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The Role of AMP-activated Protein Kinase (AMPK) in Regulating the Early Stages of Acute Pancreatitis

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I. Abstract

Acute pancreatitis is a devastating inflammatory disease, which is initiated by the activation and retention of digestive zymogens inside pancreatic acinar cells. It is proposed that adenosine monophosphate-activated protein kinase (AMPK) regulates the early responses of acute pancreatitis in pancreatic acinar cells (acini). A recent *in vitro* study shows that induction of experimental pancreatitis in isolated rat pancreatic acini with supraphysiologic cerulein (an orthologue of the hormone cholecystokinin) causes an increase in intracellular zymogen activation, but a decrease in AMPK levels. Furthermore, *in vitro* pharmacologic stimulation of AMPK reduces zymogen activation, having a protective effect. In this study, the effectiveness of two AMPK activators was examined in two separate, clinically-relevant *in vivo* pancreatitis models. In the first model, Sprague-Dawley rats received a pre-treatment of the AMPK activator metformin via intraperitoneal (IP) injection. A second IP injection of cerulein was administered one hour later. One hour following the second injection, the rat pancreata were harvested and markers of pancreatitis were measured. Intracellular zymogen activation was assessed via trypsin activity, edema was assessed via percentage wet weight, and extent of cellular damage was assessed via histological staining. In the second model, C57/Bl6 mice received a pre-treatment of either metformin or salicylate (also an AMPK activator). For each of the six hours following, mice received an IP injection of cerulein. At the seventh hour, the pancreata were harvested. The same parameters were assessed. In both models, the AMPK activators displayed a marked decrease in intracellular zymogen activation. Edema data obtained through percentage wet weight measurements were found to be insignificant. Histological staining of pre-treated sections demonstrated a considerable decrease in edema and pyknotic nuclei in rats. These results

strongly suggest that metformin and salicylate could potentially be used as prophylactic or therapeutic treatments for patients early in the course of acute pancreatitis.

II. Introduction

Acute pancreatitis is described as a sudden inflammation of the pancreas. Pain and swelling in a patient's upper left side of the abdomen is often a strong indicator of the disease. Other symptoms include nausea, vomiting, dehydration, and pain after food consumption. Clinical signs used to make a diagnosis include a swollen abdomen, fever, rapid pulse, and high amylase and lipase levels. Prognosis for acute pancreatitis ranges from mild to life-threatening, depending on cause as well as risk factors. Approximately 210, 000 patients are hospitalized with acute pancreatitis in the United States each year.[1] Treatment of this disease depends on both the cause and the severity.

A pivotal step in the initiation of acute pancreatitis is the regulated conversion of digestive zymogens, such as trypsinogen and chymotrypsinogen, to their active forms, trypsin and chymotrypsin, and their retention within the pancreatic acinar cell. Subsequent studies have demonstrated that additional pathologic acinar cell responses, including reduced apical secretion, elaboration of inflammatory mediators, and development of a paracellular leak, are also observed in the early phases of the disease.[2] Later responses include reduced perfusion, inflammation, and cell death.

The pancreatic acinar cell has the highest average rate of protein synthesis in the body.[3] This activity consumes high levels of ATP. Changes in acinar cell ATP levels have been reported in experimental acute pancreatitis models and the extent of ATP depletion in acute pancreatitis may correspond to disease severity. An important mechanism for energy sensing and conservation is AMP-activated protein kinase (AMPK).[3] AMPK is known to conserve cellular

energy by phosphorylating substrates to increase energy production or decrease activity of processes with high-energy consumption. One study has reported that acinar cell ATP levels change with pancreatitis stimuli, appearing to transiently increase, then fall.[4] Such fluctuations in ATP can potentially modulate AMPK activity during acute pancreatitis.

AMPK is a heterotrimeric protein consisting of α, β, and γ subunits. The β and γ subunits both function as regulatory domains. The α subunit, however, is the catalytic domain and serves as the site of phosphorylation for AMPK. Specifically, phosphorylation occurs at the Thr172 residue of the α subunit.

