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The Role of Nitric Oxide in Pancreatic Cell Secretion

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The Role of Nitric Oxide and Rac in Pancreatic Cell Secretion

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ABSTRACT

The pancreas is a model tissue that has been extensively studied in the lab of my research supervisor. They have shown that nitric oxide is produced by the pancreas and is necessary for sectretion; however, the mechanism of action remained to be identified. The goal of my research over the past year was to investigate the signaling role of nitric oxide, the peroxynitrite producing enzyme NADPH oxidase NOX-4, and the nitrosylation of target proteins on the zymogen granule membrane as a potential mechanism to regulate cytoskeletal reorganization and migration of the secretory vesicles. In addition, I performed research to understand the role of the small monomeric G-protein Rac in the process of secretion and cytoskeletal reorganization. Pancreatic zymogen granules were isolated from both control and CCK-stimulated pancreatic acinar cells. A reproducible nitrosylated band was identified following stimulation of the pancreatic acinar cells with CCK. In addition, these granules were probed for Rac activity by western blots. The results show a significant increase in the level of Rac G-protein following stimulation, which suggests that there is an increased level of superoxide production when cells are stimulated, thereby providing the superoxides necessary in the hypothesized mechanism of nitrosylation.

Introduction

The research involved in this project revolved around the pancreas as a model organ to study the physiological mechanisms that control cellular secretion. Therefore, it is critical to first examine the pancreas and its role in physiology. The two types of cells that dominate the mass of the pancreas are the islets of Langerhans and pancreatic acinar cells. The former function in the endocrine system, producing and secreting hormones, whereas the latter function in the process of digestion, producing and secrete digestive enzymes. The islets of Langerhans are primarily concerned with the hormones glucagon, insulin, and somatostatin. The acinar cells empty into pancreatic ducts, which lead to the small intestine. Most people generally associate the pancreas with diabetes and the endocrine role of the pancreas, which is expected, considering that the relationship between this organ and diabetes was first discovered back in 1890 by Oscar Minkowski of the University of Strasbourg, and since this time, millions of dollars have been invested into the research of diabetes and finding solutions and cures for the metabolic and hormonal condition (Silverthorn, 2010). Yet, the role of the pancreas in the endocrine system is only one facet of its functioning, and actually the mass of the pancreas that is devoted to its endocrine role makes up only 2% of the total mass—the rest is devoted to the production and secretion of digestive enzymes to the small intestine to aid in the process of digestion (Silverthorn, 2010).

This second role of secretion is critical because biological secretion plays a wide range of roles in the body from tear and saliva production to digestive enzymes and neurological communication. In all cases, the cells produce a product that must be released to the outside and this is accomplished by packaging the product into secretory vesicles inside the cell. Upon

