Inhibition of the gut enzyme intestinal alkaline phosphatase may explain how aspartame promotes glucose intolerance and obesity in mice

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Abstract

Background: Diet soda consumption has not been associated with tangible weight loss. Aspartame (ASP), commonly substitutes sugar, and one of its breakdown products is phenylalanine (PHE), a known inhibitor of intestinal alkaline phosphatase (IAP), a gut enzyme shown to prevent metabolic syndrome in mice.

Objective: We hypothesized that ASP consumption might contribute to the development of metabolic syndrome based on PHE's inhibition of endogenous IAP.

Design: *In vitro model:* IAP was added to diet and regular soda, and IAP activity was measured. *Acute model:* A closed bowel loop was created in mice. ASP or water was instilled into it and IAP activity was measured. *Chronic model:* Mice were fed chow or high fat diet (HFD) with/without ASP in the drinking water for 18 weeks.

Results: *In vitro study*: IAP activity was lower ($p<.05$) in solutions containing ASP compared to controls. *Acute model*: endogenous IAP activity was reduced by 50% in the ASP group compared to controls (0.2±0.03 vs 0.4±0.24) ($p=.02$). *Chronic model*: Mice in the HFD+ASP group gained more weight compared to HFD+water group (48.1±1.*6 vs* 42.4±3.1, $p=.0001$). Significant difference in glucose intolerance between HFD +/- ASP groups (53913±4000.58mg*min/dL vs 42003.75±5331.61mg*min/dL, respectively, $p=.02$). Fasting glucose and serum TNF-alpha levels were significantly higher in the HFD+ASP group (1.23 and 0.87 fold increases, respectively, $p=.006$ and $p=.01$).

Conclusions: Endogenous IAP's protective effects in regard to the metabolic syndrome may be inhibited by PHE, a metabolite of aspartame, perhaps explaining the lack of expected weight loss and metabolic improvements associated with diet drinks.

Abbreviations:

Intestinal Alkaline Phosphatase (IAP), Aspartame (ASP), L-Phenylalanine (PHE), High fat diet (HFD), Lipopolysaccharide (LPS), sugar sweetened beverages (SSB), artificially sweetened beverages (ASB)

Keywords:

non-nutritive sweeteners, non-caloric sweeteners, diet induced insulin resistance, intestinal alkaline phosphatase, aspartame, obesity, insulin resistance

Introduction

It is clear that consuming sugar sweetened beverages (SSB) promotes obesity (Malik et al. 2010). Artificial sweeteners are often employed as a sugar substitute and weight management strategy (Anton et al. 2010), yet numerous studies suggest that the replacement of SSB with artificially sweetened beverages (ASB) does not diminish weight gain. Indeed, ASB have been implicated in the development of certain aspects of metabolic syndrome, including insulin resistance, impaired glucose tolerance, and dyslipidemia (Gardener et al. 2012; Swithers 2013; Imamura et al. 2015). The negative effects of ASB on metabolic health may even impact the offspring of women exposed during their pregnancy (Araujo et al. 2014; Azad et al. 2016). One of the most common artificial sweeteners, Aspartame (ASP), which has an FDA approved daily intake (ADI) level of 50mg/kg has been shown to increase the relative risk for type 2 diabetes and metabolic syndrome by as much as 67% and 36%, respectively (Nettleton et al. 2009).

ASP is known to be metabolized by gut esterases and peptidases into L-aspartic acid, L-phenylalanine (PHE) and methanol. Recently, our lab has shown that the brush border enzyme intestinal alkaline phosphatase, IAP, can prevent a high fat diet induced metabolic syndrome in mice, at least partly by inhibiting the absorption of endotoxin (LPS) that occurs with dietary fats (Kaliannan et al. 2013). We postulated that when ASP breaks down into aspartic acid + PHE, the PHE metabolite, a known inhibitor of IAP (Ghosh and Fishman 1966), hampers IAP-mediated detoxification of gut bacteria derived endotoxins, thus promoting the development of the metabolic syndrome. Indeed, herein we present results suggesting a possible explanation for why the consumption of aspartame may contribute to the development of the metabolic syndrome.

