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Resistant Starch from High Amylose Maize (HAM-RS2) and Dietary Butyrate Reduce Abdominal Fat by a Different Apparent Mechanism

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Objective: Obesity is a health concern. Resistant starch (RS) type 2 from high-amylose maize (HAM-RS2) and dietary sodium butyrate (SB) reduce abdominal fat in rodents. RS treatment is associated with increased gut hormones peptide YY (PYY) and glucagon-like peptide 1 (GLP-1), but it is not known if SB increases these hormones.

Design and Methods: This was investigated in a 2 x 2 rat study with HAM-RS2 (0 or 28% weight) and dietary sodium butyrate (0 and 3.2%) resulting in isocaloric treatments: energy control (EC), sodium butyrate (SB), HAM-RS2 (RS), and the combination (SBRS).

Results: RS and SB reduced abdominal fat and the combination reduced abdominal fat compared to SB and RS. RS was associated with increased fermentation in the cecum. Serum PYY and GLP-1 total were increased with RS treatment. RS treatment was associated with increased cecal butyrate produced from fermentation of RS, but there was no cecal increase for dietary SB.

Conclusions: SB after its absorption into the blood appears to not affect production of PYY and GLP-1, while butyrate from fermentation in the cecum promotes increased PYY and GLP-1. Future studies with lower doses of RS and SB are warranted and the combination may be beneficial for human health.


Introduction

Greater than one-third of adults and almost 17% of children in the US are obese (1). Bioactive food components, such as resistant starch (RS) type 2 from high-amylose maize (HAM-RS2) and dietary sodium butyrate (SB), reduce obesity in rodents (2,3) and in the future may in humans.

RS is found in several foods including legumes and potato salad (4). It is also available as a cornstarch and used in products to replace other starches and flours. Recently, a whole-grain version has been used in a human study demonstrating increased post-prandial satiety (5). RS reaches the large intestine where it is fermented by bacteria to short chain fatty acids (SCFA), including butyrate (3). Butyrate is the major fuel of the colonocytes. It is believed to improve the health of the colon, and very little reaches the systemic circulation (6).

SB added to the diet has reduced body fat in mice (2). This dietary butyrate was absorbed in the upper GI tract (7,8) and resulted in increased serum butyrate compared to control (2). Dietary butyrate could act by a similar mechanism compared to the butyrate produced by fermentation of RS in the lower GI tract.

It is commonly known that butyrate is found in relatively high levels in milk fat and, thus, butter and cheeses are dietary sources of butyrate. However, a good source for human consumption would be synthetic triglycerides known as Salatrim, which have been extensively studied and are metabolized normally (7,8).
High dietary doses of either RS or SB have been used in rodent mechanistic studies. Lower doses will be needed for human treatment. Combining the two bioactive components is one possible way to lower the doses needed. An initial step is to combine high doses of RS and SB to determine if the combination produces greater increases in serum GLP-1 and PYY.

Additionally, to effectively study comparisons in body fat, all diets in the study were isocaloric as has been reported in several previous studies (3)(9-12).

**Methods**

**Rats and diets**

Sixty male Sprague Dawley rats were purchased from Harlan (Indianapolis, IN). The rats were assigned to four groups (n = 15) balanced for age and weight: energy control (EC), sodium butyrate (SB), resistant starch (RS) and sodium butyrate plus resistant starch (SBRS).

**TABLE 1 Diets of the four groups in the study**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Energy value (kcal g⁻¹)</th>
<th>EC</th>
<th>SB</th>
<th>RS</th>
<th>SBRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amioca³</td>
<td>3.5</td>
<td>539.9</td>
<td>539.9</td>
<td>139.9</td>
<td>139.9</td>
</tr>
<tr>
<td>HAM-RS²</td>
<td>2.8</td>
<td>0</td>
<td>0</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4.0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Casein</td>
<td>3.58</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>8.84</td>
<td>72</td>
<td>40</td>
<td>72</td>
<td>40</td>
</tr>
<tr>
<td>Sodium butyrate</td>
<td>5.923</td>
<td>0</td>
<td>32</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>Mineral mix (AIN-93M)</td>
<td>0.88</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93)</td>
<td>3.87</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>4</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Diet energy value (kcal g⁻¹)</td>
<td></td>
<td>3.504</td>
<td>3.411</td>
<td>3.504</td>
<td>3.411</td>
</tr>
</tbody>
</table>

The four dietary groups were energy control (EC), sodium butyrate (SB), resistant starch (RS) and sodium butyrate plus resistant starch (SBRS).

