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Metabolic disorders, including obesity, diabetes, and cardiovascular disease, are widespread in Westernized nations. Gut microbiota composition is a contributing factor to the susceptibility of an individual to the development of these disorders; therefore, altering a person’s microbiota may ameliorate disease. One potential microbiome-altering strategy is the incorporation of modified bacteria that express therapeutic factors into the gut microbiota. For example, N-acylphosphatidylethanolamines (NAPEs) are precursors to the N-acylthanolamide (NAE) family of lipids, which are synthesized in the small intestine in response to feeding and reduce food intake and obesity. Here, we demonstrated that administration of engineered NAPE-expressing E. coli Nissle 1917 bacteria in drinking water for 8 weeks reduced the levels of obesity in mice fed a high-fat diet. Mice that received modified bacteria had dramatically lower food intake, adiposity, insulin resistance, and hepatosteatosis compared with mice receiving standard water or control bacteria. The protective effects conferred by NAPE-expressing bacteria persisted for at least 4 weeks after their removal from the drinking water. Moreover, administration of NAPE-expressing bacteria to TallyHo mice, a polygenic mouse model of obesity, inhibited weight gain. Our results demonstrate that incorporation of appropriately modified bacteria into the gut microbiota has potential as an effective strategy to inhibit the development of metabolic disorders.

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Incorporation of therapeutically modified bacteria into gut microbiota inhibits obesity

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Metabolic disorders, including obesity, diabetes, and cardiovascular disease, are widespread in Westernized nations. Gut microbiota composition is a contributing factor to the susceptibility of an individual to the development of these disorders; therefore, altering a person’s microbiota may ameliorate disease. One potential microbiome-altering strategy is the incorporation of modified bacteria that express therapeutic factors into the gut microbiota. For example, N-acylphosphatidylethanolamines (NAPEs) are precursors to the N-acylthanolamide (NAE) family of lipids, which are synthesized in the small intestine in response to feeding and reduce food intake and obesity. Here, we demonstrated that administration of engineered NAPE-expressing E. coli Nissle 1917 bacteria in drinking water for 8 weeks reduced the levels of obesity in mice fed a high-fat diet. Mice that received modified bacteria had dramatically lower food intake, adiposity, insulin resistance, and hepatosteatosis compared with mice receiving standard water or control bacteria. The protective effects conferred by NAPE-expressing bacteria persisted for at least 4 weeks after their removal from the drinking water. Moreover, administration of NAPE-expressing bacteria to TallyHo mice, a polygenic mouse model of obesity, inhibited weight gain. Our results demonstrate that incorporation of appropriately modified bacteria into the gut microbiota has potential as an effective strategy to inhibit the development of metabolic disorders.

Introduction

Obesity dramatically increases the risks for cardiovascular disease and diabetes as well as other diseases, and rates of obesity have increased dramatically in the past 25 years (1, 2). Unfortunately, current medical and lifestyle treatments for obesity have largely failed to achieve long-term success (3–6). A major challenge is the need for lifelong adherence to maintain modest effects in the face of evolutionarily driven compensatory responses to weight loss induced by voluntary caloric restriction. Thus, alternative and novel strategies are urgently needed for long-term prevention and treatment of obesity. One such alternative is suggested by studies showing a reciprocal relationship between the composition of the gut microbiota and metabolic disorders (7–13). Transfer of gut microbiota from obese mice into germ-free mice demonstrates that this microbiota directly contributes to metabolic phenotypes (9, 11). Thus, appropriate alteration of the gut microbiota might provide long-term protection against obesity. One potential strategy to beneficially alter the gut microbiota is to incorporate bacteria that have been genetically modified to express therapeutic factors that increase satiety and sensitivity to adipose-derived negative feedback signals such as leptin. This strategy could also be used to sustainably deliver other therapeutic molecules beneficial in the treatment of obesity-related diseases or which could not be readily delivered by other therapeutic routes.

Although a number of therapeutic molecules could be potentially biosynthesized by bacteria for the treatment of obesity, we focused on N-acyl-phosphatidylethanolamines (NAPEs), the immediate precursors of N-acylthanolamides (NAEs), a family of the potent anorexigenic lipids. NAPEs are synthesized in the proximal small intestine in response to feeding (14). A high-fat diet impairs feeding-induced NAPE synthesis (15) so that bacterially-synthesized NAPEs could help compensate for this reduced NAPE synthesis. After their synthesis, NAPEs are rapidly converted to the active NAEs through hydrolysis by NAPE-hydrolyzing phospholipase D (NAPE-PLD). Intrapерitoneal administration of NAEs, where the N-acyl chains are saturated or monounsaturated (e.g., N-oleyl-ethanolamide [OEA]), or of their precursors (e.g., C16:0NAPE) markedly reduces food intake and obesity in mice fed a high-fat diet (15-24). Chronic NAE administration appears to modulate adiposity through multiple mechanisms including inhibition of fat absorption (25), delayed gastric emptying (26, 27), reduced food intake (19, 20, 28, 29), and increased fatty acid oxidation (23, 25). NAEs act as ligands for at least 3 receptors: PPARα, TRPV1, and GPR119 (23, 28, 30), and genetic ablation of PPARα or TRPV1 significantly blunts the anorectic effects of NAEs (28, 31). The goal of this study was to determine whether incorporating genetically modified bacteria

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reduce food intake to the same extent as untreated pNAPE-Ec, indicating that viable bacteria expressing NAPEs are needed for maximum effectiveness. Our follow-up studies revealed that administration of 10^11 pNAPE-Ec bacteria once a day for 7 consecutive days resulted in an approximately 2-fold increase in NAPE levels in the colon (Supplemental Figure 2A). NAPE levels were not as markedly increased in other gastrointestinal tissues such as stomach, small intestine, and cecum, nor were they increased in plasma (Supplemental Figure 2, B–E).

To examine the effect of persistent NAPE expression by gut bacteria on food intake and obesity, we transformed the probiotic wild-type strain of E. coli, Nissle 1917 (EcN), with At1g78690 (pNAPE-EcN) (Supplemental Figure 3). To facilitate monitoring of colonization by transformed EcN, we also inserted the P. luminescens luciferase operon (Lux) into the recA gene of the EcN chromosome. We analyzed phospholipid extracts from both pNAPE-EcN and pEcN by negative ion mass spectrometry (Supplemental Figure 4), and the identified NAPE species were consistent with N-acylation of the major bacterial PE species (Supplemental Table 1). We observed that pNAPE-EcN had markedly higher levels of

that biosynthesize NAPEs into the gut of mice would result in lasting attenuation of obesity induced by a high-fat diet.

