Intestinal sweet sensing pathways and metabolic changes after Roux-en-Y gastric bypass surgery

Running title: Intestinal sweet sensing pathways after RYGB

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Abstract

Introduction: Studies suggest that improvements in type 2 diabetes (T2D) post-RYGB surgery are due to decreased intestinal glucose absorption capacity mediated by exclusion of sweet taste sensing pathways in isolated proximal bowel. We probed these pathways in rat models that had undergone RYGB with catheter placement in the biliopancreatic (BP) limb to permit post-RYGB exposure of isolated bowel to sweet taste stimulants.

Methods: Lean Sprague Dawley (SD) (n=13) and obese Zucker Diabetic Fatty (ZDF) rats (n=15) underwent RYGB with BP catheter placement. On post-operative day 11 rats received catheter infusions of saccharin (sweet taste receptor (T1R2/3) agonist) or saline (control). Jejunum was analyzed for changes in glucose transporter/sensor mRNA expression and functional SGLT1-mediated glucose uptake.

Results: Saccharin infusion did not alter glucose uptake in the Roux limb of RYGB rats. Intestinal expression of glucose sensors (T1R2, SGLT3) and transporters (SGLT1, GLUT2) was similar in saccharin- vs. saline-infused rats of both strains. However, the abundance of SGLT3b mRNA, a putative glucose sensor, was higher in the common limb vs. BP/ Roux limb in both strains of bypassed rats and was significantly decreased in the Roux limb after saccharin infusion.

Conclusions: Failure of BP limb exposure to saccharin to increase Roux limb glucose uptake suggests that isolation of T1R2/3 is unlikely to be involved in metabolic benefits of RYGB as re-stimulation failed to reverse changes in intestinal glucose absorption capacity. The altered expression pattern of SGLT3 after RYGB warrants further investigation of its potential involvement in resolution of T2D after RYGB.
**Keywords:** Roux-en-Y gastric bypass; obesity; type 2 diabetes mellitus; intestinal sweet taste sensor; SGLT1
In recent decades, bariatric surgery has evolved into the foremost therapy for obesity. Moreover, several of the weight loss operation, most notably Roux-en-Y Gastric Bypass surgery (RYGB), is associated with a high rate of remission of type 2 diabetes mellitus (T2D), with an early and weight-independent mechanism that improves glucose homeostasis (2, 17). However, the mechanisms mediating the resolution of T2D remain poorly understood. Intense investigation of contributory factors has implicated several mechanisms, including alterations in incretin levels, the gut microbiome, bile acids, and an area of interest to our lab: intestinal nutrient sensing. The surgical isolation of duodenum and proximal jejunum appear to be critical in mediating resolution of T2D, supported by outcomes following endoluminal sleeve device insertion and surgical procedures (e.g. RYGB, Biliopancreatic diversion) which exclude a similar region of the intestine from the digestive process (9, 19, 20, 21). Further highlighting the importance of proximal bowel isolation are studies in RYGB models showing that the re-introduction of mixed nutrients into the excluded bowel reverses the beneficial effects of surgery on glucose tolerance (7, 8, 22). Despite recognition of the importance of the proximal small bowel in glucose regulation, a more detailed understanding of the underlying mechanisms remains elusive. RYGB removes a significant part of the proximal small bowel from the physiologic enteral tract in the formation of the biliopancreatic (BP) limb, and prevents nutrient contact with this region of the bowel. We have hypothesized that prevention of nutrient stimulation of mucosal sensors and luminal nutrient transporters in this bowel segment mediates improvements in glucose homeostasis. We previously confirmed the presence of sweet taste receptors (T1R2/3) along this region of bowel, and demonstrated that specific activation of the T1R2/3 receptor by selective agonists resulted in acute up-regulation of the critical intestinal glucose transporter SGLT1 within the region infused (23). We subsequently showed that RYGB surgery in
physiologically healthy Sprague Dawley (SD) rats leads to a decrease in SGLT1-mediated glucose uptake in the Roux limb (also known as the alimentary limb), which could contribute to the metabolic benefits of the surgery (24).