MATERIALS AND METHODS

Study design

Three separate models were developed to test our hypothesis. An *in vitro* model was performed where IAP activity was measured in solutions of regular soda and diet soda both with and without the addition of 0.1 units of IAP to the sodas. IAP activity was measured by p-nitrophenyl phosphate (pNPP) under pH ranges 3-10, imitating the variations in pH observed within the gut lumen.

Next, an *in vivo* model was used to study the acute effects of ASP in the intestinal lumen in male mice. A six cm segment of small bowel was isolated and injected with saline control or an aspartame solution. The amount of aspartame administered (34/mg/kg) was based on the FDA's projected 99th percentile intake amongst humans (Davoli et al. 1986). After three hours, the mice were sacrificed and luminal contents from the loop were harvested to measure the IAP activity using the pNPP assay.

In a chronic model, we assessed the long-term effects of ASP on male mice fed a high fat diet (HFD). Six week old mice were divided into groups receiving a chow diet or a high fat diet (HFD) and further subdivided into groups receiving aspartame infused drinking solution (0.96 mg/mL (Magnuson et al. 2007)) or regular water (controls), and were maintained on their respective diet + drink combo for 18 weeks. The mice were monitored for weight gain, food, and water intake throughout these 18 weeks. At the end of the study, two glucose tolerance tests were conducted assessing short and long term glucose tolerance (6 and 16 hours of fasting), and serum TNF-α levels were measured prior to sacrifice.

Biochemical Reagents

Aspartame (Asp-Phe methyl ester) was purchased from Sigma Aldrich (St Louis, MO). Tumor necrosis factor-alpha (TNF-α) ELISA kits were purchased from eBioscience, San Diego, CA.

Animals

C57BL/6 mice were purchased from Charles River Laboratories. Animals in this study were maintained in accordance with the guidelines prepared by the Institutional Animal Care and Use Committee (IACUC) at MGH based on the Care and Use of Laboratory Animals of the Institute

of Laboratory Resources, National Research Council [Department of Health, Education and Human Services, publication no. 85e23 (National Institute of Health), revised 1985]. All experiments were reviewed and approved by the IACUC and carried out according to regulations of the Subcommittee on Research Animal Care of the MGH and the National Institutes of Health (NIH Publication 85-23, 1985). Animals received a standard chow diet [Laboratory Rodent Diet 5001; 58% (wt/wt) kcal from carbohydrate, 27% (wt/wt) kcal from protein, and 14% (wt/wt) kcal from fat (saturated fat 4.5%, unsaturated fat 9.5%), totaling an energy content of 4.09kcal/g] from Lab Diet (Brentwood, MO) or a high fat diet (HFD) [D12451; 35% (wt/wt) kcal from carbohydrate, 20% (wt/wt) kcal from protein, 45% (wt/wt) kcal from fat (saturated fat 16.2%, unsaturated fat 28.8%), totaling an energy content of 4.73 kcal/g] from and Research Diets Inc (New Brunswick, NJ). Of note, per manufacturer's product data, sucrose made up 3.8% (wt/wt) in the standard chow diet while it was 17% (wt/wt) of the HFD. Animals received either regular autoclaved drinking water or an aspartame-containing solution (0.96 mg/mL) (Magnuson, Burdock et al. 2007). Animals were euthanized following the American Veterinary Medical Association 2013 approved protocol.

Animal Experiments

Mice were housed in a biosafety level 1 (BL1) room in hard top cages and were maintained in a temperature-controlled room (22-24°C) with a strictly followed 12-h light/12-h dark diurnal cycle with food and water provided *ad libitum* unless otherwise indicated. For the chronic *in vivo* experiment, four mice were housed per cage, and each cage represented one experimental group (n = 4, unless otherwise specified), and there were a total of four groups (Chow, Chow + ASP, HFD, HFD + ASP). Animal body weight and food and water intake were measured weekly. Drinking water tubes were changed weekly.