**Results**

We previously transformed the C41-DE3 laboratory strain of E. coli (Ec) with At1g78690, an N-acyltransferase from Arabidopsis thaliana that catalyzes the synthesis of NAPEs. This markedly increased NAPE levels in these bacteria (pNAPE-Ec) compared with those transformed with empty vector (pEc) (32, 33). To determine whether NAPEs synthesized by intestinal E. coli could markedly alter food intake, we administered a daily bolus of 10^9 CFU pNAPE-Ec or pEc bacteria by gavage to lean male C57BL/6J mice for 7 days. Additional groups of mice were administered either vehicle or pNAPE-Ec bacteria that had been killed by treatment with kanamycin prior to gavage. Cumulative food intake did not differ in mice receiving pEc compared with those receiving vehicle, but was reduced by 15% in mice receiving living pNAPE-Ec (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI72517DS1). pNAPE-Ec are sensitive to kanamycin, and pNAPE-Ec pretreated with kanamycin prior to gavage did not reduce food intake to the same extent as untreated pNAPE-Ec, indicating that viable bacteria expressing NAPEs are needed for maximum effectiveness. Our follow-up studies revealed that administration of 10^11 pNAPE-Ec bacteria once a day for 7 consecutive days resulted in an approximately 2-fold increase in NAPE levels in the colon (Supplemental Figure 2A). NAPE levels were not as markedly increased in other gastrointestinal tissues such as stomach, small intestine, and cecum, nor were they increased in plasma (Supplemental Figure 2, B–E).

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![Figure 1. Treatment with pNAPE-EcN, but not pEcN, inhibits gain in body weight and adiposity. All values are the mean ± SEM (n = 10 mice per group). Solid bars indicate time points with significant differences between pNAPE-EcN and other groups (P < 0.05 by Bonferroni’s multiple comparison test). (A) Effect of treatments on gain in body weight from start of treatment (2-way RM ANOVA, for treatment P = 0.0073, for time P < 0.0001). (B) Effect of treatments on fat mass (2-way RM ANOVA, for treatment P = 0.0127, for time P < 0.0001). (C) Effect of treatments on lean body mass (2-way RM ANOVA, for treatment P = 0.8113, for time P < 0.001). (D) Effect of treatments on cumulative food intake from start of treatment (2-way RM ANOVA, for treatment P = 0.0035, for time P < 0.0001).](image-url)
Table 1. Effects of NAPE-secreting bacteria on metabolic biomarkers during an 8-week treatment and a 4-week follow-up period

<table>
<thead>
<tr>
<th></th>
<th>Water only (n = 10)</th>
<th>Vehicle (n = 10)</th>
<th>pEcN (n = 10)</th>
<th>pNAPE-EcN (n = 10)</th>
<th>One-way ANOVA</th>
</tr>
</thead>
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<tr>
<td><strong>Fasting plasma, week 8</strong></td>
<td></td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>124 ± 17</td>
<td>114 ± 13</td>
<td>117 ± 24</td>
<td>110 ± 11</td>
<td>P = 0.356</td>
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<tr>
<td>Leptin (ng/ml)</td>
<td>40.4 ± 13.8</td>
<td>40.6 ± 12.3</td>
<td>31.0 ± 13.7</td>
<td>15.8 ± 5.8*</td>
<td>P &lt; 0.0001</td>
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<tr>
<td>Insulin (ng/ml)</td>
<td>1.41 ± 0.33</td>
<td>1.23 ± 0.47</td>
<td>0.70 ± 0.38*</td>
<td>0.42 ± 0.16*</td>
<td>P &lt; 0.0001</td>
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<tr>
<td>Oral glucose tolerance test, week 8 (AUC h mg dl⁻¹)</td>
<td>573 ± 67</td>
<td>547 ± 62</td>
<td>540 ± 76</td>
<td>470 ± 53*</td>
<td>P = 0.008</td>
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<td><strong>Body Weight (g)</strong></td>
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<tr>
<td>Week 0</td>
<td>23.3 ± 1.8</td>
<td>23.1 ± 1.6</td>
<td>23.1 ± 1.6</td>
<td>23.2 ± 1.3</td>
<td>P = 0.994</td>
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<td>Week 8</td>
<td>33.6 ± 3.7</td>
<td>33.4 ± 2.6</td>
<td>33.3 ± 2.9</td>
<td>30.4 ± 2.7</td>
<td>P = 0.070</td>
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<td>Week 12</td>
<td>36.5 ± 4.8</td>
<td>36.6 ± 3.5</td>
<td>35.7 ± 3.8</td>
<td>32.1 ± 3.4*</td>
<td>P = 0.043</td>
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<td><strong>% Body fat (g/g)</strong></td>
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<tr>
<td>Week 0</td>
<td>11.4 ± 1.6</td>
<td>11.5 ± 1.6</td>
<td>11.2 ± 1.3</td>
<td>11.0 ± 1.3</td>
<td>P = 0.876</td>
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<td>Week 8</td>
<td>27.9 ± 4.8</td>
<td>28.0 ± 2.9</td>
<td>25.2 ± 5.3</td>
<td>20.0 ± 5.5*</td>
<td>P = 0.002</td>
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<td>Week 12</td>
<td>30.9 ± 6.1</td>
<td>31.5 ± 3.3</td>
<td>27.4 ± 6.5</td>
<td>22.3 ± 5.5*</td>
<td>P = 0.002</td>
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<td><strong>Cumulative calories consumed (kcal)</strong></td>
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<td>Weeks 0–8</td>
<td>778 ± 52</td>
<td>773 ± 32</td>
<td>771 ± 36</td>
<td>711 ± 33*</td>
<td>P = 0.012</td>
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<td>Weeks 9–12</td>
<td>381 ± 35</td>
<td>388 ± 20</td>
<td>395 ± 21</td>
<td>355 ± 26*</td>
<td>P = 0.010</td>
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<td><strong>Liver triglycerides</strong></td>
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<td>Week 12 (μg/mg)</td>
<td>36.5 ± 9.2</td>
<td>41.1 ± 26.1</td>
<td>27.2 ± 9.5</td>
<td>16.1 ± 9.6*</td>
<td>P = 0.006</td>
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*P < 0.05 versus vehicle by Dunnett’s multiple comparison test; mean ± SD.

saturated and monounsaturated NAPEs expected to exert anorexigenic effects (Supplemental Figure 5). In addition to NAPEs, several species with m/z ions consistent with O-acyl-phosphatidylglycerols (acyl-PG) species (34) were also enriched in pNAPE-EcN (Supplemental Table 2).

