-PPARγ-mTORC1 pathway-mediated autophagy in the inflammatory response of of pancreatic cells in HLAP.
Materials And Methods

Animals

This study was approved by the Animal Ethics Committee of Huishan District People's Hospital of Wuxi City, China. Male Sprague-Dawley (SD) rats were provided by Wuhan Cloud Clone Animal Co., LTD (Wuhan, China) and animal work was performed in our Hospital's Central Laboratory. The rats were randomized into six groups of eight rats each, including the control (C) group fed a normal diet for 4 weeks, acute pancreatitis (AP) group fed a normal diet for 4 weeks and then intraperitoneally injected with caerulein to induce AP, hyperlipidemia (HL) group fed a high-fat diet for 4 weeks, and HLAP group fed a high-fat diet for 4 weeks and then intraperitoneally injected with caerulein to induce AP. Thirty minutes before induction of AP, 1.5 mg/kg rapamycin (an inducer of autophagy) or 2 mg/kg 3-MA (an blockers of autophagy) was intraperitoneally injected in the HLAP group, yielding HLAP A1 and HLAP AB groups. All rats were euthanized with sodium pentobarbital (dose > 50 mg/kg) and blood samples were collected from the abdominal aorta, and serum was separated and pancreatic tissues were obtained.

Cell culture and treatment

Pancreatic AR42J cells were thawed and resuspended in culture medium. Cells were incubated as a semi-adherent culture in RPMI 1640 medium supplemented with 0.1 mol/L palmitic acid for 3 h at 37°C in a 5% CO2 atmosphere. After that, caerulein was added to the medium to a final concentration of 10 nmol/L and mixed uniformly with the medium to induce HLAP.

mTORC1 inhibition and cell transfection

Rapamycin was dissolved in 140 uL of DMSO to a concentration of 2 mol/L, which was diluted to a final concentration of 200 uM with serum-free RPMI640 immediately before use. The AR42J cells treated with caerulein were seeded in a six-well plate, and when 95% confluence was reached, rapamycin, a specific inhibitor of the target of rapamycin complex 1 (mTORC1), was added to the medium and cells were cultured for another 6 h, 12 h and 24 h, respectively.

siHIF-1α and siPPARγ were constructed and verified by restriction mapping and sequencing. AR42J cells were transiently transfected with plasmids containing HIF-1α-siRNA and PPARγ-siRNA using Lipofectamine 2000 according to the manufacturer’s instructions.

ELISA analysis

The levels of Triglyceride (TG), Total cholesterol (TC), Amylase (AMY), Lipase (LPS), TNF-α and IL-6 in serum and supernatant were determined by ELISA according to the manufacturer’s instructions.

Morphology analysis

A small piece of pancreatic tissues were fixed in 4% paraformaldehyde, dehydrated in graded ethanol, cleared in dimethylbenzene, and then embedded in paraffin. After that, samples were sliced into sections,
which were dewaxed, stained with hematoxylin for 10-30 min and then counterstained with 0.5% eosin in alcohol for observation of pathological changes of pancreatic cells under a microscope and determination of pathological scores of pancreatic injury. The pathological scores criteria: 0 points: normal histological morphology. 1 points: slight pathological changes of pancreatic tissue. 2 points: focal acinar tissue necrosis, interstitial hyperemia and inflammatory cell infiltration. 3 points: medium area of acinar tissue necrosis, pancreatic tissue was replaced by adipose tissue, interstitial hyperemia and inflammatory cell infiltration. 4 points: pancreatic tissue was severely damaged with acinar tissue coagulative necrosis.

Ultrastructure analysis

Pancreatic tissue and AR42J cell suspension was fixed with 2.5% glutaraldehyde for 24 h, washed three times with 2% sodium cacodylate buffer, fixed again with 1% osmic acid for 2 h, washed three times with double distilled water, dehydrated in graded ethanol, infiltrated in an acetone /EPON-812 mixture (1: 1) for 2 h, embedded at 35℃ for 24 h and 45℃ for 24 h, and polymerized at 68℃ for 24 h. ultrathin sections were double stained with lead citrate and uranyl acetate and observed under a transmission electron microscope.