A recent study performed by Shugrue et al. demonstrates the potential protective effect AMPK has in the pancreatic acinar cell.[5] This study explored the pharmacological modulation of AMPK and its effects on intracellular zymogen activation in cell culture. First, a group of isolated rat pancreatic acinar cells was treated with a hyperstimulatory concentration of cerulein (an orthologue of the hormone cholecystokinin), a substance commonly used to experimentally induce pancreatitis. A second group of acinar cells was first pre-treated with agents that enhance AMPK activity, namely AICAR and metformin. Then, the same hyperstimulatory dose of cerulein was introduced to these pre-treated acinar cells to induce pancreatitis. By using trypsin activity as a measure of intracellular zymogen activation, it was discovered that cells pre-treated with AMPK activating drugs displayed a striking reduction of cerulein-induced zymogen activation. This result was further supported through the pre-treatment of acinar cells with Compound C, an AMPK inhibitor. As anticipated, Compound C produced the opposite effect of AICAR and metformin; it increased cerulein-induced zymogen activation.[5]

Given that this rat *in vitro* model exhibited a decrease in zymogen activation, a rat *in vivo* model was used to determine whether the effects of the AMPK activators would translate to

decreased severity of acute pancreatitis. Although the rat model is a well-characterized model that is useful for pancreatitis studies, the use of genetic knockout mice has been steadily increasing over recent years. For this reason, a mouse model was also developed as a part of this study. Attaining comparable results in mice could lay the foundation for transgenic studies.

The two AMPK activators used in this study are metformin and salicylate. Although metformin and salicylate both activate AMPK, they do so using two completely different mechanisms of action. Metformin works indirectly on AMPK by mildly reducing the activity of the mitochondrial respiratory chain complex I.[6] The resulting decrease in ATP production puts the cell into a hepatic energy state. In this state, the amount of AMP and ADP in the cell are increased. The elevated AMP-to-ATP ratio causes the activation of AMPK. Salicylate, on the other hand, has a direct effect on AMPK. After AMPK is phosphorylated at Thr172, salicylate binds to the β subunit and acts as an allosteric activator.[7] The binding of salicylate also inhibits certain phosphatases from dephosphorylating AMPK.

The results of this study provide evidence that pharmacological activation of AMPK in both rats and mice causes a decrease in cerulein-induced zymogen activation, edema, and cellular damage.

III. Methods

All experiments and procedures using animals were approved by the Veterans Affairs Healthcare System's Institutional Animal Care and Use Committee (IACUC) in West Haven, CT.

1a. In Vivo Rat Model. A 2-hour *in vivo* time-course was developed **(Figure 1)** whereby each Sprague-Dawley rat was given one initial IP injection and an additional IP injection one hour later. In the initial injection, each rat received either phosphate buffered saline (PBS control) or a

pre-treatment of metformin (MET) (120mg/kg). In the second injection, each rat received either PBS (control) or cerulein (40μg/kg). An hour following the second injection, the animals were euthanized; their pancreata were harvested and used to assess the following parameters of pancreatitis: intracellular zymogen activation, edema, and histological damage. This time-course resulted in four separate treatment groups requiring a total of 16 rats (4 per treatment group): **1. PBS/PBS, 2. PBS/CER, 3. MET/PBS, 4. MET/CER.** For simplicity, PBS/PBS will be referred to as ***PBS***, MET/PBS as ***MET***, and PBS/CER as ***CER***. MET/CER will still be referred to as such.

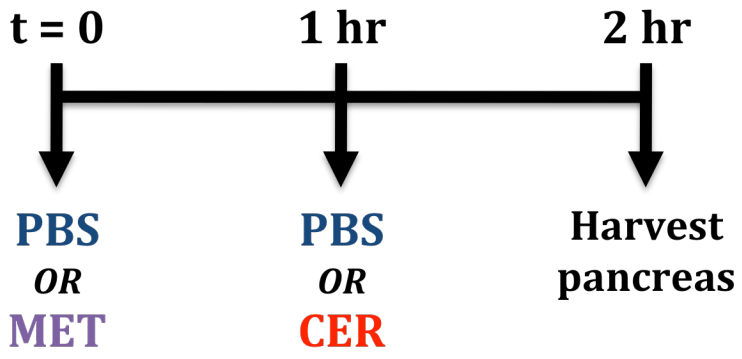

Figure 1. Time-course for in vivo rat model consists of two hourly IP injections. Pre-treated rats are injected with metformin for one hour.

