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# Targeting of the receptor for advanced glycation end products regulates neutrophil infiltration and extravascular recruitment in mice acute pancreatitis.

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**Abstract**

**Infiltration of leukocytes and pancreatic acinar cell damaging are good indicators of extreme Acute Pancreatitis (AP). The signaling pathways for inflammation and tissue damage of the pancreas not been elucidated yet. In this study, we evaluated the role of targeting of the Receptor for Advanced Glycation End products (RAGE) signaling in AP. Moreover, we investigated the role of signaling RAGE in AP. C57BL/6 mice were injected with a RAGE inhibitor (anti-RAGE) (500 µg/kg) before induction of taurocholate into the pancreatic duct to induce pancreatitis. Treatment anti-RAGE decreased blood amylase concentration, neutrophil recruitment in the pancreas, hemorrhage and edema formation in pancreatitis decreased by taurocholate. Additionally, anti-RAGE administration decreased the MPO activity in the pancreas and lung induced by taurocholate. Intraperitoneal (IP) injection of anti-RAGE significantly decreased concentrations of CXCL2 and IL-6 in the pancreas and plasma respectively in response to challenges of taurocholate. Finally, RAGE inhibition did not have a direct impact on secretagogue-induced trypsinogen activation in pancreatic acinar cells *in vitro*. Thus, these findings show new signaling pathways in AP and suggest that RAGE targeting may be an efficient way to improve extreme AP.**

**Keywords**: Amylase, Chemokines, Inflammation, Leukocytes, Pancreas.
**Abbreviations:** AP: Acute Pancreatitis; CXCL2/MIP2: Macrophage Inflammatory protein-2; IL-6: Interleukin-6; Mac-1: Membrane Activated Antigen-1; MNL: Monomorphonuclear Leukocyte; MPO: Myeloperoxidase; PBS: Phosphate Bufferd Saline; PMNL: Polymorhoneuclear Leukocytes; WT: Wild-Type

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## Introduction

Severe Acute Pancreatitis (AP) is linked to morbidity and death [1]. Patient with severe AP is a major challenge for clinicians and is limited to supporting therapies because of a limited understanding of the pathophysiology. Activation of trypsinogen is generally considered; recruitment of leukocytes and damaged microvascular perfusion are integral components in severe AP pathophysiology [2,3]. In view of the fact that trypsinogen activation appears to be an early and transient event, however, pancreatic inflammation continues for longer time and could be a more useful target for therapies [4,5]. Infiltration of neutrophil is an important characteristic of the inflammatory response and several studies have found neutrophils to be potential in severe AP [6,7]. Neutrophil infiltration is a multisequential steps supported by particular adhesion molecules, including P-selectin and Lymphocyte Antigen-1 (LFA-1) [8,9]. Secreted chemokines co-ordinate the extravascular direction of leukocytes. For example chemokines CXC, like CXCL1 and CXCL2, are important stimulants for accumulation of neutrophils [2,10,11]. However, CXCR2 is a receptor on CXCL1 and CXCL2 neutrophils that has been shown to mediate pancreatic neutrophil extravasation [2,12]. The receptor for advanced glycation end-products (RAGE) is a member of the superfamily immunoglobulin

gene, plays a crucial role in the regulation of nucleosomal proinflammatory behavior in macrophages [13]. The knockout of RAGE in macrophages suppresses the nucleosome-induced absence of inflammatory Activation In Melanoma 2 (AIM2) and followed by proinflammatory mediator release [14]. Targeted ablation of the expression RAGE or AIM2 in mice protects against L-arginine-or cerulein-induced AP in animal experiments.

Based on the mentioned considerations we hypothesize that RAGE signaling could play a role in severe AP. So, Taurocholate induction model of severe AP was used in mice, and use of anti-RAGE was achieved to inhibit RAGE activity.

## Materials and Methods

### *Animals*

Male mice C57BL/6 (20-25 g) were housed in a 12 light/12 dark cycle and fed a diet and water within the laboratory. The ethical committee at hawler Medical University-College of Pharmacy approved all of the experimental procedures. The 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine per kg body weight (Janssen Pharmaceutica, Beerse, Belgium) were used to anesthetize mice were intraperitoneally (i.p.)