The aim of this study was to extend our previous work by assessing the role of the sweet taste receptor T1R2/3 in the down-regulation of intestinal SGLT1-mediated glucose uptake after RYGB in the SD rat and to investigate changes in the intestinal nutrient sensing pathway following RYGB in the obese Zucker diabetic fatty (ZDF) rat. We hypothesized that decreased SGLT1-mediated intestinal glucose absorption in the Roux limb after RYGB is due to exclusion of the sweet taste receptor complex in the isolated BP limb. To test this, we developed a model in which isolated BP mucosa can be re-exposed to sweet taste receptor stimulating agonists via an indwelling catheter. Thus, contrary to most previous studies involving infusion of mixed meals, we re-stimulated the BP limb with a specific stimulus that has no caloric value to help isolate the contributions of a specific pathway in the anti-diabetic effect of RYGB.
Methods

Animals. The Harvard Medical Area Standing Committee on Animals prospectively approved all studies. Thirteen week old SD (Harlan) and obese (fa/fa) ZDF rats (Charles River Laboratories) were acclimatized for 5 days at constant temperature and humidity (22°C and 70% respectively), with a fixed 12:12 light:dark cycle (lights on at 7am). Standard chow (Carbohydrate 58.0%, Protein 28.5%, Fat 13.5%) and Purina 5008 (Carbohydrate 56.5%, Protein 26.8%, Fat 16.7%) were provided ad libitum to each group respectively. Animals were caged in pairs pre-operatively and individually after surgery.

Animal model. Animals were fasted from 7pm the evening prior to surgery, with ad libitum access to water. Under inhalation anesthesia (isoflurane 2-3% in oxygen), rats were given a dose of 0.1ml Penicillin G and RYGB was undertaken as follows: through a midline laparotomy the stomach was divided using the Endo GIA stapling device, (2-2.5mm staples) just below the gastroesophageal junction from greater to lesser curve, taking care to preserve the vagus nerve in this region. The jejunum was divided 16cm distal to the Ligament of Treitz and the distal cut end of the jejunum anastomosed to a 1cm gastrotomy on the anterior surface of the gastric pouch (interrupted, 6/0 PDS). A 1cm enterotomy was made on the antimesenteric aspect of the jejunum 10cm distal to the gastrojejunostomy and anastomosed to the proximal cut end of jejunum as an end-to-side anastomosis (interrupted, 6/0 PDS), forming the BP limb.

A soft polyurethane cannula (0.05 inch external diameter) was inserted into the gastric remnant at the time of RYGB, and passed into the 2nd part of the duodenum (tip of catheter in proximal BP limb) before securing with a 4/0 silk suture. The cannula was exteriorized through the left anterolateral abdominal wall, and tunnelled subcutaneously to emerge at the dorsal neck. It was cut short and capped, and secured in place with 3/0 vicryl. The abdomen was closed in 2 layers with 3/0 vicryl. 10-15ml 0.9% normal saline was administered subcutaneously to maintain intra-operative hydration. Post-operatively,
animals had free access to tap water, and a liquid diet was provided *ad libitum* from day 2 post-operatively (CVS Liquid Nutrition 8oz: 250kcal, 40g carbohydrate, 9g protein, 6g fat). After 5 days animals were returned to pre-operative solid diet (standard chow or Purina 5008). Catheters were inspected and flushed daily to ensure the site of exteriorization was clean, and to maintain patency.

*Experiment Protocol.* Body weights (commencing on the day of surgery) and food intake (pre-operatively and once solid diet was resumed post-operatively) of animals were documented on a daily basis. Blood glucose levels were measured before commencing RYGB surgery and immediately prior to harvest. On the 11th post-operative day (by which time point the animals’ weights have stabilized with no ongoing weight loss and they have been re-established on their regular pre-operative diet) rats were randomized into treatment and control groups. Treatment animals received a 9ml infusion of 0.3% saccharin via the BP catheter, over a 3 hour period commencing at 8am (SD n=6, ZDF n=10). Control animals received 9ml of 0.9% normal saline following the same infusion regime (SD n=3, ZDF n=5). Infusions were delivered via an electronically programmed pump. At harvest, delivery of infusate into the BP limb was confirmed by ensuring the tip of the indwelling catheter was correctly positioned in the duodenum.

