Regulation of vesicular nucleotide transporter (VNUT) in pancreatic β cells

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by

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Abstract

Vesicular nucleotide transporter (VNUT) is a protein found in insulin granules that facilitates the uptake of ATP and release of ATP and insulin. Studies show that suppressed VNUT results in reduced insulin secretion. Currently, the regulation of VNUT expression in β cells is unknown. The objectives of this study were to determine the effect of high fat diet (HFD) on VNUT levels and test if free radicals generated from HFD are involved in HFD modulation of VNUT expression in β cells. A mouse insulin-producing cell line (MIN6) was used for this study. The control group received growth medium treatment, and the test group received growth medium containing fatty acids palmitate, oleate, and linoleate (1:2:1 ratio). The cells were lysed 48 hours after treatment, and protein was extracted for western blot analysis for VNUT. To further determine the effect of HFD in VNUT expression, adult male mice were fed either a control diet or HFD (n=3/group) for one week. We sacrificed the mice and isolated islets from the pancreas for protein content. Western blot was run in both models for VNUT expression, normalized against the housekeeping gene α-tubulin. Results validated a correlation between HFD and reduced VNUT levels compared to control group. Secondly, we introduced the drug BAM15, a mitochondrial uncoupler which has been shown to reduce reactive oxygen species production from mitochondia. Three trials were conducted with MIN6 cells in the following treatment groups: control, HFD, HFD + BAM15, and BAM15 only. After treatment for two days, western blot was run to determine VNUT expression. BAM15 showed a trend to restore VNUT levels in HFD-fed cells. Additional replications are necessary, as results with the drug were not statistically significant. Results of this study and the role of free radicals on modulating VNUT expression could have implications for managing diseases where insulin regulation is impaired.

Introduction

The protein vesicular nucleotide transporter (VNUT) was discovered relatively recently and is involved in ATP uptake and release from secretory vesicles including insulin secretory granules in pancreatic β cells. After a meal, glucose enters β cells and is metabolized to generate ATP. ATP then binds and closes ATP-sensitive potassium channels, which triggers calcium to move through calcium channels, ultimately resulting in insulin and ATP secretion from secretory vesicles. Recent study has suggested that VNUT resides within insulin granules to modulate cellular ATP levels. Previous studies showed that suppression of endogenous VNUT resulted directly in a reduction of basal and glucose-induced ATP and insulin release. Likewise, overexpression of VNUT resulted in greater ATP release. Thus, this protein could be essential in regulating insulin secretion from pancreatic β cells. However, the regulation of VNUT expression in β cells is unknown thus far. One possible mechanism by which VNUT is regulated involves reactive oxygen species (ROS) as they are generated by the mitochondria in the presence of a high energy source. Results of recent experiments indicate a correlation between

high-fat diets and reduced VNUT expression; therefore, we hypothesized that ROS may negatively modulate VNUT levels in pancreatic β cells under nutrient excessive conditions.

Problem Identification and Justification

Previous studies show that treating pancreatic cells with saturated and unsaturated fatty acids results in reduced VNUT expression compared to non-treated cells. It is unknown why a high fat diet (HFD) lowers VNUT levels. In order to further study this relationship, it is necessary to gain knowledge of the mechanism by which VNUT expression is altered.

Through this research project, the effect of fatty acid treatment on VNUT was evaluated. Understanding the mechanism of VNUT regulation will provide knowledge to manipulate this protein and potentially control ATP release and insulin secretion. Knowledge gained from this study will have implications for management of diseases such as diabetes where insulin release from β cells is impaired or where insulin resistance in cells is an issue.

Hypotheses and Objectives

Previously, western blot analyses on a mouse insulin-producing cell line (MIN6) revealed that feeding cells fatty acids reduced VNUT expression. An objective of this study is to validate these previous findings in the MIN6 cell line. The level of VNUT present after cells receive fatty acid treatment will be measured and compared to controls that receive only the growth medium with minimal fatty acids. The hypothesis is that VNUT expression will be reduced by high fat treatment compared to cells treated with control media.