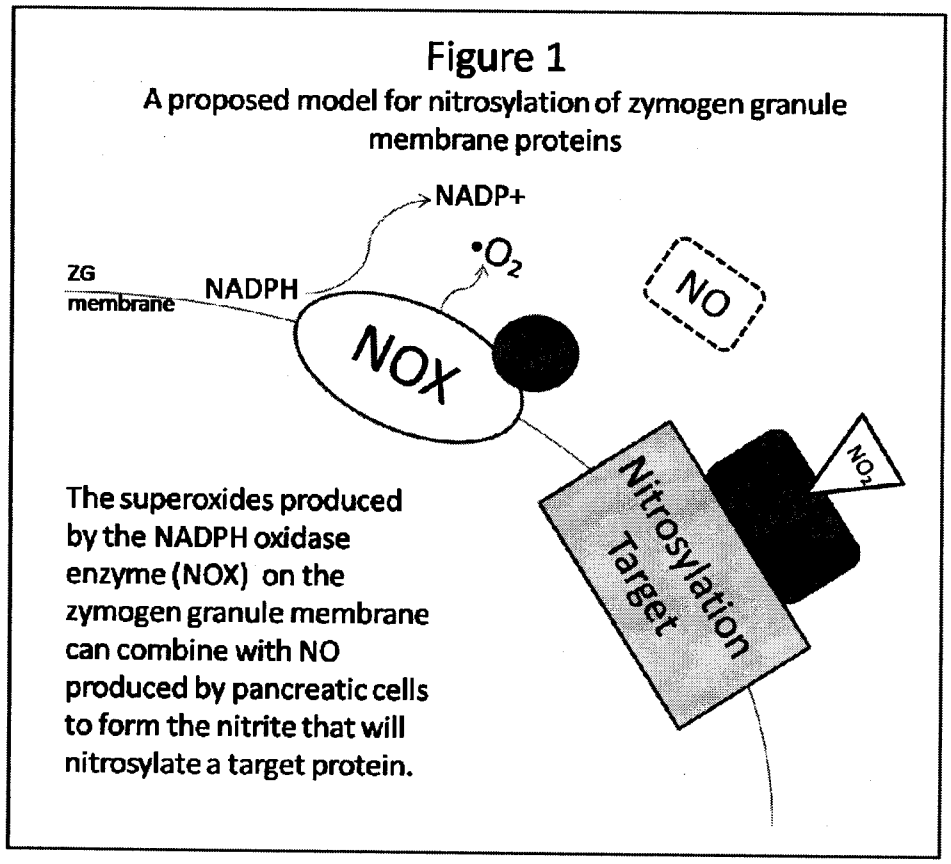
stimulation, these vesicles will fuse with the exterior plasma membrane and release the contents. The compounds then can act either locally on neighboring cells or can be transported in the blood (ie, hormones) where they can act at distant sites in the body. From the experimental perspective, the pancreas is an ideal secretory representative since the tissue focuses almost exclusively on this process. Physiological procedures are redundant in the body, and therefore knowledge of the mechanism of secretion in the pancreas is easily applicable to all other secretory tissues as well, with the only change being the substance to be released. Thus, the pancreatic cell serves as a great model to perform research on secretion.

The process of secretion is dependent upon signal transduction pathways. These pathways are located at the plasma membrane and allow for signals to be communicated from the inside of the cell to the outside of the cell, or vice versa. Various intra- or extra-cellular molecules can function as the signal, but as a rule, these molecules involved in the pathway are never passed directly through the membrane. Instead, these signal molecules bind to receptors, which then undergo conformational changes to pass along the message to the next intermediate (called secondary messengers), and this process continues until the desired response is triggered on the opposite side of the membrane. This allows for the cell to maintain its selective permeability, yet also allow messages to be sent within the cell without the physical presence of the signal molecule. In addition, the various steps involved in a signal transduction cascade make possible the amplification of the signal. For example, each receptor can influence many G-proteins, and each G-protein can influence many effectors, resulting in a massive amplification, which is very useful in the process of secretion in the pancreas, since the pancreas must secrete massive amounts of digestive enzymes in a simultaneous fashion to aid the small intestine in digestion.

The process of secretion is one of the most well known examples of signal transduction, involving a specific signal transduction cascade. This cascade first involves a signal molecule binding to a seven transmembrane domain receptor, which is bound to a cytoplasmic G-protein. This G-protein will then undergo a conformational change due to the binding of the molecule to the receptor, and this in turn causes the G-protein to release GDP and bind GTP, causing yet another conformational change to result in the detachment of various subunits of the G-protein. These subunits are then free to diffuse within the plane of the membrane and can interact allosterically with the target effector protein (the secondary messenger). After this initial binding, a specific signal transduction cascade relates the signal to the interior of the cell, where the response is processed. The stimulation of this cascade and the proteins involved in the cascade were important in the research of the project because nitric oxide functions as a signaling molecule to stimulate secretion.