1b. Zymogen Activation Assay. Following harvest of pancreatic tissue, samples were homogenized in homogenization buffer [1.5 M Tris (pH 6.4), 0.3 M sucrose] and centrifuged at 600*g* (4°C) for 10 minutes. The post-nuclear supernatant (PNS) (containing zymogen granules) of each sample was stored for assaying. For each sample, 10 μL of the PNS and 440 μL of zymogen assay buffer [50 mM Tris (pH 8.1), 150 mM NaCl, 1 mM CaCl2, 0.01% BSA] were both added to a well of a 24-well plate (Greiner Bio-one *Cellstar* TC-Plate). The assay was initiated by the addition of 50 μL of 400 mM trypsin substrate (fluorometric trypsin substrate;

Peptides International, Louisville, KY) diluted in zymogen assay buffer (40 mM final). The plate was read using a fluorometric microtiter plate reader (model HTS 7000; Perkin-Elmer Analytical Instruments, Shelton, CT. 380-nm excitation; 440-nm emission; 20 reads /10 min.).

1c. Edema. Following the harvest of pancreatic tissue, each sample was blotted and its corresponding wet weight determined. The samples were then incubated at 60°C for 72 hours and reweighed. Edema was expressed as percent wet weight using the following equation:
(Wet weight − Dry weight)/Wet weight × 100%. [8]

1d. Histological Assessment. A 1-millimeter piece of pancreatic tissue was removed from each animal and was immersion fixed in 4% formalin. Then, the tissue was dehydrated, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin. Slides were viewed using an Axiophot microscope (Carl Zeiss, Thornwood, NY) at 40x and 60x magnification and images were collected with a Spot Digital camera (Diagnostic Instruments, Sterling Heights, MI). Samples were assessed using a histological scoring system and representative images of each treatment group were selected.

2a. In Vivo Mouse Model. A 7-hour *in vivo* time-course was developed **(Figure 2)** whereby each C57/Bl6 (Wild type or Wt) mouse was given a single IP injection every hour for 7 hours. In the initial injection, each mouse received either PBS control, a pre-treatment of MET (200mg/kg), or a pre-treatment of salicylate (SAL) (120mg/kg). For each of the six hours following, each mouse received either PBS (control) or CER (40μg/kg). At the seventh hour, the mice were euthanized; their pancreata were harvested and used to assess the following parameters of pancreatitis:

intracellular zymogen activation, edema, and histological damage. This time-course resulted in six separate treatment groups: **1. PBS/PBS, 2. MET/PBS, 3. SAL/PBS, 4. PBS/CER 5. MET/CER 6. SAL/CER.** For simplicity, PBS/PBS will be referred to as ***PBS***, MET/PBS as ***MET***, SAL/PBS as ***SAL***, and PBS/CER as ***CER***. MET/CER and SAL/CER will still be referred to as such.

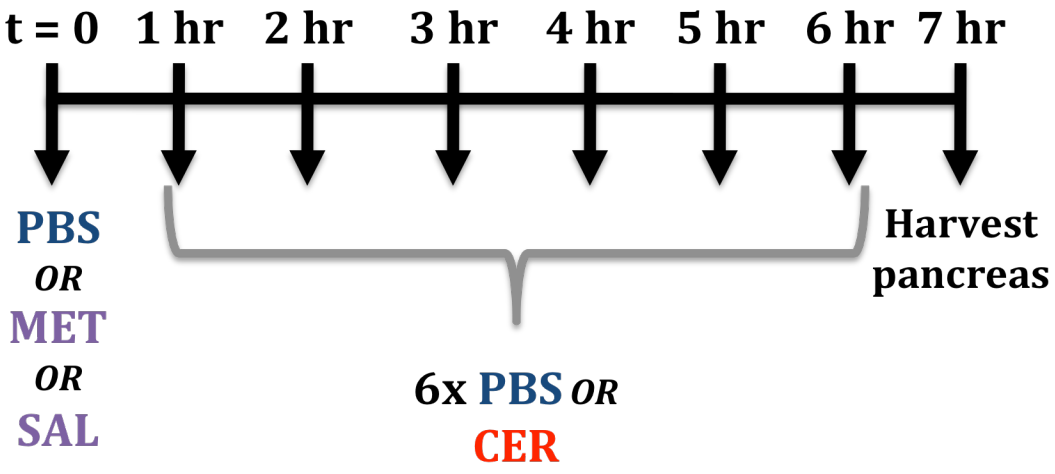

Figure 2. Time-course for in vivo mouse model consists of seven hourly IP injections. Pre-treated mice are injected with either metformin or salicylate for one hour.