We then tested whether incorporating pNAPE-EcN into the gut microbiota would protect against the development of obesity, insulin resistance, and liver steatosis that occurs in C57BL6 mice fed an ad libitum high-fat diet (60% of calories from fat). For these experiments, we administered pNAPE-EcN via the drinking water at 5 × 10⁹ CFU bacteria/ml with 0.125% gelatin added to the water to keep the bacteria suspended and viable for at least 48 hours. In addition to NAPEs, several species with m/z ions consistent with O-acyl-phosphatidylglycerols (acyl-PG) species (34) were also enriched in pNAPE-EcN (Supplemental Table 2).

During the 8-week treatment period, pNAPE-EcN–treated mice gained less body weight (Figure 1A) and accumulated less fat mass (Figure 1B) compared with control mice. Consistent with their reduced adiposity, pNAPE-EcN mice also had lower plasma leptin and insulin levels (Table 1). In contrast to the striking differences in adiposity, we observed no significant differences in lean body mass between the groups (Figure 1C). One of the major bioactivities of the NAE metabolites of NAPEs is the reduction of food intake, and pNAPE-EcN–treated mice had markedly lower cumulative food intake than those treated with standard water, vehicle, or pEcN (Figure 1D).

Importantly, we found no evidence that changes in body weight and adiposity were the result of an adverse health effect of treatment with pNAPE-EcN. For instance, there was no difference between treatment groups in the consumption of kaolin (Supplemental Figure 9), so bacterial administration did not produce gastrointestinal distress. Nor did bacterial overgrowth appear to be the cause of reduced food intake and adiposity in pNAPE-EcN–treated mice, as levels of EcN retained in the intestinal tract did not significantly differ between pNAPE-EcN– and pEcN-treated mice (Supplemental Figure 10). Scores on simple tests of muscle strength, coordination, and speed either did not differ or were improved with pNAPE-EcN treatment (Supplemental Table 3). Perhaps most importantly, since it is well established that endotoxemia impairs glucose tolerance and insulin sensitivity (12, 13), mice administered pNAPE-EcN had significantly improved glucose tolerance compared with that observed in the other groups (Table 1). Overall, these findings support the conclusion that the reduced adiposity found in high-fat diet–fed mice receiving pNAPE-EcN results from the action of NAPEs rather than from an adverse effect of bacterial administration.

Our larger goal was to incorporate NAPE-secreting bacteria into the gut microbiota in order to endow their host with persistent resistance to obesity. Mice initially treated with either pNAPE-EcN or pEcN excreted luminescent bacteria in their feces for at least 4 weeks after the cessation of bacterial administration (Supplemental Figure 11), consistent with persistence of pNAPE-EcN in the gut. We therefore investigated whether the beneficial effects of pNAPE-EcN on adiposity would persist after stopping its administration. Food intake, body weight, and fat mass during the
oluminescence was no longer detectable in feces at this time, suggesting that the loss of inhibited weight gain at this time was the result of loss of pNAPE-EcN from the gut.

Molecules absorbed through the colon enter the portal circulation, so that bacterially secreted NAPEs that had been subsequently converted to the biologically active NAEs by NAPE-PLD might exert effects in the liver in addition to their effects within the intestinal tract. When we examined liver NAE levels in mice that were euthanized 2 days after ending bacterial administration, we found that pNAPE-EcN treatment increased the levels of total NAE in liver by 44% (Figure 3). Treatment with pNAPE-EcN did not result in a follow-up period (experimental weeks 9–12) remained significantly lower in mice initially treated with pNAPE-EcN than was the case in the other groups (Table 1), consistent with the notion that incorporation of pNAPE-EcN in the gut lumen leads to long-lasting delivery of NAPEs and concomitant inhibition of obesity. Subsequent studies in a separate cohort of mice found that body weight was still significantly lower than that of vehicle- or pEcN-treated mice even 12 weeks after ending administration of pNAPE-EcN (Figure 2). The difference in body weight between vehicle- and pNAPE-EcN-treated mice continued to increase in magnitude up until 6 weeks after cessation of administration. Bioluminescence was no longer detectable in feces at this time, suggesting that the loss of inhibited weight gain at this time was the result of loss of pNAPE-EcN from the gut.

Figure 2. Effects of pNAPE-EcN persist for more than 6 weeks after ending administration. Groups of mice were fed a high-fat diet for a total of 20 weeks (n = 10 mice per group). For the first 8 weeks, mice were administered either vehicle (0.125% gelatin), 5 × 10⁹ CFU pEcN/ml, or 5 × 10⁹ CFU pNAPE-EcN/ml. For the remaining 12 weeks, all mice received standard drinking water. (A) Effect of treatment on total body weight. (B) Body weight of group treated with pNAPE-EcN normalized to the vehicle-treated group. (C) Percentage of body fat for all 3 groups. (D) Percentage of body fat of pNAPE-EcN mice normalized to the vehicle-treated mice. (E) Daily average food intake for each group as a 2-week rolling average. A rolling average was used to minimize day-to-day variations to better determine the overall trends. The dip in food intake that occurred in all groups after day 70 is likely the result of changing the housing location of all the mice due to institutional requirements. (F) Daily average food intake of pNAPE-EcN–treated mice normalized to the vehicle-treated mice.
Figure 3. Treatment with pNAPE-EcN increases hepatic NAE levels. NAEs were measured by LC/MS in liver collected from mice 2 days after ending a 9-week treatment with pNAPE-EcN or from untreated mice receiving standard drinking water (Water) during this same period (n = 10 mice per group; mean ± SEM). NAE levels were significantly different (2-way ANOVA, for treatment P < 0.0001, for NAE species P < 0.0001). Summed NAE levels were 1.70 ± 0.18 versus 2.45 ± 0.21 nmol/g liver for untreated versus pNAPE-EcN-treated mice, respectively.

