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Role of NOS and NOX-4 on pancreatic signal transduction

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Role of NOS and NOX-4 on Pancreatic Signal Transduction

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Abstract:

Nitric oxide is a signaling molecule which has been shown to be a necessary component for secretion. The actual process of exocytosis that leads to secretion is of yet unknown. This project attempted to understand the protein complex NOX-4 as well as NO, a product of NOS, in their signaling roles that lead to the exocytosis and secretion of digestive enzymes from pancreatic acinar cells. There were several objectives of this project which included determining the location of NOX-4, how hormonal stimulation affected recruitment to the zymogen granules and if NOX-4 was necessary for secretion to occur. Also investigated was the presence of nitrosylated proteins on zymogen granules, an activation strategy which utilizes the product of NOS and NOX-4. This project was completed using lysed pancreatic cells which had been stimulated with CCK. The liberated zymogen granules were collected, lysed and subsequently subjected to SDS-PAGE and western blotting procedures. Antibodies specific for the protein in question, NOX-4, were applied, allowing us to definitively conclude the presence and location of NOX-4. Western blotting procedures also assayed for nitrosylated zymogen granule proteins. The results of these procedures could be quantified using Scion software to identify how CCK stimulation affected NOX-4 recruitment. These experiments allowed us to conclude that NOX-4 resides on the zymogen granule and that there is an increased recruitment for a limited period of time in vitro when subjected to CCK. Also discovered was that, upon inhibition of NOX-4, secretion decreases. This leads to the conclusion that NOX-4 is in fact a vital component in the process of secretion. Finally, western blotting revealed the presence of nitrosylated proteins on the zymogen granule, allowing us to hypothesize how the actual event of exocytosis occurs.

We are using the pancreas as a model system of secretion because it utilizes NO, as so many other physiological systems do as well. The findings obtained therefore provide information for systems such as the nervous, cardiovascular and immune systems which also utilize secretion. Though this project has allowed us to learn how NOX-4 and NOS contribute to secretion, the many complex components of the exocytosis and secretion process must be further investigated to obtain a full understanding of the exocrine functions of the pancreas.

Introduction:

Secretion is the process whereby cells release hormones, antibodies, neurotransmitters, and/or enzymes into the environment. This process occurs when stimulation of a cell causes resident secretory vesicles to bind to the cell's plasma membrane, causing an exocytotic release of the contents. The pancreas is involved in this exocrine secretion process. Specifically, acinar cells within the pancreas regulate secretion by using a complex signal transduction cascade, causing the migration of zymogen granules (secretory vesicles) to the plasma membrane where they fuse and release stored digestive macromolecules into the lumen of a duct that leads to the small intestine. This cascade occurs in response to the intestinal hormone cholecystokinin (CCK). Coupled with the release of digestive enzymes is the process of secreting a fluid rich in sodium bicarbonate. This fluid neutralizes stomach acid and provides the optimal pH for enzymatic digestion.

Previous research has shown that acinar cells produce and respond to nitric oxide (NO) in their signaling cascade triggered by stimulation with CCK (Ervin 2005). NO is made through a series of chemical reactions which are catalyzed by the enzyme nitric oxide synthase (NOS) (Eu 1999). NADPH oxidase 4 (NOX-4) is another important component in the secretion process. NOX-4 reduces molecular oxygen, producing a superoxide (O_2^-). Together, the NO and O_2^- products rapidly combine to form a peroxynitrate (Eu 1999). This peroxynitrate then may nitrosylate different molecules in the acinar cell. Our hypothesis suggests that peroxynitrosylation leads to exocytosis and secretion.

The aim of our research is to understand NO as well as NOX-4 in their signaling roles leading to secretion of digestive enzymes. In particular, we hope to determine if NOX-4 can promote the loss (through the utilization during production of peroxynitrates) of NO, where NOX-4 is localized, and if zymogen granule proteins are being nitrosylated in order to initiate the exocytosis process. The nitrosylation of these proteins may have roles in cytoskeletal reorganization (Sollner, Sequeira 2003), regulation of calcium receptors (Jamieson, et al. 1997; Favre, et al. 1998), and/or regulation of potassium channels (calcium and potassium are necessary ion components to secretion) (Duprat, et al. 2005). We also anticipate providing evidence that NOX-4 is in fact required for pancreatic secretion.