2b. Zymogen Activation Assay. See Section 3.1b for procedure.

2c. Edema. See Section 3.2c for procedure.

2d. Histological Assessment. See Section 3.2d for procedure.

IV. Results

1a. Zymogen activation (Rat). Metformin pre-treatment of the rats was found to significantly decrease cerulein-induced intracellular zymogen activation, as measured by trypsin activity **(Figure 3)**. Although the MET rats presented a slight decrease in trypsin activity in comparison to PBS rats, this decrease was statistically insignificant.

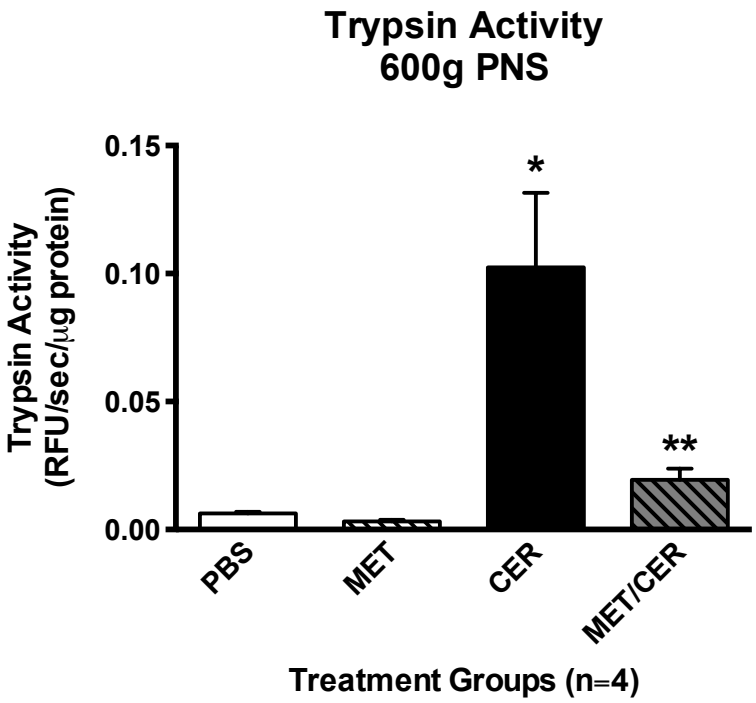

Figure 3. Metformin pre-treatment in rats results in a significant 5-fold decrease in zymogen activation (as measured by trypsin activity) in the cerulein model.

*p<0.05 vs PBS
**p<0.05 vs CER

1b. Edema (Rat). CER rats exhibited significant amounts of edema in comparison to PBS (control) rats **(Figure 4)**. Specifically, CER rats had a percent wet weight of 80% whereas the PBS rats had approximately 65%. MET/CER rats experienced a 5% reduction in edema at 75% when compared to CER rats. The difference in edema between CER and MET/CER rats is statistically insignificant. Since this measure of edema is limited to the wet weight of the pancreas, histology was carefully examined to determine whether this result was conclusive.