### *Taurocholate perfusion*

Anaesthetized animals were subjected to midline laparotomy, and the duodenum's second part and vater papilla was identified. Traction sutures were placed one cm from the papilla, and a small cut was made in parallel to the vater papilla through the duodenal wall with a 23 G needle. A polyethylene catheter connected to a microinfusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) was inserted into the common bile duct through a perforated hole in the duodenum and one mm. The hepatic duct was established, and the liver hilum was clamped. Ten µl of 5 percent sodium taurocholate (Sigma, St. Louis, MO, USA) was impregnated for 10 min in the pancreatic duct. The catheter along with the hepatic duct clamp was removed once completed. After sealing the duodenal puncture with a suture with a purse string, traction sutures were removed, and two layers of the abdomen closed. Mice have allowed to wake up and accessed to food and water freely. Vehicle or the, The Abs to RAGE were obtained from Abcam (Cambridge, MA), was given (500 µg/kg) i.p. prior to bile duct cannulation. Sham mice underwent infusion into the pancreatic duct with laparotomy and Phosphate Buffered Saline (PBS) and were pretreated with i.p. with vehicle (PBS n=6). All mice were killed 24 hours after induction of pancreatitis, and all parameters that included in this study were evaluated.

### *Amylase activity*

Amylase activity concentration were detected in blood which collected from vein in mice tail using a commercially existed assay (Reflotron®, Roche Diagnostics GmbH, Mannheim, Germany).

### *Leukocyte counts*

Systemic differential counts were performed from the taken blood from the tail vein. The blood was diluted with Turks solution (Merck, Damnstadt, Germany) in a 1:20 dilution. Leucocytes were recognize as monomorphonuclear and polymorphonuclear cells in a Burker chamber.

### *Myeloperoxidase (MPO) activity*

A small piece of the pancreatic tissue (pancreatic head) and lung tissue were collected for MPO evaluations. The tissues before freezing were weighted and homogenized in 1 ml mixture (4:1) of PBS and aprotinin 10 000 KIE per ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenate samples were centrifuged (15339 × g, 10 min) and after storing the supernatant at −20 °C, the MPO assay was performed using pellet. After all pellets were mixed with 1 ml of 0.5% hexadecyltrimethylammonium bromide. Then, the sample was putted in the freezer for 24 h and then thawed, sonicated for 90 s, incubated in a water bath at 60 °C for 2 h, then the activity of MPO in the supernatant was measured. Spectrophotometric method used to determine the enzyme activity as the MPO-catalysed in the redox reaction of $H_2O_2$ (450 nm, with a reference filter 540 nm, 25 °C) change its absorbance the considered values are MPO units per g tissue.

### *Morphologic evaluation of pancreas*

The 4% formaldehyde phosphate buffer used to fix pancreas tissue and then the fixation was followed by dehydration and embedding in paraffin. The embedded samples were cut into Six µm sections and stained with (hematoxylin and eosin), then light microscopy used for examining the prepared slides. The pancreatitis severity was determined in a blinded manner depending on former scoring system, including edema, acinar cell necrosis, hemorrhage and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scales as previously described [15].

### *Enzyme-Linked Immunosorbent Assay (ELISA)*

CXCL2 concentration of the pancreas tissue and plasma concentration of IL-6 were evaluated using a double-antibody quantizing enzyme linked immunosorbent assay kits (R and D Systems Europe, Abingdon, UK) by utilizing recombinant murine CXCL2 and IL-6 as standards. The minimum concentration of detectable protein is less than 0.5 pg/ml.

### *Acinar cell isolation*

Collagenase digestion and gentle shearing used to prepare pancreatic acini cells as described in former studies. Cells have been suspended in O2 saturated HEPES-Ringer buffer (pH 7.4) and passed through a 150 µm cell strainer (Partec, Cörlitz, Germany). The acinar cells ($1 \times 10^7$

cells per well) which were isolated, they were incubated previously with PBS or GGTI-2133 (100 µM, 30 min) and stimulated with 100 nM cerulein (37 °C, 30 min) in duplicates. After discarding the buffer, the cells were washed twice with another buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3-(morpholino) propanesulphonic acid (MOPS) and 1 mM MgSO4. Then, in cold (4 °C) MOPS buffer cell were homogenized using a potter Elvehjem-type glass homogenizer. The homogenized cells with buffer was centrifuged (56 xg, 5 min), and supernatant was isolated and used for assay. The activity trypsin was evaluated fluorometrically in which Boc-Glu-Ala-Arg-AMC used as substrate as described previously. This was done by adding 200 µl aliquot of the homogenate acinar cell to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM CaCl2 and 0.1% BSA, pH 8.0). However, addition of substrate led the reaction to start, and the reaction was monitored on fluorescence emitted at 440 nm in response to excitation at 380 nm. By using a standard curve which generated by assaying purified trypsin, the level of trypsin (pg/ml) were calculated. The pancreatic acinar cells viability was higher than 95% as determined by trypan blue dye exclusion.