We are using the pancreas as a model system of secretion. Since NO is used by several physiological systems, the findings we obtain may be applicable to many areas of the body that utilize secretion including, but not limited to, the nervous, cardiovascular and immune systems (Maxey, Johnson 2002).

Materials and Methods:

Pancreatic acinar cells were rinsed with a Krebs-Ringer HEPES buffer solution and placed in a 37° C water bath. The cells were subsequently transferred into separate tubes for control and CCK treatment (2 pM). After homogenization, the acinar cell mixture was placed in a nitrogen cavitation bomb (at 750 p.s.i.) in order for the cells to be lysed by depressurization. The liberated cellular contents were collected and mixed with a Percoll solution (1.7 ml 10x Percoll buffer, 2.2 ml sucrose, 16 ml Percoll). The mixture was centrifuged at 20,000 x g for 20 minutes. The zymogen granules, which formed a

dense layer toward the bottom of the centrifugation tube, were collected and added to cold homogenization buffer (250 mM sucrose, 40 mM MOPS, 0.1 mM EGTA, 0.1 mM $MgSO_4$). The sample was again centrifuged for 15 minutes at 2,000 x g, where the zymogen granules formed a pellet. The samples were lysed and diluted with laemelli buffer to be used subsequently for SDS Polyacrylamide Gel Electrophoresis (PAGE) and western blotting procedures.

SDS-PAGE is a technique which involves applying an electrical current to a polyacrylamide gel, thereby separating mixtures of proteins based on their size and charge. After boiling and denaturing the zymogen granule samples, they were cooled. 20 ul of each sample were loaded into a 10% PAGE gel and electrophoresed in running buffer (750 ml ddH_2O, 3.03 g Tris, 14.4 g glycine, 1.0 g SDS) at 110 V for 70 minutes. After the zymogen granule proteins were separated, they were transferred onto nitrocellulose paper by electroblotting at 110 V for 70 minutes in transfer buffer (750 ml ddH_2O, 3.03 g Tris, 14.4 g glycine, 200 ml ethanol).

Once the samples had been successfully transferred onto the nitrocellulose paper, they were then subjected to a blocking procedure, using a 10% solution of nonfat dry milk and Tween-Tris Buffer Solution (TTBS; 750 ml ddH_2O, 1.21 g Tris, 14.6 g NaCl, 0.5 ml Tween, pH 7.6). This incubation blocked any exposed reactive sites on the nitrocellulose paper to restrict the probing to the protein of interest, NOX-4. After blocking the nitrocellulose paper on a rocker for 60 minutes at room temperature, the nitrocellulose paper was washed with TTBS 6 times, 5 minutes for each wash, changing the TTBS rinse between each wash. The nitrocellulose was then incubated (on a rocker) for 60 minutes at room temperature with a 1:2000 dilution of rabbit polyclonal primary

antibody solution (3.8 ml TTBS, 200 ul 10% milk solution, 2 ul antibody). This antibody was specific for NOX-4. The nitrocellulose paper was again subjected to 6 TTBS washes on a rocker, each 5 minutes in length, again changing the TTBS solution between rinses. A 1:4000 dilution of secondary antibody solution (3.8 ml TTBS, 200 ul 10% milk solution, 1 ul secondary antibody) was applied; the nitrocellulose rocked at room temperature for 60 minutes. The secondary antibody was an anti-rabbit antibody that was ligated to an enzyme called horseradish peroxidase. This antibody will selectively bind to the primary antibody, which in turn is bound to the NOX-4.

