

Research Article

Guoliang Wang, Yan Liu, Danhua Dui*, Liang Bai, Yao Liu, Fei Tian, Wei Wei

Trypsinogen activation peptide induces HMGB1 release from rat pancreatic acinar cells

<https://doi.org/10.1515/med-2017-0043>

received June 11, 2017; accepted August 3, 2017

Keywords: Pancreatic acinar cell; Trypsinogen activation peptide; High mobility group box-1; Ethyl pyruvate; Rat

Abstract: Background: The development of acute pancreatitis (AP) is associated with intracellular events in pancreatic cells, as well as with early and late inflammatory responses; however, their underlying mechanisms remain unclear. This study investigated trypsinogen activation peptide (TAP)-induced release of high mobility group box-1 (HMGB1) from pancreatic acinar cells and how ethyl pyruvate (EP) affects this release.

Methodology: Pancreatic acinar cells from Sprague Dawley rats were divided into control, TAP (administered TAP), and EP (administered TAP and EP) groups. Cells were collected at 3, 6, 12, and 24 hours after TAP administration to detect HMGB1 mRNA and protein levels using quantitative PCR (qPCR) and Western blotting, respectively.

Results: The TAP and EP groups exhibited higher levels of HMGB1 mRNA and protein expression ($P < 0.05$) than the control group. The HMGB1 mRNA and protein expression levels also increased with prolonged TAP activity ($P < 0.05$)—especially at 12 and 24 hours ($P < 0.01$)—and showed positive correlations with TAP activity duration (3, 6, 12, and 24 hours) ($r = 0.971$, $P < 0.01$; $r = 0.966$, $P < 0.01$, respectively).

Conclusion: TAP induces HMGB1 release from pancreatic acinar cells. A positive temporal link exists between early TAP activity and late HMGB1 expression in AP, and EP inhibits HMGB1 release.

1 Introduction

The pathophysiological mechanism of acute pancreatitis (AP) is highly complex. Recent studies have shown that this mechanism is closely correlated with initial intracellular events in pancreatic cells, early inflammatory reactions, and late inflammatory responses [1]. The initial intracellular events in pancreatic cells primarily refer to abnormal secretion and activation of trypsinogen; these changes initiate signaling cascades that facilitate the development of pancreatitis, which can then progress to severe pancreatitis, systematic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndrome (MODS).

As pancreatitis progresses, trypsinogen is activated and generates trypsinogen activation peptide (TAP). TAP causes the self-digestion and necrosis of pancreatic acinar cells, which in turn induce the release of various inflammatory mediators—a significant feature in AP [2]. Some studies have shown that TAP is critical to the pathological mechanism of AP and that mild and severe pancreatitis can be distinguished using TAP concentrations in blood, urine, and ascites [3,4]. During AP, the levels of early inflammatory mediators such as TNF- α and IL-1 peak soon after their release and then decline rapidly as the inflammatory response progresses. Despite this observed decrease, clinical application of either TNF- α or IL-1 receptor antagonists did not ameliorate disease progression, which indicates that other late-stage inflammatory mediators are likely involved in the pathology of AP [5]. High mobility group box-1 (HMGB1), a late inflammatory factor, is highly stable under normal conditions and does not exhibit strong biological activity. However, once secreted, HMGB1 rapidly exerts its activity and causes the subsequent release of large quantities of the protein, thus resulting in a diffuse inflammatory response [6]. Studies have observed that the serum HMGB1 levels in rats with severe

*Corresponding author: Danhua Dui, Department of Hepatopancreatobiliary Surgery, Affiliated Hospital of Zunyi Medical College, Zunyi 563003, China, Tel: 86-0852-8608573; Email: yuedh_cn@163.com

Guoliang Wang, Yan Liu, Department of Hepatobiliaropancreatic Surgery, Guizhou Provincial People's Hospital, Guiyang, 550002, China

Liang Bai, Yao Liu, Fei Tian, Wei Wei, China Department of Hepatobiliaropancreatic Surgery, Affiliated Hospital of Zunyi Medical College, Zunyi 563003, China

AP (SAP) increase significantly at 12 h after disease initiation and remain elevated at 24 h and 48 h after disease initiation [7-9]. These results suggest that HMGB1 is a widespread late-stage inflammatory mediator. However, the relationship between TAP (an important early predictor of AP severity) and HMGB1 (a late inflammatory mediator) has not been clearly elucidated to date.