### *Statistical analysis*

Since the data was non-parametric, Mann–Whitney test was used in order to statistically evaluate the data and the significant level considered as $P \leq 0.05$ and the number of animals represented with "n". Moreover, the data presented as mean values ± SEM.

## Results

### *RAGE controls pancreatic tissue damage*

In order to assess the role of RAGE in severe AP, the amylase activities were determined as tissue damage indicator in pancreatitis. We noticed that taurocholate infusion into the pancreatic duct dramatically increased the concentration of blood amylase by 7-fold (Table 1). Treatment with the RAGE inhibitor decreased retrograde infusion of taurocholate-induced blood amylase activity levels from 579.6 ± 28.7 µKat/L to 215.0 ± 36.5 µKat/L, it corresponded to a 70% reduction (Table 1).

***Table 1.*** *Blood amylase (µKat/L) in sham mice and taurocholate-exposed mice pretreated with vehicle or the RAGE inhibitor (500 µ/kg).*

| Parameters | Mean | SEM |
|---|---|---|
| Sham | 66 | 11 |
| Taurocholate | 468* | 34 |
| Anti-RAGE | 386# | 25 |

Blood samples were obtained 24 h after pancreatitis induction. Data represent means ± SEM and n=5. *P<0.05 vs. PBS and #P<0.05 vs. Vehicle+Taurocholate

Tissue morphology analysis showed normal pancreatic microarchitecture in the negative control mice, on the other hand, taurocholate induction result in significant structural degradation of the pancreatic tissue which characterized by acinar cell necrosis, formation of edema and accumulation of neutrophils (Table 2). The results indicate that RAGE inhibition protected against destruction of tissue caused by taurocholate induction. Administration of anti-RAGE reduced taurocholate-evoked haemorrhage by 60% and edema by 30% in the pancreas (Table 2). Acinar necrosis with anti-RAGE decreased by 50% and the number of extravascular leukocytes in pancreatitis mice by 75% (Table 2).

***Table 2.*** *RAGE regulates tissue damage in AP.*

| Parameters | Hemorrhage (Scores) | Acinar cell necrosis (Scores) | Edema formation (Scores) | Neutrophil infiltration (Scores) |
|---|---|---|---|---|
| Sham | 1.0 | 0.5 | 0.5 | 1.5 |
| Taurocholate | 3.5* | 2.5* | 3.5* | 3.5* |
| Anti-RAGE+ Taurocholate | 2.0# | 1.5# | 2.5# | 2.0# |

Haemorrhage, acinar necrosis, edema formation and neutrophil infiltration. In sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the RAGE inhibitor (500 µg/kg). Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM and n=5. *P<0.05 vs. PBS and #P<0.05 vs. Vehicle+Taurocholate.

Taurocholate challenge declined the amount of PMNLs and MNLs circulating, indicating systemic activation continued (Table 3). RAGE inhibition significantly prevented the reduction in systemic leucocytes caused by taurocholate (Table 3).

***Table 3.*** *Systemic leukocyte differential counts.*

| | PMNL | MNL | Total |
|---|---|---|---|
| PBS | 1.4 ± 0.6 | 12.1 ± 0.4 | 13.5 ± 1.0 |
| Taurocholate | 0.5 ± 0.4* | 6.1 ± 0.2* | 6.6 ± 0.6* |
| Anti-RAGE+ Taurocholate | 0.7 ± 0.4# | 8.1 ± 0.3# | 8.8 ± 0.7# |

Blood was collected from sham mice and taurocholate-treated animals pretreated with vehicle or the RAGE inhibitor (500 µg/kg). Cells were identified as monomorphonuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMNL). Data represent mean ± SEM, 106 cells/ml and n=5. #P<0.05 vs. PBS and *P<0.05 vs. Vehicle+Taurocholate.

### *Regulation of neutrophils by RAGE*

The levels of MPO in pancreas and lung tissues have been used as an indicator of extravasation by neutrophils. Our result indicated that taurocholate infusion causes increasing in pancreatic MPO levels by 9.4-fold (Table 4). RAGE inhibition decreased the levels of MPO pancreas caused by taurocholate by 69% (Table 4).

