AWARD NUMBER:  W81XWH-17-1-0138

TITLE:  Statins prevent pancreatic diseases through mitophagy activation

PRINCIPAL INVESTIGATOR:  Stephen Pandol, MD

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles, CA 90048

REPORT DATE: May 2018

TYPE OF REPORT:  Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland, 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
This proposal addressing FY16 PRMRP topic area Pancreatitis is designed to determine the mechanisms of statin mediated protection from pancreatitis, an observation recently reported using a retrospective cohort analysis of large clinical datasets. Prevention of pancreatitis, especially cases that progress to recurrent acute and chronic pancreatitis is of high relevance because there is no treatment for these disorders and because the progression is associated with an extremely high risk of pancreatic cancer, one of the most lethal human cancers.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>3</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>3</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>3</td>
</tr>
<tr>
<td>4. Impact</td>
<td>15</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>15</td>
</tr>
<tr>
<td>6. Products</td>
<td>16</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>17</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>N/A</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>19</td>
</tr>
</tbody>
</table>
INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

A recent retrospective cohort analysis showed a reduced occurrence of pancreatitis with statin intake (especially simvastatin) in the Southern California Kaiser Permanente Health Care System. We hypothesized that the beneficial effects of simvastatin were due to its ability to enhance removal of failing mitochondria from the pancreas to improve the ability of the pancreas to withstand stressors that cause pancreatitis. In this report we describe our experiments to test this hypothesis in an experimental model of pancreatitis. We posit that further work using simvastatin in combination with other anti-inflammatory agents could be tested for clinical efficacy in humans.

1. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Pancreatitis, mitochondria, mitophagy, simvastatin, statin

2. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Specific Aim 1: Determine the effects of simvastatin treatment in vitro and in vivo on expression of sXBP1 and pathways of mitophagy in acinar cells of the pancreas using human and murine acinar cells. Yet to be completed.

Specific Aim 2. Determine the effect of the simvastatin treatments on Ca\(^{2+}\) signaling and mitochondrial responses to a Ca\(^{2+}\) stress. Completed and result shown below.

Specific Aim 3. Determine the roles of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and sXBP1 on the pathways of mitophagy, and mitochondrial and pancreatitis responses to Ca\(^{2+}\) stress. Completed and result shown below.

What was accomplished under these goals?

Progress in the Project

Simvastatin pretreatment reduces inflammation and cell death during acute pancreatitis

A recent retrospective cohort analysis showed a reduced occurrence of pancreatitis with statin intake (especially simvastatin) in the Southern California Kaiser Permanente Health Care System.(1) To determine if simvastatin treatment could reduce pancreatitis severity of pancreatitis and the mechanism of any beneficial effect we employed an experimental pancreatitis model in mice using cerulein hyperstimulation as previously described.(2) We pretreated the mice with simvastatin (20 mg/kg b.w.) for 24 hours or DMSO (vehicle control), followed by 7 hourly
intraperitoneal injections of cerulein (50 µg/kg) to induce acute pancreatitis as illustrated (Figure 1a). The animals were sacrificed 1 hour after the last cerulein injection and tissue was collected for histology and other measurements. We found necrotic cell death, infiltration of inflammatory cells, and edema in the cerulein-treated pancreas, whereas simvastatin pretreatment attenuated this histopathology (Fig. 1b and Table 1). Furthermore, we also found that simvastatin pretreatment reduced the vacuole accumulation commonly seen in the pancreatic acinar cells during acute pancreatitis. Although necrotic cell death was prominent, we also determined the effect of simvastatin on apoptotic cell death. TUNEL staining of the pancreatic tissue sections showed reduced apoptotic cell death in pancreatic acinar cells of mice pretreated with simvastatin (Fig. 1c). Trypsin activation is one of the hallmarks of acute pancreatitis. We observed a significant reduction in trypsin activity with simvastatin pretreatment (Fig. 1d).