The particular objective of this project was to study nitric oxide, a signaling molecule present in the pancreas that is now known to be involved in a wide range of physiological events. Over-production of this compound has several complications: it can lead to the death of cells during stress, it is associated with neurological pathologies such as Alzheimer Disease, and it can also be utilized in a positive fashion by immune cells to kill pathogens or cancer cells (Szabo et al., 2007). Alternatively underproduction of nitric oxide can lead to clinical health problems such as impaired blood flow to tissues. Therefore, strict control of nitric oxide is necessary and it is believed that the pancreas is a good model to study its action. Dr. Kenneth Gasser's laboratory has previously demonstrated the ability of the pancreas to produce nitric oxide as well as superoxides via the NADPH oxidase enzyme (NOX) (Connelly, et al., 2006). The production of nitric oxide is simple: the enzyme nitric oxide synthase (NOS) helps synthesize nitric oxide

with the substrates of oxygen and the amino acid arginine. The localization of the NOX enzyme, responsible for the production of superoxides, a reactive oxygen species, to the secretory granules is significant in that this provides the spatial opportunity for nitric oxide molecules and superoxides to chemically combine at this site and form peroxynitrites, as shown in the cartoon in Figure 1 below:

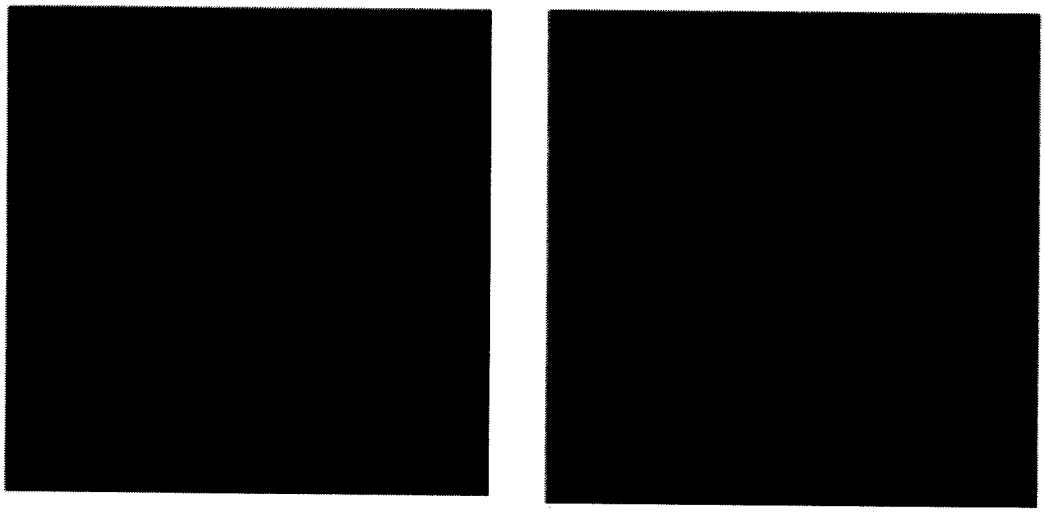


Consistent with this suggestion, previous work in Dr. Gasser's lab also shows a decrease in nitric oxide concentration in the vicinity of the secretory vesicles, suggesting that the nitric oxide is being consumed or modified (such as into a peroxynitrite) by the vesicles. The hypothesis is that this peroxynitrite produced at the surface of the pancreatic secretory vesicle binds to the amino acid tyrosine on specific proteins that regulate secretion. Prior research in Dr. Gasser's lab has shown the increase in peroxynitrite in pancreatic cells upon stimulation with the

physiological hormone, cholecystokinin (CCK), as shown below (note, the increase in fluorescence indicates an increase in peroxynitrite):

Figure 2

Confocal images of peroxynitrite location in pancreatic acniar cells under control and CCK conditions



Control CCK stimulation

Similarly to the tyrosine kinase signal transduction pathway, the nitrosylation of a tyrosine residue could have the same function to initiate the cascade for secretion. Thus, this nitrosylation, as proposed in this project, may cause a physiologically relevant change in protein structure, and this change in structure will alter the protein's ability to facilitate secretion. Clearly, peroxynitrites are very reactive compounds and must be regulated or else they will cause extensive cellular damage and cell death (Murphy 1999). Therefore, the system must target the peroxynitrite to specific tyrosines and they must not be made available to proteins at random. The targeted nature of the peroxynitrosylation could be achieved by the direct association of the

NOX enzyme with the targeted secretory protein. In this way, the superoxide and peroxynitrite would be produced immediately adjacent to the tyrosine to be modified, preventing any consequences that a highly oxidative molecule such as a peroxynitrite could have if it needed to diffuse a large distance.

