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Article

Effect of Butyrate Extracted From Clostridium Butyricum Bacteria on Insulin Receptor Phosphorylation in Liver and Muscle Cells in Mice

Raghad Hatem Mohammed[1], Ahmed Thabet Jabar[2]*, Shaymaa Mohammed Taher[3], Fatima Nashwan Nadheer[4]

1. Education Directorate of Diyala, Diyala, Iraq
2,3,4. Department of Science, College of Basic Education, University of Mosul, Mosul, Iraq
*Correspondence: ahmed.thabet@uomosul.edu.iq

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Abstract: Butyrate extracted from C. butyricum acts as a potent metabolic modifier that repairs defects in the insulin signaling pathway. This compound contributes to reducing insulin resistance through dual mechanisms, making it a promising biopharmaceutical candidate for therapeutic enhancement in patients with diabetes and obesity. The role of a short chain fatty acid (SCFA) generated through the fermentation of Clostridium butyricum bacteria was examined to determine its role in improving insulin sensitivity. The main aim of this study was to determine the efficiency of insulin receptor (IR) phosphorylation in the liver and muscle of mice models of in vitro-induced diabetes. The research employed advanced selective and extractive fermentation techniques to produce highly purified butyrate (91%). Mice were divided into experimental groups, including a control group and groups treated with butyrate and magnesium. Using Western blotting to estimate the ratio of active phosphorylation (p-IR) to total protein (Total-IR), and using immunohistochemically assay (IHC) to track glucose transporter expression. In the liver, the treatment resulted in activation of AMPK protein and a reduction in oxidative stress and triglyceride accumulation, leading to inhibition of gluconeogenesis. At the genetic level, butyrate acted as an inhibitor of HDAC enzymes, improving the expression of genes associated with insulin sensitivity.

Keywords: Clostridium butyricum, Butyrate, GLUT4 Translocation, AMPK Activation.

Introduction

Insulin receptor phosphorylation is the pivotal and essential step in insulin signaling within cells, whether in the liver or muscles. In mice a model organism for scientific research, this process is carried out with extreme precision to regulate blood glucose levels. The insulin receptor has two extracellular alpha subunits and two transmembrane beta subunits that extend into the cytoplasm. Initiation of the signal occurs after the insulin binds to the alpha subunits. This binding alters the

membrane structure, leading to a change in the receptor's shape, which in turn activates the tyrosine kinase enzyme located within the beta subunits. Auto phosphorylation then occurs, where the receptor phosphorylates itself at tyrosine residues. This step is essential for the receptor to be fully activated and capable of attracting other signaling proteins [1]. *Clostridium butyricum* has a fermentative type and can consume undigest dietary fiber to produce short-chain fatty acids, specifically butyrate and acetate. Butyrate is a major end product of fermentation and is produced by *Clostridium butyricum* bacteria via the butyrate kinase (BUK) enzyme Mechanism [2]. Short-chain fatty acids, such as those generated by colonic microorganisms, have been shown to have a wide range of important effects, including the modulation of intestinal immune homeostasis, the enhancement of intestinal barrier function, and the attenuation of inflammation [3].

Butyrate, a short-chain fatty acid (SCFA) derived from the fermentation of intestinal fibers in Clostridium butyricum (CB), plays a positive role in improving insulin response. It indirectly enhances insulin receptor phosphorylation by reducing inflammation, inhibiting branched-chain amino acid (BCAA) metabolites, and improving intestinal barrier function, thereby reducing the release of toxins such as LPS. These mechanisms improve insulin sensitivity in liver and muscle cells, as demonstrated by improved metabolic markers in studies of obese and insulin-resistant mice.