The nitrocellulose was washed with TTBS in an identical procedure as previously stated to remove unbound secondary antibody. When complete, 0.75 ml of the chemiluminescent chemical, Luminol, was added to the nitrocellulose and allowed to incubate for 1 minute. Horseradish peroxidase oxidizes the Luminol resulting in the emission of photons of light. The presence of these photons were detected by exposure of the nitrocellulose paper to X-ray film for 15 to 25 minutes. The film was subsequently developed using an automated film developer. These resulting bands then allow us to conclude whether or not NOX-4 is associated with the pancreatic zymogen granules.

Results:

The first goal of this project was to show that acinar cells use NO. In a previous experiment, NO induced DAF fluorescence was identified in acinar cells that had been stimulated with CCK (figure 1). The cells fluoresced brightly on the outer edges, but in regions where stimulation had caused a high concentration of zymogen granules, there was an absence of NO induced fluorescence. Therefore, it was concluded that zymogen

granules were utilizing the NO. This conclusion led us to further research what was using the NO. Since NOX-4 was known to create reactive oxygen species that combine with NO, we further investigated if NOX-4 resided on zymogen granules. It is known that NOX-4 is made of a complex of proteins that includes gp91 phox, p22 phox, p47 phox and p67 phox subunits. However, the one that we assayed by western blots was the p47 phox subunit (figure 2). To determine the location of NOX-4, the zymogen granule samples were subjected to SDS-PAGE and western blotting. Figure 3 shows the result of this western blot. The bands clearly show that NOX-4 was present on isolated zymogen granules. Therefore, since we used a NOX-4 specific antibody, we can concretely conclude that NOX-4 and the p47 phox subunit resides on the zymogen granule.

CCK has long been recognized as one of the main hormonal stimulants for pancreatic enzyme secretion. Originally, one of the goals of this project was to determine how CCK affected the recruitment of NOX-4 to the zymogen granule. Since it was confirmed that NOX-4 resided on the secretory vesicle, research then focused on how CCK affected the recruitment to zymogen granules. By assaying NOX-4 concentration on zymogen granules in a timescale of 0 (control), 1, 3, 5, 10, and 20 minutes after stimulation with CCK, it was proven that the recruitment of NOX-4 increased progressively until its peak at 5 minutes. After 5 minutes, there was an observable decrease (time 1-10 minutes shown in figure 4). The western blot data in figure 4 was then quantified (figure 5). The quantified data shows that the bands were most dense at the 5 minute time point. This means that CCK had stimulated the largest amount of NOX-4 recruitment to zymogen granule 5 minutes after exposure.

The next goal of this project was to determine if NOX-4 is necessary for secretion. In a previous experiment, apocyanine, a NOX-4 inhibitor, was used to observe its effects on amylase secretion. Figure 6 shows that with the addition of the inhibitor, secretion decreases. Because of this data, we have evidence that NOX-4 activity is crucial for secretion to occur.

With the knowledge that NOX-4 is present on zymogen granules and that it is utilizing NO, we propose that peroxynitrates are being formed. The suggested targets of nitrosylation by these peroxynitrates was proteins on the zymogen granule. A western blot was performed to assay for the presence of nitrosylated proteins on the zymogen granule. Figure 7 demonstrates the results that there are indeed nitrosylated proteins on the zymogen granule, however the identity of the proteins are as of yet unknown.

Discussion:

In this study, pancreatic acinar cells were stimulated to induce zymogen granule production, migration, and exocytosis. A major question that hinders the research of the pancreas is how zymogen granules, when stimulated by the intestinal hormone CCK, move from one area of the cell to another to release their contents into the lumen of a duct that leads to the small intestine. The p47 phox subunit causes NOX-4 to be of interest because it associates with cortactin, a protein which induces cytoskeletal rearrangement which can facilitate the movement of molecules and organelles within a cell (Touyz, et al. 2005). Since this association protein is part of NOX-4 and this project has proven that NOX-4 resides on the zymogen granule, we now have a clearer picture of

how the zymogen granules may migrate to the plasma membrane of the acinar cell where exocytosis occurs.