Figure 4. Treatment with pNAPE-EcN induces expression of genes encoding for fatty acid oxidation, but not fatty acid synthesis, and reduces expression of inflammatory genes in the liver. Liver mRNA was measured by qRT-PCR using primers specific for each gene. β-actin (Actb) was used as a control, and all values were normalized to the vehicle group (mean ± SEM, n = 10 mice per group). *P < 0.05 by 1-way ANOVA for individual gene expression and by Dunnett’s multiple comparison test versus vehicle for pNAPE-EcN, but not for pEcN.
The Journal of Clinical Investigation

Figure 5. Treatment with pNAPE-EcN reduces infiltration of F4/80 and CD11b immunopositive leukocytes into liver. Slides were immunostained with either anti-F4/80 (A–D) or anti-CD11b (E–H) antibodies (DAB brown stain) and counterstained with hematoxylin. Representative photomicrographs are shown. Scale bars: 10 μm. (A and E) Standard drinking water only. (B and F) Vehicle-treated (0.125% gelatin) mice. (C and G) pEcN–treated mice. (D and H) pNAPE-EcN–treated mice.

Position of the gut microbiota. Because the mice colonized with pNAPE-EcN continued to show reduced food intake and body weight (Figure 2), these results support the notion that global changes in microbiota composition are not required for the beneficial effects of pNAPE-EcN treatment and that these effects are instead the result of the direct action of NAEs on target host tissues.

To further characterize alterations in feeding behavior and energy expenditure induced by pNAPE-EcN, we performed metabolic monitoring after 4 weeks of treatment with pNAPE-EcN, pEcN, or standard drinking water. Mice were placed for 7 days in cages equipped with the Promethion system for monitoring indirect calorimetry, physical activity, and food and water intake. The mice were maintained on a high-fat diet and their designated treatment throughout these metabolic monitoring experiments. All groups had some body weight and fat loss during their time in the Promethion system, likely due to the novel environment (Supplemental Figure 15). Analysis was performed using data from the final 3 days of monitoring when mice had acclimated to the novel environment. Examination of the pattern of food intake over the 24 hours of the light-dark cycle showed that food intake primarily diverged during the dark phase, when mice typically are most active and consume the majority of their food (Figure 9A and Supplemental Figure 16). During the dark phase, we observed that meal duration was significantly shorter for mice treated with pNAPE-EcN (Figure 9B). We found no significant differences in meal duration between groups during the light phase. Intermeal intervals did not significantly differ between the groups in either the light or dark phase (Figure 9C). When meal size was categorized by food weight consumed during each 5-minute time block of monitoring, the pNAPE-EcN–treated mice consumed significantly fewer large meals (Figure 9D). These results suggest that treatment with pNAPE-EcN lowered the threshold of the high-fat diet consumption required to achieve satiation. The pattern of water intake was similar to that of food intake: pNAPE-EcN–treated mice consumed less water overall, and the greatest divergence in water intake occurred during the dark phase (Supplemental Figure 17).

In addition to changes in energy intake, pNAPE-EcN treatment induced subtle changes in energy expenditure. While the lower body fat of pNAPE-EcN mice complicated direct comparisons of energy expenditure between groups, during the light phase of the 24-hour light-dark cycle (when mice are least active), the linear slope of the relationship between energy expenditure versus body weight increased more steeply for pNAPE-EcN mice than for the other 2 groups (Figure 10A and Supplemental Figure 18A). The slopes during the dark phase were similar (Figure 10B and Supplemental Figure 18B). The increase in energy expenditure we observed for pNAPE-EcN–treated mice during the light phase was not due to increased physical activity, because pNAPE-EcN–treated mice did not show an increase in distance...
traveled within the cage, as calculated from beam breaks (Supplementary Figure 19). These results suggest that pNAPE-EcN might slightly increase resting metabolic rate.

To examine whether the increased energy expenditure during the light phase resulted from increased fatty acid oxidation while resting, we analyzed the effect of pNAPE-EcN treatment on the respiratory quotient (RQ). Because all mice ate the same high-fat diet, no large-scale changes in the RQ would be expected, but animals with an increased reliance on their fat reserves might have a slightly lower RQ (i.e., favoring fatty acid oxidation over carbohydrate and protein oxidation) during periods of rest. Indeed, the pNAPE-EcN–treated mice consistently showed a slightly lower RQ than did the other 2 groups, although this apparent difference failed to reach statistical significance (Supplemental Figure 20).

To further characterize the effects of pNAPE-EcN treatment on glucose intolerance and insulin resistance, we performed several follow-up studies. To estimate the extent of protection from glucose intolerance afforded by pNAPE-EcN treatment, we treated mice with a high-fat diet, a high-fat diet and pNAPE-EcN, or a low-fat chow diet for 8 weeks. While administering pNAPE-EcN to mice fed a high-fat diet significantly improved homeostatic model assessment–estimated insulin resistance (HOMA-IR) index scores and the insulin response to glucose when compared with untreated mice receiving this diet, impairment in glucose tolerance was still evident in the pNAPE-EcN–treated mice when compared with mice fed a low-fat chow diet (Figure 11).

The improvement in glucose tolerance raised the possibility that pNAPE-EcN treatment increased insulin sensitivity. Insulin sensitivity could be improved if pNAPE-EcN treatment inhibited high-fat diet–induced phosphorylation of insulin receptor substrates that reduce activation of downstream signals in response to insulin. We therefore examined this signaling in response to both endogenous and exogenous insulin. As expected, the 4-hour fasting plasma insulin levels were lower in mice treated with pNAPE-EcN (1.14 ± 0.12 ng/ml) compared with levels in mice treated with pEcN (1.78 ± 0.17) or standard drinking water (1.96 ± 0.24; mean ± SEM, 1-way ANOVA, *P* < 0.0102), which is consistent with increased insulin sensitivity. Phosphorylation of the serine-threonine protein kinase AKT at serine 473 (p-S473-AKT) is a sensitive indicator of downstream activation of insulin targets. Consistent with pNAPE-EcN preserving sensitivity to insulin action during high-fat feeding, mice treated with pNAPE-EcN had higher levels of p-S473-AKT in their liver per unit of circulating insulin (p-AKT-INS) than did control mice (Figure 12A). When stimulated with a bolus of 0.75 IU/kg