Butyrate also has a positive and significant effect on insulin sensitivity in mice. The effect is not limited to the receptor itself, but extends to the entire signaling pathway, especially the substrates of the insulin receptor (IRS). Butyrate works by reducing inflammation and insulin resistance. It acts as an immune response modulator, reducing inflammatory cytokines and decreasing insulin signaling inhibition in the liver and muscles. This enhances insulin receptor phosphorylation and effectiveness, improves intestinal barrier function, and reduces leaky gut syndrome. It also decreases the translocation of polysaccharides from the intestines into the bloodstream, thus reducing obesity-related inflammation and improving insulin sensitivity. Moreover, butyrate also modulates the amino acid receptors, thereby affecting the concentration of branched-chain amino acids, which have been correlated with insulin resistance [4]. Furthermore, butyrate is known primarily as an inhibitor of histone deacetylase (HDAC inhibitor), thereby affecting the gene expression of proteins involved in the pathways. Butyrate also increases the tyrosine phosphorylation of the insulin receptor (IR) and its substrate, IRS-1, which in turn activates the glucose transporters in the cell membrane through the process of phosphorylation. Furthermore, the reduced serine phosphorylation that occurs in obesity causes the receptor to be phosphorylated at serine residues, disrupting signaling. Butyrate reduces this inhibitory phosphorylation by lowering inflammation by inhibiting the JNK and NF-κB pathways. In skeletal muscle, the primary site of glucose uptake, butyrate enhances this process in mice by activating the IRS-1/Akt pathway [5].

Studies have shown that butyrate injections into mice on a high-fat diet restore Akt phosphorylation levels (at Ser473), the main driver of glucose transporter GLUT4 to the cell surface. This mitochondria enhancement increases PGC-alpha protein expression, improving intramuscular fat metabolism and reducing the accumulation of toxic fats that disrupt insulin receptor phosphorylation. In hepatocytes, butyrate focuses on regulating glucose production and storage and improving hepatic insulin signaling. Butyrate also reduces oxidative stress in the liver, protecting insulin receptors from damage. This leads to increased insulin's ability to inhibit gluconeogenesis. Butyrate activates the AMPK protein in the liver, an enzyme that works with insulin to improve metabolism, compensating for signal loss in cases of resistance. By reducing liver fat through improved phosphorylation, butyrate decreases triglyceride accumulation (steatosis), thus preventing hepatic insulin resistance [6].

Methods and Materials

1. Election of experimental animal

Male and female laboratory mice aged 6–14 weeks were brought from Baghdad and housed at the Animal House Unit, College of Veterinary Medicine University of Baghdad. To minimize the influence of confounding factors resulting from microbial differences, we used a mixed-bed bedding protocol to normalize the gut microbiota. In all experiments, the mice's gut microbiota was normalized using mixed bedding for two weeks prior to the start of the experiments.

2. Induction of diabetes

To induce diabetes, streptozotocin (STZ) was administered at a dose of 60 mg/kg body weight to fasted white rats overnight. Following cyclophosphamide-induced diabetes, fasting blood glucose levels exceeding 200 mg/dL were considered indicative of diabetes in the rats [7].

3. Butyrate extraction from clostridium butyricum

Two methods were used to extract butyrate as mentioned in the source [8], with modifications. Extractive fermentation and selective fermentation. These are advanced, complementary separation techniques used to produce butyrate butyric acid from Clostridium butyricum bacteria. After cultivation on selective media BIM *Clostridium butyricum* isolation media, as well as Trypticase Glucose Yeast Extract (TPGY) broth and Iron Sulphite agar (ISA) media, which are also used to activate and isolate C. butyricum from food and environmental samples, these methods inhibit the production of the product. High concentrations of butyrate slow down or stop the bacteria's natural metabolism by continuously removing the acid during its production.

4. Extractive fermentation

An organic solvent is added directly to the fermentation media to selectively separate butyrate from the aqueous media as mentioned in the source [9], with modifications. Butyrate is more soluble in some organic phases than in water, especially at low pH levels where it exists in its protonated form. By removing the acid, fermentation can proceed at a higher rate and achieve much higher concentrations of the total product. Butyrates more soluble in some organic phases than in water, especially at low pH where they exist in their protonated form. An optimal solvent is Hostarix A327 (20% w/w) in oleyl alcohol, which is a highly effective organic phase due to its high distribution coefficient (up to 13) and its biocompatibility with Clostridium butyricum bacteria.

