PKM2 Influences the Metabolic Fate of Butyrate in Colorectal Cancer Cells

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PKM2 Influences the Metabolic Fate of Butyrate in Colorectal Cancer Cells

UNDERGRADUATE THESIS

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1. INTRODUCTION

1.1 Need for Colorectal Cancer Treatment

Cancer is the second leading cause of death of people ages 1-85 in the United States today, and the number of new cases is expected to rise by 70% over the next two decades (Organization, 2017). Of the 8.8 million worldwide cancer deaths in 2015, colorectal cancer accounted for 774,000 of the deaths. The need for effective colorectal cancer treatment is present through these statistics and is growing in urgency. Development of these life-saving colorectal cancer treatment and prevention techniques are dependent on innovative research.

1.2 Colorectal Cancer and Metabolism

Cancer is now accepted as a metabolic disease, where changes in energy metabolism in the cancer cell promote its progression, development, and metastasis (Seyfried et al., 2014). Identifying the factors that impact changes in metabolism during progression of colorectal cancer is pivotal. Pyruvate kinase is a protein that catalyzes the rate limiting, final step of glycolysis to convert phosphoenolpyruvate to pyruvate by addition of a phosphate group, yielding one pyruvate and one molecule of ATP (Gupta and Bamezai, 2010). This step is vital for cell growth and survival. It has been shown that pyruvate kinase exists in four isozymic forms, and that pyruvate kinase M2 (PKM2) is upregulated in colorectal cancer (Wong et al., 2015). PKM2, a 58-60 kDa member of the PK enzyme family, is believed to promote the transformation toward glucose-driven cancer cell metabolism and support cancer cell progression (Dong et al., 2016; Luo and Semenza, 2012; Wong et al., 2015). If the mechanism of this transformation could be regulated or manipulated, it could serve as a therapeutic cancer treatment in the future.
1.3 Changes in Butyrate Oxidation

Non-cancerous colorectal cells (colonocytes) utilize a bacterial product called butyrate as their primary energetic source through oxidation (Goncalves and Martel, 2013). Butyrate is a short chain fatty acid derived from dietary fiber and is produced by resident microbiota in the colon. In contrast, colorectal cancer cells shift from utilizing butyrate to instead using glucose which is known as the Warburg effect (Donohoe et al., 2012; Goncalves and Martel, 2013). As colorectal cancer tumors reach later stages and progress towards metastasis, glucose uptake is increased exponentially in order to sustain the rapid growth.

1.4 Purpose of Research

These metabolic changes and the shift away from butyrate utilization in colorectal cancer cells are likely driven by increased expression of PKM2, but it has yet to be demonstrated. This has created new questions regarding manipulation techniques to effect cancer progression. Specifically, it was hypothesized that the presence or absence of PKM2 effects the oxidative fate of butyrate in colorectal cancer cells. While butyrate may encourage the growth of normal colonocytes because it functions as an oxidative source, via β-oxidation and tricarboxylic acid cycle, it cannot be used efficiently when the Warburg effect is occurring. Therefore, colorectal cancer cells cannot oxidize butyrate to use as an energy source and it may inhibit growth of cells. In contrast, colorectal cancer cells that lack PKM2 and lack the Warburg effect should be able to better oxidize butyrate as an energy source and should not exhibit cancerous growth patterns, as demonstrated in Figure 1. To support these hypotheses, characterizations of the metabolism of butyrate in both colorectal cancer cells containing and lacking PKM2 were performed.
2. EXPERIMENTAL PROCEDURES

2.1 Reagents

DMEM, penicillin/streptomycin, puromycin, ammonium persulfate, fetal bovine serum, trypsin, pyruvate kinase M2 antibodies, β-actin antibodies, agarose gel for electrophoresis, various buffers.

2.2 Cell Culture and Treatment Conditions

HCT 116 cells (ATCC, CCL-247) were grown in DMEM prepared with 5 mM glucose and 10% FBS. HCT 116 PKM2 mRNA scramble cells and HCT 116 PKM2 Knockout cells were grown in DMEM prepared with 5 mM glucose, 10% FBS, and 1 μg/ml Puromycin.
2.3 Western Blotting

Proteins from the three HCT116 cell types were extracted using RIPA buffer. Bradford assay was used to measure protein concentration. Standard Western blot protocol was used in gel electrophoresis and blotting transfer experiments. Antibodies used in this experiment were β-actin and PKM2.

2.4 Flux Experiments

XF24 Analyzer (Seahorse Bioscience) was used to measure the change in the oxygen consumption rate (OCR). Cells were split and seeded with 60,000 cells per well into XF24 cell culture microplates. Prior to assay, cells were incubated in their respective treatments in a non-CO₂ incubator at 37°C for 45 minutes. For the treatments, half of the cell received 30 μl of butyrate at 100mM injected during assay while the other half received 30 μl glucose free media, and the oxygen consumption rate (OCR) was measured in pmol/min/cell concentration. The second injection for all cells was 75 μl of 2-Deoxy-D-glucose which inhibits glycolysis. Sodium azide was injected last to halt all oxidative metabolism. OCR data was normalized by Bradford absorbance assay on cell concentration.