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**Figure 6. Pair-feeding fails to fully recapitulate the effect of pNAPE-EcN treatment on body weight and body fat gain.** A group of mice treated with pEcN (E) were pair-fed (PF) by restricting them to the same number of calories of food as the pNAPE-EcN mice (N). An ad libitum–fed control group received standard drinking water (W). All values are the mean ± SEM; *n* = 9–10 mice per group. (A) Body weight gain during 4 weeks of treatment and pair-feeding. Two-way RM ANOVA, *P* < 0.0001 for time, *P* = 0.0003 for treatment. *P* < 0.05 by Bonferroni’s multiple comparison test for pair-fed versus pNAPE-EcN for days 16 to 25. *P* < 0.05 for pair-fed versus pEcN and versus standard water for day 28. (B) Body fat at week 0 (before beginning treatment) and week 4 (28 days after beginning treatment). No group differences were observed at week 0. Week 4 1-way ANOVA, *P* < 0.0001 by 1-way ANOVA; *P* < 0.05 by Dunnett’s multiple comparison test for pair-fed versus water or versus pEcN. Pair-fed versus pNAPE-EcN, not significant. (C) Calculated feeding efficiency. *P* < 0.0001 by 1-way ANOVA; *P* < 0.05 by Dunnett’s multiple comparison test for pair-fed versus pEcN; pair-fed versus standard water or versus pNAPE-EcN, not significant.
obesity more typical of human disease has led to the development of mice with polygenic obesity such as TallyHo/Jng (TallyHo) mice (42–45). Female TallyHo mice have been reported to rapidly gain excess weight and body fat (relative to C57BL6J mice) in the first 12 weeks of life, even while consuming a standard chow diet and without displaying the hyperglycemia seen in male TallyHo mice (45). We therefore tested whether pNAPE-EcN treatment could inhibit weight gain in female TallyHo mice fed a standard chow diet. TallyHo mice treated with pNAPE-EcN beginning at 5 weeks of age gained significantly less weight than did mice treated with vehicle or pEcN (Figure 13). This reduced body weight persisted for at least 4 weeks following the end of pNAPE-EcN treatment. The obesity induced by the low-fat chow diet in the vehicle-treated TallyHo mice was relatively modest in this study, for this reason, along with the difficulty of obtaining a large number of young TallyHo mice of similar age and body weight, our study was underpowered to detect statistically significant improvements in many of the metabolic parameters previously examined for C57BL6J mice fed a high-fat diet. Nevertheless, treatment of TallyHo mice with pNAPE-EcN tended to improve the same parameters such as lowering body fat, liver triglyceride levels, plasma leptin levels, expression of inflammatory genes in the liver, and infiltration of macrophages, while increasing expression of fatty acid oxidation genes in the liver (Supplemental Table 5 and Supplemental Figure 22). These data demonstrate that pNAPE-EcN can prevent obesity arising from a variety of causes and not simply obesity induced by high-fat diets.

Discussion

Our results demonstrate the feasibility of incorporating genetically modified bacteria that secrete NAPEs into the intestinal microbiota to provide sustained treatment for a chronic condition, obesity, and thus relieve the need for continuing daily administration of

Figure 7. Pair-feeding does not induce hepatic expression of fatty acid oxidation genes but does reduce lipid accumulation. All values are the mean ± SEM; n = 9–10 mice per group. (A) Liver triglyceride (TG) levels in mice given standard drinking water (W), or drinking water with pEcN (E), or pNAPE-EcN (N), or pEcN with pair-feeding to pNAPE-EcN mice (PF). P = 0.0012 by 1-way ANOVA; *P < 0.05 versus standard water by Dunnett’s multiple comparison test. (B) Expression of mRNA encoding for proteins related to fatty acid oxidation. Ppara, P < 0.0001 by 1-way ANOVA; *P < 0.05 versus standard water by Dunnett’s multiple comparison test; Cpt1a, P = 0.0233 by 1-way ANOVA; *P < 0.05 versus standard water by Dunnett’s multiple comparison test; AOX, P = 0.1326 by 1-way ANOVA.

Figure 8. Relative abundance in feces of major bacterial phyla. Bacterial composition of feces collected during experimental week 8 (final week of treatment) and week 12 (fourth week of post-treatment follow-up) was determined by 16S rRNA sequencing (n = 9–10 mice per group). Treatment with either pNAPE-EcN (N) or pEcN (E) significantly decreased the abundance of Firmicutes compared with vehicle (V) and increased the abundance of Proteobacteria in excreted feces (week 8), but microbial composition reverted to that of the vehicle-treated animals by 4 weeks after ending bacterial administration (week 12).
administration primarily increased NAPE levels in the colon, consistent with the predominant localization of *E. coli* in the colon. We also found significant elevation of liver NAE (but not NAPE) after sustained pNAPE-EcN administration, suggesting that NAPE absorbed from the colon is converted to NAE by NAPE-PLD and then enters the portal circulation. We did not find increased plasma levels of NAE (or NAPE), consistent with previous findings that elevations in liver NAE levels do not result in significant elevations in plasma NAE (14). The rapid metabolism of NAE by FAAH and NAAA likely accounts for this observation (46, 47). That the modest elevations in hepatic levels of NAE generated by pNAPE-EcN treatment were sufficient to induce beneficial effects was evident by the increased hepatic expression of genes involved in fatty acid oxidation (*Ppara*, *Cpt1*, and *AOX*) and the decreased infiltration of leukocytes and expression of genes involved in inflammation (*Cd36*, *Ccl2*, and *Cd68*). These changes in hepatic gene expression were the result of NAE action rather than of a downstream effect of altered body composition, because fatty acid oxidation gene expression was not increased in pair-fed mice. Thus, our studies suggest that the interval between booster administrations would likely be at least 4 weeks. Thus, even without further optimization, pNAPE-EcN could represent a significantly less onerous treatment regimen than daily medication or voluntary caloric restriction.