Immunohistochemistry analysis

The expressions of Beclin-1, LC3, HIF-1α and PPARγ in pancreatic tissues were examined by immunohistochemistry. Paraffin-embedded sections of pancreatic tissues underwent deparaffinization, hydration, antigen retrieval and blocking. The primary antibody(Abcam) was added dropwise and incubated in a wet chamber at room temperature for 1 h. After washing with PBS, the secondary antibody (UltraPolymer Goat anti-Mouse IgG-HRP, Proteintech Group, Inc) was added dropwise and incubated in a wet chamber at room temperature for 20~30 min. After that, the sections were washed with PBS, developed with DAB, counterstained with hematoxylin, dehydrated with alcohol, washed and then dried. Subsequently, they were cleared in xylene, blocked with neutral gum, dried and photographed under a microscope. A semiquantitative analysis was performed using Image-pro plus 6.0.

Western blot analysis

The protein levels of mTORC1, Beclin-1, LC3, HIF-1α and PPARγ in AR42J cells were assessed by Western blot. Proteins were loaded at a concentration of 10-20 μg per well, separated on a 8% SDS-polyacrylamide gel, transferred to the membrane, and incubated with 5% defatted milk powder at room temperature for 1 h. The membrane was washed with TBST and then incubated with the primary antibody(Abcam) at 4℃ overnight, washed again with TBST, and then incubated with the secondary antibody(Abcam) at 37℃ for 2 h. Protein bands were visualized by ECL and photographed using the Gel imager system. The images were analyzed using the ImageJ software.

Statistical analysis
Data were expressed as mean ± SD and all statistical analyses were performed using SPSS 19.0 software. A comparison between different groups was carried out using one-way analysis of variance followed by least significant difference (LSD) test for post hoc comparisons. \( P < 0.05 \) was considered statistically significant.

**Results**

**Inflammatory response of pancreatic tissues in HLAP**

The levels of TG, TC, AMY, LPS, TNF-\( \alpha \) and IL-6 in the serum were determined by ELISA, as shown in Figure 1A. It is found that the TG, TC, AMY, LPS, IL-6 and TNF-\( \alpha \) levels are significantly increased in HLAP groups compared with the other groups, the rat model of HLAP has been successfully established, and all of them are further increased with rapamycin treatment but decreased with 3-MA treatment.

**Pathological changes of pancreatic tissues in HLAP**

As shown in Figure 1B, focal pancreatic necrosis occurs in rats with HLAP concurrently with fatty degeneration and infiltration of inflammatory cells. However, the pathological changes of pancreatic tissue become more remarkable in the HLAP AI group, while in the HLAP AB group, pancreatic acinar cells have a normal morphology. It is also seen that the pathological score is higher in the HLAP group than in the AP group (Figure 1C).

Figure 1D shows that in the HLAP group, chromatin is also slightly condensed with some lipid droplets but more autophagy-related structures, in the HLAP AI group, chromatin condensation and margination that are are characteristic of apoptosis are clearly observed, while in the HLAP AB group, chromatin is slightly condensed with some lipid droplets, and autophagy-related structures are occasionally seen. It is concluded that more pronounced cellular damage occurs in rats with HLAP compared to rats with AP. Induction of autophagy by rapamycin leads to exacerbation of cellular damage and even occurrence of apoptosis, while inhibition of autophagy by 3-MA contributes to relieving cellular damage.

**Expressions of Beclin-1, LC3-II, HIF-1\( \alpha \), PPAR\( \gamma \) in pancreatic tissues**

Figure 2A, 2B reveals that compared with the C group, the expressions of Beclin-1 and LC3 are up-regulated in AP, HL and HLAP groups, the expressions were highest in HLAP groups. It is also noted that treatment with rapamycin leads to further up-regulation of Beclin-1, LC3, while treatment with 3-MA leads to further down-regulation. The expression of HIF-1\( \alpha \) is up-regulated, while that of PPAR\( \gamma \) is down regulated in AP, HL and HLAP groups, the HLAP group showed the most significant changes (Figure 2C, 2D).

**Changes of mTORC1 inhibition for pancreatic cells in HLAP**

The changes of rapamycin-induced inhibition of mTORC1 on the TG, TC, AMY, LPS, IL-6 and TNF-\( \alpha \) levels in the supernatant are shown in Figure 3A. It is found that compared with the C group, the levels of TG,
TC, AMY, LPS, IL-6 and TNF-α are higher in the HLAP group but even higher in the HLAP anti-mTORC1 group at each time point. The results reveal that inhibition of mTORC1 by rapamycin can exacerbate the inflammatory response and pancreatic injury.