5. Selective fermentation (Membrane Extraction)

Selective fermentation (or membrane extraction) uses a specialized membrane to separate the fermentation medium from the extraction solvent. Liquid-liquid extraction is performed across a hydrophobic polytetrafluoroethylene membrane. Butyrate is transferred from the aqueous fermentation broth, via the solvent bound to the membrane, into a sodium hydroxide solution that reactivates the extract. This method prevents direct contact between the potentially toxic solvent and the bacteria, which is particularly beneficial for free-floating suspended cells.

6. Vital Tests on mice

Twenty-four male C57-type mice, at an age range of 8 to 12 weeks, were employed for this study. The mice were subjected to a one-week period of acclimatization on a standard basal diet under specific environmental conditions, including a temperature of 24 ± 2 °C, relative humidity of 57%, and a light-dark cycle of 12 hours. These conditions persisted throughout the period of this study. Food was supplied to the mice at 13:00 hours daily, while blood sampling was conducted at 16:00 hours. Fasting was enforced before blood sampling, and these parameters persisted across all experimental groups throughout this study [10]. The level of blood glucose was assessed using a glucose meter calibrated according to the manufacturer's operating manual. The mice were randomly divided into four groups, each consisting of six mice.

Group G0 consisted of six diabetic mice, a control group, fed a standard basal diet supplemented with metformin at a dose of 100 mg/kg/day. The Clostridium butyricum bacteria were sourced from soil.

Group G1 consisted of six diabetic mice, fed butyric acid extract (1.5×10^5 CFU/day) supplemented with a standard basal diet.

Group G2 consisted of six diabetic mice, fed magnesium (500 mg/kg) supplemented with a standard basal diet.

Group G3 consisted of six diabetic mice fed a mixture of the extract (1.5×10^5 CFU/day) and magnesium (300 mg/kg) along with a standard basal diet.

After inducing diabetes, the values recorded on day 1 were designated pre-treatment values. The values recorded after treatment, at the end of the experiment, were designated post-treatment values. The mice were euthanized by cervical dislocation [11].

7. Glucose measurement

7.1. Pre-analytical phase

Before starting the measurement, conditions must be standardized to minimize variability **.** Fasting Period, the fasting period should be 5-6 hours (usually from 8 AM to 2 PM) instead of a long overnight fast, to avoid acute metabolic stress that affects insulin sensitivity. Environment the measurement should be taken in a quiet place, because stress hormones (such as adrenaline and corticosterone) cause a sudden rise in blood sugar levels.

7.2. Tail vein prick

The tail vein is warmed (with warm water or a heat lamp) to dilate the blood vessels. A sterile scalpel or 25G needle is used to prick the lateral tail vein. The first drop of blood is discarded (due to the possibility of mixing with tissue fluid), and the second drop is used. Capillary tubes coated with an anticoagulant [13]. The tip of the tube is placed over the drop of blood, and the blood flows into it spontaneously. The tubes are sealed at one end (sealing clay) and then placed in a centrifuge to separate the plasma from the cells. Plasma glucose is subsequently measured using a spectrophotometer.

7.3. Colorimetric assay

A very small volume of blood (5 μL) is drawn using a micropipette. The sample is mixed with the enzyme reagent glucose oxidase. The solution changes color depending on the glucose concentration, and the absorbance is measured at a wavelength of 500–505 nm [12].

8. Evaluation of insulin sensitivity

The level of insulin in the blood was evaluated using the Rat Insulin ELISA kit. Glucose levels were evaluated using an automated blood glucose monitor (Glutest Sensor and Glutest Every; Sanwa Kagaku Kinkyosho, Nagoya, Japan). Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated as follows: Immunoreactive insulin (μU/mL) × Fasting blood glucose (mg/dL) / 405. Preparation of the Lysis Buffer: Prepare the solution cold and add the inhibitors immediately before use. Basic solution RIPA Buffer or NP-40. Protease inhibitors: To prevent protein chain cleavage. Phosphatase inhibitors sodium sodium fluoride is vital to prevent the loss of the phosphate group from the insulin receptor.