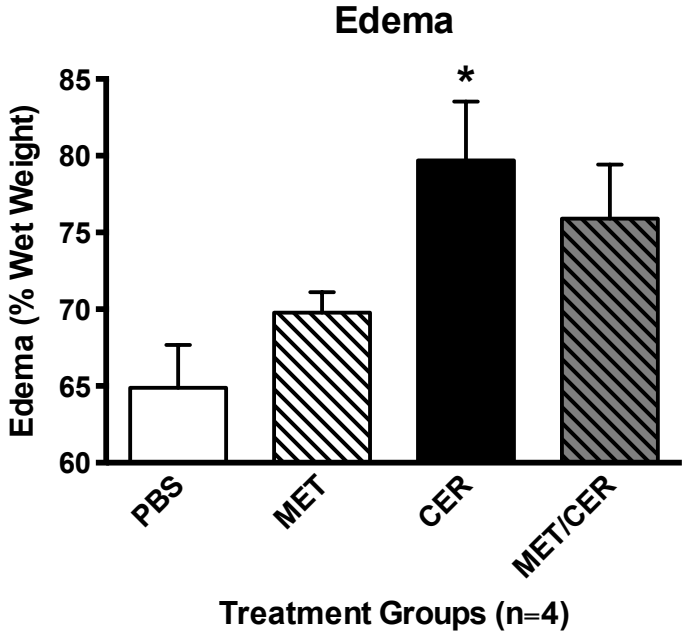

Figure 4. Metformin pre-treatment in rats does not significantly reduce edema, as measured by percentage wet weight.

*p<0.05 vs PBS

1c. Histology (Rat). Histological assessment of sections gathered from pancreatic tissue from each of the four treatment groups is not consistent with the edema studies described above. It is evident that unlike the CER sections, the MET/CER sections show a substantial decrease in edema, pyknotic nuclei, and plasma membrane blebbing **(Figure 5)**. PBS and MET are nearly identical and exhibit no cellular damage, as expected. The visual evidence of cellular damage (including edema) was found to be more reliable than the edema measurement carried out in Section 4.1b.

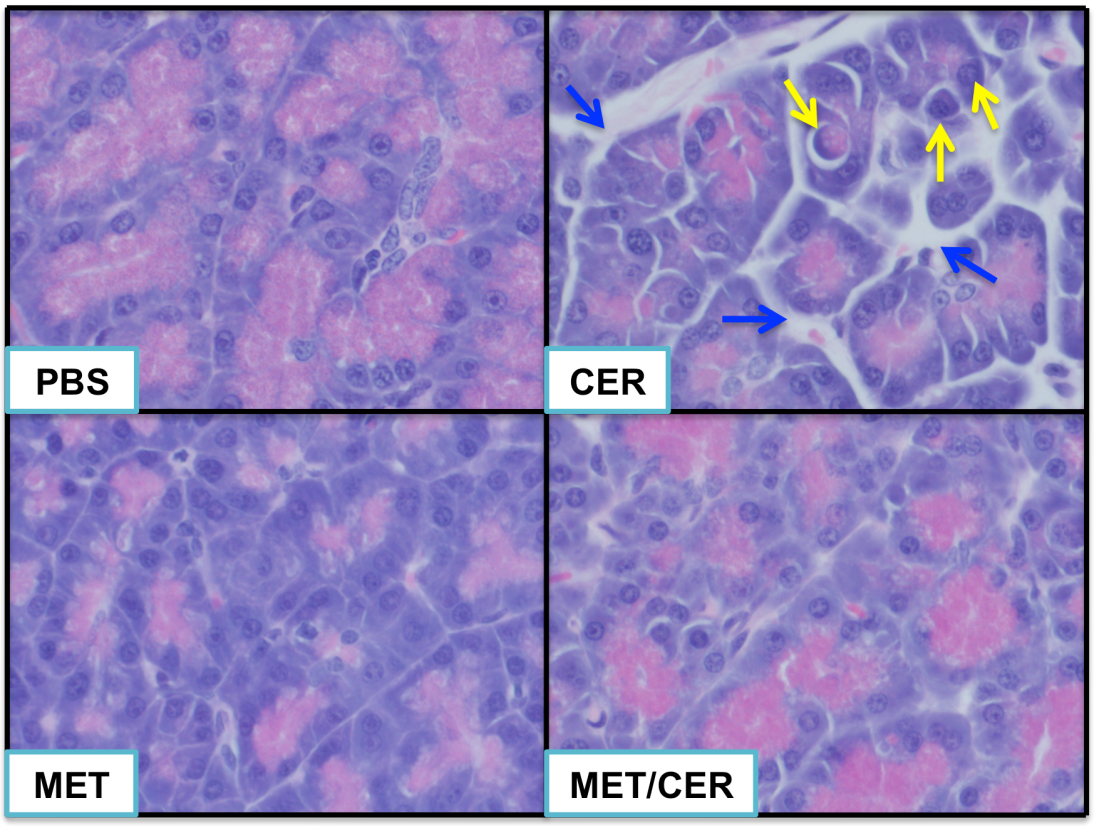

Figure 5. Histological pancreatitis responses (pyknotic nuclei-yellow arrows, and edema- blue arrows) in rats are reduced by metformin pre-treatment.