This study investigated the TAP-induced release of HMGB1 from rat pancreatic acinar cells and the effects of ethyl pyruvate (EP), an HMGB1 inhibitor, on this release, with the goal of identifying new treatment strategies for AP.

2 Materials and methods

2.1 Animals

Twelve male and female adult Sprague Dawley (SD) rats (8–10 weeks old, 200–250 g) were provided by the Third Military Medical University of Chongqing (certificate number Scxk (Chongqing) 2007-0005). The animals were administered chow away from light and maintained at 18–26 °C in the animal laboratory.

All surgical procedures performed were approved by the Institute of Animal Ethics at Zunyi Medical College (approval no. 2010032).

2.2 Cell experiments

The rats were euthanized by cervical dislocation and then soaked in 75% ethanol for 30 min. Pancreatic tissues were removed under sterile conditions and then rinsed three times with 100 U/ml penicillin-streptomycin, after which the tissues were washed three times with serum-free F12-K medium. The extra tissues were removed, and the pancreatic tissues were cut into approximately 1 mm³ pieces. These pieces were transferred into a polypropylene culture bottle until the remaining rinsing fluid was absorbed, after which 10 ml of collagenase II was added to promote cellular digestion and isolation. The bottles were sealed with a cap and incubated in a 37 °C water bath. The samples were then oscillated at 120 r/min, and the bottle was removed when the solution became turbid. The turbid liquid was removed, and 20 ml of fresh digestive solution was added. The samples were treated with the above-men-

tioned method and then oscillated at 120 r/min for 30 min until most of the pancreatic tissues were digested. The bottle was left undisturbed until large tissue pieces sank; the upper cell suspension was then extracted and filtered through a 200-mesh screen into F12-K medium, after which the digestion was terminated. The filtered samples were centrifuged at 1000 r/min for 5 min, the upper culture solution was removed, and the samples were re-suspended in fresh culture solution. Cell suspensions were centrifuged (800 r/min, $r=16$ cm, 5 min) to purify the acinar cells, and cell morphology was observed under an inverted microscope at different time points (5 min, 3 h, 24 h, and 5 d). Using the lot method, we randomly divided the rats into 3 groups: control, TAP, and EP groups. Each of these groups was further divided into the following 4 subgroups: 3 h, 6 h, 12 h, and 24 h. A prepared TAP solution (1000 nmol/L) was added to the cell culture medium of the TAP and EP groups to a final concentration of 3 nmol/L, as previously described [6]. Rats in the EP group were administered an additional EP solution (56 mmol/L) to a final concentration of 28 mmol/L. After the treatments were administered, pancreatic acini samples were collected at 3 h, 6 h, 12 h, and 24 h (corresponding to the subgroups), and the HMGB1 mRNA and protein expression levels were measured in the collected cells.

2.3 Detection of HMGB1 mRNA expression in rat pancreatic acinar cells

Total mRNA was extracted from pancreatic acinar cells using a TaKaRa RNAiso reagent kit (BioTechnology (Dalian) Co., Ltd.) according to the manufacturer's instructions, and cDNA was synthesized using a PrimeScript[®] 1st Strand cDNA synthesis kit (BioTechnology (Dalian) Co., Ltd.). Afterward, quantitative PCR (qPCR) was performed using a SYBR[®] Premix Ex Taq[™] kit (BioTechnology (Dalian) Co., Ltd.) based on the manufacturer's guidelines using the following primers to amplify HMGB1: 5'-TGTCACACACCCTGCATATT-3' (upstream) and 5'-GAATCCCATGGTGTGACAGAATTGA-3' (downstream). Primers targeting β -actin were used as an internal reference. Reverse transcription was performed under the following reaction conditions: 37 °C for 15 min followed by 85 °C for 5 sec and 4 °C indefinitely for sample preservation. The relative expression of HMGB1 mRNA was defined as the expression of HMGB1 divided by the expression of β -actin; it was calculated in each sample using the 2^{- $\Delta\Delta$ CT} method based on the obtained Ct value.