³Amioca is a 100% amylopectin cornstarch product from Ingredion (Bridgewater, NJ).

²HAM-RS2 is high amylose maize cornstarch product from Ingredion. It was estimated by the company to have 56% resistant starch.

⁴Sodium butyrate was added to the SB and SBRS diets at 3.2% and this value was calculated using metabolic body size for rats from a higher success study (5).

**GI tract collections and culture bacterial analyses**

At study end, rats were euthanized and the GI tract with contents was collected from the base of the esophagus to the anus and weighed. This amount was subtracted from body weight to determine disemboweled body weight (DBW). Fermentation variables (weight of empty cecum, amount of contents in cecum and their pH, and butyrate in contents of cecum) were measured in twelve rats and three rats from each group had ceca collected for bacterial analyses. Both ends of the ceca were tied off with sutures before removal from GI tract in preparing for bacterial analyses. The full cecum was weighed and transferred to a Whirl pak bag that was placed in a double zip lock bag with an anaerobic GasPak™ EZ...

**TABLE 2 Results for rats in a 2 x 2 factorial design with factors resistant starch (RS, RS = HAM-RS2; ×) and dietary sodium butyrate (SB, ×): the four dietary groups were energy control (EC), sodium butyrate (SB), resistant starch (RS) and sodium butyrate plus resistant starch (SBRS)**

<table>
<thead>
<tr>
<th>Group</th>
<th>EC</th>
<th>SB</th>
<th>RS</th>
<th>SBRS</th>
<th>Pooled SEM</th>
<th>RS</th>
<th>SB</th>
<th>RS*SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal fat⁴ (abfat), g</td>
<td>10.9</td>
<td>8.9</td>
<td>8.6</td>
<td>7.1</td>
<td>0.5</td>
<td>0.0003</td>
<td>0.002</td>
<td>NS</td>
</tr>
<tr>
<td>Disemboweled body weight (DBW), g</td>
<td>402.2</td>
<td>380.5</td>
<td>358.7</td>
<td>367.7</td>
<td>7.2</td>
<td>0.0003</td>
<td>NS</td>
<td>0.04</td>
</tr>
<tr>
<td>Abfat/DBW, %⁵</td>
<td>2.7²</td>
<td>2.4²</td>
<td>2.3²</td>
<td>1.9³</td>
<td>0.1</td>
<td>0.003</td>
<td>0.0006</td>
<td>NS</td>
</tr>
<tr>
<td>Food intake, g⁶</td>
<td>1622.5</td>
<td>1611.6</td>
<td>1605.6</td>
<td>1544.5</td>
<td>22.1</td>
<td>0.06</td>
<td>0.10</td>
<td>NS</td>
</tr>
</tbody>
</table>

⁴Abdominal fat includes three fat groups: epididymal (attached to testes), perirenal (attached to kidney), and retroperitoneal (fat in abdominal cavity not attached to a particular organ).

⁵As the major goal of this study was to determine if combination of SB and RS was more effective than their individual use and since there was no significant interaction, a mean post hoc comparison LSD was performed to estimate if there was a combination effect. The results were: EC vs. SB, P < 0.02; EC vs. RS, P < 0.05; SB vs. RS, P < 0.02; RS vs. SBRS, P > 0.008; and SB vs. SBRS, P < 0.02. Different superscripts designate significant differences.

⁶The four treatment diets were isocaloric with 3.504 kcal g⁻¹ for EC and RS diets, and 3.411 kcal g⁻¹ for the SB and SBRS diets.