Figure 1. Simvastatin reduces edema, cell death, and trypsin activation during acute pancreatitis. (a) Schematic representation of mice model of acute pancreatitis. Mice received DMSO or Simvastatin (20mg/kg, i.p.) pretreatment for 24 hours. Acute pancreatitis was induced by intraperitoneal injections cerulein (50µg/kg) every hour for 7 hours. Control mice received same amount of vehicle control (saline). Mice were sacrificed one hour after the last cerulein injection. Pancreata were harvested for histology and other measurements. (b) Representative images of H&E staining of pancreatic tissue (n=5). (c) Apoptotic cell death was detected using TUNEL staining kit. Representative images show the TUNEL positive cells (red) and DAPI staining (blue) (n=3). (d) Trypsin activity was measured using calorimetric assay. Bar graphs represent the trypsin activity (nmol/mg) for
Cerulein-induced acute pancreatitis stimulates mitophagy in mice

As we have previously shown that oxidative stress induces mitophagy as a cell survival defense mechanism(3), we next sought to determine if there is a mitophagy induction in our model of acute pancreatitis. Consistent with previous publications(4), we found a significant increase in the levels of total (post-nuclear supernatant) p62 and lipided LC3 after cerulein injections suggesting autophagy effects of cerulein (Fig. 2a, c) as have been discussed recently.(5) Next, we measured the mitophagy marker, parkin, and found that parkin abundance was significantly reduced during acute pancreatitis (Fig. 2a, b). Furthermore, we observed increased translocation of parkin to the mitochondria after cerulein exposure (Fig. 2d-f), which suggests the induction of mitophagy during pancreatitis. Interestingly, simvastatin treatment (Simva-Cerulein) significantly increased p62 compared to DMSO-Cerulein.

![Figure 2. Cerulein-induced pancreatitis triggers mitophagy. Mice pancreatic tissue was collected and tissue lysate was fractionated for post-nuclear supernatant and crude mitochondrial fraction. (a) Western blot of post-nuclear supernatant for parkin, p62 and LC3. Ponceau S staining was used to normalize the protein expression. Scatter dot plot]
representing the Ponceau S stain normalized expression of parkin (b) and p62 (c). (d) Western blot showing the protein expression of parkin, p62 and LC3 in crude mitochondrial extract. (e) Scatter dot plot representing the Ponceau S stain normalized expression of parkin (e) and p62 (f) present in crude mitochondrial fraction. Values in graphs are represented as Mean ± SD, *p< 0.05, **p< 0.01, ***p<0.001 vs DMSO Saline; ^p< 0.05 vs DMSO Cerulein

Cerulein impairs and simvastatin treatment preserves autophagosome-lysosome fusion

As shown previously, pancreatitis causes an accumulation of vacuoles in the acinar cells, associated with dysfunctional autophagy(6). We next determined effects of simvastatin on mediators of autophagosome function. Syntaxin 17 (Stx17) is located on the outer membrane of the autophagosome(7) and necessary for autophagic processing; and its depletion causes autophagosome accumulation and lack of processing of autophagosome contents. Interestingly, we found a decrease in the expression of syntaxin 17 after cerulein injections although simvastatin didn’t modify Stx17 abundance during pancreatitis (Fig. 3a, c), suggesting that cerulein effects on autophagy are in part due to decreased Stx17 but that simvastatin’s protective effects are Stx17 independent. We next examined LAMP-1, ULK-1 and AMPK, which are essential for autophagy(8,9) and mitochondrial targeting to lysosomes(10). We found that cerulein treatment significantly reduced LAMP-1 and ULK-1 protein expression, while AMPK levels didn’t change (Fig. 3a, b, d-f). As AMPK phosphorylates ULK-1(10) to regulate the fusion process in autophagosome formation and activity of both proteins is largely dependent on their phosphorylation states, we next determined the phosphorylation levels of AMPK$^{T172}$ and ULK-1$^{S555}$ and found that both AMPK$^{T172}$ and ULK-1$^{S555}$ were dephosphorylated after cerulein treatment (Fig. 3g-h), suggesting the possibility that with experimental pancreatitis the dephosphorylated state of these regulators could lead to disorders in autophagosome-lysosome fusion. Simvastatin pretreatment upregulated LAMP-1, AMPK-1 and ULK-1 as well as the phosphorylated forms of AMPK and ULK-1 (Fig. 3a-h), suggesting that simvastatin preserves autophagosome-lysosome fusion to promote autophagy function. To confirm our findings, we next determined the extent of vacuolization in the pancreatic acinar cells, as these vacuoles accumulate when autophagic flux is impaired. Using electron microscopy, we found that the total number of vacuoles in the simvastatin pretreatment group was reduced compared to DMSO control during acute pancreatitis (Fig. 4a). Next, we counted the number of autophagosomes (recognizable cargo), intermediate vacuoles and autolysosomes
digested cargo) as a percent of the total number of vacuoles (Fig. 4b). Simvastatin pretreatment increased the number of intermediate vacuoles and autolysosomes, consistent with enhanced clearance of autophagosomes after cerulein exposure (Fig. 4c).