The goal of this project deals with the relation between the pancreas and nitric oxide in order to study the method of secretion. Specifically, the goal was to monitor the level of nitrosylation in actively secreting pancreatic cells versus quiescent control cells. In this project, as stated before, nitrosylation was suggested as an unexplored possibility for the mechanism by which pancreatic cells are induced to secrete digestive enzymes. While nitric oxide is involved in many major physiological systems throughout the body, it is also specifically of importance in the pancreas where it is produced in the mechanism of secretion. In addition, as also stated before, the pancreas also has the ability to produce superoxides via the NADPH oxidase enzyme, NOX. Since the nitric oxide and the superoxides produced by the NADPH oxidase enzyme are both localized to the secretory granules of the pancreatic cells, there is a spatial opportunity for these two products to combine and form a peroxynitrite, which can then bind to the amino acid tyrosine, thereby nitrosylating the granule and inducing it to be secreted. Tyrosine nitrosylation has already been demonstrated as chemically possible and has been previously measured and reported (Reiter et al, 2000). While superoxides could be dangerous to a cell, their production can also function in an effective regulatory system for cellular secretion, and thus be useful in pancreatic cells.

Another protein, Rac, a small G-protein in cells, seems to play several roles in pancreatic secretion. It has been demonstrated that Rac is an essential component in superoxide formation (Sohn et al., 2000). This suggests that Rac should also be measured in higher levels in those

cells stimulated for secretion, further supporting the hypothesis of nitrosylation as a regulation mechanism of secretion in pancreatic cells. In addition, Rac has also been known to be an essential part of the mechanism in cytoskeletal rearrangement, and could thus be an essential part of cytoskeletal reorganization during exocytitic processes such as secretion of the digestive enzymes from the migrated zymogen granules (Price et al., 1995). Yet another apparent role is Rac's ability to regulate NOX activity (Valentin, et al., 2001). Given these roles of Rac in the cell, by measuring this protein under normal and stimulated conditions its role in cellular secretion could be verified. Once verified, future experimentation could be organized to determine whether the activity of Rac in these three areas happens simultaneously and is interdependent or whether each process is independent.

This project used two separate experimental approaches to determine whether superoxides are indeed used as a means of nitrosylating zymogen granules, and thus secretion:

1) Determine whether or not the level of nitrosylated proteins on zymogen granules increases when treated and stimulated with the physiological hormone, cholecystokinin (CCK).
2) Determine whether or not the level of the small G-protein Rac, essential in superoxide formation and cytoskeletal rearrangement, increases in zymogen granules when the granules are treated and stimulated with CCK.