2.4 Detection of HMGB1 protein biosynthesis in rat pancreatic acinar cells

HMGB1 protein expression in pancreatic acinar cells was semi-quantitatively analyzed using Western blotting. Total protein was extracted from lysed cells from the culture suspension and quantified using the bicinchoninic acid (BCA) method. Equal amounts of protein from each sample were then mixed with 5× sodium dodecyl sulfate (SDS) loading buffer (containing 2 ml of 0.5 mol/L Tris-HCl (pH=6.8), 2 ml of glycerol, 2 ml of 20% SDS, 0.5 ml of 0.1% bromophenol blue, 1 ml of β-mercaptoethanol, and 2.5 ml of ddH₂O) at a 4:1 ratio and placed into boiling water for 5 min to denature the proteins. These protein samples were separated using electrophoresis (80 V when the sample was in stacking gel and 120 V when the sample was in the separation gel; electrophoresis was terminated when the bromophenol blue stain reached the bottom edge of the separation gel) and then transferred to polyvinylidene fluoride (PVDF) membranes (220 mA for approximately 0.1 h). The membranes were blocked overnight with a blocking solution and then incubated with primary antibody (goat anti-HMG-1 (K-12): sc-26351; Santa Cruz Co., USA; 1:200 dilution) for 2 hours. Afterward, the membranes were washed 3 times with Tris-buffered saline containing Tween 20 (TBST) and incubated with secondary antibody (horseradish peroxidase-conjugated rabbit anti-goat IgG (H+L); Beijing Zhongsheng Jinqiao Biotechnology Co., Ltd.; 1:10,000 dilution) for 2 hours. After the membranes were washed 3 more times with TBST, they were subjected to chemiluminescence and exposed onto film. The developed film was fixed, scanned, and analyzed for grayscale values using a LabWorks 4.6 UVP Gel Image Processing System (SYNGENE, Cambridge, UK). Relative HMGB1 protein expression was defined as the HMGB1 grayscale value divided by the β-actin grayscale value. The films were read by senior laboratory technicians from the Central Laboratory of Zunyi Medical College who were blinded to the treatment conditions.

2.5 Assessment of the purity and viability of pancreatic acinar cells

The number of pancreatic acinar cells per milliliter of culture broth was counted, and the purity was determined using a cell counting plate under an inverted microscope (cell purity=number of pancreatic acinar cells/number of total cells). Cell viability was assessed using Trypan blue staining [10].

2.6 Statistical analysis

Data were processed using the SPSS 17.0 statistical software package, and categorical data were expressed as the mean ± standard deviation ($\bar{x} \pm s$). The relationship between duration of TAP treatment and HMGB1 expression was assessed using Spearman rank correlation analysis and repeated measures analysis of variance. A positive r value indicates a positive correlation with $0.7 \leq r < 1$ reflecting a high degree of correlation, $0.4 \leq r < 0.7$ reflecting a moderate degree of correlation, and $r < 0.4$ reflecting a low degree of correlation. The significance level was set at $\alpha = 0.05$.

3 Results

3.1 Relative HMGB1 mRNA expression in pancreatic acinar cells

The relative mRNA expression of HMGB1 was higher in the pancreatic acinar cell lysates from rats exposed to 3 nmol/L TAP for 3 h ($P < 0.05$) than in those from the control rats (Table 1). Furthermore, this expression gradually increased with extended TAP activity, especially at 12 h and 24 h ($P < 0.01$). Compared with the control group, the TAP group exhibited increases in the relative HMGB1 mRNA expression in pancreatic acinar cells by 1.3- ($P < 0.05$), 5.5- ($P < 0.01$), 81.2- ($P < 0.01$), and 87.7-fold ($P < 0.01$) at 3 h, 6 h, 12 h, and 24 h after TAP administration, respectively. Compared with the TAP group, the EP group showed decreases in the relative HMGB1 mRNA expression in pancreatic acinar cells by 0.7- ($P < 0.05$), 3.6- ($P < 0.01$), 40- ($P < 0.01$), and 25-fold ($P < 0.01$) at 3 h, 6 h, 12 h, and 24 h after TAP administration, respectively. The Spearman rank correlation analysis indicated that HMGB1 mRNA expression in pancreatic acinar cells was positively correlated with duration of TAP action ($r = 0.971$, $P < 0.01$).