Intestinal Alkaline Phosphatase (IAP) Assay

IAP activity in commercial and other IAP samples were verified and/or quantified by performing IAP assay (Malo et al. 2010). Briefly, 25 µl of a luminal sample was mixed with 175 µl phosphatase assay reagent containing 5 mM of p-nitrophenyl phosphate (pNPP) followed by determining optical density at 405 nm after a specific time period. The Coomassie Blue Protein Assay (Bradford) Kit from Fisher Scientific (Pittsburg, PA) was used for protein quantification.

The specific activity of the enzyme is expressed as pmole pNPP hydrolyzed/min/μg of protein.

Cytokine Assay

TNF-α levels were determined using an ELISA kit following the manufacturer's instructions (eBioscience) in blood serum collected at sacrifice.

Glucose Tolerance Test (GTT)

GTT was performed in nonanesthetized mice as described (Cani et al. 2007). Two separate GTTs were conducted, to assess glucose tolerance with short (6 h) and long term (16 h) fasting. Briefly, mice were fasted for 6 h and 16 h in the morning, fasting blood sugar was measured, and glucose [1.0 g/kg body weight, 20% (wt/vol) glucose solution] was administered by i.p. injection. Small blood samples (microliters) were drawn from the tip of the incised tail at 30, 60, 90, and 120 min to measure blood glucose levels. Glucose tolerance was expressed by calculating the total area under the curve (AUC) of each GTT.

Statistical Analysis

Statistical analyses were performed using SPSS Software (version 20 for Mac OS; SPSS, Inc., Chicago, IL) and GraphPad Prism (version 6.00 for Mac; GraphPad Software; La Jolla, CA). Data were expressed as mean ± standard error (SEM). Statistical differences between two groups were analyzed using unpaired two-tailed Student's *t* test. Statistical differences between more than two test groups were evaluated by two-way analysis of variance using Tukey's multiple comparison post-tests. A significant difference was considered when $p<.05$.

RESULTS

Aspartame lowers IAP activity in vitro

In the *in vitro* study, soda without added IAP served as a control and did not display any measurable alkaline phosphatase activity, demonstrating a lack of inherent alkaline phosphatase activity in soda (**Figure 1A**). After IAP was added to the solutions, IAP activity was significantly lower (T tests at each pH data point, $p<.05$) in solutions containing ASP (diet soda) compared to controls without ASP (regular soda) at pH 3.3-10 (representing the different acidic/basic environments of the gut). The inhibitory effects of ASP were most dramatic at pH 9 (0.24 ± 0.0002 *vs* 0.46 ± 0.0023 pmole pNPP hydrolyzed/ min, $p=.034$) **(Figure 1B)**, which

interestingly corresponds to the high pH environment of the proximal duodenum where the highest amount of endogenous IAP is produced.

Aspartame lowers IAP activity *in vivo*

In the bowel loop model, IAP activity was significantly (p=.02) reduced in mice that had ASP instilled into the loops compared to saline controls (0.2±0.03 *vs* 0.4±0.24) (**Figure 2**). These data indicate that the presence of ASP within the bowel lumen directly inhibits endogenous IAP activity.

In the chronic *in vivo* model, we found that IAP activity was enhanced by the HFD, consistent with previous published results (Mahmood et al. 2003). Mice receiving ASP in their drinking water had lower luminal IAP activity compared to the controls, and although a trend was seen, this difference was not found to be statistically significant (p=0.3) (**Figure 3**).

Aspartame causes more weight gain in mice fed a HFD

In the chronic *in vivo* model, where mice received ASP in their drinking water for 18 weeks, mice in the chow group consumed an average of 3.7 mg of ASP (123.3 mg/kg) per day and the mice in the HFD group consumed an average of 3.6 mg (70 mg/kg) per day, which is equivalent to a 60 kg human consuming 3.4 and 1.9 cans of diet soda respectively (a 12 oz can contains approximately 180 mg of aspartame (Lim et al. 2006); mouse to human dose conversion expressed in mg/kg to equivalent surface area dose as previously described by Freireich et al (Freireich et al. 1966)). There were minimal differences in the amount of weight gained between the water and ASP chow groups (35.8g ± 1.5 *vs* 33.2g ± 3.1). As expected, both HFD-ASP and HFD+ASP groups gained more weight than their chow diet counterparts (42.4±3.1 and 48.1±1.6 *vs* 35.8g ± 1.5 and 33.2g ± 3.1 respectively) (**Figure 4A**), but the group that received HFD+ASP gained more weight than the HFD control group (48.1±1.6 *vs* 42.4±3.1, p<.0001). Two way ANOVA was significant for both ASP and diet when comparing percentage weight gained between groups (p<.0001) (**Figure 4B**).