Figure 3B reveals that at 6 h, cells in the HLAP group are irregularly shaped and a small number of apoptotic cells are observed, while chromatin is condensed into compact masses and autophagy-related structures are also observed in the HLAP anti-mTORC1 group. At 12 h, the nuclei in the HLAP group are irregularly shaped with lipid droplets and swelling of some mitochondria, but no apoptotic cells are observed, while in the HLAP anti-mTORC1 group, chromatin condensation and margination that are characteristic ultrastructural attributes of apoptosis are observed. At 24 h, cells are swollen in the HLAP group with high chromatin condensation and apparent autophagy-related structures, while in the HLAP anti-mTORC1 group, chromatin is highly condensed with the presence of apoptotic cells and bodies but no autophagy-related structures. It is clear that as the time increases, the autophagy and cellular damage become more evident in HLAP rats, which however are further exacerbated with the inhibition of mTORC1 by rapamycin.

Figure 3C,3D reveals that compared with the HLAP group, the expression of Beclin-1 and LC3 is significantly increased in the HLAP anti-mTORC1 group, indicating that inhibition of mTORC1 by rapamycin can up regulate the expression of autophagy-related proteins.

**Changes of HIF-1α and PPARγ knockdown for pancreatic cells in HLAP**

AR42J cells were transiently transfected with plasmids containing HIF-1α-siRNA and PPARγ-siRNA. It is seen in Figure 4A that the levels of TG, TC, AMY, LPS, IL-6 and TNF-α in the HLAP group are significantly higher than those in the control group, which in turn are reduced by transfection with HIF-1α-siRNA but increased by transfection with PPARγ-siRNA. Thus, the inflammatory response of AR42J cells would be relieved by HIF-1α interference but further exacerbated by PPARγ interference.

Figure 4B reveals that compared with the 0h groups, at 6 h, most cells in the HLAP group are swollen with a small number of autophagy-related structures and chromatin margination, cells in the HLAP siHIF-1α group are swollen with a small number of autophagy-related structures but no apoptotic cells, while cells in the HLAP siPPARγ group are irregularly shaped with more lipid droplets, and chromatin margination and autophagy-related structures are occasionally seen. At 12 h, cells in the HLAP group are irregularly shaped with chromatin condensation, margination and autophagy-related structures, cells in the HLAP siHIF-1α group remain largely unchanged with a few autophagy-related structures but no apoptotic cells, while cells in the HLAP siPPARγ show slight chromatin condensation with more autophagy-related structures but no apoptotic cells. At 24 h, cells in the HLAP group are irregularly shaped with chromatin condensation and margination, a small number of lipid droplets and obvious mitochondrial swelling, cells in the HLAP siHIF-1α group are swollen with incomplete cellular structures but no autophagy-related structures and apoptotic cells, while cells in the HLAP siPPARγ are irregularly shaped with chromatin condensation and margination and only a small number of autophagy-related structures. It can be
concluded that autophagy and cellular damage of AR42J cells are obviously exacerbated in HLAP rats, which however could be relieved by HIF-1α interference but further exacerbated by PPARγ interference.

Figure 4C,4D reveals that compared with the control group, the mTORC1 activity is decreased with up regulation of HIF-1α and down regulation of PPARγ in the HLAP group, and accordingly the levels of both Beclin-1 and LC3 are increased. Transfection with HIF-1α-siRNA leads to up regulation of PPARγ and thus an increase in mTORC1 activity and a decrease in Beclin-1 and LC3 levels, indicating that HIF-1α interference can reduce the autophagy of pancreatic acinar cells by activating mTORC1 via up regulation of PPARγ. However, transfection with PPARγ-siRNA leads to an obvious reduction in mTORC1 activity and a significant increase in Beclin-1 and LC3 levels, indicating that PPARγ interference can increase the autophagy of pancreatic acinar cells by inhibiting the activity of mTORC1.