3.2 Relative HMGB1 protein expression in pancreatic acinar cells

The relative levels of HMGB1 protein expression in the rat pancreatic acinar cell lysates were increased in rats exposed to 3 nmol/L TAP for 24 h ($P < 0.01$) compared with rats in the control group, whereas the relative HMGB1 protein expression in the EP group showed no significant change compared to that in the control group at 24 h ($P = 0.099$, Table 2 and Figure 1). Compared with the TAP

Table 1: Relative mRNA expression levels of HMGB1 in pancreatic acinar cells from the different rat groups at various time points ($\bar{X} \pm s$, $n=4$ per treatment group).

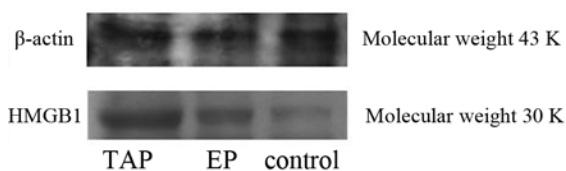
Group	Sampling time			
	3 h	6 h	12 h	24 h
Control group	1.2651±0.1066	1.2651±0.0693	1.2267±0.118	1.2718±0.1062
TAP group	2.8628±0.1132*	8.2286±0.1468**	100.8388±8.869**	112.844 ±12.4390**
EP group	1.7144±0.2752△	1.7710±0.2331△△	2.4363±0.2884**△△	4.304 5±0.0925**△△

* $P<0.05$, ** $P<0.01$ compared with the control group; △ $P<0.05$, △△ $P<0.01$ compared with the TAP group.

Table 2: Relative protein expression levels of HMGB1 in pancreatic acinar cells from the different rat groups at various time points ($\bar{X} \pm s$, $n=4$ per treatment group)

Group	Sampling time			
	3 h	6 h	12 h	24 h
Control group	—	—	—	0.352±0.088
TAP group	0.435±0.132	0.748±0.129	1.675±0.385	2.715±0.380*
EP group	—	—	0.573±0.356△	0.738±0.134△

* $P<0.01$ compared with the control group; △ $P<0.01$ compared with the TAP group.

**Figure 1:** HMGB1 protein expression in pancreatic acinar cells at 24 h after TAP exposure.

group, the EP group exhibited lower levels of HMGB1 protein at 12 h and 24 h ($P<0.01$). This result indicates that TAP induces HMGB1 protein biosynthesis in pancreatic acinar cells in rats, whereas EP inhibits this induction to some extent. The Spearman rank correlation analysis showed that the HMGB1 protein levels in pancreatic acinar cells were positively correlated with the duration of TAP action ($r=0.966$, $P<0.01$).

3.3 Purity and viability of pancreatic acinar cells

Pancreatic acinar cells were observed under an inverted microscope after purification. The cells were either oval or irregular in shape, with a rounded nucleus that was not centered within the cell (e.g., biased toward one side). Zymogen granules were evident in the cytoplasm. Both individual acinar cells and cell clumps were observed (Figure 2A). The cell count was 10^6 – 10^8 cells/ml with a purity of 95%. Pancreatic acinar cell viability was assessed using Trypan blue staining. No significant morphological changes were observed at the 3-h and 24-h time points (Figure 2B, 2C); the cell membrane was intact, and more than 90% of pancreatic acinar cells exhibited viability. After 24 h, the cells began to die at a more rapid rate, and no viable cells were identified by inverted microscopy on day 5 (Figure 2D).