Currently the mechanism underlying the effect of high fat feeding on VNUT expression in β cells is unknown. A second objective of this study is to gain a better understanding of the possible mechanism by which VNUT expression is regulated. The high energy source from a nutrient-rich diet causes the mitochondria to pump out more ATP. In this process, ROS is generated. The hypothesis being tested is that free radicals generated from HFD play a role in the mechanism to regulate VNUT expression in β cells. If the accumulation of free radicals does affect VNUT expression, then treatment of cells with HFD along with compounds that reduce ROS production such as drug BAM15, a mitochondrial uncoupler that reduces free radicals, should reverse the effect HFD has on VNUT levels.

Procedures and Methodology

For the first part of this study, cells from a frozen mouse insulin-producing cell line (MIN6) were grown in a tissue culture plate and then passaged into additional plates before they reached confluence. These cells were divided into six separate wells (~$1x10^6$ cells/well). The wells were assigned to either the control group or the test group (n=3 wells/group).

The control group received cell growth medium (GM) treatment that contained necessary nutrients such as vitamins, minerals, and essential amino acids, as well as some glucose and phenol red indicator (1:4 dilution of 10% BSA:Gibco®DMEM 1X). The cells in the test group, or HFD group, received GM with the addition of palmitate, oleate, and linoleate in a 1:2:1 ratio. The control group treatment used 1.2 ml 10%BSA, 4.8 ml GM, and 60µl ethanol to account for the additional volume in the treatment group. The HFD treatment used 1.2 ml 10% BSA, 4.8 ml GM, and 15µl:30µl:15µl of the respective fatty acids. The control treatment and HFD treatment were both filtered separately and 2 ml of the appropriate medium was pipetted into each of 3 wells for that treatment group. Both groups received treatment for 48 hours in a cell culture incubator (held at 37°C and 5% CO_2).

At the end of this incubation period, the old GM was suctioned away and wells were washed twice with phosphate buffer (PBS; Gibco®DPBS 1X) solution. The cells in both groups were lysed by adding ~200µl of lyse buffer (cocktail of RIPA and protease inhibitor 1:100). The lysate from each well was transferred into separate 1.5-ml centrifuge tubes, vortexed, and placed on ice for 30 minutes. The tubes were then centrifuged at 4-6°C for 15 minutes at 13,000 rpm. The supernatant from each tube was transferred into a new 1.5-ml centrifuge tube.

Series of protein standards were prepared for the Bradford protein assay in order to calculate the protein concentration of each sample. The standard 1st micro-well used 5µl RIPA buffer and 250µl Quickstart Bradford reagent (room temperature). Each subsequent micro-well contained 5µl of sample from the respective centrifuge tube and 250µl Bradford reagent. Based on the spectrometer readings, calculations were made for preparation of samples with equal protein content and volume to be loaded for gel electrophoresis. Appropriate amounts of sample buffer (5X SB) and 3µl 1M DTT (as reducing agent) were added to each sample.

The sample buffer contains glycerol and bromophenol blue tracking dye. 7µl of the protein standard and 30µl of each sample were loaded into separate lanes of an SDS-PAGE gel. Gel electrophoresis was run to separate the proteins in the lysate by molecular weight. Then, proteins in the gel were transferred onto a PVDF membrane via an electric field over 2 hours in a cold room. The membrane was washed in TBST washing buffer (Tris-Buffered Saline and Tween 20), placed into a milk blocking buffer, and placed on a shaker for 30 minutes.

The membrane was washed with TBST and incubated overnight in the cold room with primary antibody anti-α VNUT produced in rabbit (1:1000 dilution; 8µl antibody in 8ml 2.5% milk solution). The membrane was then washed and further incubated for 2 hours on a slow shaker with secondary antibody anti-rabbit IgG conjugated with HRP (1:5000 dilution). Lastly, Pierce ECL Western blotting substrate was added so the antibody-antigen complex could be visualized through a digital camera. Image reader software was used to analyze and quantify the amount of protein stained on the membrane by optical density.