A further control group of SD rats underwent RYGB with catheter placement but received no infusion at harvest to exclude the effects of mechanical distension in the BP limb (n=4). At the end of the 3 hour infusion period (11am), animals were harvested and tissues collected for analysis.

*Tissue harvest.* Under inhalation anesthesia using isoflurane 2-3% in oxygen, a midline laparotomy was performed and the Roux limb flushed with ice-cold phosphate-buffered saline before harvesting the first 5cm for everted sleeve studies. This segment was divided into equal thirds, which were everted and mounted onto a glass rod for functional glucose uptake assays. The remainder of the Roux limb as well as the common and BP limbs were harvested for mucosal scrapings, with samples being immediately snap frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.
Functional glucose transport assays. Glucose uptake was measured in everted sleeves as previously described (1, 12), using triplicate sleeves from the Roux limb. Uptake experiments were performed with Mammalian Ringer’s (128mM NaCl, 4.7mM KCl, 2.5mM CaCl₂, 1.2mM KH₂PO₄, 1.2mM MgSO₄, 20mM NaHCO₃) containing 50mM D-glucose (saturating concentration) and trace quantities of [¹⁴C]D-glucose (Perkin Elmer, Waltham, MA) and [³H]L-glucose (Moravek Biochemicals, Brea, CA) (1 min, 37°C). Another set of sleeves was similarly incubated in uptake solution containing 500μM phloridzin, an SGLT1-specific inhibitor (Sigma, St. Louis, MO). The glucose uptake rate was calculated from the difference between the uptake of [¹⁴C]D-glucose and that of the non-transported [³H]L-glucose used as a passive control.

RNA analysis. RNA was extracted from frozen tissue samples with a mirVana mRNA Isolation Kit (Ambion, Austin, TX) and quantified with a microplate reader (Spectramax M5; Molecular Devices, Sunnyvale, CA). Reverse transcription was performed simultaneously on 0.5-1.0 μg of RNA from each rat with Superscript III and oligo-dT (Invitrogen, Carlsbad, CA). To facilitate inter- and intra-group comparisons of gene expression, quantitative PCR of all cDNA samples were run on a single 384-well plate. The cDNA product was diluted and added to forward and reverse primers (Sglt1, T1r2, Glut2, Sglt3b, Cyclophylin A (housekeeping gene) Invitrogen; Supplementary Table 1), together with SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in duplicate or triplicate with diluted cDNA primers and SYBR green master mix using an Applied Biosystems ABI 7900HT Thermal Cycler (2 min, 50°C; 10 min, 95°C; 40 cycles of 15 s, 95°Cand 1 min, 60°C).

Statistical analyses. All data are presented as means ± standard error of the mean. Data were analyzed using Student’s t-test or ANOVA with post-hoc correction. Differences were considered significant at p≤0.05. Analyses were undertaken using SPSS 16 software or Excel.
Results

Body weight, food intake and blood glucose:

SD rats. Mean body weight of rats decreased from 364.8 ± 3.3g at baseline (day of surgery) to 315.1 ± 7.2g at harvest on POD 11 (13.5 ± 2.1% decrease in baseline body weight) (Figure 1). There was no difference in weight loss between those randomized to saline vs. saccharin infusion (p=0.7). Food intake was decreased in the post-operative period (24 ± 0.5 vs. 19.1 ± 1.5 g/rat/24hr standard chow pre and post-op respectively, p<0.05) although this decrease was not evident when corrected for body weight (0.064 ± 0.001 vs. 0.060 ± 0.004 g/gram body weight/24hr pre and post-op respectively, p=0.4). Blood glucose did not change significantly in physiologically healthy SD rats after RYGB.

ZDF rats. ZDF body weights were lowest at POD 9, and remained at this level until harvest on POD 11 (mean body weight 390.4 ± 11.4g at baseline vs. 337.6 ± 8.2g at harvest; 13.1 ± 1.8% decrease in baseline body weight) (Figure 1). There was no difference in weight loss between the 2 infusion arms (p=0.9). Food intake was decreased in the post-operative period (34.5 ± 0.9 pre-op vs. 22.2 ± 2.3 post-op g/rat/24hr Purina 5008, p<0.01). This decrease remained significant when corrected for body weight (0.085 ± 0.004 pre-op vs. 0.064 ± 0.007 post-op g/gram body weight/24hr, p<0.05). Rats in the 2 infusion arms consumed similar amounts of chow in the post-operative period (p=0.2). Mean blood glucose decreased by more than 30% in all ZDF groups after surgery (331 ± 37 pre-op vs. 200 ± 29mg/dl post-op; p=0.02).