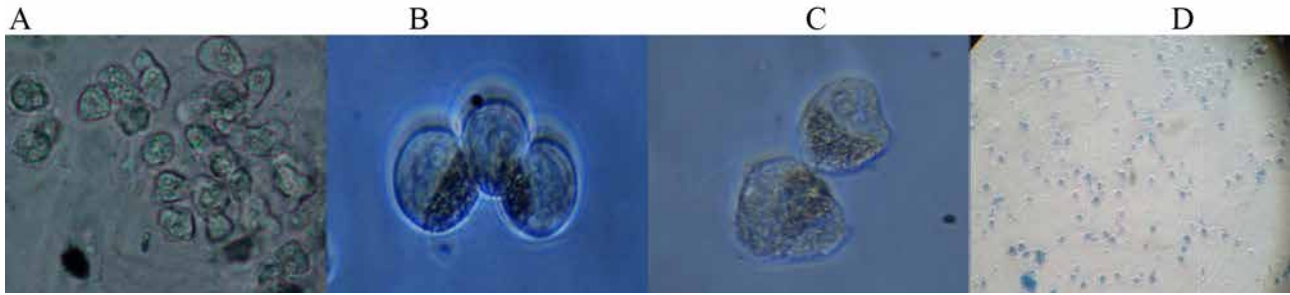


Figure 2: Micrographs of isolated rat pancreatic acinar cells at different time points (Trypan blue staining). 1A: 5 min ($\times 200$); 1B: 3 h ($\times 400$); 1C: 24 h ($\times 400$); 1D: 5 d ($\times 40$).

4 Discussion

AP is a formidable disease that progresses rapidly and often leads to SIRS and MODS. Therefore, AP has become a focal point in recent studies; however, its pathogenesis has not been fully elucidated to date. The aim of this study was to explore the pathogenesis of AP and identify correlations among its significant factors, such as pancreatic intracellular events, early inflammatory reactions and late inflammatory responses, from a cellular and molecular viewpoint.

The qPCR results showed that exposure to 3 ng/ml TAP increased HMGB1 mRNA expression at all tested time points compared with the expression in untreated rats. Additionally, the relative expression of HMGB1 mRNA at each time point was lower in the inhibitor group (EP group) than the corresponding values in the positive control group (TAP group). TAP is a critical molecule in the pathology of AP, and mild and severe pancreatitis can be distinguished based on the TAP concentrations in blood, urine, and ascites [11, 12]. Early inflammatory mediators can stimulate the production of HMGB1 [13], which mediates an inflammatory reaction upon its release from cells [14]. Furthermore, a prior study showed that HMGB1 is involved in the pathophysiological process of SAP and is associated with disease severity [15]. Another study reported that intravenous injection of 28 mmol/L EP into SAP rats markedly inhibited HMGB1 expression [16]. Our results are consistent with those reported in the literature [13-16]. In the current study, Western blotting revealed that HMGB1 protein expression significantly increased at 24 hours after TAP stimulation compared to the levels observed in untreated animals ($P < 0.01$), whereas HMGB1 protein expression at 12 h and 24 h was significantly decreased ($P < 0.01$). These findings further confirmed the qPCR results. From these experiments, we can infer that TAP induces the release of HMGB1 from pancreatic acinar cells (i.e., early-stage inflammatory factors are able to induce late-stage inflammatory factors), thereby aggra-

vating pancreatitis, and that EP inhibits the TAP-mediated effects to a certain degree, which reduces the damage caused by pancreatitis.

In addition, HMGB1 is a late inflammatory mediator that promotes the robust release of various early inflammatory mediators and activates the coagulation system to influence platelet function [17, 18]. Prior studies have shown that HMGB1, as a late inflammatory mediator, is involved in the pathogenesis of SAP and SIRS [19, 20]. Given the characteristics of HMGB1 and its important role in the development and progression of inflammation, we speculate that HMGB1 plays an important role in AP pathogenesis. Furthermore, we hypothesize that inhibiting HMGB1 expression to decrease serum HMGB1 levels will reduce damage to important organs outside the pancreas and exert a protective effect that can prevent or delay the development of SIRS and MODS. Confirmation of this activity would be highly beneficial for the treatment of pancreatitis.

This study was performed using cell culture, which exhibits different characteristics than the *in vivo* microenvironment. As such, some of the results of this study may not translate to an *in vivo* setting. To address this limitation, we plan to confirm our results in future animal experiments.

In summary, our results suggested that the early inflammatory factor TAP causes the release of the late inflammatory factor HMGB1; concomitant presence of both molecules affect the progression and severity of pancreatitis, including whether the pathology progresses to SIRS or MODS. Based on these assumptions, inhibiting HMGB1 release could serve as a new treatment strategy for individuals with AP. However, the detailed mechanism presented here must still be confirmed in both basic and clinical studies.

Conflict of interest: The authors declare no conflicts of interest.

Acknowledgements: The authors thank the colleagues from the Department of Hepatobiliary Pancreatic Surgery at the Affiliated Hospital of Zunyi Medical College, for their support during the work.