***Table 4.*** *MPO levels in the Pancreas (U/g/Tissue).*

| Parameters | Mean | SEM |
|---|---|---|
| Sham | 0.58 | 0.126 |
| Taurocholate | 5.46* | 0.466 |
| Anti-RAGE+ Taurocholate | 2.065# | 0.43 |

RAGE controls taurocholate-induced neutrophil accumulation. MPO levels in the pancreas in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the RAGE inhibitor (500 µg/kg). Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM and n=5. *P<0.05 vs. PBS and #P<0.05 vs. Vehicle+Taurocholate.

However, neutrophils activation accumulated in the pulmonary microvasculature as part of a systemic inflammatory response in severe AP. Furthermore, we observed that the taurocholate infusion has significantly increased MPO activity in the lung (Table 5). RAGE inhibition results in reduction of MPO activity in the lung by more than 65% in mice that have treated with taurocholate (Table 5).

***Table 5.*** *RMPO levels in the Lung (U/g/Tissue).*

| Parameters | Mean | SEM |
|---|---|---|
| Sham | 0.191 | 0.04 |
| Taurocholate | 6.33* | 1.2 |
| Anti-RAGE+ Taurocholate | 2.33# | 0.4 |

RAGE controls taurocholate-induced neutrophil accumulation. MPO levels in the Lung in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the RAGE inhibitor (500 µg/kg). Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM and n=5. *P<0.05 vs. PBS and #P<0.05 vs. Vehicle+Taurocholate.

We have also demonstrated that the challenge with taurocholate would increase the concentration of CXCL2 in the pancreas (Table 6). Anti-RAGE administration significantly reduced levels of CXCL2 in inflamed pancreas tissue in taurocholate treated mice (Table 6).

***Table 6.*** *CXCL2 levels in the pancreas (pg/mg).*

| Parameters | Mean | SEM |
|---|---|---|
| Sham | 48.5 | 0. 6 |
| Taurocholate | 136.4* | 0.8 |
| Anti-RAGE+ Taurocholate | 67.6# | 0.5 |

Levels of CXCL2 in the pancreas. Levels were determined in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the RAGE inhibitor (500 µg/kg). Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM and n=5 *P<0.05 vs. PBS and #P<0.05 vs. Vehicle+Taurocholate.

Additionally, taurocholate challenges significantly increased IL-6 plasma concentration and the administration of anti-RAGE normalized IL-6 plasma levels in mice with pancreatitis (Table 7).

***Table 7.*** *IL-6 levels in plasma (ng/ml).*

| Parameters | Mean | SEM |
|---|---|---|
| Sham | 5.27 | 2.6 |
| Taurocholate | 104* | 5.2 |
| Anti-RAGE+ Taurocholate | 69.2# | 8.5 |

Levels were determined in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the RAGE inhibitor (500 µg/kg). Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM and n=5 *P<0.05 vs. PBS and #P<0.05 vs. Vehicle+Taurocholate.

### *Trypsinogen activation in vitro*

Next, we asked whether trypsinogen activation would be controlled by RAGE in pancreatic acinar cells *in vitro*. For this end, the acinar cells from mice pancreas were isolated and incubated with cerulein. We found that trypsinogen activation in mice acinar cell increased when stimulated by cerulein by more than 8-fold in compare with unstimulated acinar cells (Table 8). Moreover, secretagogue-induced activation of trypsinogen has not affected by preincubation of the mice acinar cells RAGE (Table 8).

***Table 8.*** *Trypsinogen levels in pancreas in-vitro.*

| Parameters | Mean | SEM |
|---|---|---|
| Sham | 2100 | 8.4 |
| Taurocholate | 6000* | 4.3 |
| Anti-RAGE+ cerulein | 5800 | 2.9 |

Acinar cell activation of trypsinogen was measured in negative control cells and in cells exposed to cerulein with PBS or Anti-RAGE (100 µM). Activation of trypsinogen activation was quantified by measuring enzymatic activity of trypsin fluorometrically by using Boc-Gln-Ala-Arg-MCA as the substrate as described in detail in Materials and Methods. Trypsin levels were calculated using a standard curve generated by assaying purified trypsin. Data represent means ± SEM and n=5. *P<0.05 vs. negative control.