Functional glucose uptake:

SD rats. Mean functional glucose uptake rates in the Roux limb of the rats were 6.65 ± 0.77, 7.10 ± 0.10, and 5.76 ± 1.53 nmol/mg/min in the saccharin-infused, saline-infused and non-infused groups.
respectively. Differences were not significant. Approximately 90% reduction of glucose uptake in the presence of phloridzin confirmed that SGLT1 accounted for the majority of glucose absorption (Figure 2).

ZDF rats. There was no difference in SGLT1-mediated glucose uptake in the Roux limb of RYGB rats receiving BP infusions of saccharin (n=10) vs. normal saline (n=5) (9.03 ± 0.68 vs. 7.57 ± 0.65 nmol/mg/min, p=0.15) (Figure 2). Phloridzin inhibition was >90%.

Intestinal gene expression:

Glucose transporters- Sglt1 and Glut2. 11 days after RYGB, there was no difference in mRNA expression of the glucose transporters Sglt1 and Glut2 between rats receiving a BP infusion of saccharin and the groups receiving control saline infusions in either SD rats or the ZDF model (assessed regionally per bypass limb or average expression levels throughout bowel). Topographic variation of mRNA expression of these transporters was not evident in SD or ZDF rats after RYGB (Figure 3a and b).

Glucose sensors- T1r2 and Sglt3b. Topographic variation of mRNA expression of the sweet taste receptor T1r2 was not evident in SD or ZDF rats after RYGB. There were no differences in expression levels overall or regionally (within BP/ Roux/ common limb) between saccharin and saline-infused rats of either strain (Figure 3c).

Topographic variation of Sglt3b was apparent in both SD and ZDF rats, but was more pronounced in the ZDF rats with 7- to 10-fold higher levels of Sglt3b in the common limb than in the Roux and BP limbs (p≤0.01 for both infusion groups) (Figure 4a). Levels of Sglt3b were approximately 3- to 5-fold higher in the common limb of bypassed SD rats compared to Roux and BP limb levels (p<0.05 for all SD groups combined) (Figure 4b). There was no difference in SGLT3b expression between infusion groups of the SD
rats. However, in ZDF rats there was a 2.4-fold down-regulation on infusion of saccharin vs. normal saline (p<0.05).
Despite widespread acceptance of RYGB as a treatment for obesity and T2D, the mechanisms through which improvements in glycemic control are achieved are not well understood. Numerous studies have examined feeding patterns, weight and hormonal changes after RYGB in either obese or diabetic rodent models (4, 13, 25), but few have analyzed changes in the obese and diabetic rat or attempted to delineate the mechanisms mediating change. To this end, we elected to study changes in healthy SD as well as obese ZDF rats. The obese ZDF rat carries an autosomal recessive mutation in the leptin receptor gene leading to hyperphagia, obesity and progressive insulin resistance, closely paralleling the metabolic syndrome in humans.

We had previously shown that RYGB leads to down regulation of intestinal glucose absorption capacity, through changes in SGLT1 function (24). We hypothesized that this post-RYGB reduction in intestinal SGLT1-mediated glucose uptake is due to exclusion of the sweet taste receptor T1R2/3 otherwise positioned to detect the presence of glucose in the anatomically normal bowel. We confirmed the presence of the sensor in both models, and observed no changes in mRNA expression of the T1R2 component or of the glucose transporter genes SGLT1 and GLUT2 in response to a sweet taste receptor agonist infused into the surgically isolated bowel in either rat strain. However we did find changes in the putative glucose sensor SGLT3b.