2a. Zymogen Activation (Mouse). Both metformin and salicylate pre-treatment of the rats were found to decrease cerulein-induced intracellular zymogen activation, as measured by trypsin activity **(Figure 6)**. The two-fold decrease in trypsin activity between CER mice and SAL/CER mice was determined to be statistically significant. However, the decrease in trypsin activity between CER mice and MET/CER mice was found to only be approaching significance ($p = 0.0553$). Increasing the number of animals in the MET/CER treatment group might have produced a statistically significant decrease.

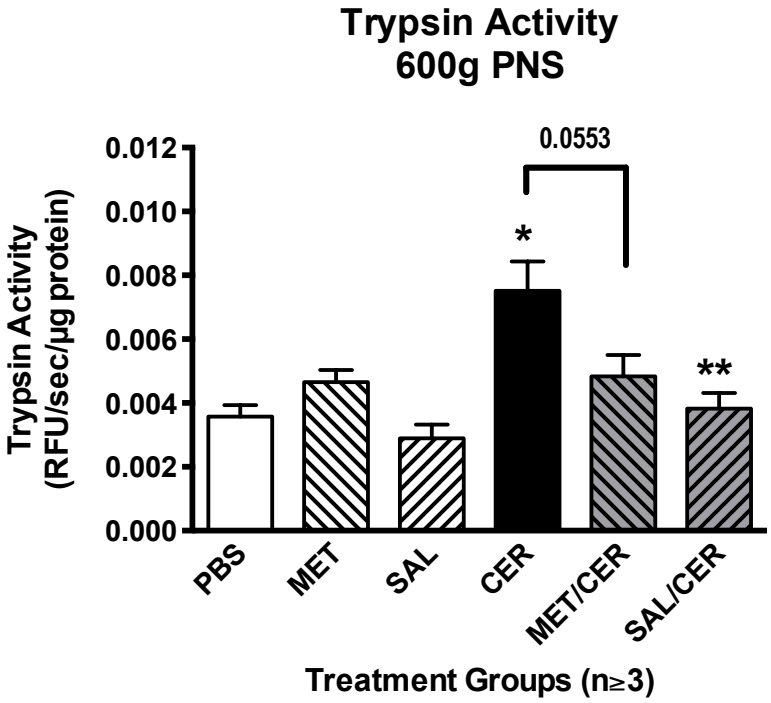

Figure 6. Salicylate pre-treatment in mice results in a significant 2-fold decrease in zymogen activation (as measured by trypsin activity) in the cerulein model. Metformin pre-treatment also exhibits a decrease (nearly significant) in zymogen activation.

*p<0.05 vs PBS
**p<0.05 vs CER

2b. Edema (Mouse). CER rats exhibited significant amounts of edema in comparison to PBS (control) rats **(Figure 7)**. Specifically, CER rats had a percent wet weight of 83% whereas the PBS rats had approximately 65%. MET/CER rats experienced an 8% reduction in edema at 75% when compared to CER rats. SAL/CER rats experienced a 10% reduction in edema at 83% when compared to CER rats. Neither MET/CER nor SAL/CER rats displayed a significant difference in percent wet weight from that of CER rats.