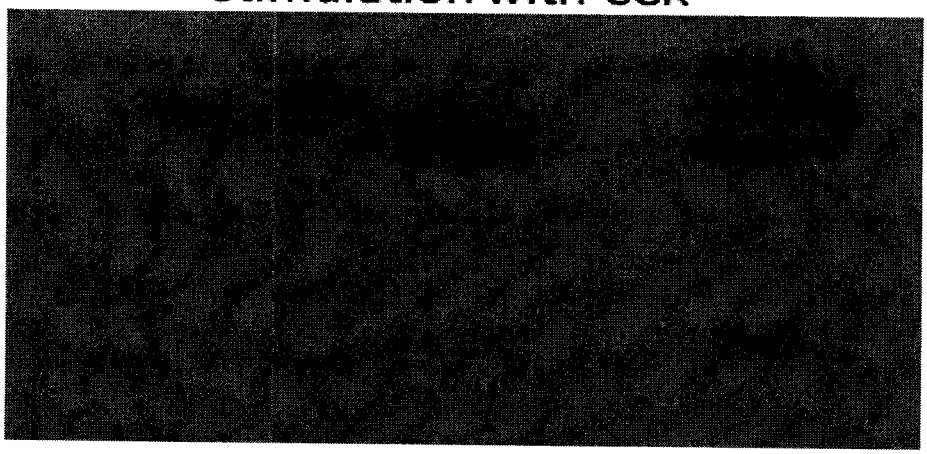
Methods and Results

The first step in the experiments involved isolating the pancreas from the rat. This technique was performed under controlled and stimulated conditions (treated with a 2.0nM

concentration of the physiological hormone cholecystokinin, CCK). The samples were taken from two different rats on separate days, and the samples labeled accordingly (04.07.2010 and 04.15.2010, each with a control and stimulated sample). After this step, the zymogen granules from the pancreas were isolated using density gradient centrifugation, a technique that allows for isolation of pancreatic zymogen granules from pancreatic acinar cells into a solution of Percoll buffer. After isolation of the pancreatic cells from the rats, they were transferred to a culture tube and washed with 20ml ice-cold homogenization buffer. After mincing the pancreas with a razor, the cells were ground in a glass-teflon potter. Then, the cells were placed in a nitrogen cavitation bomb to be pressurized for one minute to 750 p.s.i. Finally, the homogenized and lysed cells were mixed with 16 ml of Percoll solution. Then, the purified granules were placed in a detergent and the amount of protein normalized within each sample via a protein assay. The samples were divided into 20μL aliquots and frozen at -80°C. For each experiment, the aliquots were boiled for seven minutes and then run through gel electrophoresis and western blotting to separate the proteins and transfer them to nitrocellulose paper. The running buffer used was made with 25mM Tris, 191.82 mM glycine, 3.47 mM SDS) and electrophoresis was set at 110 Volts for approximately 45 minutes, or until the blue tracking dye in the samples reached the bottom of the gel. The transfer buffer used was made with 20mM Tris, 192 mM glycine, and 200mL ethanol and the transfer was also set at 110 Volts for 70 minutes. Once transferred, the paper was placed in a container with a Tween Tris Buffered Saline (TTBS) and milk dilution for blocking for 1.5 hours. The dilution of TTBs used consisted of 10mM Tris-HCL, 250 mM NaCl, and 0.05% Tween-20 (pH 7.6). The paper was rinsed with TTBS and a new solution containing TTBS, 10mL milk, and a specific amount of antibody (either Santa Cruz Biotechnology Rac-1 rabbit polyclonal or Invitrogen anti-nitrotyrosine rabbit IgG fraction) was placed on the paper

and left on for at least 1.5 hours. After this time, the paper was rinsed six times for five minutes with TTBS and then the final antibody solution was applied to the paper, containing TTBS, milk, and the appropriate secondary antibody (Santa Cruz goat anti-rabbit). This solution was left on for at least 1.5 hours and then rinsed six times for five minutes with TTBS. Then, a total of .8mL of the developing chemical, Pierce SuperSignal West Pico Chemiluminescent was dropped onto the nitrocellulose paper and left on for 1.5 minutes (equal parts of the stable peroxide solution and luminal enhancer solution). Then, the paper was placed in a protective clear sheet in order to develop it on the Syngene Chemiluminescence Imager. Figure 3 shows the effects adding an anti-nitrotyrosine antibody to the western blot, which is a standard process to identify this particular modification (nitrosylated tyrosine residues on the secretory granules). The dilution used was 1:2000 (2.0 μL of antibody, 400 mL milk solution, and 3.5 mL of TTBS) Both samples show that the level of nitrosylation significantly increased in the samples stimulated with CCK to induce secretion compared to the control samples.