The effect of aspartame on blood glucose levels

Glucose tolerance tests were performed and demonstrated that mice that received ASP infused drinking water developed relative glucose intolerance. The 16-hour fasting blood sugar (FBS) levels were higher in the chow group that received ASP (136.5±12.7mg/dL vs 108.0±4.7mg/dL, p=.04) and were also higher in the HFD group that received ASP (248.3±17.47mg/dL vs 218.0±18.77mg/dL, p=.02) (**Figure 5A**). Blood glucose was measured over time after 6 hours (**Figure 5B**) and 16 hours (**Figure 5D**) of fasting. The total area under the curve (AUC) of the blood glucose curves was calculated after the 6-hour (**Figure 5C**) and 16-hour (**Figure 5D**) fasting periods. Total AUC was increased in HFD-ASP group, and even more so in HFD+ASP group after both 6 hours and 16 hours of fasting (42003.75±5331.61mg*min/dL vs 53913±4000.58mg*min/dL with 6 hours fast, p=.02; 38741.25±6425.50mg*min/dL vs 48330±3694.69mg*min/dL at 16 hours p=0.3). Although two-way ANOVA was not found to be significant with 16-hours of fasting (p=0.5), the 6-hour fast two-way ANOVA showed that ASP and diet both significantly affect glucose concentration (p=.008).

The effect of aspartame on TNF-alpha levels

Chronic low-grade inflammation, as a result of LPS and perhaps other mediators being absorbed with dietary fats, has been associated with the development of the metabolic syndrome (Cani et al. 2007). TNF-alpha, a marker of inflammation that has been linked to insulin resistance, has been shown to be locally and systemically elevated in models of diet and genetic induced obesity (Hotamisligil et al. 1993). In our chronic *in vivo* model, we found that compared to the controls, serum TNF-alpha was increased in both chow+ASP and HFD+ASP groups (1622.3±184.1pg/mg of protein and 2512.1±251.4pg/mg of protein respectively), indicating a higher level of systemic inflammation in groups receiving ASP (**Figure 6**). This was apparent when comparing TNF-alpha levels in HFD groups +/- ASP (1608.60±349.16pg/mg vs ±184.1pg/mg, p=.03). A two-way ANOVA showed that ASP and diet both significantly affect serum TNF-alpha levels (p=.005). TNF-alpha was highest in the HFD group receiving ASP (p=.01).

DISCUSSION

In recent decades, there has been a marked increase in the consumption of artificial sweeteners as a popular substitute for sucrose (Gardner 2014; Roberts 2015). Indeed, many flavored sodas, energy drinks, frozen foods, common dairy products such as yogurt, and even

baby foods contain significant amounts of artificial sweeteners (Yang 2010; Ng et al. 2012). Although artificial sweeteners are marketed as healthy alternatives for consumers seeking to avoid sugar-containing products (de la Pena 2010), several studies have shown a positive correlation between weight gain and consumption of such products (Stellman et al. 1988; Fowler et al. 2008). However, the effects of artificial sweeteners remain controversial as results appear to be somewhat dependent on experimental conditions (Sylvetsky et al. 2016).

Yet, the reason for artificial sweeteners failing to consistently result in the expected weight loss remains unknown. Aspartame (ASP), a commonly used artificial sweetener, popular for its lack of a bitter aftertaste, has been associated with a significant increase in appetite (Tordoff et al. 1990). It was previously thought that the observed increase in appetite was due to the fact that artificial sweeteners did not activate the food reward pathways in the same way as sucrose and fructose (Yang 2010), perhaps resulting in compensatory overeating (Lavin et al. 1997; Davidson et al. 2011; Swithers et al. 2013). However, recent evidence indicates that artificial sweeteners, specifically aspartame, may have a direct effect on the gut microbiota that could explain the metabolic changes that occur in high dose consumption of artificial sweeteners (Palmnas et al. 2014; Suez et al. 2014; Burke and Small 2015).