The membrane was stripped and re-incubated to test for expression of the housekeeping gene α-tubulin. For this portion of the study, the primary antibody used was monoclonal anti-mouse tubulin (1:4000 dilution), incubated overnight. The secondary antibody was anti-mouse IgG conjugated with HRP (1:5000), incubated for 2 hours. Image analysis was conducted, and VNUT levels were normalized against tubulin expression.

The control and HFD treatments were duplicated in an in vivo model as well. Adult male mice (weighed between 25-30g; Strain C57BL/6J) were fed either a normal chow diet (Purina LabDiet®) or HFD (Research Diets Rodent Diet with 45% kcal %fat) for one week (n=3/group). The mice were sacrificed and islets were isolated from the pancreas. Islets were lysed for protein analysis, and quantitative western blot analysis was conducted in the same way as was described for the in vitro model.

For the second part of the study, the drug BAM15 was introduced. It has been shown that BAM15 functions as a mitochondrial uncoupler to reduce ROS production (Kenwood et al., 2014). A total of four groups were assigned: control, HFD, HFD + BAM15, and control + BAM15. The mitochondrial uncoupler drug was added to the respective treatments in a 1:1000 dilution (n = 3 wells/group). Cells were lysed after 48 hours of treatment and protein was extracted. The same quantitative western blot procedure was followed for the four treatment groups as with the first part of the study with two treatment groups. The results from the western blot tests indicated the relative amount of VNUT that was present in each group of cells receiving the specified treatments.

Timeline

During the summer of 2014, the aforementioned experiments were run and test results analyzed. More studies into VNUT regulation and HFD modulation of VNUT expression could continue in the future.

Findings

The intensity of the band from each western blot test is representative of the amount of VNUT within the lysed cell. Results of western blot analyses were compared to see the relative levels of VNUT expressed in fat-treated and untreated cells as well as drug-treated cells, with and without fat treatment.

The data from the first part of this experiment validated previous findings that a high-fat diet treatment decreases expression of endogenous VNUT in pancreatic β cells for both in vitro (Fig. 1) and in vivo (Fig. 2) models.

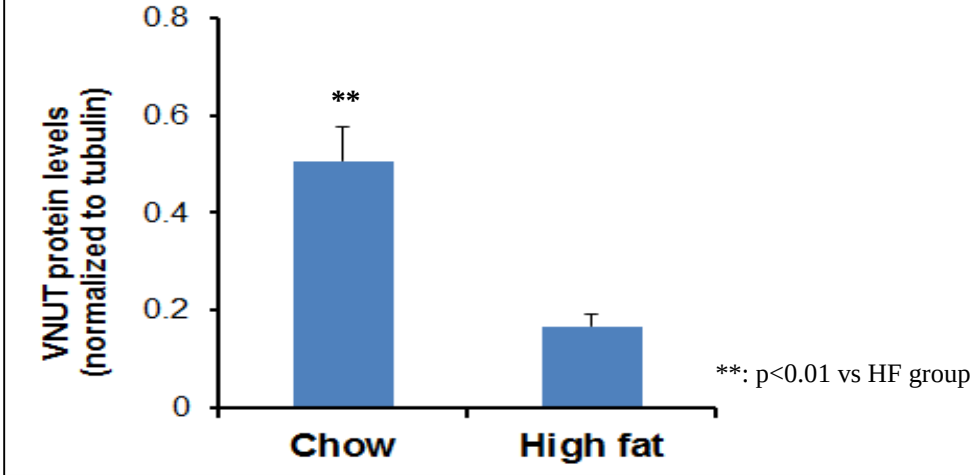

Figure 1: Levels of VNUT in MIN6 β Cells Fed with Control Medium or High Fat Medium for 48 Hours