Our studies also show that therapeutic compounds produced by colonic bacteria can be delivered to tissues beyond the intestinal tract. Within the intestinal tract, we found that pNAPE-EcN administration primarily increased NAPE levels in the colon, consistent with the predominant localization of *E. coli* in the colon. We also found significant elevation of liver NAE (but not NAPE) after sustained pNAPE-EcN administration, suggesting that NAPE absorbed from the colon is converted to NAE by NAPE-PLD and then enters the portal circulation. We did not find increased plasma levels of NAE (or NAPE), consistent with previous findings that elevations in liver NAE levels do not result in significant elevations in plasma NAE (14). The rapid metabolism of NAE by FAAH and NAAA likely accounts for this observation (46, 47). That the modest elevations in hepatic levels of NAE generated by pNAPE-EcN treatment were sufficient to induce beneficial effects was evident by the increased hepatic expression of genes involved in fatty acid oxidation (*Ppara*, *Cpt1*, and *AOX*) and the decreased infiltration of leukocytes and expression of genes involved in inflammation (*Cd36*, *Ccl2*, and *Cd68*). These changes in hepatic gene expression were the result of NAE action rather than of a downstream effect of altered body composition, because fatty acid oxidation gene expression was not increased in pair-fed mice. Thus, our studies demonstrate that genetically modified bacteria are capable of producing sufficient amounts of secreted small molecules to induce significant therapeutic effects,
els decreased food intake primarily by increasing post-meal intervals from previous reports in chow-fed rats, in which increasing NAE levels are known to influence eating behavior. However, this effect on meal patterning for high-fat feeding differs somewhat without an effect on intermeal intervals. This effect of pNAPE-EcN treatment was accompanied by shorter meal bouts and smaller meal size reductions in food intake during the dark phase (the active period), indicating that the lower overall food intake appeared to be particularly driven by the earlier onset of feeding stress. For instance, the increase in feeding stress experienced by mice on the high-fat diet was associated with increased energy expenditure, which was associated with shorter meal bouts and smaller meal size reductions in food intake during the dark phase (the active period).

The Journal of Clinical Investigation

**Figure 10. Effect of pNAPE-EcN treatment on energy expenditure.** (A) Energy expenditure during 12-hour light phase of the 24-hour light-dark cycle. Slopes for each group differed: pNAPE-EcN, \( y = 0.01016x + 0.02295 \); pEcN, \( y = 0.006475x + 0.1201 \); standard water, \( y = 0.02076 + 0.001879 \); differences in slope, \( P = 0.04038 \), \( F = 3.702 \). (n = 9–10 mice per group). (B) Energy expenditure during the dark phase of the 24-hour light cycle. Slopes for each group did not differ: pNAPE-EcN, \( y = 0.002704 + 0.3448 \); pEcN, \( y = 0.003438 + 0.3304 \); standard water, \( y = 0.004455 + 0.2857 \); differences in slope, \( P = 0.8734 \), \( F = 0.1362 \).}

**Figure 11. Treatment with pNAPE-EcN reduces the insulin resistance index and increases insulin response in mice fed a high-fat diet.** All values are the mean ± SEM; \( n = 10 \) mice per group. (A) Treatment with pNAPE-EcN (HF + N) improved basal glucose levels and glucose tolerance compared with mice treated with standard drinking water (HF + W), but not a low-fat chow diet (LF + W). Fasting glucose levels differed significantly between groups: \( P < 0.001 \) by 1-way ANOVA; \( P < 0.05 \) by Bonferroni’s post-hoc multiple comparison for HF + W versus HF + N or LF + W, and HF + N versus LF + W. Glucose AUC differed significantly between each treatment group: \( P < 0.0009 \) by 1-way ANOVA; \( P < 0.05 \) by Bonferroni’s post-hoc multiple comparison for HF + W (50,841 ± 2,191 mg/dl/min) versus either HF + N (39,463 ± 1,800) or LF + W (24,573 ± 517), and for HF + N versus LF + W. (B) Treatment with pNAPE-EcN increased insulin responsiveness. Fasting levels of insulin were significantly higher in HF + W versus HF + N or LF + W mice: \( P < 0.0001 \) by 1-way ANOVA; \( P < 0.05 \) by Bonferroni’s post-hoc multiple comparison for HF + W (193.9 ± 17.0) or HF + N (158.7 ± 8.4, \( P < 0.05 \)). (C) Treatment with pNAPE-EcN improved the HOMA-IR score compared with a high-fat diet–only treatment: \( P < 0.0001 \) by 1-way ANOVA; \( P < 0.05 \) by Bonferroni’s post-hoc multiple comparison for HF + W versus HF + N or LF + W, but not for HF + N versus LF + W.
diture is most likely the result of an increased metabolic rate during rest. We also found an increased expression of fatty acid oxidation genes and a small but consistent tendency toward a lower RQ in pNAPE-EcN–treated mice. Even small changes in resting metabolic rate and fatty acid oxidation may result in marked long-term differences in adiposity. A reduced RQ when switching to high-fat diets predicts lower long-term weight gain and body fat in humans (61). Conversely, individuals prone to obesity have blunted lowering of their RQ in response to deliberate short-term overeating of a balanced diet (62). Therefore, the ability of pNAPE-EcN treatment to increase expression of fatty acid oxidation genes and resting energy expenditure may be important mechanisms contributing to its long-term efficacy in lowering adiposity.

Of note, our finding that elevations of NAEs in the liver corresponded with slightly increased resting energy expenditure contrasts with a recent study that found decreased resting energy expenditure when NAE levels were elevated systematically by genetic ablation of fatty acid amide hydrolase or by subcutaneous infusion of synthetic NAE (63). Thus, the failure of the bacterially synthesized NAPE metabolites to lower RQ may in part result from NAPEs being endogenous phospholipids of E. coli (68), rather than a foreign peptide. Because NAPEs are also endogenous mammalian phospholipids that exert antiinflammatory effects after conversion to NAEs, NAPEs seem unlikely to invoke an immune response even when biosynthesized chronically by gut bacteria.

In addition to our genetically modified EcN, a number of unmodified probiotic bacteria also show antiobesity effects in mice (69–73). One clear advantage of using genetically modified gut microbiota rather than wild-type probiotics is the ability to choose both an appropriate carrier bacteria that can colonize the gut of the diseased individual and an appropriate therapeutic compound, whereas most probiotic bacteria are poor colonizers and the actual bioactive metabolites that confer their benefit poorly characterized. Without appropriate characterization of the bioactive metabolites, quality control during production and continuous culture of these probiotics may be very difficult. Another final possible advantage of genetically modified gut bacteria is that they could be made responsive to temporal cues such as food intake by use of appropriate promoters. Such food-dependent biosynthesis would mimic physiological regulation of many metabolic responses, potentially improving efficacy. Thus, if appropriate regulatory guidelines for use of genetically modified gut bacteria can be established, this approach may allow highly customized long-term
treatments for individuals with different chronic medical conditions including obesity. Our finding that biosynthesis of NAPEs by EcN inhibits obesity induced by a high-fat diet and does so for an extended period after ending administration of bacteria illustrates the potential efficacy of this approach.