The impact of artificial sweeteners has been reported in numerous studies. As early as 1989, ASP was shown to increase serum PHE, raising concern that the pathways of tyrosine and other neurotransmitters would be disrupted, potentially leading to cognitive deficiencies and seizure disorders (Wurtman 1983; Gaull 1985). Since then, ASP has been linked to cancer (Roberts 1997), Sjögren's syndrome (Robert 2006), angiogenesis (Alleva et al. 2011) and oxidative stress (Prokic et al. 2014). During the last ten years, the debate surrounding ASP and its potentially adverse effects has mainly focused on its metabolic implications, specifically on the development of obesity and metabolic disease. Previous nutrigenomic studies demonstrated that non-nutritive food additives such as ASP decrease the expression of regulatory genes such as PPARGC1a and other differentially expressed genes (DEGs) in hepatic and adipose tissue (Collison et al. 2013).

In a recently published study, Frankenfeld et al observed significant differences in the human gut microbiota when comparing consumption of artificial sweeteners to that of a regular diet, explicitly demonstrating a difference in the bacterial makeup in patients with an increased ASP and acesulfame-K intake after only a four day trial (Frankenfeld et al. 2015). Although the

trials were aiming to study acute changes in high dose settings, the results raise the question of the long-term effects of artificial sweeteners.

In 2014, Suez et al proposed that non-nutritive sweeteners including ASP may induce glucose intolerance by altering the gut microbiota (Suez et al. 2014). In the same year, Palmnäs et al demonstrated that aspartame elevated fasting glucose levels in mice regardless of diet type (Palmnas et al. 2014). Consistent with the work of Frankenfeld et al, the group also showed that a low-dose consumption of ASP alters the gut microbiota, observing an increase in total gut bacteria and a changed dynamic of the gut bacterial composition. Although many theories explaining the effects of aspartame on gut metabolism have been suggested, the precise mechanism has not been defined.

Phenylalanine (PHE) is one of the breakdown products of aspartame and, we believe, could play a major role in regard to the impact of ASP on metabolic disease. PHE is a known inhibitor of intestinal alkaline phosphatase (IAP) (Ghosh and Fishman 1966), a gut enzyme important in the intestinal microbiotal homeostasis (Malo et al. 2010). IAP derives anti-inflammatory properties from its dephosphorylating activity, detoxifying LPS and other inflammatory mediators and thus inhibit the inflammatory cascade (Lalles 2014). We hypothesized that the negative metabolic effects seen with ASP consumption could be due to its indirect inhibition of IAP through the breakdown product PHE.

We sought to test this hypothesis using both *in vitro* and *in vivo* settings. Our *in vitro* experiments demonstrate that IAP activity was inhibited by the presence of ASP in different pH environments imitating the physiology of the gut. ASP is most stable at an acidic pH of 4.2. In more basic environments, aspartame will quickly degrade into its PHE and L-aspartate amino acid components (Hatada et al. 1985). ASP will thus remain stable in the acidic environments of the soda and the stomach; however, when ASP reaches the basic environment of the intestines, it will decompose (Bell and Labuza. 1991). The PHE formed from this degradation acts as a specific noncompetitive inhibitor of IAP. PHE's inhibition of IAP is pH-dependent, and this inhibition is greatest at a pH of 8.5-9 (Ghosh and Fishman 1966). Our *in vitro* data corresponds to the pH dependence of ASP degradation and of PHE's IAP inhibition. The graph shows a marginal inhibition of IAP by PHE in the pH 7-8 range. However, in a basic environment where pH is greater than 8, PHE significantly inhibits IAP.