Studies have shown that the T1R2/3 complex is necessary for SGLT1 up-regulation in response to an increased carbohydrate load (18), and that non-nutritive sweeteners acting at the sweet taste receptor as an alternative to glucose can reproduce this up-regulation as well as elicit an incretin hormone response (15). In our study of the post-RYGB model however, the sweet taste receptor stimulant saccharin did not alter Roux limb glucose uptake when infused into the isolated BP limb. The lack of response to saccharin infusion could be due to one of several reasons. Firstly, the T1R2/3-SGLT1
signaling pathway may be irrecoverably disrupted by the surgical intervention, e.g. due to disruption of vagal nerve branches or division of enteric nerve plexus within the bowel wall. These factors may also explain why others have found no effect of sweet taste receptor agonists on intestinal glucose absorption in isolated segments of bowel that had been ligated proximally and distally (5). Alternatively, T1R2/3 may exert a more local effect on SGLT1 (autocrine/paracrine) but not one that reaches as far distally as the jejunum of the Roux limb. Given reports of diminished desire for sweet tasting food post-RYGB and descriptions of altered perception of sweet foods on the palate, it is even plausible that either sensitivity or ability of intestinal T1R2/3 to perceive sweet substrates as it did pre-operatively is altered post-RYGB (3, 27). To pre-empt reduced sensitivity post-RYGB we infused a dose of saccharin that is 10 times greater than that in saccharin-sweetened sodas.

Another possibility for our negative result is that the relevant sweet sensing pathway is one that is independent of the T1R2/3 complex and one for which saccharin is not an agonist. This possibility made us explore SGLT3, another putative glucose sensor. SGLT3 is a neuronally located member of the solute carrier family of proteins that appears to function as a sensor rather than a transporter of glucose (6, 10). Binding of glucose (but not saccharin) to SGLT3 leads to membrane depolarization. Thus if SGLT3 is an important nutrient sensor after bariatric surgery, our infusion of saccharine to the BP limb would not reverse the anti-glucose absorptive effects of surgery, consistent with what we have seen. We found higher levels of the putative glucose sensor SGLT3 in the common limb of bowel vs. the BP and Roux limbs in both SD and ZDF rats. The decrease in SGLT3 mRNA level in the Roux limb of RYGB ZDF rats after saccharin infusion are interesting findings and should prompt further investigation of its role in glucose sensing, the pathophysiology of metabolic disease and resolution of T2D after RYGB, including measurement of both its known isomeric forms in rodents (SGLT3a and b). Other groups have also studied changes in glucose transporters and sensors after RYGB in other rodent models. In a study to assess taste preference after RYGB, Bueter et al. found decreased T1R2 levels in the BP limb of bypassed
Wistar rats but increased levels in the Roux limb (3). In their study on SD rats, Taqi et al. found an increase in GLUT2 mRNA levels in both the Roux and common limbs, with decreased SGLT1 in the Roux limb (26). These studies differ from ours in several ways however including the fact that they compared RYGB rats to sham animals rather than expression levels of transporters in RYGB limbs after BP limb infusion with different agents.

To clarify the role of the foregut in diabetes amelioration, a limited number of studies with catheter placement into the gastric remnant or BP limb have been done. In separate studies, one group conducted glucose tolerance tests post-RYGB in obese SD and ZDF rats and compared results from orally administered glucose to administration via gastrostomy tube in the gastric remnant (8, 22). RYGB led to a significant decrease in glucose area under the curve following orally administered glucose. This post-RYGB improvement was lost when glucose was administered per gastrostomy. In these studies, administration of glucose rather than a specific sweet taste receptor agonist as in our study may exert a different effect on glucose sensors in the isolated BP limb, e.g. also activate SGLT3. In humans, single case studies report improved glucose, incretin and insulin responses with liquid mixed meals administered orally vs. via gastrostomy tube in the remnant (7, 16). Most recently, Lindqvist et al. showed an increased insulin and GLP-1 response to a mixed meal test delivered orally vs. via the gastric remnant in 4 female patients post-RYGB (14). Although Hansen et al. found no difference in glucose homeostasis parameters in human subjects post-RYGB following mixed meals administered orally or into the isolated gut (11), most studies suggest that re-stimulation of the proximal bowel with glucose or mixed meal leads to deterioration of glucose tolerance. Our studies take these observations further, attempting to identify the mechanisms involved in these observations by testing specific nutrient sensing pathways.
In humans, resolution of T2D occurs early post-RYGB, before substantial weight loss. For this reason, we conducted catheter infusion studies in rats early (POD 11) in order to investigate changes in the sweet taste sensing pathway in this early post-operative period. We chose to selectively interrogate one pathway at a time, using saccharin which is specific for T1R2/3 activation rather than challenging the isolated limb of bowel with a mixed meal which may activate multiple pathways and introduce confounding. We have previously demonstrated an acute response (within 3 hours) of intestinal glucose uptake to saccharin stimulation of T1R2/3 and designed the current study based on these data and did not pursue models of longer infusions or repeated infusions over a longer period.