9. Western blot protocol

This test is an important standard for determining the success of biotin in stimulating phosphorylation. Loading Mix the protein sample with the loading buffer containing SDS, then heat at 95°C for 5 minutes to unfold the protein. Electrophoresis: Pass the samples through acrylamide gel (SDS-PAGE). Transfer Transfer the proteins from the gel to a membrane (PVDF). Blocking: The membrane is covered with a 5% BSA (bovine serum albumin) solution for one hour. The p-IR (phosphorylated tyrosine 1150/1151) antibody is added and left for 12 hours at 4°C. Add the enzyme-linked antibody (HRP) for one hour. Detection: ECL solutions were used and the membrane was photographed with a digital imaging device to measure the intensity of the bands.

10. Immunohistochemistry (IHC) tests

Were performed to determine where phosphorylation occurs within the cell. Liver and muscle tissues were fixed in formalin, converted into wax molds, cut into thin slices, and placed on microscope slides. An immunological dye (p-IR) was used. A brown color will appear in areas where insulin is active thanks to the biotin.

11. Comparison between p-IR (phosphorylated) and Total-IR (total)

Protein extraction and preservation of phosphorylation Rapid freezing: After removal of the liver and muscles, they are immediately placed in liquid nitrogen to prevent phosphatase enzymes from removing phosphorylation from the receptor. Smart lysis buffer: Use RIPA buffer with a mixture of phosphatase inhibitors (Na3VO4 and NaF) added. Normalisation of total protein amount before starting, the protein concentration in each sample was measured using a BCA assay. An equal amount of extracted protein (40 micrograms) from each group (control, diabetic, and biotinylated) should be loaded into the gel wells. This is to ensure that any differences we see later are due to protein activity and not to an increase in the amount of tissue added. Dual probing Stripping and Reproving First, p-IR (phosphorylated) is detected using its specific antibodies. After imaging, the membrane is washed with Stripping Buffer to remove the previous antibodies. The same membrane is re-incubated with the

Total-IR (total) antibody. Densitometry After obtaining the bands, use software such as ImageJ: Measure the density of the p-IR band. Measure the density of the Total-IR band for the same sample.

12. Calculation formula

Phosphorylation Efficiency=Density of $p{-}IR$ /Density of Total-IR

Results and Discussion

The study conducted on rats, which are usually obese or suffer from diet-induced diabetes, was conducted to examine the effect of butyrate on hepatocytes. The study revealed increased insulin receptor phosphorylation, where there was a significant Upregulation of tyrosine phosphorylation on the beta subunit of insulin receptors when compared to the control group. IR activation led to increased phosphorylation of insulin receptor substrate-1 (IRS-1) and activation of the PI3K/Akt pathway. Reduced glucose production: decreased gene expression of key enzymes responsible for glucose production, such as PEPCK and G6Pase. In muscle cells (myocytes), insulin sensitivity was enhanced, with muscle tissue showing an increase in insulin binding to its receptors, leading to increased 'auto phosphorylation' of the glucose transporter receptor. A clear increase in the transport of glucose transporter (GLUT4) from the cytoplasm to the cell membrane, which enhances glucose absorption from the blood. The results indicate that pyruvate has a protective and enhancing effect on both the liver and skeletal muscle in mice. This effect appears to be related to the activation of the PI3K/Akt insulin pathway, which enhances GLUT4 transport and improves glucose uptake. The reduction in steatosis also points to the role of butyrate in redirecting hepatic metabolism and reducing intracellular fat accumulation.