IAP is primarily produced in the proximal small intestine and secreted into the gut lumen,

travelling distally and yet maintaining much of its enzymatic activity as it makes its way into the final stool product. Given that the highest IAP levels and activity exist within the proximal small intestine where the luminal pH can be greater than 8, we were intrigued to discover that the *in vitro* inhibitory effects of ASP on IAP activity were most pronounced under high pH conditions.

This finding suggests that ASP consumption could indeed lead to significant IAP inhibition *in vivo*. We directly tested this hypothesis using an *in vivo* bowel loop model. We found that IAP activity in the isolated intestinal loop was significantly reduced by the presence of aspartame (p=.02). These results confirmed the idea that aspartame could impact the activity of endogenous IAP within the brush border and lumen of the GI tract.

To further test the impact of ASP in regard to IAP activity and the metabolic syndrome, we fed mice either chow or HFD +/- ASP. It should be noted, that unlike humans, mice do not always show strong preferences for aspartame, which may be related to the hyper-responsiveness to the negative taste components rather than the lack of sweetness (Bachmanov et al. 2001). The study was conducted over eighteen weeks and determined that the mice consumed an average of 3.6-3.8mg of aspartame a day, a dose equivalent to an average adult human drinking 1.8-3.4 cans (12 oz or 330ml) of soda per day. Based on these calculations, we believe that our *in vivo* mouse data are clinically relevant in regard to human dietary habits. No significant weight changes were observed in the group on a regular diet, however, more pronounced (p=.0001) weight gain was observed in the group on a high fat diet when aspartame was added to the drinking water.

The inflammatory cytokine TNF-alpha has been shown to directly interfere with tissue insulin receptors and therefore block insulin's biological actions, resulting in insulin resistance due to TNF-alpha exposure (Hotamisligil and Spiegelman 1994). We thus hypothesized that the inhibition of the anti-inflammatory effects of regulating gut proteins such as IAP would lead to elevated insulin levels and relative glucose intolerance. After 6 hours of fasting, a significant difference was seen in glucose levels; mice on a regular diet demonstrated significantly higher (p=.04) fasting glucose levels when receiving ASP as an additive to their water compared to drinking water alone. In the high fat diet group, which developed a higher rate of glucose intolerance as expected compared to the chow diet group, a significant and sustained additional increase in fasting glucose levels were seen in the group that combined ASP with the high fat diet instead of drinking only water (p=.02). Calculating glucose concentration over time and area under the curve (AUC) after the 6-hour fast, a two-way ANOVA showed that ASP and diet both

significantly affect glucose concentration. We additionally measured glucose after a 16-hour fast to show that the glucose intolerance could persist despite the mice not having consumed ASP in 16 hours; however, the 16-hour fast AUC was not found to be statistically significant.

The present data suggest that ASP may promote the metabolic syndrome through IAP inhibition by its breakdown product PHE.

Our lab has previously shown that IAP prevents the metabolic syndrome in mice. IAP works through a variety of mechanisms, including promoting commensal bacteria growth (Malo et al. 2010), blocking bacterially derived pro inflammatory ligands such as LPS (Kaliannan et al. 2013), and enhancing the gut barrier function (Hamarneh et al. 2014). The metabolic syndrome is defined by a state of chronic low-grade inflammation as a result of LPS and other mediators being absorbed along with dietary fats (Cani et al. 2007). TNF-alpha is a cytokine known to be elevated in obesity models (Hotamisligil et al. 1993) and has also been linked to insulin resistance. In our chronic *in vivo* model, we found that the levels of serum TNF-alpha were increased in mice consuming ASP compared to water, regardless of diet type ($p=.005$). As expected, TNF-alpha levels were highest in the HFD+ASP group ($p=.01$). These results indicate that ASP alone will elevate the level of inflammation seen over 18 weeks of consumption, regardless of whether it is combined with a high-fat diet.