3. RESULTS

3.1 Confirmation of PKM2 Knockout in Colorectal Cancer Cells

In order to confirm the success of the knockout of pyruvate kinase M2 (PKM2) in the HCT116 PKM2 Knockout colorectal cancer cells, a Western Blot was performed. Three cell types were used, HCT116 Wild Type (WT), HCT116 PKM2 mRNA Scramble (Scram), and HCT116 PKM2 Knockout (KO). The outcome was considered successful if two criteria were met. First, all samples must have shown a present β-actin control protein band. Second, the WT and Scram cells must have exhibited bands indicating presence of PKM2 while the PKM2 KO showed no PKM2 band presence. As shown in
Figure 2, all three cell types exhibited present β-actin and only the HCT116 PKM2 KO cells lacked PKM2. Therefore, this experiment confirmed the successful knockout of PKM2 in the HCT116 PKM2 KO cells and allowed further experiments to proceed.

<table>
<thead>
<tr>
<th>HCT116</th>
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<tbody>
<tr>
<td>WT</td>
<td>PKM2</td>
<td>PKM2</td>
</tr>
<tr>
<td>Scram</td>
<td>KO</td>
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**Figure 2**: Immunoblot analyses of PKM2 and β-actin from HCT116 WT, HCT116 PKM2 Scramble, and HCT116 PKM2 KO.
3.2 Schematic of Cellular Metabolism analysis with Mitochondrial Stress Assay

Cellular metabolism of the three cell types was investigated using a The Seahorse XF Extracellular Flux Analyzer, an instrument that measures oxidative and non-oxidative metabolism. The three treatments used were as follows: (1) half of the cell wells received 30 μl of butyrate at 100 mM injected during assay while the other half received 30 μl glucose free media, (2) all cells received 75 μ of 2-Deoxy-D-glucose (2DG) which inhibited glycolysis, and (3) all cells received 30 μl sodium azide which halted all oxidative metabolism. Oxygen consumption rate (OCR) was measured in pmol/min/cell concentration. Since 2DG inhibits glycolysis, this sequence of treatment injection allowed the metabolism and OCR of butyrate to be analyzed independently of the other substrates, as shown in Figure 3. OCR data was normalized based on cell concentration found by Bradford absorbance assay.

![Figure 3: Illustration of rational of Seahorse XF Assay. Shading under line shows where butyrate oxidation will be isolated.](image-url)
3.3 Analysis of Metabolism in Response to Butyrate in Three Cell Types

The oxygen consumption rates of HCT116 WT, HCT116 PKM2 KO and HCT116 PKM2 SCR cells treated with butyrate were analyzed with a mitochondrial stress assay. In the results, all three cell types began with a basal OCR of ~300 - 420 pmol/min/cell concentration (Fig. 4). When treated with 30 μl butyrate, the oxygen consumption rate of all three cell types increased (Fig. 4). When treated with 75 μl 2-Deoxy-D-glucose (2DG), the OCR of the HCT116 WT cells decreased while the OCR of the HCT116 PKM2 KO and HCT116 PKM2 SCR cells continued to increase (Fig. 4). The Azide injection resulted in a sharp decrease in oxygen consumption and halt of metabolism. Figure 5 shows the maximum OCR of only the area under the curve between injections 2 (2DG) and 3 (Azide) which was earlier demonstrated to contain only OCR from butyrate oxidation. Results show (Fig. 5-a) HCT116 WT butyrate oxidation maximum OCR ~250 pmol/min/cell concentration, (Fig. 5-b) HCT116 PKM2 KO butyrate oxidation maximum OCR ~325 pmol/min/cell concentration, and (Fig. 5-c) HCT 116 PKM2 Scramble butyrate oxidation maximum OCR ~350 pmol/min/cell concentration (Fig. 5).
Figure 4: Change in oxygen consumption (OCR) in HCT116 cells with PKM2 (WT) and without PKM2 (KO) treated with injection of butyrate. Isolated contribution of butyrate metabolism to OCR is observed after injection of 2DG.

Figure 5: Maximum oxygen consumption (OCR) from oxidation of butyrate in HCT116 cells with PKM2 (WT) and without PKM2 (KO) after treated with injection of butyrate and 2DG.
3.4 Analysis of Metabolism in Response to Butyrate or Media in Two Cell Types

Additionally, OCR of HCT116 WT and HCT116 PKM2 KO treated with (+) and without (-) butyrate were directly analyzed against each other. It was notable that the basal OCR of HCT116 WT (-) butyrate was much higher than the basal OCR of HCT116 WT (+) butyrate, likely caused by inconsistent cell numbers in the different wells.

Results showed that after the first injection of butyrate, the OCR of HCT116 PKM2 KO (+) BUT and HCT116 WT (+) BUT increased slightly (Fig. 6). The same cell types treated with glucose free media maintained their basal OCR and saw no increase upon treatment. Once treated with 2DG, the HCT116 WT cells with and without butyrate showed a similar trend of a rapid increase of OCR followed by a sharp drop in OCR to below their respective basal OCR values (Fig. 6). In contrast, once treated with 2DG, the HCT116 PKM2 KO cells with and without butyrate showed a steady increase in OCR until Azide was added.