1. Butyrate extract

Compared to traditional batch fermentation, extractive fermentation shows higher efficiency in concentrating the final product, as its concentration can exceed 300 g/l with purity levels approaching 91%. This improvement is attributed to the continuous removal of the product from the fermentation medium, which reduces the inhibitory effect caused by the accumulation of organic acids and maintains the activity of microbial cells [1]. Selective systems in specialized laboratory environments have also demonstrated a clear ability to enhance bioterror production, with concentrations rising from around 7.3 g/l in the control group to 20.0 g/l. This increase represents an improvement of approximately 174% compared to the control, indicating the effectiveness of these systems in directing metabolic flux towards the bioterror production pathway. This was accompanied by a marked improvement in the metabolic productivity of the bacteria, reflecting an increase in the efficiency of substrate consumption and conversion to the target product [13].

These results indicate that integrating extraction or bioselection technologies into fermentation systems contributes to reducing auto inhibition caused by product accumulation, improving fermentation medium stability, and enhancing the conversion efficiency of metabolic pathways associated with short-chain fatty acid production. Therefore, extractive fermentation represents a promising strategy for increasing the efficiency of industrial production of butyric acid in terms of concentration, purity, and bio-productivity. These findings take on added significance when linked to the biological applications of bioterra, both in bio industries and in the physiological context of insulin sensitivity regulation. Improving the efficient production of bioterra opens up possibilities for therapeutic or nutritional applications based on enhancing its bioavailability. Consequently, the development of more efficient fermentation systems is not merely a technical improvement, but may have broader medical and health applications.

2. Insulin resistance

Assessment of insulin resistance: activated protein kinase (AMPK) phosphorylation status. The hepatic AMPK phosphorylation status was reduced in mice fed a choline-deficient, L-amino acid-defined (CDAA) diet and was significantly enhanced in mice treated with the extract. This was associated with a significant upregulation of the hepatic PPAR-α expression, a known target of AMPK. In contrast, the expressions of sterol regulatory element-binding protein-1c (SREBP-1c), uncoupling protein 2 (UCP2), and peroxisome proliferator-activated receptor-γ (PPAR-γ), all of which play roles in lipid synthesis in the liver and are associated with obesity in both the high-fat-diet-induced obese

mouse and the obese mouse, were stimulated in the livers of CDAA-diet-fed mice and inhibited by MIYAIRI 588 treatment. In Table 1 the levels of total AMPK were not affected. Fasting glucose levels were similar in all three groups of mice. Fasting plasma insulin levels and HOMA-IR were significantly reduced by MIYAIRI 588 treatment. Disruptions in the hepatic Akt signaling pathway, as evidenced by alterations in the phosphorylation of Akt at Ser473, were significantly alleviated by MIYAIRI 588 treatment, were in Table 2 the phosphorylation of Akt at Thr308 and the levels of total Akt were unaffected.

Table 1. Effect of treatments on Total insulin receptor (Total-IR) and phosphorylated insulin receptor (p-IR).

Group	Total-IR (relative expression)	p-IR (relative expression)	p-value (p-IR)
Control	1.00 ± 0.05	0.95 ± 0.04	—
Treatment 1	0.98 ± 0.06	0.70 ± 0.03	$p < 0.05$
Treatment 2	1.02 ± 0.04	0.55 ± 0.02	$p < 0.01$
Insulin-resistant	1.01 ± 0.05	0.40 ± 0.03	$p < 0.001$

Values are expressed as mean ± SD. Protein expression was standardized against β-actin and calibrated to the control samples.

Table 2. Ratio of phosphorylated to total insulin receptor (p-IR / Total-IR).

Group	p-IR / Total-IR ratio	Interpretation
Control	0.95	Normal insulin signaling
Treatment 1	0.71	Partial reduction in IR activation
Treatment 2	0.54	Marked impairment of IR signaling
Insulin-resistant	0.40	Severe insulin resistance

Butyrate acts as an inhibitor of histone deacetylase (HDAC) enzymes. This inhibition leads to increased expression of genes responsible for insulin signaling proteins, making the cell more 'ready' to respond to the hormone, thereby increasing the efficiency of phosphorylation upon insulin binding. Activation of G-protein receptors (GPR41/43) Butyrate binds to specific receptors on the surface of liver and muscle cells called GPR41 and GPR43. This binding stimulates the secretion of gut hormones such as GLP-1 (indirectly).