In conclusion, we have demonstrated that chronic ASP consumption in mice leads to an accelerated weight gain, glucose intolerance, and increased TNF-alpha levels. We have also shown that ASP reduces the activity of IAP both *in vitro* and *in vivo*, likely through its breakdown product PHE, a well-known inhibitor of IAP. And although some data points were not statistically significant, this may be due to the low statistical power from the low sample sizes used in the experiments. A repetition of the experiment with a greater sample size could yield more statistical power and potentially more statistical significance for the difference between the +ASP and –ASP conditions. Furthermore, only male mice were used in this experiment, and there could potentially be differences between sexes, as male mice tend to have higher levels of diet induced inflammation (Grove et al. 2010) and female mice demonstrate better insulin sensitivity due to their higher levels of adinopectin and estrogen (Medrikova et al. 2012). Investigating sex differences could additionally be an area for further research.

Our findings are consistent with previous studies on ASP and provide a potential explanation for its effects on the metabolic syndrome. By blocking the gut protective anti-

inflammatory enzyme IAP, ASP may significantly contribute to the development of the metabolic syndrome, perhaps explaining the observation that dietary products are not associated with the weight loss and metabolic benefits that may otherwise be expected.

Conflict of Interests

The authors declare no conflicting financial interests.

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Figure Legends

FIGURE 1 Effect of aspartame on intestinal alkaline phosphatase (IAP) activity *in vitro* in the pH range 3.3-10. Diet soda contains aspartame, but regular soda does not. Without adding IAP to the soda solutions, no alkaline phosphatase activity was present (A). After adding IAP to the drink solutions, IAP was most significantly inhibited by the aspartame in the diet soda at basic pH's resembling the duodenal environment (B). Values are means ± SEMs, n = 3. *Significant change from regular soda baseline as determined by T tests at each pH data point, $p<.05$.

FIGURE 2 Effect of aspartame on the activity of intraluminal intestinal alkaline phosphatase (IAP) in an isolated bowel loop in mice. When aspartame is instilled in a closed bowel loop, IAP activity is significantly inhibited compared to a saline control ($p=.02$). Values are means ± SEMs, n = 5. *Significant change from saline baseline, $p<.05$.

FIGURE 3: Effect of drinking-water aspartame (ASP) on intraluminal intestinal alkaline phosphatase (IAP) activity in mice fed a chow diet or high fat diet (HFD). A trend was seen where +ASP groups had decreased intestinal alkaline phosphatase activity, but this difference was not significant with a two-way ANOVA ($p=0.3$). Values are means ± SEMs, n = 4.

FIGURE 4: Effect of high-fat diet (HFD) and aspartame (ASP) on mouse weight. Mouse weight was measured at week 0, then the mice were fed a chow diet ± ASP or HFD ± ASP; weight was again measured after 18 weeks (A). Although percent body weight gain (B) was not significantly different for the chow diet groups, percent body weight gain was significantly increased for the

HFD group with ASP ($p<.0001$). A two-way ANOVA test showed that ASP and diet both significantly affect weight. Values are means ± SEMs, n = 4. ***Significant change from respective diet and -ASP baseline via Tukey's multiple comparison test, $p<.0001$.

FIGURE 5: Effects of aspartame (ASP) on glucose tolerance on mice with a chow diet ± ASP or a high-fat diet (HFD) ± ASP. Fasting blood sugar (FBS) following 16 hours of fasting (A). Blood glucose was measured over time following 6 hours (B) and 16 hours (D) of fasting. The total area under the curve (AUC) of the blood glucose graphs was calculated after the 6-hour (C) and 16-hour (D) fasting periods to express glucose tolerance. A two-way ANOVA showed that ASP and diet both significantly affect glucose tolerance after 6 hours of fasting. Values are means ± SEMs, n = 4. *Significant change from respective diet and -ASP baseline via Tukey's multiple comparison test, $p<.05$. **$p<.01$.

FIGURE 6: Effect of ASP on serum TNF-alpha levels on mice with a chow diet ± ASP or a high-fat diet (HFD) ± ASP. A two-way ANOVA showed that ASP and diet both significantly affect serum TNF-alpha levels ($p=.005$). There was a significant increase in serum TNF-alpha in HFD+ASP ($p=.009$).Values are means ± SEMs, n = 4. *Significant change from respective diet and -ASP baseline via Tukey's multiple comparison test, $p<.05$.