Ketone Bodies and Signaling in Pancreatic Cancer Cell Lines

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Ketone Bodies and Signaling in Pancreatic Cancer Cell Lines

University Honors Program Thesis/Capstone/Creative Project

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Submitted by

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ABSTRACT OF THESIS:
Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States, and 95% of these cases are caused by PDAC (pancreatic ductal adenocarcinoma). Ketone bodies have previously been shown to decrease cell proliferation and cancer-induced cachexia. The molecular mechanism of ketone body-mediated growth inhibition of pancreatic cancer cells is not well understood. Research conducted thus far has not explored which molecular pathways are affected by ketone body treatment in pancreatic cancer cells. In the current study, the effect of the ketone body sodium hydroxybutyrate on the JAK-STAT and mTOR pathways and cell migration was explored. A decrease in cell migration was observed in a dose dependent manner. Levels of p-STAT3 and p-p70 S6K were decreased after 72 hour treatment with 5 and 10 mM sodium hydroxybutyrate. These proteins regulate transcription and translation of several genes involved in cellular growth and proliferation.
**Key Words**

Pancreatic cancer, ketone bodies, sodium hydroxybutyrate, migration, signaling

**Introduction and Background**

Pancreatic cancer is one of the leading types of cancer related deaths in the United States, with pancreatic ductal adenocarcinoma (PDAC) accounting for 95% of pancreatic cancer cases.\(^1\),\(^2\) Cancer cells have several genetic mutations and metabolic pathways altered, and most of these cells also show increased uptake of glucose.\(^3\),\(^4\) This leads to an increased rate of glycolysis and increased lactate secretion, even in the presence of oxygen, which is known as the Warburg effect.\(^5\) The increase in conversion from pyruvate to lactate leads to increased acid levels in the tumor, which helps to facilitate invasion and migration of cancer cells to other parts of the body.\(^6\)

Ketone bodies are compounds produced in the metabolism of fat. There are three ketone bodies: acetone, acetoacetic acid, or beta-hydroxybutyrate.\(^7\) A ketogenic diet is a high-fat, low-carbohydrate diet. This kind of diet has anticonvulsant and antiinflammatory activities and is also produces important metabolic changes.\(^8\),\(^9\) This makes ketone bodies a novel potential therapy due to high levels of inflammation and metabolic changes associated with pancreatic cancer.

It has previously been shown that treatment of pancreatic cancer cell lines Capan 1 and S2-013 with ketone bodies (sodium hydroxybutyrate and lithium acetoacetate) inhibited cell survival in a dose dependent manner.\(^10\) It was also demonstrated that this inhibition was not due to the sodium or lithium ions, and was therefore due to ketone bodies. Significant inhibition of cell growth was observed with concentrations of 10 and 20 mM after 72-hour treatment. This
inhibition was specific to cancer cells; treatment of non-transformed pancreatic epithelial cell lines HPNE and RAPAN with ketone bodies showed no significant effect on survival. A decrease in cellular pH levels were observed upon treatment with ketone bodies. Decrease in cellular pH (acidification) of cells, however, was not the primary cause of cell death; this pH decrease was observed even in non-cancerous cell lines that did not show significant cell death with ketone body treatment.

Metabolic alterations were observed in pancreatic cancer cell lines upon treatment with ketone bodies. Capan 1 and S2-013 cells showed a decrease in glucose and glutamine uptake and release of lactate following ketone body treatment. There was also a decrease in ATP and ROS (reactive oxygen species) levels after treatment with ketone bodies. All of these alterations indicate that the energy levels of the cells have been diminished by treatment with ketone bodies, which in turn leads to decreased proliferation and increased cell death.

The effects of ketone body treatment on the progression of cachexia was also tested via observation of differentiated myotube degradation and adipose deposit depletion induced by cancer cell-conditioned medium. Treatment of differentiated myotubes and adipose deposits with Capan 1 and S2-013 cancer cell-conditioned medium increased the degradation of myotubes and decreased adipose deposits. However, upon treatment with ketone bodies, myotube degradation was prevented, as was depletion of adipocyte deposits. Proteins normally upregulated during cancer-induced cachexia (such as muscle-specific ring finger protein 1 and Atrogin in myotubes and zinc alpha-2-glycoprotein 1 and hormone-sensitive lipase in adipocytes) showed decreased expression after treatment with ketone bodies. These results lend to the conclusion that ketone bodies inhibit cancer-induced cachexia. Results obtained in vitro were replicated in vivo using
mouse models, which demonstrates that ketone body treatment has significant potential for use in humans.\textsuperscript{10}