The oxidation of butyrate is further investigated in figure 7 which compares the OCR after 2DG injection, measuring either butyrate metabolism, or the metabolism in the absence of butyrate, in the four conditions. The maximum OCR of HCT116 WT without butyrate (Fig. 7-a) after the 2DG injection is higher than the maximum OCR of HCT116 WT with butyrate (Fig. 7-b), although this is likely the result of difference in cell number between the two wells. Interestingly, the maximum OCR after the 2DG injection in HCT116 PKM2 KO cells with butyrate (Fig. 7-d) was higher than the maximum OCR in HCT116 PKM2 KO cells without butyrate (Fig. 7-c). This is consistent with cells utilizing butyrate when glucose utilization is inhibited by 2DG.
Figure 6: Change in oxygen consumption (OCR) in four conditions: HCT116 WT (-) butyrate, HCT116 WT (+) butyrate, HCT116 PKM2 KO (-) butyrate, and HCT116 PKM2 KO (+) butyrate. Isolated contribution of butyrate metabolism to OCR is observed after injection of 2DG.

Figure 7: Maximum consumption (OCR) from oxidation of butyrate in HCT116 WT (-) butyrate, HCT116 WT (+) butyrate, HCT116 PKM2 KO (-) butyrate, and HCT116 PKM2 KO (+) butyrate. Isolated contribution of butyrate metabolism to OCR is observed after injection of 2DG.
4. Discussion

The effect of PKM2 on cellular metabolism of butyrate in the cancerous colonocyte has not been investigated in detail. While non-cancerous colonocytes utilize butyrate as a primary energy source, cancerous colonocytes shift towards an elevated glycolysis to supply their rapid energy needs with glucose (Delbeke and Martin, 2011; van Kouwen et al., 2006). PKM2 increases the capacity of cancerous colonocytes to use glucose for energy, and may represent a targetable mechanism of cancer growth progression.

In this study, we measured metabolic response to removal of PKM2 and subsequent stimulation by butyrate in live HCT116 colorectal cancer cells. The difference in metabolism trends of colonocytes containing pyruvate kinase M2 and lacking pyruvate kinase M2 suggest that this enzyme is indeed a contributing factor to the fate of butyrate in the cell. It was shown that cancer cells that expressed PKM2, and were likewise driven by glycolysis for energy, exhibited a decrease in oxygen consumption when treated with butyrate (Fig. 4). This showed that these cells were not readily using butyrate as an energy source since they normally were driven by glycolysis via PKM2. In contrast, cancer cells that lacked PKM2 actually increased their oxygen consumption when treated with butyrate (Fig. 4). This increase in oxygen consumption was likely the result of the cell’s ability to undergo β-oxidation/TCA cycle and use the butyrate for energy. This supported the proposed hypothesis that removing PKM2 from a cancerous colonocyte plays a role in shifting its metabolism from a cancerous, glycolysis driven metabolism, to a non-cancerous β-oxidation driven metabolism.

These results were confirmed by comparing the metabolism of cancerous colonocytes with and without PKM2 when treated with butyrate or glucose free-media (Fig. 6). In both treatments, the cells expressing PKM2 showed a sharp decrease in oxygen consumption. This finding agreed with the first experiment and showed that these cells do not readily use butyrate for energy. In contrast, the cells
lacking PKM2 showed a steady increase in oxygen consumption when treated with butyrate or glucose free media, with a higher increase in the butyrate treatment. This again showed that cancerous colonocytes that lack PKM2 exhibit a shift towards non-cancerous metabolism by readily using butyrate as a primary energy source.

5. Conclusion and Future Work

In conclusion, these findings supported the proposed hypothesis that butyrate cannot be used efficiently when the Warburg effect is occurring (PKM2 present) and therefore normal colorectal cancer cells cannot oxidize butyrate efficiently. The findings also support that colorectal cancer cells that lack PKM2 and lack the Warburg effect are better able to oxidize butyrate as an energy source, and showed a shift towards non-cancerous metabolism by readily using butyrate as a primary energy source. It was not determined how this would affect cancerous growth patterns and proliferation.

This research identified a novel mechanism to control the metabolic activity of colorectal cancer cells in response to butyrate, and could potentially be applied in therapeutic cancer treatment to inhibit colorectal cancer progression in the future. These finding warrant further research to determine the effect of butyrate and PKM2 on the growth and metastasis of colorectal cancer cells, as well as further research to confirm and challenge these results.

6. Acknowledgements

Special thanks to Dr. Dallas Donohoe, the advising professor who made this project possible. Also thanks to Ana Han and Boyhe Park for help in the lab, and to Chris Qualls for technological assistance. Particular gratitude is due to the University of Tennessee-Knoxville Cellular and Molecular Nutrition Department and the Chancellor’s Honors Program.
7. References