Because this research shows that NOX-4 resides on zymogen granules, it is necessary to investigate its function as well as the importance of its role in secretion. Previous experiments have shown that NOX-4 produces superoxides (Ago, et al. 2004). NO, a product of the enzyme NOS, has been shown to be produced in other areas of the cell rather than at the zymogen granule itself. It was observed that NO levels were very low in areas of high zymogen granule concentration (figure 1). Purportedly, the superoxide produced by NOX-4 and NO rapidly combine, forming a peroxynitrate. This in turn caused a decrease in the fluorescent signal from the NO-sensitive probe, DAF

Our results led us to the conclusion that NOX-4, located on the zymogen granule, must be the cause of the NO sink. Because the greater the CCK stimulation (and greater amount of zymogen granules present) shows progressively smaller presence of NO, we can propose that NOX-4 is indeed contributing to the decrease of NO in the region of zymogen granules.

NOX-4 has been proven to be necessary in secretion, due to experiments with the NOX inhibitor, apocyanine. Amylase, an enzyme secreted by the pancreas, is an important protein which helps break down starches. Using amylase secretion to determine the importance of NOX-4 to this process is ideal because we can measure the rate of its secretion when NOX-4 is not functioning. When NOX-4 was inhibited, a marked decrease in the amount of amylase secretion was observed (figure 6). Therefore, we know NOX-4 must be functioning (and present) in order for secretion to occur.

Because we have confirmed the presence of nitrosylated proteins on the zymogen granule (figure 7), we can hypothesize how the actual event of exocytosis occurs. After peroxynitrates nitrosylate certain proteins on the zymogen granule, the plasma membrane may undergo cytoskeletal reorganization necessary for exocytosis. Other possible consequences of nitrosylation may be regulation of ryanodine receptors (Ca^{2+} receptors), and/or regulation of K^+ channels; Ca^{2+} and K^+ being necessary ions for secretion cascades to occur.

K^+ is relevant to pancreatic exocytosis because zymogen granules have K^+ channels on their membranes. Ca^{2+}, whose release may be stimulated with NO, can activate these K^+ channels (Favre, et al. 1998). Because K^+ concentrations are higher in the zymogen granule, when Ca^{2+} opens the channels, the gradient will cause K^+ to flow into the zymogen granule lumen. This causes water to follow and the zymogen granule will swell. With the zymogen granule being near the cell plasma membrane, the increased surface tension will eventually cause the zymogen granule membrane to bubble out, fusing with the cell plasma membrane and exocytosis occurs. Ca^{2+} may also have other roles in the exocytosis process, triggering the activation of ion channels elsewhere (Eu, et al. 1999; Nadin, et al. 1989).

Although there is good evidence that exocytosis may occur because these mechanisms, we are unable to conclusively state that the peroxynitrates are nitrosylating proteins on the cell plasma membrane, ryanodine receptors and/or K^+ channels. Therefore, identifying the proteins that are being nitrosylated will be the target of future research projects.

Though much has been learned about how NOX-4 and NOS contribute to secretion through this study thus far, many complex components of this process must continue to be investigated to obtain a full understanding of the exocrine functions of the pancreas.

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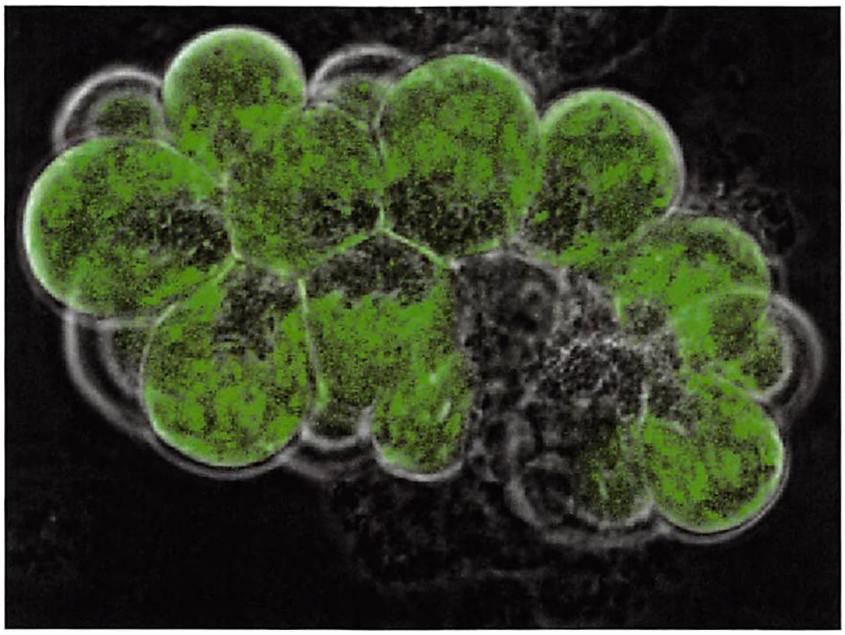