Discussion

This study reveals that the inflammatory response and cellular damage of pancreatic tissues are more evident in HLAP rats than in AP rats because of the presence of hyperlipidemia. It is known that excessive autophagy may lead to massive degradation of intracellular proteins and organelles and subsequent apoptotic cell death[6], and the increased formation of autophagosomes in AP leads to activation of trypsinogen in pancreatic acinar cells and subsequent progression of AP[7]. There is mounting evidence that excessive autophagy mediated by different signaling pathways plays a crucial role in the onset and progression of AP, which may provide potential targets for the treatment of AP [8-13]. Nevertheless, little is known about the role and molecular mechanism of autophagy in the pathogenesis of HLAP. It is found that lipid metabolism disorder may activate autophagy via the AMPK-mTOR pathway[14], and autophagy is involved in activation of trypsinogen and disruption of calcium homeostasis in pancreatic acinar cells, which ultimately leads to apoptotic death of pancreatic acinar cells. It is found that autophagy is activated in HLAP that can have an effect on the removal of metabolic waste products from the pancreas and the local inflammatory necrosis of pancreatic tissues[15]. This study has revealed that the increased autophagy in HLAP can exacerbate the inflammatory response of pancreatic cells, which however is further exacerbated with the induction of autophagy by rapamycin but relieved with the inhibition of autophagy by 3-MA. Autophagy promote or protect against the inflammatory pathogenesis of pancreatitis depending on the level of autophagy, excessive autophagy -induced inflammatory response is an important pathogenesis mechanism of HLAP.

Multiple signaling pathways have been implicated in the regulation of autophagy. The mTOR pathway plays an important role in the up-regulation of autophagy under ischemic/hypoxic conditions [16], which are mainly associated with PI3K /Akt, HIF-1α and ROS. Autophagy is regulated by the mTOR pathway in AP with pancreatic microcirculation disturbance and hypoxia/ischemia of pancreatic tissues. HIF-1α is a transcription factor activated under hypoxic conditions[17], and the activation of downstream target genes would be regulated by hypoxia/ischemia-induced expression of HIF-1α. In various cell types, hypoxia induces autophagy in a HIF-1α-dependent fashion[18]. Hyperlipidemia leads to accumulation of free fatty acids and local microcirculation disturbance and hypoxia/ischemia, which in turn can up
regulate the expression of HIF-1α, increase microvascular permeability and consequently lead to microvascular pathological changes[19]. In a mouse model of AP, the HIF-1α level was significantly increased in pancreatic tissues, indicating that HIF-1α could promote inflammatory response in the onset and progression of AP[20]. The peroxisome proliferators-activated receptor γ (PPARγ) is mainly expressed in liver and adipose tissues and it plays a role in promoting the differentiation and maturation of adipocytes and regulating the activation of signaling pathways responsible for lipid metabolism and the expression of related genes [21]. It is believed that hyperlipidemia can exacerbate the inflammatory response and pathological injury of AP, the mechanisms of which may be related to the down regulation of PPARγ mRNA expression[22, 23]. For this reason, treatment with PPARγ agonist can relieve the inflammatory response of pancreatic tissues in HLAP. Both PPARγ and HIF-1α are implicated in the regulation of lipid metabolism, and the HIF-1α/ PPARγ pathway plays a critical role in hepatocyte damage in fatty liver [24, 25]. Under simulated hypoxic conditions, HIF-1α can directly regulate the expression of PPARγ[26, 27]. It is also reported that mTORC1 is a downstream target of PPARγ, and thus the mTOR-dependent autophagy pathway can be regulated by regulating PPARγ gene transcription and expression. PPARγ deficiency results in severe, accelerated osteoarthritis associated with aberrant mTOR signaling in the articular cartilage[28], and telmisartan-induced PPARγ activity attenuates lipid accumulation in vascular smooth muscle lipid cells via induction of autophagy[29]. This study reveals that the mTORC1 activity is decreased with up regulation of HIF-1α and down regulation of PPARγ in HLAP rats. Transfection with HIF-1α-siRNA leads to increased mTORC1 activity, thus causing a decrease in autophagy and consequently relief of inflammatory response, while transfection with PPARγ-siRNA leads to decreased mTORC1 activity, thus causing an increase in autophagy and consequently exacerbation of inflammatory injury. It is evident that hyperlipidemia up regulates HIF-1α but down regulates PPARγ, which in turn decrease mTORC1 activity and then increase the autophagy and consequently the inflammatory response of pancreatic cells. Thus, the HIF-1α-PPARγ- mTOR pathway-mediated autophagy plays an important regulation role in the inflammatory response of pancreatic cells in HLAP.