The possibility of a type 2 statistical error should be considered due to use of ZDF rats with less predictable disease phenotype expression. To account for this however we used an n=10 in the ZDF saccharin-infused group and n=5 in the ZDF saline-infused group. We would expect this to provide adequate power to detect any meaningful and significant differences.

Transcription and post-translational modifications mean that mRNA results cannot be extrapolated/interpreted to make assumptions about protein levels. However, technical difficulties with antibodies against SGLT1 and T1R2 precluded protein assays in this study. We believe that measurement of functional SGLT1-mediated glucose uptake is an acceptable and likely better surrogate for SGLT1 protein levels as it measures the final step in the process and the extent of functional protein.

Changes in intestinal glucose sensing in the pathophysiology of obesity and T2D, and following RYGB require further research to enhance understanding of the gut as an endocrine organ, the effects of RYGB, and its scope as a metabolic as well as bariatric procedure. Analysis of changes in intestinal sensors and transporters at the protein level, and challenging the isolated gut after RYGB with different agonists in a selective manner will add valuable insight into the mechanisms effecting improvements in T2D after surgery.
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Author disclosure:
A Tavakkoli has an equity interest in Avaxia Biologics, a company that is developing oral antibodies for treatment of intestinal disorders, with potential applications for treatment of diabetes and obesity. AT’s interests were reviewed and are managed by the Brigham and Women’s Hospital and Partners HealthCare in accordance with their conflict of interest policies.
References


Figure Legend

Figure 1. Weight change in SD and ZDF rats after RYGB with indwelling catheter placement in the BP limb.

Figure 2. Mean functional glucose uptake in Roux limb of RYGB SD and ZDF rats following BP limb infusion of 0.3% saccharin vs. 0.9% normal saline vs. no infusion (SDs only), as measured by the everted sleeve technique. Phloridzin inhibition of glucose uptake demonstrates SGLT1-mediated uptake.

Figure 3a. SGLT1 mRNA expression in BP, common and Roux limbs of RYGB SD and ZDF rats 11 days after surgery. Black bar- no infusion, white bar- Normal Saline infusion, shaded bar- Saccharin infusion.

Figure 3b. GLUT2 mRNA expression in BP, common and Roux limbs of RYGB SD and ZDF rats 11 days after surgery. Black bar- no infusion, white bar- Normal Saline infusion, shaded bar- Saccharin infusion.

Figure 3c. T1R2 mRNA expression in BP, common and Roux limbs of RYGB SD and ZDF rats 11 days after surgery. Black bar- no infusion, white bar- Normal Saline infusion, shaded bar- Saccharin infusion.

Figure 4a. SGLT3b mRNA expression in BP, common and Roux limbs of RYGB ZDF rats 11 days after surgery. White bar- Normal Saline infusion, shaded bar- Saccharin infusion.

Figure 4b. SGLT3b mRNA expression in BP, common and Roux limbs of RYGB SD rats 11 days after surgery. Black bar- no infusion, white bar- Normal Saline infusion, shaded bar- Saccharin infusion.

Table 1. RT-PCR primers for rat genes; the sodium glucose co-transporter Sglt1, the glucose transporter Glut2, the sweet taste receptor component T1r2, the sodium glucose co-transporter Sglt3b, and housekeeping gene Cyclophylin A. Thermal Cycler conditions used were 2 min, 50°C; 10 min, 95°C; 40 cycles of 15 s, 95°C and 1 min, 60°C. Dissociation curves were obtained to ensure a single amplicon.
Roux

BP

Common

Normalized GLUT2 mRNA expression

SD  ZDF

Normalized GLUT2 mRNA expression

SD  ZDF
Table 1. RT-PCR primers for rat genes

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<th>Gene of interest</th>
<th>Primer Sequence- Forward</th>
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