References

- [1] Hines O.J., Reber H.A., *Acute pancreatitis*, 2nd ed., Oxford textbook of surgery, Oxford, 2000
- [2] Chen S.S., Kong L.M., Zhang Y.Z., *Medical Biochemistry*. Shanghai, Fudan University Press, 1999, pp, 43-44 (in Chinese)
- [3] Hurley P.R., Cook A., Jehanli A., Austen B.M., Hermon-Taylor J., Development of radioimmunoassays for free tetra-L-Lysine trypsinogen activation peptides (TAP), *J. Immunol. Methods*, 1988, 111, 195-203
- [4] Frossard J.L., Trypsin activation peptide (TAP) in acute pancreatitis: from pathophysiology to clinical usefulness, *JOP*, 2003, 26, 230-234
- [5] Ulloa L., Messmer D., High-mobility group box 1 (HMGB1) protein: friend and foe, *Cytokine Growth Factor Rev.*, 2006, 17, 189-201
- [6] Harris H.E., Andersson U., Pisetsky D.S., HMGB1: a multi-functional alarmin driving autoimmune and inflammatory disease, *Nat. Rev. Rheumatol.*, 2012, 8, 195-202
- [7] Wang H., Bloom O., Zhang M., Vishnubhakat J.M., Ombrellino M., Che J., et al., HMG-1 as a late mediator of endotoxin lethality in mice, *Science*, 1999, 285, 248-251
- [8] Aoun E., Chen J., Reighard D., Gleeson F.C., Whitcomb D.C., Papachristou G.I., Diagnostic accuracy of interleukin-6 and interleukin-8 in predicting severe acute pancreatitis: a meta-analysis, *Pancreatology*, 2009, 9, 777-785
- [9] Wang H., Li W., Richard G., Tracey K.J., Sama A.E., HMGB1 as a potential therapeutic target, *Novartis Found. Symp.*, 2007, 280, 73-85
- [10] Ma Y., Yun S.R., Nam S.Y., Kim Y.B., Hong J.T., Kim Y., et al., Protective Effects of sanjoinine Against N-methgtd-aspartate-induced seizure, *Biol. Pharm. Bull.*, 2008, 31, 1749-1754
- [11] Jiao J., Wang Y., The relationship between the concentration of plasma trypsinogen activation peptides and pancreatitis, *J. Chin. Med. Univ.*, 2005, 34, 359-360 (in Chinese)
- [12] Gudgeon A.M., Heath D.I., Trypsinogen activation peptides assay in the early prediction of severity of acute pancreatitis, *Lancet*, 1990, 335, 4-8
- [13] Wang H., Yang H., Tracey K.J., Extra cellular role of HMGB1 in inflammation and sepsis, *J. Inter. Med.*, 2004, 255, 320-331
- [14] Bianchi M.E., Manfredi A.A., *Immunology: Dangers in and out*, Science, 2009, 323, 1683-1684
- [15] Yasuda T., Ueda T., Takeyama Y., Shinzeki M., Sawa H., Nakajima T., et al., Significant increase of serum high2mobility group box chromosomal protein 1 levels in patients with severe acute pancreatitis, *Pancreas*, 2006, 33, 359-363
- [16] Yang R., Uchiyama T., Alber S.M., Han X., Watkins S.K., Delude S.K., et al., Ethyl pyruvate ameliorates distant organ injury in a routine model of acute necrotizing pancreatitis, *Crit. Care Med.*, 2004, 32, 1453-1459
- [17] Andersson U., Wang H., Palmblad K., Aveberger A.C., Bloom O., Erlandsson-Harris H., et al., High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes, *J. Exp. Med.*, 2000, 192, 565-570
- [18] Rouhiainen A., Imai S., Rauvala H., Parkkinen J., Occurrence of amphoterin (HMG1) as an endogenous protein of human platelets that is exported to the cell surface upon platelet activation, *Thromb Haemost*, 2000, 84, 1087-1094
- [19] Magna M., Pisetsky D.S., The role of HMGB1 in the pathogenesis of inflammatory and autoimmune diseases, *Mol Med*. 2014, 20, 138-146
- [20] Shen X., Li W.Q., High-mobility group box 1 protein and its role in severe acute pancreatitis, *World J Gastroenterol.*, 2015, 21, 1424-1435