Earlier studies have examined the effect of ketone bodies on cell survival, but the molecular mechanism of ketone body-mediated growth inhibition of pancreatic cancer cells is not well understood. In this current project, I have explored the effect of ketone bodies on growth signaling and migration of pancreatic cancer cells. The two signaling pathways investigated were the JAK-STAT and mTOR pathways (Figure 1). Both of these pathways have been shown to be altered in many cancer types, including PDAC.
The JAK-STAT pathway has been recognized recently as an oncogenic signaling pathway. STAT is a protein found in the cytoplasm that functions as a transcription factor. \(^\text{11}\) STATs respond to cytokines and growth factors, and they are activated by phosphorylation of a conserved tyrosine residue. \(^\text{11}\) Once activated, they relocate to the nucleus, where they bind to DNA and activate target genes. Shortly after growth factors or cytokines bind to their receptors,
receptor tyrosine kinases or receptor-associated kinases (including JAK) are activated. STAT3 can also be phosphorylated at a serine residue on their COOH terminus, which isn’t needed for dimerization, nuclear translocation and DNA binding, but is needed for maximal transcription of certain genes.\textsuperscript{11} After STAT activation, many genes regulating cancer progression processes (such as uncontrolled proliferation, resistance to apoptosis, angiogenesis, and immune surveillance evasion) are altered.\textsuperscript{11} STAT3, specifically, has been implicated in selectively inducing and maintaining an inflammatory microenvironment during malignant transformation and cancer progression.\textsuperscript{11}

The other pathway that was investigated was the mTOR pathway. The mammalian target of rapamycin (mTOR) is a protein serine/threonine kinase that was first identified as the cellular target of the drug rapamycin, and it has been found to regulate cell growth, proliferation, motility, and survival.\textsuperscript{12} Activation of transmembrane receptors by binding a ligand leads to activation of PI3K. PI3K then phosphorylates Akt, which is dephosphorylated by PTEN. There are two mTOR complexes, mTORC1 and mTORC2, which are both involved in cell growth. Activated mTORC1 regulates several molecular processes such as: phosphorylation of downstream targets that lead to translation of mRNA, suppression of autophagy through Atg13 and ULK1, ribosome synthesis, and transcription activation.\textsuperscript{12} mTORC2 promotes cell survival by means of Akt activation. Other processes regulated by mTORC2 include dynamics of the cytoskeleton and ion transport and growth.
Materials and Methods

**Cell Culture:** T3M4 and CFPAC-1 cells were cultured in DMEM with 10% FBS at 37°C with 5% CO\textsubscript{2} and allowed to proliferate. Cells were subcultured routinely upon reaching 80% confluence.

**Ketone Body Treatment:** A stock solution of sodium hydroxybutyrate was prepared at a concentration of 1 M. Sodium hydroxybutyrate was dissolved in sterile milliQ water. T3M4 and CFPAC-1 cells were seeded in dishes or plates depending on the requirements of the experiment. 12-16 hours after seeding (or after cells had become attached), culture media of the cells was changed and cells were treated with increasing doses of sodium hydroxybutyrate (2.5 mM, 5mM and 10mM) for different time points such as 24h, 48h, 72h, and 96h. Untreated cells were used as control. The media was changed every 24 hours and cells retreated with the appropriate doses.

**Scratch Assay:** To study the effect of ketone bodies on cellular migration, 3000-5000 cells/well were seeded in 6 well plates. After cells were 100% confluent, a scratch was made down the middle of the well using a pipet tip. Cells were then treated with increasing doses of sodium hydroxybutyrate for 48 hours and incubated at 37°C with 5% CO\textsubscript{2}. Images of the same area were taken at 12 hour intervals to observe migration.

**Immunoblotting:** After ketone body treatment of cells for different time points, plates were washed twice with cold PBS, cells were lysed using RIPA lysis buffer, and were incubated on a shaker for 20 minutes. Lysed cells were harvested from the plate and centrifuged at 13,000 rpm at 4°C for 10 minutes. The supernatant containing proteins was collected in a fresh tube. Protein content was measured using a Bradford assay. Proteins were then run through a polyacrylamide gel by gel electrophoresis and transferred onto a nitrocellulose membrane. Membranes were
probed with primary antibodies of cellular signaling molecules involved in the JAK-STAT and mTOR pathways. Membranes were then incubated in chemiluminescence solution and developed on X-ray film in a dark room.

Results

**Ketone body treatment decreases pancreatic cancer cell migration**

The effect of ketone bodies on pancreatic cancer cell migration was investigated using a scratch assay. After the scratch was made, cells were treated with increasing concentrations of sodium hydroxybutyrate (Control, 5 mM and 10 mM) for 24 hours. Pancreatic cancer cells treated with higher doses of sodium hydroxybutyrate showed a decrease in cell migration. Both cell lines showed a significant gap immediately after the scratch was made. The control group for both cell lines showed significant gap-closing after 24 hours. However, after 12 hours in 5 mM and 10 mM sodium hydroxybutyrate treatments, there was still a visible gap observed (Fig. 2a,b). 24 hours showed even less gap closing (Fig. 2a,b). Although both cell lines showed diminished cell migration upon treatment with ketone bodies, the effect observed was much greater in T3M4 cells. While T3M4 cells showed some gap closing with 5 mM and 10 mM sodium hydroxybutyrate treatments (Fig. 2b), CFPAC-1 cells showed much more gap closing with the same treatments (Fig. 2a).
a.