**Diet (Table 1)** were based on the AIN-93M diet and formulated to contain 3.504 kcal g⁻¹ of metabolizable energy (ME) for the EC and RS diets, and 3.411 kcal g⁻¹ for the SB and SBRS diets. A 100% amylopectin cornstarch (54 wt% of diet), Amioca (Ingredion Incorporated, Bridgewater, NJ), and increased cellulose (10%) were used in the EC and SB diets. The cellulose was used to match the ME content to the RS and SBRS diets. Thus, all four diets were isocaloric so that body fat comparisons could be made. Hi-maize 260⁷ (Ingredion Incorporated, Bridgewater, NJ) cornstarch (50%) was used as the source of HAM-RS2. This ingredient was estimated by Ingredion to contain 56% HAM-RS2. The RS and SBRS diets were estimated to contain 28% HAM-RS2. The metabolizable energy content of Hi-maize 260⁷ has been estimated to be 2.8 kcal g⁻¹ (13). SB was added to the SB and SBRS diets at 3.2% and this value was calculated using metabolic body size for rats from a higher successful dose (5%) in a previous mouse study with SB (2).
Gas generating Pouch System (Voiglobal Distribution INC., Lawrence, Kansas), and immersed in ice.

Total anaerobic bacteria (clostridia and other culturable anaerobes), lactic acid producing species (Lactobacillus, Streptococcus, Pediococcus and Leuconostoc and Bifidobacterium), and total Bifidobacterium species were cultured from ceca. Ceca and contents were ground in a stomacher (Seward Limited, London, UK) and initially diluted 1:4 with peptone buffer solutions (PBS) and serial dilutions were made. Lactic acid bacteria were cultured using de Man-Rogosa-Sharpe Agar (MRS agar) (Difco, Laboratories, Detroit, Michigan). Reinforced Clostridial agar (Oxoid, Basingstoke, UK) plates were used for total anaerobes, and bifidobacterium agar was used for Bifidobacterium species. The MRS and bifidobacterium agar (BD-BBL, Le Pont de Claix, France) were anaerobically incubated at 30°C for 48 h and reinforced Clostridial agar plates were anaerobically incubated at 37°C for 2-4 days in a chemically generated anaerobic system using anaerobic GasPak™ EZ in an anaerobic box (Mitsubishi Gas Chemical America, New York, NY). Total colony forming units (CFU) were determined and verified by gram staining.

Analyses
For measurements of butyrate and pH, the cecal contents were collected and frozen for later analysis. After thawing, cecal contents were homogenized in distilled water (0.5 g wet sample to 5 ml of water) and pH was measured using a combination electrode. Samples were then acidified with 1 mL of a 25% (w/w) solution of metaphosphoric acid that contained 2 g L\(^{-1}\) 2-ethyl-butyric acid as an internal standard for butyrate content. Solids in the homogenized samples were separated by centrifugation at 8,000g for 10 min. Before use, the sample was filtered through a Millipore filter (MILX HA 33 mm, 0.45 µm MCE STRL; Fisher SLHA 033SS). Butyrate in the effluent was analyzed by gas–liquid chromatography by a method similar to Barry et al. (14). Briefly, the column was an Alltech (Nicholasville, KY) Econo-cap EC-1000, 100% polyethylene glycol acid modified with dimensions of 15 m \(\times\) 0.53 mm with a film thickness of 1.20 µm. The program for temperature control was: 115°C for 0.1 min, then there was an increased rate of temperature of 10°C per minute up to 150°C and held for 0.1 min. Then the temperature was increased at 11°C per minute up to 170°C and held for 2 min. The injector temperature was 250°C.

Serum total GLP-1 (7-36 and 9-36) was measured using ELISA kit (ALPCO Diagnostics, Salem, NH). The human kit is effective in measuring the rodent hormone and the intra-assay variation for the kit is 3.7-4.7%. Rat/mouse PYY (1-36 and 3-36) from serum was measured using a radioimmunoassay kit (Millipore Corporation, St. Charles, MO) with an intra-assay variation of 3.2-3.9%.

Statistical analysis
Data were analyzed as a 2 \(\times\) 2 factorial with RS (±) and SB (±) as factors. All non-log-dependent variables (all but bacteria and pH)
Results

Rats fed RS or SB and the combination had decreased weights of abdominal fat normalized for DBW compared to control. The combination of the two bioactive food compounds had lower normalized abdominal fat compared to SB and RS (Table 2). Part of the effect on reducing abdominal fat may have been due to reduced food intake (SBRS < RS < SB < EC), but this reduction only approached significance.