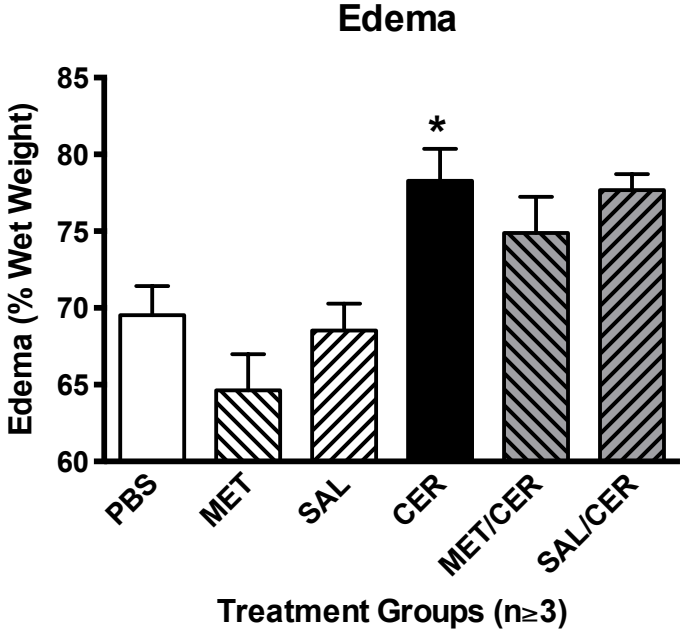

Figure 7. Metformin and salicylate pre-treatment in mice do not significantly reduce edema, as measured by percentage wet weight.

*p<0.05 vs PBS

2c. Histology (Mouse). Histological assessment of sections gathered from pancreatic tissue demonstrates clear histological damage in all sections of animals treated with CER (including MET/CER and SAL/CER) **(Figure 8)**. This damage included severe edema, plasma membrane blebbing, and pyknotic nuclei. PBS, MET, and SAL showed no cellular damage. Damage in the pre-treated sections is thought to be the result of stress on the animals, as they received six injections in a matter of six hours. This amount of stress alone might have cause the damage in the pre-treated sections.