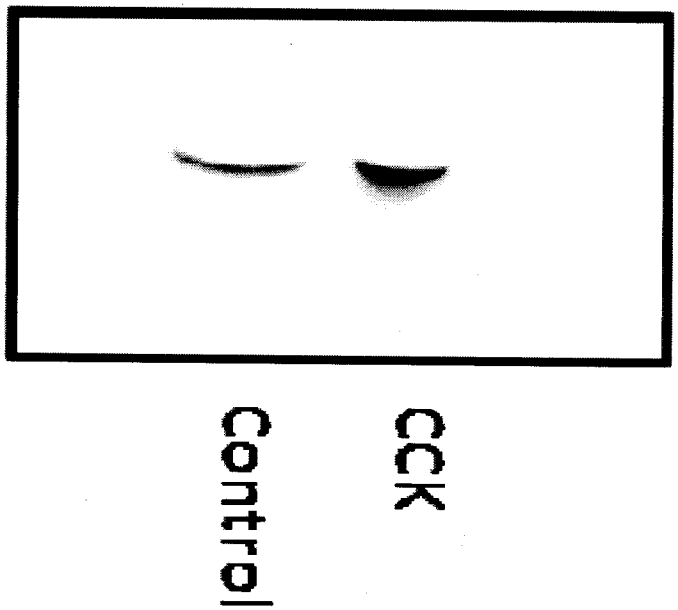
Figure 3
A Western blot showing an increase in nitrosylation after stimulation with CCK



Additionally, the presence of Rac was tested by adding a Rac antibody in a 1:1000 dilution (4.0 μL antibody, 400 mL milk solution, and 3.6 mL TTBS). The results were similar, where the sample stimulated with CCK displaying a higher level of the Rac protein present than the controlled sample, as shown in Figure 4 below.

Figure 4

A Western blot showing an increase in Rac after stimulation with CCK



Conclusions

Although the identity of the protein being nitrosylated was not determined as originally proposed, the results of this project show that 1) there is a significant increase in nitrosylation upon stimulation for secretion of a zymogen granule with CCK and that 2) there is also a significant increase in the amount of Rac produced when a zymogen granule is stimulated for secretion with CCK. Both of these experiments compared stimulated zymogen granules with those under controlled, unstimulated conditions. The increase in Rac present under stimulated conditions suggests that there is an increased level of superoxide production in these conditions, thereby providing the superoxides necessary in the hypothesized mechanism of nitrosylation. Thus, the nitric oxide produced by the pancreatic cells could combine with the superoxides localized on the zymogen granule membrane in order to form the peroxynitrite. This would then nitroyslate tyrosine residues on the granule membrane, and this process was supported by the results from Figure 1 regarding nitrosylation.

Once stimulation occurs for migration and secretion of the zymogen granules, there must be a mechanism involving cystoskeletal reorganization in order to physically move these granules, and this is where a second role for Rac seems to be important in pancreatic secretion. Since Rac GTPase is known to be critical in actin polymerization, it seems that the Rac protein has an important role as well in the actual migration of the zymogen granule (Johnson, 2006). Generally, Rac GTPase is known to induce lamellipodial protrusions on the cell membrane surface, serving as a vehicle for migration and movement of the cell (Waterman-Storer, 1999). Thus, the localization of Rac on the zymogen granule, as demonstrated in this project, would suggest that Rac carries out a similar purpose on the ZG membrane: it helps to migrate the

zymogen granule to the membrane for secretion. Thus, the physiological importance of Rac being localized on the ZG membrane includes its roles in superoxide production (a precursor to the proposed method of nitrosylation as stimulation), cystoskeletal reorganization, and actin polymerization (direct participation in migration).

Future experimentation could be organized to determine whether the activity of Rac in these three areas happens simultaneously and is interdependent or whether each process is independent. In addition, it would be interesting to determine whether the zymogen granule is pushed along the newly formed microfilaments or if myosin works to pull the zymogen granule toward the membrane for fusion.

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