Figure 3. Simvastatin pretreatment improves autophagosome-lysosome fusion by enhanced autophagy signaling and preservation of LAMP-1 protein. Western blot showing the protein expression of (a) Stx17, LAMP-1 and parkin; (b) AMPK, pAMPK (Thr172), ULK1 and pULK1 (S555) in post-nuclear supernatant. Scatter dot plot representing the Ponceau S stain normalized expression of Stx17 (c), LAMP-1 (d), AMPK (e), ULK1 (f), pAMPK (T172) (g), pULK1 (S555) (h). Values in bar graphs are represented as Mean ± SD (n=3), *p<0.05, **p<0.01, ***p<0.001 vs DMSO Saline; ^p<0.05, ^^p<0.01 vs DMSO Cerulein.
Figure 4. Simvastatin pretreatment reduces autophagosomes accumulation. (a) Electron microscopy was done on pancreatic tissue to count for different vacuoles presence in the sections. Representative images show Amphisome, Intermediate vacuoles and autolysosomes in pancreatic sections. (b) Bar graph represents the total number of vacuoles during acute pancreatitis with DMSO (DMSO Cerulein) or Simvastatin (Simva Cerulein) pretreatment (n=2). Vacuoles were counted from at least 30 different fields for each group. (c) Different types of vacuoles (Amphisomes, Intermediate and Autolysosomes were counted and expressed as percentage of total vacuoles. Values in graphs are represented as Mean ± SD.

Mitochondrial biogenesis is an early event in acute pancreatitis

Previous studies have shown that stress signaling pathways can increase mitochondrial biogenesis to meet increased energy demand(11). To determine if pancreatitis induces mitochondrial biogenesis, we determined the abundance of representative OXPHOS subunits (CI-NDUFB8, CII-SDHB, CIII-UQCR2, CIV-MTCO1, CV-ATP5A) and COXIV using Western blot. We found that cerulein administration significantly increased the abundance of mitochondrial proteins (Fig. 5a-b) in both DMSO- and simvastatin-treated mice, although there was a trend of slight decrease with simvastatin treatment. To understand the mechanism of mitochondrial biogenesis during pancreatitis, we performed a time-course study where mice were sacrificed at various times after hourly cerulein injections (2, 4, or 8 hr. after the first injection). We detected an increase in mitochondrial proteins as early as 1 hour after the first cerulein injection (Fig. 5c-d), suggesting
that mitochondrial biogenesis in pancreatitis is a translationally-controlled process, probably regulated through microRNAs.

Figure 5. Upregulation of mitochondrial proteins occurs rapidly after cerulein exposure. (a) Graphs representing the ponceau S stained normalized protein expression of ATP5A (Complex V), MTCO1 (Complex IV), UQRC2 (Complex III), SDHB (Complex II), NDUFB8 (Complex I) and (b) COX IV. (c) Different number of (1, 3 or 7) Cerulein injections (50ug/kg, i.p.) were given to mice and mice was sacrificed after 1 hour of indicated cerulein injections to collect pancreatic tissue. Control mice received no injection (0 hour). Western blot showing the protein expression of ATP5A (Complex V), MTCO1 (Complex IV), UQRC2 (Complex III), SDHB (Complex II), NDUFB8 (Complex I) involved in OXPHOS subunits assembly. (d) Line graph represents the ponceau S staining normalized expression of these proteins at different time points after cerulein injections. Values in graphs are represented as Mean ± SD (n=3), *p< 0.05, **p< 0.01, ***p<0.001 vs DMSO Saline.

Simvastatin-induced mitophagy increases stress resistant mitochondrial population

Previously, we reported that simvastatin induces mitophagy to provide cardioprotection during ischemia-reperfusion injury(12). We next questioned if simvastatin induced mitophagy to remove dysfunctional mitochondria and thereby prevent acinar cell injury. We assessed mitophagy at various times after simvastatin administration, using parkin and mfn2 as well as autophagy-related markers p62 and LC3. We observed an early decrease in the levels of mfn2 and p62 followed by subsequent increase by 6 hours while parkin and LC3 levels were lower at 6 hours and returned to
baseline by 12 hours (Fig. 6a). Mitochondrial protein abundance reached a nadir at 12 hours, followed by partial recovery (Fig. 6b). To determine if simvastatin depended on autophagic flux to induce clearance of dysfunctional mitochondria, we used chloroquine to prevent lysosomal acidification and fusion of autophagosomes to lysosomes. We then assessed the levels of p62 and OXPHOS proteins. Consistent with our earlier results, simvastatin triggered a decrease in OXPHOS proteins and p62, which was abolished by chloroquine administration (Fig. 6c-d). We hypothesized that simvastatin-mediated mitophagy would leave behind a population of mitochondria that were more stress-resistant. We performed mitochondrial swelling assays on mitochondria isolated from pancreas 24 hours after treating the mice with DMSO or simvastatin. Mitochondria were resuspended in mitochondria swelling buffer and the decrease in absorbance was monitored after the addition of calcium (100 µM). Mitochondria from simvastatin-treated mice showed a smaller decrease in absorbance over the 20-minute observation (Fig. 6e), suggesting that simvastatin-induced mitophagy resulted in a mitochondrial population that was more resistant to calcium overload.