This study makes a first attempt to investigate the molecular mechanism of the HIF-1α- PPARγ- mTOR pathway-mediated autophagy in the pathogenesis of HLAP. It is concluded that hyperlipidemia induces up regulation of HIF-1α but down regulation of PPARγ, which in turn leads to an increase in autophagy and consequently exacerbation of inflammatory response of pancreatic cells. Interference with the HIF-1α-PPARγ-mTOR pathway can provide new targets for prevention and treatment of HLAP. Clinical studies are needed to further evaluate the value of this study in prevention and therapy for HLAP.

**Abbreviations**

HLAP, Hyperlipidemic acute pancreatitis; HIF-1α, Hypoxia-inducible factor 1-alpha; mTORC1, Mammalian Target of Rapamycin Complex 1; PPARγ, Peroxisome proliferator-activated receptor-γ; Beclin-1, myosin-like BCL-2 interacting protein; LC3, Microtubule-associated protein 1 light chain 3; siRNA, Small Interfering Ribonucleic Acid; 3-MA, 3-methyladenine; TG, triglyceride; TC, Total cholesterol; AMY, amylase; TNF-α, Tumor necrosis factor-α; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; ROS, reactive oxygen species; ATG, Autophagy related gene.
Declarations

AUTHOR CONTRIBUTIONS

MYM and LXL contributed to the conception and design of the study, acquired the majority of the data, and drafted the manuscript. MYC contributed to the conception and design of the study and acquired some of the data. LZL and XXH contributed to the design of the study and interpretation of the data. WYP contributed to the design of the study, interpretation of data, and substantively revised the manuscript. All the authors reviewed and approved the final draft of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

The dataset supporting the conclusions of this article are included within the article.

Ethical Approval and Consent to Participate This study was conducted in accordance with the guidelines of the Declaration of Helsinki. All animal experimental protocols applied in this study were conducted in accordance with the standards of the Animal Ethics Committee of Huishan District People's Hospital of Wuxi City, China.

Competing Interests The authors declare no competing interests.

References


Figure 1

Inflammatory response and pathological changes of pancreatic tissues in HLAP

(A) Levels of TG, TC, AMY, LPS, IL-6 and TNF-α. (B) The morphology changes (200×). (C) The pathological scores. (D) The ultrastructure and autophagy changes (30000×). C: control; AP: acute pancreatitis; HL: hyperlipidemia; HLAP: hyperlipidemic acute pancreatitis; HLAP+AI: HLAP plus rapamycin treatment; HLAP+AB: HLAP plus 3-MA. Data were expressed as mean ± SD (N=8). *: vs. C, p < 0.05, △: vs. HLAP, p < 0.05. Note: (B) Blue arrows indicate islet cells oedema, Yellow arrows indicate inflammatory cell infiltration, red arrows indicate red cell infiltration and black arrows indicate necrotic tissue was replaced by adipose tissue.
Figure 2

Expressions of Beclin-1, LC3-II, HIF-1α, PPARγ in pancreatic tissues (400×). (A) Beclin-1 expressions. (B) LC3 expressions. (C) HIF-1α expressions. (D) PPARγ expressions. (E) Protein expressions. Data were expressed as mean ± SD (N=3). *: vs. C, p < 0.05, △: vs. HLAP, p < 0.05.
Figure 3

Changes of mTORC1 inhibition for pancreatic cells in HLAP.

(A) Levels of TG, TC, AMY, LPS, IL-6 and TNF-α. (B) The ultrastructure and autophagy changes (30000x). (C) Beclin-1, LC3 and mTORC1 expressions. (D) Protein expressions. C: control; HLAP: hyperlipidemic
acute pancreatitis; HLAP anti-mTORC1: HLAP specific inhibitor of mTORC1. Data were expressed as mean ± SD (N = 3). *: vs. C, \( p < 0.05 \); △: vs. HLAP, \( p < 0.05 \).

Figure 4

Changes of HIF-1α and PPARγ knockdown for pancreatic cells in HLAP.
(A). Levels of TG, TC, AMY, LPS, IL-6 and TNF-α. (B) The ultrastructure and autophagy changes (30000×). (C) Beclin-1, LC3, mTORC1, HIF-1α and PPARγ expressions. (D) Protein expressions. C: control; HLAP: hyperlipidemic acute pancreatitis; HLAP siHIF-1α: HLAP small interfering RNA for siHIF-1α; HLAP si PPARγ: HLAP small interfering RNA for si PPARγ. Data were expressed as mean ± SD (N = 3). *: vs. C, p < 0.05; △: vs. HLAP, p < 0.05.