Figure 1: NO induced DAF fluorescence in CCK stimulated pancreatic acinar cells. The darker regions towards the inner part of the cells displays the absence of NO due to high zymogen granule concentration. NOX-4 on the zymogen granules is producing a superoxide which combines with NO, thereby reducing the fluorescence of that area.

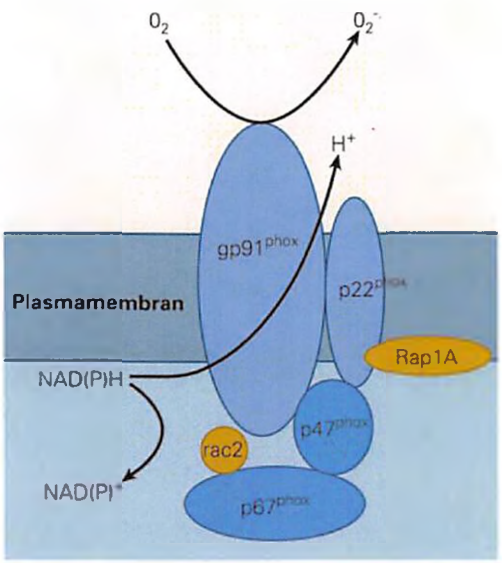

Figure 2: Subunits of NOX-4; of particular importance is the p47 phox protein. p47 phox is the subunit that was assayed for by western blotting during this project. This subunit has the ability to associate with cortactin, a protein which promotes movement of molecules and organelles within a cell.

Figure 3: Western Blot of Demonstrating the Presence of NOX-4 on Isolated Pancreatic Zymogen Granules.

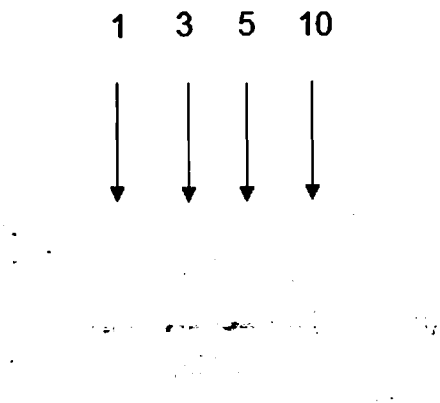

Figure 4: Western blot of NOX-4 recruitment during CCK stimulation timescale of 0, 1, 3, 5, 10, and 20 minutes. Times 1-10 minutes can be seen in this western blot.