## Discussion

OMechanisms which regulate pathological inflammation in pancreatitis are not fully understood. For the first time, our present study demonstrates that RAGE is an important pathophysiological regulator in SAP. These findings indicate that RAGE is included in regulation of neutrophils and in the production of CXC chemokine in the pancreas. In addition, our result showed that RAGE inhibition is not only reduces neutrophil recruitment in the pancreas of mice, but also lowers the rates of mice acinar cell necrosis and blood amylase in SAP. Furthermore, the results indicate that RAGE inhibition eradicates the formation of IL-6 and the extravasation of neutrophils in the lung, suggesting that RAGE regulates inflammation both locally and systemically in SAP. Previous study demonstrated that depletion of RAGE or AIM2 mice regulated tissue injury, decreased systemic inflammation, and protected against severe inflammation mice [14]. In the current study, we demonstrated that when specific RAGE inhibitor is used

for inhibition of RAGE, it clear cut reduced tissue injury in SAP. In addition, IP administration of anti-RAGE reduced taurocholate-provoked results in increasing of blood amylase by 70% and necrosis of acinar cell by 50%. This data is suggesting that RAGE controls the tissue damage's major part in SAP. Such findings represent the first proof in the literature that the signaling mechanism for RAGE is implicated in AP pathophysiology. Therefore, our current findings will also help the understanding the recorded anti-inflammatory effects of RAGE in pancreatitis.

There is sufficient proof that recruitment of neutrophils is a central component of AP [5,11]. However, it has been shown that reduction of neutrophil or inhibition of neutrophil recruitment protects against pancreatitis tissue injury [6]. Here, taurocholate challenge was observed to have increased concentrations of MPO and the number of recruited neutrophils in the inflamed pancreas. Treatment with anti-RAGE significantly reduced MPO activity 69% and the amount of extravascular neutrophils 75% in the pancreatic tissues, suggesting that the signaling of RAGE is an obvious regulator of pancreatic neutrophil recruitment. Considering the main function of neutrophils in pancreatitis pathophysiology, it could be proposed that the impact of RAGE inhibitory on the activation and infiltration of neutrophil could help to understand the protective impact of RAGE on the inflamed tissue. In addition, systemic complications of SAP include inflammatory cell extravasation of the lung [2]. We found that MPO activity in lung was improved in response to taurocholate. Interestingly, in taurocholate-exposed mice, anti-RAGE reduced lung levels of MPO, indicating that RAGE also regulates systemic neutrophil extravasation in the lung in SAP. Our finding also support the concept which RAGE regulates systemic inflammation, hence anti-RAGE significantly reduced the IL-6 plasma levels caused by taurocholate, because increasing of IL-6 is considered as a main indicator of systemic inflammation and correlates with septic patient mortality [16].

Secreted chemokines CXCL1 and CXCL2 co-ordinate the movement of leukocytes to inflammatory sites. A specific role of chemokines in pancreatitis has been suggested and taurocholate was observed to induce a significantly increase in pancreatic CXCL2 levels. Anti-RAGE administration of dramatically decreased CXCL2 pancreatic levels in AP, indicating that RAGE controls the production of CXCL2 in the mice inflamed pancreas tissue. Our finding would help explaining the inhibitory effect of RAGE on infiltration of neutrophils in SAP, suggesting that RAGE may controls pancreatic neutrophil infiltration via pancreatic CXCL2 formation.

## Conclusion

It is commonly thought that trypsinogen activation is a crucial function of AP's pathophysiology. We then asked if RAGE can be involved in activating trypsin in acinar cells. Nonetheless, it was found that in isolated acinar cells RAGE has no effect on trypsin activation that is caused by secretagogoue, indicating that RAGE activity is not specifically involved in trypsin activation in acinar cells.

Taken together, data results show that for severe AP, RAGE signaling regulates tissue injury in mice. Our findings show that RAGE inhibition in the pancreas decreases the formation of CXCL2 neutrophil up-regulation. RAGE inhibition also decreased recruitment of neutrophils and damage to tissue in the pancreas. Finally, blocking the RAGE activity in animals with pancreatitis attenuated systemic inflammation and pulmonary neutrophils. This research, however, not only delineates a novel signaling pathway in AP. but also indicates that interaction with RAGE may be a valuable tool for increasing local and systemic inflammation in extreme AP.

## Authorship

All authors are contributed equally

## Conflict of Interest

The authors state no conflict of interest.

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