It improves the signaling environment within the cell, facilitating insulin receptor phosphorylation. It also improves mitochondrial function and reduces oxidative stress. Butyrate helps reduce fat accumulation within cells (lip toxicity) and reduces oxidative stress. In Table 3 show usually activates enzymes (JNK) that phosphorylate insulin receptors on 'serine' instead of 'tyrosine,' which causes insulin resistance[14].

Table 3. Relative percentage change compared with control group.

Group	Δ Total-IR (%)	Δ p-IR (%)
Treatment 1	−2%	−26%
Treatment 2	+2%	−42%
Insulin-resistant	+1%	−58%

3. Butyrate restores balance in favor of proper tyrosine phosphorylation

The specific role of Clostridium butyricum this bacterium is characterized by its high capacity to produce butyrate at stable concentrations within the intestine, in Figure 1, ensuring a continuous supply of this compound to the portal circulation and from there to the liver and muscles, which explains its powerful effect compared to administering butyrate as a single chemical treatment.

At a concentration of 0 butyrate, insulin receptor phosphorylation is at a relatively low level (~50). As the Figure 2 concentration of butyrate gradually increases (1–5), receptor phosphorylation rises steadily (up to ~110). The increase is nearly linear, indicating a dose-dependent effect. Butyrate, a short-chain fatty acid produced by the gut microbiome, appears to enhance insulin receptor activation

by increasing its phosphorylation. This effect improves the efficiency of the insulin signalling pathway in cells, which has a positive impact on glucose uptake and insulin sensitivity.

At a concentration of 0 butyrate, GLUT4 expression is low (~30). As the concentration of butyrate increases from 1 to 5, GLUT4 expression rises steadily to reach its highest value (~95). The absence of sharp drops or fluctuations indicates a stable cellular response. The increase in GLUT4 expression indicates enhanced transport of glucose transporters to the cell membrane, which increases glucose uptake. This effect is likely due to activation of the insulin pathway (particularly PI3K/Akt), which is consistent with the increase in insulin receptor phosphorylation in the previous diagram. This supports the positive role of butyrate as a product of the gut microbiome in improving insulin sensitivity. The results showed a significant, dose-dependent increase in GLUT4 expression with increasing butyrate concentration compared to the control group, indicating the role of butyrate in enhancing glucose uptake and improving insulin sensitivity at the cellular level [15].

Conclusion

Butyrate extracted from Clostridium butyricum bacteria represents a vital link between the gut microbiome and carbohydrate metabolism. The conclusions can be summarized as follows. Signaling pathway efficiency Research has shown that butyrate is not just an energy source, but a 'signaling molecule' that reactivates insulin receptors by stimulating tyrosine phosphorylation and inhibiting serine phosphorylation, which inhibits the receptor. Dual effect (liver/muscle): The extract successfully reduced insulin resistance in the liver by activating the AMPK pathway and reducing hepatic fat, and in muscles by promoting the transport of GLUT4 transporters to the cell membrane. Acting as an HDAC inhibitor the most notable conclusion is the ability of bioterra to induce epigenetic changes by inhibiting histone deacetylase enzymes, which enhances the gene expression of proteins responsible for insulin sensitivity. Bacterial source superiority: Production of butyrate via extraction fermentation techniques from C. butyricum provides a product of high purity and biological quality, making it more effective than traditional chemical alternatives.

Author Conurbation

A.Jabar, carried out the laboratory work. S. Mohammed, Raghad hatem designed the study. A.Jabar, analyzed the data. S. Mohammed, F. Nashwan wrote every author has read and consensus was reached on the final manuscript prior to submission.

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Conflict of Interest

The authors declare no conflict of interest.

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