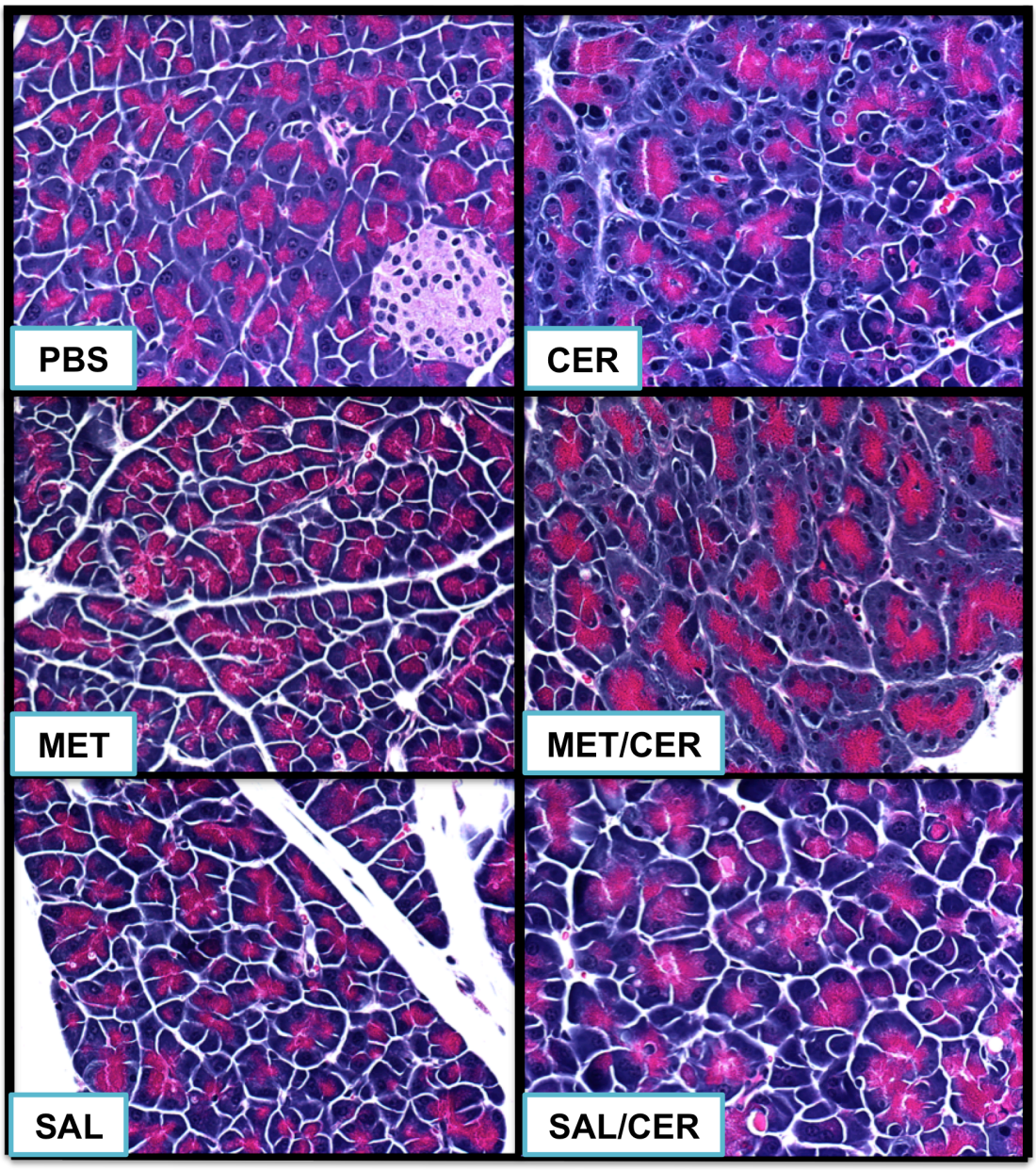

Figure 8. Histological damage is present in the CER sections as well as the pre-treated sections. Edema and pyknotic nuclei are abundant in the CER, MET/CER, and SAL/CER sections. 40x magnification.

V. Discussion

This study demonstrates that pre-treatment with the AMPK activators metformin and salicylate can lessen the severity of the early stages of acute pancreatitis in *in vivo* models. Although metformin and salicylate are able to lessen the pancreatitis responses by activating AMPK, cerulein causes an increase in pathological responses despite being able to activate AMPK. It has been shown that when cerulein activates AMPK, AMPK is translocated to an unidentified Triton X-100 insoluble compartment in the acinar cell, preventing AMPK from exerting its protective effects.[5] Now, a new set of questions arises: Do metformin and salicylate prevent this translocation and, if so, how?

Three potential answers to this question are being considered. First, it is possible that metformin and salicylate are directly preventing the translocation of AMPK. By altering the conformation of one of the AMPK subunits, metformin and salicylate might prevent the translocation of AMPK. A chaperonin might also play a role in this potential mechanism. Second, cerulein might be stimulating additional pathways that move AMPK. Metformin and salicylate might be preventing these pathways from translocating active AMPK. Third, metformin and salicylate might be activating different AMPK proteins than cerulein, which could explain why AMPK activated by cerulein translocates away while AMPK activated by these drugs does not.

An important point to note is the difference between prophylactic and therapeutic treatment. In this study, salicylate and metformin were considered to be prophylactic treatments, or pre-treatments. Prophylactic treatments such as this one could be useful in patients with hereditary pancreatitis.[9] Patients with hereditary pancreatitis often experience recurrent problems and undergoing prophylactic treatments of salicylate and metformin in low doses could

potentially be useful as a preventive measure. In addition, patients who undergo invasive procedures such as Endoscopic Retrograde Cholangeopancreatography (ERCP) could benefit from prophylactic treatment.[10] ERCP is used when a patient's pancreatic or bile ducts are narrowed or fully blocked. The procedure makes use of an endoscope and X-rays for treatment. A major complication that frequently arises from this procedure is pancreatitis.[11] By administering prophylactic treatments to patients prior to undergoing ERCP, post-ERCP pancreatitis may be avoided. Future studies should also consider the therapeutic effect of salicylate, metformin, and additional AMPK activating drugs.

It has also been discovered that different isoforms for each of the three AMPK subunits exist in the acinar cell. For instance, the α subunit can exist as α_1 or α_2, the β subunit can exist as β_1 or β_2, and the γ subunit can exist as γ_1, γ_2, or γ_3 [12]. Theoretically, there are twelve possible isoform combinations for the protein AMPK (ex. $\alpha_1\beta_1\gamma_1$, $\alpha_1\beta_2\gamma_2$, etc.). Drugs that target a specific subunit of AMPK could be of greater therapeutic value if the abundance of each subunit is better determined. The use of AMPK-α_1 and AMPK-α_2 knockout mice should also be considered in future studies, as genetically reducing levels of AMPK in mice should confirm that salicylate and metformin are acting through AMPK.

Since metformin and salicylate employ different mechanisms of action in activating AMPK, it may of great importance to consider the combination of MET and SAL as a potential prophylactic and therapeutic agent, as this combination may further reduce the severity of early acute pancreatitis responses.

VI. References

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