CFPAC-1

0 hours | 12 hours | 24 hours
--- | --- | ---
Control

5 mM SHB

10 mM SHB
Figure 2. a) Scratch assay of CFPAC-2 cells. Confluent cells were treated with increasing concentrations of sodium hydroxybutyrate. A scratch was made and cells were imaged at 0, 12 and 24 hours to observe migration. b) Scratch assay of T3M4 cells. Confluent cells were treated with increasing concentrations of sodium hydroxybutyrate. A scratch was made and cells were imaged at 0, 12, and 24 hours to observe migration.
Levels of key transcription and translation proteins were diminished after sodium hydroxybutyrate treatments

Levels of key components of the JAK-STAT and mTOR pathways were examined using Western blot. Proteins looked at include: mTOR, STAT3, p-STAT3, p70 S6K, and p-p70 S6K. Actin was used as a loading control. It was shown that there was a decrease in levels of p70 S6K and p-p70 S6K in T3M4 cells after treatment of cells with increasing concentrations of sodium hydroxybutyrate for 72 hours (Fig. 3). Decrease in levels of p70 S6K and p-p70 S6K would lead to diminished protein synthesis. p70 S6K is activated (phosphorylated) by PI3K, which in turn activates protein synthesis through the 40S ribosomal subunit (Fig. 4). This activation leads to synthesis of necessary components of translation, including elongation factors, ribosomal proteins, and poly(A)-binding protein. Less activation of p70 S6K would then in turn yield less protein synthesis. p70 S6K is also involved in cell cycle control and cell migration. It has been hypothesized that p70 S6K activation is needed for cells to progress from S phase to G1 phase in the cell cycle, which would have a negative impact on cell proliferation. It has been found that p70 S6K associates with Rac1 and cdc42, which are small GTPases that assist with regulation of membrane ruffling, migration and actin polymerization. These events are needed in order for cells to migrate, and a decrease in the activating of p70 S6K would in turn decrease cellular migration. CFPAC-1 cells did not show prominent changes to p70 S6K and p-p70 S6K protein expression at the concentrations tested.

There was a significant decrease in p-STAT3 levels in T3M4 cells and marginal decrease in CFPAC-1 cells after treatment with sodium hydroxybutyrate (Fig. 3). Phosphorylated STAT3
activates transcription in the nucleus. This decrease in p-STAT3 would decrease activation of transcription, which would in turn affect protein synthesis. This global decrease in transcription would have a negative impact on expression of proteins necessary to carry out cellular functions and promote survival. Again, no prominent changes were observed in expression of these proteins in CFPAC-1 cells at these treatment concentrations.

**Figure 3.** Western blot of proteins found in the JAK-STAT and mTOR pathways. Levels of p70 S6K, STAT3, and their phosphorylated counterparts were analyzed. Beta-actin was used as a loading control.
**Figure 4.** Outline of p70 S6K signalling in cancer cells. p70 S6K is activated by PI3K, and activated p70 S6K in turn activates protein synthesis via the small 40S ribosomal subunit. Other targets of activated p70 S6K include cellular components involved in regulation of cell migration and movement through the cell cycle (Berven and Crouch, 2000).

**Conclusion and Future Directions**

Treatment of pancreatic cancer cell lines CFPAC-1 and T3M4 with the ketone body sodium hydroxybutyrate had a negative impact on cell migration in a dose dependent manner, which is consistent with previous findings. It was shown that migration of CFPAC-1 and T3M4 cells was diminished with 5 mM and 10 mM SHB treatments; T3M4 cells showed more diminished migration than CFPAC-1 cells. Diminished levels of p70 S6K, p-p70 S6K, and p-STAT3 were observed in T3M4 cells. A decrease in expression of p70 S6K and p-p70 S6K would decrease protein synthesis due to the role of p-p70 S6K in the activation of the ribosomal 40S subunit. Less activation of the 40S subunit would in turn decrease expression of proteins globally, including ones that are vital for carrying out cellular functions. When phosphorylated, STAT3
activates transcription in the nucleus. A decrease in p-STAT3 would diminish activation of transcription, which could also have a negative effect on the expression of genes necessary for cell survival and proliferation. Future directions for the project are to continue to identify key components of oncogenic signaling pathway(s) that are altered upon treatment with ketone bodies, and to perform qRT-PCR to evaluate the effect of ketone bodies on expression of key genes linked to cell proliferation, migration, and death.

All research was conducted at the University of Nebraska Medical Center.
References


Supplemental Figures and Data

Western Blot films (whole)