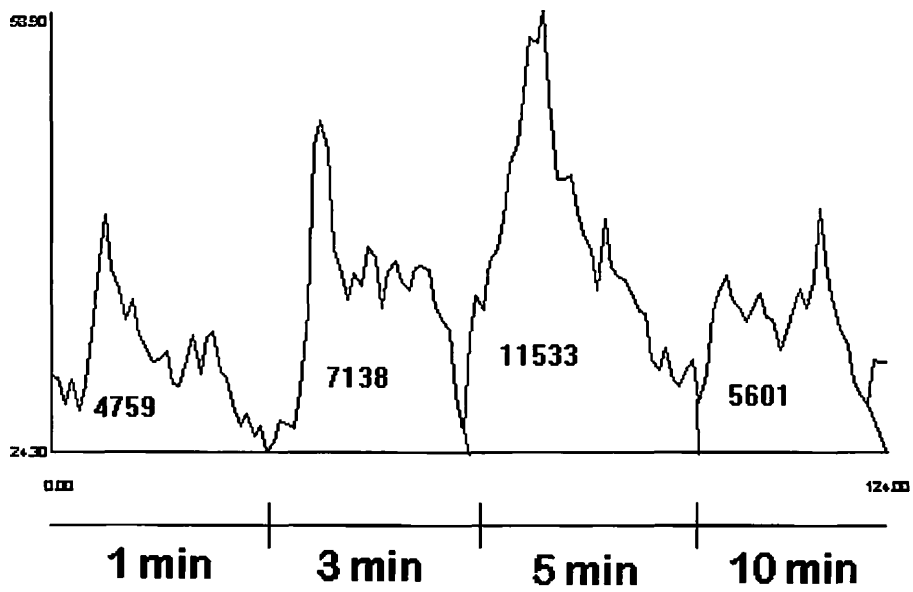

Figure 5: Quantified Data of Western Blot of a CCK Stimulation Timescale. At 5 minutes, band density is at its greatest, NOX-4 recruitment is at its highest point.

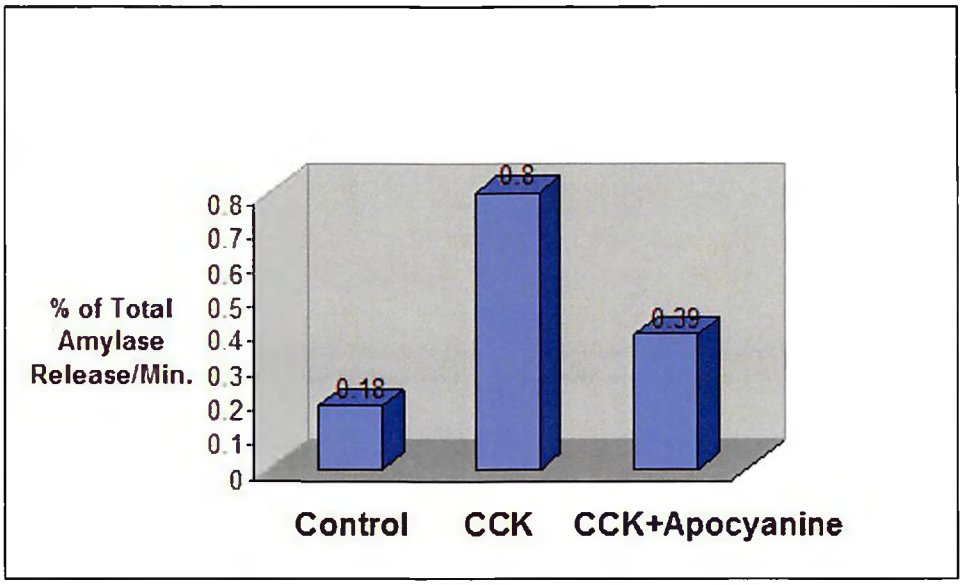

Figure 6: NOX-4 inhibition reduces CCK induced amylase secretion. With the addition of the NOX-4 inhibitor apocyanine, amylase secretion decreases. Measuring the rate amylase secretion is ideal to determine how secretion is affected when NOX-4 is not functioning.

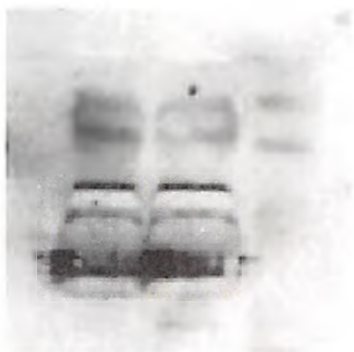

Figure 7: Western blot of displaying evidence of nitrosylated proteins on pancreatic zymogen granules.

University Honors Program

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