Methods

Bacterial strains and preparation. The pDEST-At1g78690 expression plasmid and transformation into Ec have been previously described (32). EcN was obtained from ArdeyPharm, GmbH and the P. luminescens luciferase operon cloned from the pXen5 plasmid (Xenogen) inserted into the RecA gene. For expression of At1g78690 in EcN, pQE-80L (QIAGEN) was modified by removing one lac operator to enable basal expression of inserted genes without IPTG induction. This was accomplished by digesting pQE-80L with Xhol and EcoRI and then annealing 2 pairs of oligonucleotides that had previously been annealed and then digested with the same enzymes. The sequences of these oligonucleotides are as follows: Pair 1, sense: TCGTCTTCAC TCGGAGAAAT CATAACAAAAAAAAAAT TTATTTGGTT TGAAGCGGGA TAAAAATTTAT TAAAGATCCCA ATACACAGAT AACCATTTAAA; antisense: TTTTAATGGAAT TCTGCTGTATG TGAATCTATT ATTATTTTATGAT TTTCCTCGAG GTGAAGACGA. Pair 2, sense: TCGTCTTCAC TCGGAGAAAT CATTTAAAAAT TTATTTGGTT TCGAGAAATT TTTTGTATT AATGATTCA ATACACAGAT ATACATTTAAA; antisense: TTATTTGGAAT TCTGCTGTATG TGAATCTATT ATTATTTTATGAT TTTCCTCGAG GTGAAGACGA. This was accomplished by digesting pQE-80L with Xhol and EcoRI and then annealing 2 pairs of oligonucleotides that had previously been annealed and then digested with the same enzymes. The sequences of these oligonucleotides are as follows: Pair 1, sense: TCGTCTTCAC TCGGAGAAAT CATAACAAAAAAAAAAT TTATTTGGTT TGAAGCGGGA TAAAAATTTAT TAAAGATCCCA ATACACAGAT AACCATTTAAA; antisense: TTTTAATGGAAT TCTGCTGTATG TGAATCTATT ATTATTTTATGAT TTTCCTCGAG GTGAAGACGA. Pair 2, sense: TCGTCTTCAC TCGGAGAAAT CATTTAAAAAT TTATTTGGTT TCGAGAAATT TTTTGTATT AATGATTCA ATACACAGAT ATACATTTAAA; antisense: TTATTTGGAAT TCTGCTGTATG TGAATCTATT ATTATTTTATGAT TTTCCTCGAG GTGAAGACGA. For the main diet-induced obesity study, 40 male C57BL/6J mice were obtained at approximately 4 weeks of age and initially fed a standard chow diet (LabDiet 5001: 13.5% kcal from fat, 60% kcal from carbohydrate, 28.5% kcal from protein). After 1 week of adaptation to the animal facility (experimental day −7), the mice were separated into individual cages and were treated with 500 mg/l ampicillin in drinking water for 7 days. During this time, food intake, body weight, and body composition were determined for each mouse, and then the mice were divided into 4 groups of 10 mice each so that the mean and variation in body weight and body fat for each group were as similar as possible (Table 1). Each group was then randomly assigned to 1 of the 4 treatment groups. On experimental day 0, ampicillin treatment was stopped, mice began receiving their assigned treatment for 8 weeks and began the high-fat diet (TestDiet 58Y1, containing 60% fat by kcal), which they received for the remainder of the study. The 4 treatment groups received standard drinking water either with no additives (water), with 0.125% gelatin (vehicle), with 0.125% gelatin and 5 × 10^5 CFU/ml pEcN (pNAPE-EcN), or with 0.125% gelatin and 5 × 10^6 CFU/ml pEcN (pEcN). Additionally, all mice were given preweighed kaolin pellets (Research Diets), and the change in kaolin pellet weight was measured once a week as an indicator of intestinal distress. On the final day of the study (day 87), mice were euthanized and blood and tissue collected.

Body weight was measured using a portable electronic scale. For body composition, mice were scanned by MRI using a Bruker Minispec MQ10 NMR Analyzer to determine fat mass, lean mass, and free fluid. All bioluminescence imaging (BLI) was performed using a Xenogen IVIS 200 CCD camera (Caliper Life Sciences). Equal areas for each region of interest (ROI) were centered over the bioluminescent region. Photon counting measurements summed bioluminescent intensity for all pixels within the ROI over the integration time.
For glucose tolerance testing after 8 weeks of bacterial treatment, all mice were returned to standard drinking water, fasted overnight, weighed, and then given a bolus of glucose (2 g/kg body weight) by oral gastric gavage. Blood samples were taken 0, 15, 30, 60, and 120 minutes after gavage.

The 4 follow-up mouse studies were performed as described for the main study except that for the first follow-up study, no untreated (water-only) group of mice was used, and the follow-up period after ending bacterial administration was extended to 12 weeks rather than only 4 weeks as in the main study. Feces were collected for 16S RNA analysis at the end of the eighth week of treatment and the fourth week of follow-up (week 12). Mice were euthanized on experimental day 140.

For the second follow-up study, the groups of mice were ad libitum fed a Chow diet, a high-fat diet and standard drinking water, or a high-fat diet and drinking water supplemented with 5 x 10^9 CFU/ml pNAPE-EcN. After 9 weeks of treatment, the pNAPE-EcN group was returned to standard drinking water for 1 day, the mice were fasted for 6 hours, and an oral glucose tolerance test was performed. Mice were euthanized the following day (experimental day 65), and livers were returned to standard drinking water for 1 day, the mice were fasted for 4 hours and blood samples drawn. One week after their return to standard cages, the mice were fasted for 4 hours starting at lights on prior to receiving either saline or 0.75 IU/kg body weight insulin and then euthanized 15 minutes later.

For the fourth follow-up study, TallyHo mice were used instead of C57BL6 mice. Because of the limited number of TallyHo mice available for purchase from the commercial vendor (The Jackson Laboratory), 2 different cohorts of mice were used. Only mice weighing between 21 and 24 g at 5 weeks of age were included in the study. The 3 treatment groups received: vehicle (0.125% gelatin in water), 5 x 10^9 CFU/ml pECN, and 5 x 10^10 CFU/ml pECN in drinking water. All TallyHo mice received a standard Chow diet for the entire study. Ampicillin (500 mg/l) was given in the same drinking water as the EcN or the vehicle for 1 week at the beginning of the treatment. Treatment was continued for 11 weeks, and mice were euthanized and tissue harvested 4 weeks after stopping administration of EcN. Mice were fasted for 4 to 6 hours prior to being euthanized.