The groups fed RS demonstrated increased fermentation demonstrated by increased weight of empty cecum (Figure 1A), decreased pH of cecal contents (Figure 1B), increased weight of cecal contents (Figure 1C), and increased butyrate in cecal contents (Figure 1D). Dietary butyrate did not increase butyrate in the cecum indicating the dietary SB was absorbed in the upper gut as expected (7,8). The increased amounts of butyrate in the cecum of rats fed RS came from the fermentation of RS.

Serum total GLP-1 (Figure 2A) and PYY (Figure 2B) were elevated in groups fed RS. However, the SBRs group had significantly reduced amounts of these hormones compared to RS.

RS increased cecal bacteria measured by culture (Figure 3A-C). Dietary SB was associated with increased amounts of Bifidobacterium species (Figure 3B) and decreased amounts of lactic acid producing bacteria (Figure 3C).

Discussion

Results from this study indicate that RS and dietary SB reduce body fat normalized for body weight when fed to rats. Previous studies demonstrated this in rats and mice (3,11,12,15,16), and the major aim of this study was to investigate the use of the two bioactive compounds in combination. Although the two compounds at relatively high doses (used to study mechanism in past studies), did not demonstrate a statistically factorial interaction (additive or
synergistic), the combination was more effective in reducing abdominal fat normalized for DBW than the use of the individual compounds (post hoc means comparison test). This result indicates that future studies investigating combinations of SB and RS at lower dietary doses are warranted. If a combination of the compounds is as effective as a higher dose of the individual compounds, this may allow for practical doses to give to humans.

There are dietary sources of RS and SB. Sources of RS include common foods as legumes and potato salad or starch or whole-grain flour sources of RS sold to companies. The latter have been previously used in research (3,5,17). The most effective sources of dietary butyrate would be previously studied synthetic triacylglycerols known as Salatrim (7,8).

Previous studies demonstrated that beneficial effects of dietary RS are associated with increased serum levels of GLP-1 and PYY. The increased amounts of these hormones appeared to be associated with increased fermentation and increased cecal butyrate (3). In one study, primary cultures of cecal cells increased gene expression for the two hormones when butyrate was included in the media (15). Theoretically it is possible that dietary SB absorbed in the upper gut could interact with lower gut endocrine cells at the blood vessel surface to promote decreased production of GLP-1 and PYY. Results from the current study indicate that dietary SB does not increase production of GLP-1 and PYY. However, the significant interaction observed with the SBRS group to decrease serum levels of GLP-1 and PYY and cecal butyrate amounts compared to RS may be speculated to result from some type of negative feedback to prevent greater reductions in abdominal fat. This may have been done by a change in the stool bacteria reducing fermentation and lowering gut hormone stimulation by butyrate.

The association of increased cultured amounts of Bifidobacteria with dietary butyrate is surprising and future research is needed to elucidate the mechanism. However, it does indicate that dietary butyrate absorbed in the upper GI tract may affect the health of the lower GI tract.

In conclusion, isocaloric diets were used to compare effects of dietary sodium butyrate, dietary resistant starch and their combination on body fat in rats. As expected, it appeared that dietary sodium butyrate was absorbed in the upper GI tract as cecal butyrate was not increased with dietary sodium butyrate. Dietary resistant starch was fermented in the lower GI tract and resulted in increased serum gut hormones, GLP-1 and PYY, which are produced in the lower GI tract. This indicated a possible different mechanism for the two dietary components. Each bioactive dietary component was associated with reduced abdominal fat and the combination resulted in a greater decrease in abdominal fat. These results merit further investigation with lower dietary doses that can be tolerated in humans and may indicate that lower combined doses of dietary sodium butyrate and resistant starch will be as effective as high doses of the individual compounds.

Acknowledgments

Amioca® cornstarch and Hi-maize® cornstarch were gifts from Ingredion Incorporated (Bridgewater, NJ).

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References