Figure 6. Simvastatin induces mitophagy for mitochondrial quality control. Mice were treated with Simvastatin for different hours viz. 3-hour, 6-hour, 12 hours and 24 hours and sacrificed after the indicated time point. (a) Western blot for pancreatic tissue lysate showing the expression of mitophagy markers, Parkin and Mfn2, and autophagy markers, p62 and LC3. COX IV is used as mitochondrial content marker in the lysate. Ponceau S staining was used as loading control. (b) Western blot for different mitochondrial proteins
involved in OXPHOS complex assembly (ATP5A, UQRC2, MTCO1, SDHB and NDUFB8) and used as total mitochondrial content present in the lysate at the indicated time points. (c) Mice were given DMSO or Simvastatin (20mg/kg, i.p.) for 6 hours followed by Saline or Chloroquine (40mg/kg, i.p.) for 4 hours and pancreatic tissue was collected for post-nuclear supernatant. Representative Western blot showing the protein expression of p62, ATP5A, UQCRC2, MTCO1, SDHB and NDUFB8. (d) Bar graph represents the ratio of Ponceaus S staining normalized p62 protein expression from Saline and Chloroquine treated mice given either DMSO or Simvastatin. (e) Mitochondrial swelling was used to assess the pancreatic mitochondrial quality from the mice treated with DMSO or Simvastatin (20 mg/kg) for 24 hours. Bar graph represents the decrease in absorbance (540 nm) after incubating the isolated mitochondria with 100 uM Ca2+ for 20 minutes. Values in graphs are represented as Mean ± SD, *p< 0.05, **p< 0.01 vs DMSO control.

**mtDNA release during acute pancreatitis**

Cells such as neutrophils with dysfunctional autophagosome-lysosome fusion are known to release mtDNA and associated mitochondrial transcription factor, TFAM, into the blood(13). As pancreatitis showed a similar impaired autophagic flux, we next tested if there is increased mtDNA release with pancreatitis and whether simvastatin can abrogate these effects. Using plasma obtained from healthy individuals and patients with acute pancreatitis, we determined mtDNA content assessed by amplification of ND4 normalized to a spiked in control plasmid (GFP). Our findings revealed higher levels of mtDNA in plasma from acute pancreatitis patients compared to normal controls (Fig. 7a). We next determined if simvastatin could reduce mtDNA release into plasma in the cerulein-induced acute pancreatitis model in mice. We detected a tendential increase (p=0.xx) in mtDNA in plasma samples from cerulein-exposed mice, which was attenuated with simvastatin treatment (Fig. 7b). These findings support the importance of efficient mitophagy and phagosome-lysosome fusion in preventing the release of inflammatory molecules such as mtDNA.
Figure 7. Effects of pancreatitis and simvastatin treatment on mtDNA release. (a) Quantification of mtDNA release in blood assessed by qRT-PCR of mitochondrial gene ND4 (fold change) present in plasma of normal control and acute pancreatitis patients (n=8). Spiking GFP was used as normalized control. (b) Bar graph represents the quantification of ND4 gene, normalized to spiking GFP control, from plasma of mice treated with DMSO or Simvastatin for 24 hours followed by Saline or Cerulein (7 hourly) injections. Values in graphs are represented as Mean ± SD, *p< 0.05 vs Control.

Simvastatin protective effects are abolished with parkin deletion

As previously shown by our group(12) in ischemia-reperfusion model of cardiac injury, simvastatin protection is parkin-dependent. We elucidated the role of parkin in pancreatic injury and whether parkin is required for simvastatin-induced protection. We used the same model as previously described in Fig. 1a and compared the effects of simvastatin in preventing pancreatic injury in parkin knockout (PKO) mice as compared to wild type mice. Histopathological analysis revealed that while at baseline, pancreatic tissue had normal appearance, cerulein administration resulted in severe edema, infiltration of inflammatory cells, and necrotic cell death in parkin knockout mice compared to wild type mice. Moreover, simvastatin’s protective effects against cerulein-induced pancreatitis were abolished with parkin deletion (Fig. 8a). Interestingly, there was increased infiltration of inflammatory cells in Parkin knockout mice treated with simvastatin compared to PKO
mice treated with DMSO+cerulein (Fig. 8a). Examination of p62 and AMPK revealed that both proteins were upregulated in parkin knockout mice even in control saline groups, while simvastatin pretreatment further increased p62 and AMPK during pancreatitis (Fig. 8b). We also noted increased accumulation of LC3 in parkin knockout mice pretreated with simvastatin.