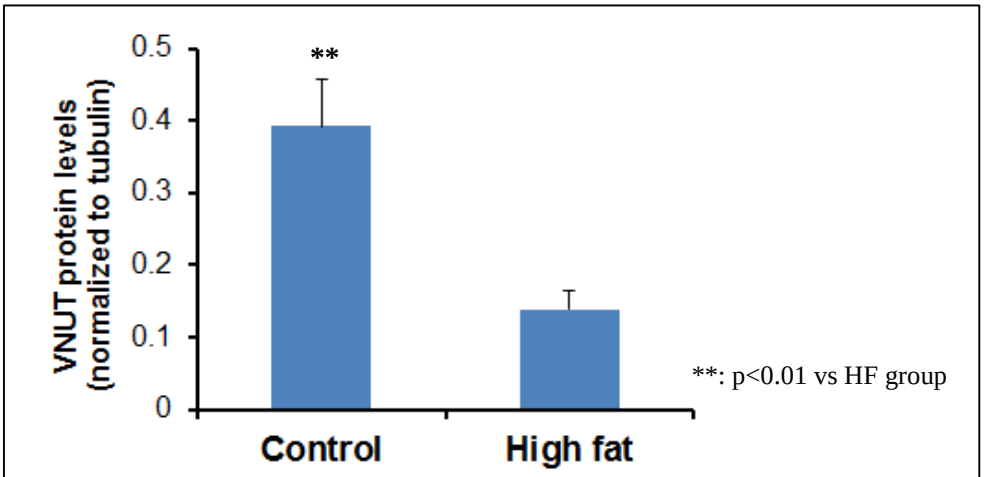

Figure 2: Levels of VNUT in Islets Isolated from Mice Fed with Chow Diet or High Fat Diet for Seven Days

Furthermore, in the second part of the experiment, BAM15 treatment showed a trend to restore VNUT levels in cells fed high fat (Fig. 3). This supports the hypothesis that free radicals are involved in HFD modulation of VNUT expression in pancreatic β cells. However, results with the drug were not statistically significant.

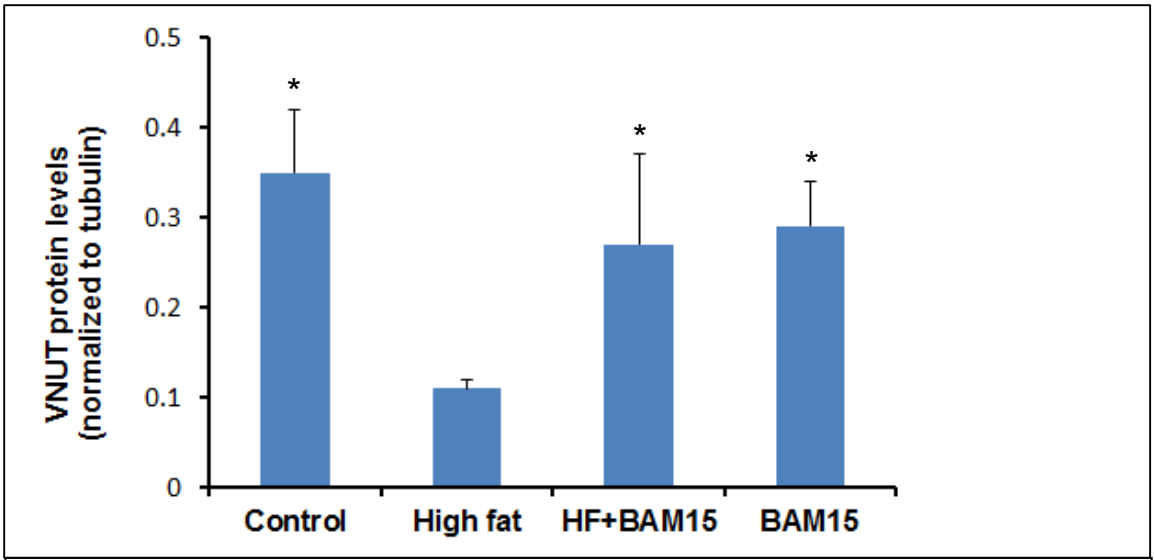

Figure 3: Effect of BAM15, a Mitochondrial Uncoupler, on VNUT Expression in MIN6 Beta Cells Fed with High Fat Medium for 48 Hours

Future direction for this study would be to replicate the methodology of the four treatment groups one or two more times to test for statistical significance ($p<0.01$ vs HF group). If this is determined, the next steps would be publication and studying the effect of BAM15 or similar drugs on insulin secretion in animal models fed HFD.

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