Biochemical measurements. Blood glucose was measured using the Accu-Chek Diabetes monitoring kit (Roche). Insulin and leptin were measured by the Vanderbilt Hormone Assay and Analytical Services Core using radioimmunoassay. For triglyceride measurements in liver, lipids were extracted using chloroform/methanol (2:1), and individual lipid classes were separated by thin-layer chromatography. The triglycerides were scraped from the plate, and the fatty acids methylated using BF3/methanol. Fatty acid methyl esters were analyzed using an Agilent 7890A gas chromatograph equipped with flame ionization detectors and a capillary column (SP2380, 0.25 mm x 30 m, 0.25 μm film; Supelco). The esters were identified by comparing the retention times to those of known standards. Triclosan (C20:1) was added to the total lipid extract, serving as an internal standard and permitting quantitation of the amount of triglycerides in the sample.

For measurement of gene expression in liver, total RNA was extracted from liver tissue using the RNeasy Micro Kit (QIAGEN), and the concentration was measured spectrophotometrically. The extracted RNA was reverse transcribed into cDNA using a cDNA reverse transcription kit (Applied Biosystems), and the RNA expression level was quantified by quantitative RT-PCR (qRT-PCR) using SYBR Green PCR Master Mix (QIAGEN) and the 7500 Real Time PCR system (Applied Biosystems) according to the manufacturer’s protocol. For Ppara, the primers pair used was GTACGGTGTGTATGAAGCCTT and GCCGATCAGCAT. For Ppard, the primers were GCCATATTCCGTCAACAGAGC-3 TC TTAAAGGAATCCCCGTGT and TGCATTTGCCAATGTCTAGC.

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For Scd1, the primers were TTCTTACAGCACACCA and CCGAAGGAGCCGGTAG. For Cd68, the primers were ACT- CCGAGTCCTCTCCCTC and TTCTTCTGGGG GTCA- CACAGC. For Cds68, the primers were CCATCTCAGATCGA- CACCT and GAGAGTATAGTGACAGTT. For β-actin (Actb), the primers were GAGCGCAAGTACTCTGTGTG and CGGACT- CATCGTAATCCCTTG. Relative quantification of gene expression with real-time PCR data was calculated relative to β-actin.

Fecal DNA extraction, pyrosequencing, and bacterial composition analysis. Bacterial DNA was extracted by a bead-beating method using a commercial DNA extraction kit (Mobio Powersoil Kit) following the manufacturer’s instructions. The bead-beating step was performed on a homogenizer for 60 seconds at a speed of 4 m/s. Amplification of the 16S rRNA genes was carried out using universal bacterial primers (530F-1100R) to amplify DNA in a single-step, 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (QIAGEN) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads. Samples were sequenced using Roche 454 FLX titanium instruments and reagents according to the manufacturer’s guidelines. The Q25 sequence data were processed using a proprietary analysis pipeline (www.mrdnalab.com) (74, 75). Briefly, sequences were depleted of barcodes and primers, then sequences less than 150 bp were removed, as were sequences with ambiguous base calls and homopolymer runs exceeding 6 bp. Operational taxonomic units (OTUs) were generated by clustering at 97% similarity from de-noised sequences, and chimeras were removed. Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (76) and compiled into each taxonomic level as the percentage of sequences within each sample that map to the designated taxonomic classification. A total of 423,513 sequences, 5,361 ± 262 per sample, were analyzed. Mean differences at each taxonomic level were evaluated by T-testing (Microsoft Excel) against vehicle controls. The Sequence Read Archive (SRA) accession number for our study is SRP042014.

Western blot analysis. Frozen tissues were made into a powder using a tissue pulverizer, and then approximately 50 mg of powered tissue was sonicated with a Vironic 10 Ultrasonic Cell Disruptor (at setting 8, for less than 10 seconds; Viritis) in cold T-Per Tissue Protein Extraction Buffer (10 μl/mg tissue; Thermoscientific) with added protease and phosphatase inhibitor cocktails (P8340, P5726, and P0044; Sigma-Al drich). Diluted protein samples (10.5 μg total protein per lane) were subjected to denaturing electrophoresis on a 4%-12% Tris Acetate gel with XT MES Running Buffer using the Bio-Rad XT Criterion System. Proteins were transferred to 0.2-μm nitrocellulose membranes (Millipore), blocked in 5% BSA and 0.2% Tween-20, and incubated with primary antibodies diluted 1:1,000 in blocking buffer overnight at 4°C with gentle rocking. Primary antibodies used allowed the detection of AKT and its Ser473 and Thr308 phosphorylated forms (4691, 9271, and 9275, respectively; Cell Signaling Technology), the phosphorylated (Thr183/ Tyr185) form of JNK (4668; Cell Signaling Technology), the phosphorylated (Ser307) form of IRS1 (2381; Cell Signaling Technology), and a loading control, HSC70/HSP73 (ADI-SPA-816; Enzo Life Sciences), was used. Blots were washed and incubated with a species-specific HRP-conjugated secondary antibody (goat anti-rabbit, W401B; Promega) in 50/50 blocking buffer and Starting Block T20 Blocking Buffer (37543; Thermo Scientific). After washing, membranes were developed using the PerkinElmer Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate Kit (NEL105001; Amersham Biosciences) and GeneMate Blue Ultra Autorad Film (BioExpress). Films were scanned on an Epson 3200 scanner and band density quantified using ImageJ software (NIH). Densitometric values were reported after normalization for gel-loading differences and relative to the average value obtained from protein extracts of vehicle-treated control mice.

Statistics. Statistical analysis was performed using GraphPad Prism 5.04 (GraphPad Software). A P value of less than 0.05 was considered statistically significant. For 2-way repeated-measures (RM) ANOVA, when overall significance for treatment effect was found, the Bonferroni’s multiple comparison post-hoc test was used to determine differences between treatment groups. For 1-way ANOVA, when overall significance for treatment effect was found, the Dunnett’s multiple comparison post-hoc test was used to determine groups that differed from the control group. A 2-tailed Student’s t test was used for studies with a fixed endpoint comparing pNPE-ECN treatment to the control.

Study approval. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of Vanderbilt University.

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