**Figure 8. Simvastatin-mediated protection is dependent on Parkin.** Acute pancreatitis was induced in wild type (wt-mice) and Parkin knock out mice (Parkin KO mice) using cerulein 7 hourly injection model. DMSO or Simvastatin (20 mg/kg) pretreatment was given for 24 hours before inducing acute pancreatitis in mice. (a) Representative images of H&E staining of pancreatic tissue from different groups. (b) Western blot showing the protein expression of LC3, p62, COX IV and AMPK.

**Network analysis for biological processes**

Our results support the role of impaired autophagic flux in pancreatitis and the effect of simvastatin to resolve the impaired flux by activating AMPK and ULK-1. We next tried to determine the biological processes which can be affected by AMPK and ULK-1 dysregulation. We initially generated a protein-protein interaction network from whole human proteome showing 156 and 55 interacting proteins of AMPK and ULK-1 respectively (Figure 9). Based on the network, biological processes involved were extracted using ClueGo-CluePedia. Shared interacting proteins with ULK1 and PRKAA2 are shown as small circles in red font, relatively small or big hubs for enriched pathway terms and biological processes. The size of the nodes refers to significance through p-
values and the links indicate gene participation to the pathway terms. The latter are clustered and colored to emphasize similarly annotated proteins and biological processes. Many of the enriched biological processes especially mTOR signaling, p53 signaling, and energy homeostasis are the key hallmarks of pancreatic cancer progression, underscoring the importance of activating AMPK and ULK-1 by simvastatin to mitigate pancreatitis and potentially pancreatic cancer.

**Figure 9.** BINGO/ClueGo Network analysis for protein-protein interaction of AMPK (PRKAA2) and ULK1 to identify different biological processes generated using Cytoscape. Some of these processes viz. TOR signaling, energy homeostasis and autophagy are known to have a role in progression to pancreatic cancer.

**What opportunities for training and professional development has the project provided?**

The project provided training for postdoctoral fellow, Honit Piplani, PhD, in a novel area in pancreatic research with Drs. Pandol and Gottlieb who are experts in pancreatitis and mitophagy, respectively. The project will allow him to be a leader in this new field or research.
What do you plan to do during the next reporting period to accomplish the goals?

| We are currently completing experiments planned and preparing and manuscript for publication of the results. We also plan to prepare abstracts for submission so that the work can be considered for presentations at national meetings. |

### 4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

| This project will have an impact on the clinical treatment of pancreatitis where there are no currently available treatments. |

What was the impact on other disciplines?

| This project will have an impact on the clinical treatment of other inflammatory disorders where there are no currently available treatments. |

What was the impact on technology transfer?

| Nothing to report |

What was the impact on society beyond science and technology?

| Nothing to report |

### 5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

| Nothing to report |

Actual or anticipated problems or delays and actions or plans to resolve them

| Nothing to report |
Changes that had a significant impact on expenditures

| Nothing to report |

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

| Nothing to report |

Significant changes in use or care of vertebrate animals

| Nothing to report |

Significant changes in use of biohazards and/or select agents

| Nothing to report |

6. PRODUCTS:

- Publications, conference papers, and presentations

  | Journal publications. |
  | Nothing to report |
  | Books or other non-periodical, one-time publications. |
  | Nothing to report |

- Other publications, conference papers and presentations.

  | Nothing to report |

- Website(s) or other Internet site(s)

  | Nothing to report |
7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Stephen Pandol, MD
Site PI
Months 2
Develops goals and direction for the project. Oversees direction and analyzes results.

Roberta Gottlieb, MD
Site PI
Months 2
Provides expertise in cell biology, mitophagy and autophagic function. Oversees direction and analyzes results.

Honit Piplani PhD
Postdoctoral fellow
Months 12
Performs experiments, organizes and analyzes results interacting with site PIs and Dr. Lugea.

Aurelia Lugea PhD
Research Scientist
Months 2
Provides expertise on animal models, treatments, measurements of pathways.
Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES:

References


