Intestinal alkaline phosphatase prevents metabolic syndrome in mice

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Metabolic syndrome comprises a cluster of related disorders that includes obesity, glucose intolerance, insulin resistance, dyslipidemia, and fatty liver. Recently, gut-derived chronic endotoxia has been identified as a primary mediator for triggering the low-grade inflammation responsible for the development of metabolic syndrome. In the present study we examined the role of the small intestinal brush-border enzyme, intestinal alkaline phosphatase (IAP), in preventing a high-fat-diet–induced metabolic syndrome in mice. We found that both endogenous and orally supplemented IAP inhibits absorption of endotoxin (lipopolysaccharides) that occurs with dietary fat, and oral IAP supplementation prevents as well as reverses metabolic syndrome. Furthermore, IAP supplementation improves the lipid profile in mice fed a standard, low-fat chow diet. These results point to a potentially unique therapy against metabolic syndrome in at-risk humans.

Metabolic syndrome is a complex syndrome composed of a cluster of disorders that includes obesity, glucose intolerance, insulin resistance, abnormal lipid profile (dyslipidemia), fatty liver, and hypertension (1, 2). Metabolic syndrome leads to type 2 diabetes, atherosclerosis, and nonalcoholic fatty liver disease (1, 2). Approximately 35–39% of the US population suffers from the syndrome (3). This epidemic of metabolic syndrome has devastating consequences in terms of mortality, morbidity, and total healthcare expenditures (4).

Recently, “metabolic endotoxia” has been proposed to be central to the pathogenesis of metabolic syndrome. The Gram-negative bacterial cell wall component lipopolysaccharide (LPS) is known as endotoxin, and metabolic endotoxia is defined as a two- to threefold persistent increase in circulating endotoxin concentrations above the normal levels (5). Metabolic endotoxia leads to low-grade systemic inflammation as evidenced by increased serum levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, and IL-6 (5). It is well recognized that chronic inflammation causes damage to pancreatic beta cells (6), hepatocytes (7), and vascular endothelial cells (8), and dysfunction of these cells is thought to contribute to metabolic syndrome.

A high-fat diet (HFD) has been shown to cause metabolic endotoxia in animals and humans (5, 9), but the underlying molecular mechanisms remain incompletely understood. Ghoshal et al. (10) demonstrated that intestinal epithelial cells (enterocytes) internalize LPS from the apical surface, which is then transported to the Golgi apparatus where it complexes with chylomicrons, the lipoproteins that transport the absorbed long-chain fatty acids in enterocytes. The chylomicron–LPS complex is then secreted into mesenteric lymph and makes its way into the systemic circulation. Excess chylomicron formation during high-fat feeding leads to prolonged chylomicronemia (complexed with LPS) that ultimately induces systemic inflammation. Also, it has been shown that an HFD causes local intestinal inflammation (11). Systemic and local inflammation lead to overexpression of proinflammatory cytokines (12), which cause increased gut permeability (13) and an acceleration of endotoxin translocation (14), resulting in a vicious cycle of endotoxia. A central role of LPS in the pathogenesis of metabolic syndrome is also supported by the observation that mice lacking toll-like receptor 4 (TLR4), the receptor for LPS, are resistant to HFD-induced inflammation, obesity, and insulin resistance (15).

We and others have shown that the brush-border enzyme intestinal alkaline phosphatase (IAP) detoxifies a variety of bacterial toxins, including LPS, CpG DNA, and flagellin (16). Furthermore, it has been reported that inhibition of endogenous IAP by t-pheylalanine (Phe) increases serum endotoxin levels (17). Based upon these previous observations regarding IAP function, we hypothesized that this enzyme could play an important role in preventing gut-derived systemic inflammation. Here we report that endogenous IAP plays a critical role in reducing endotoxia, and oral supplementation with IAP prevents HFD-induced endotoxia, as well as metabolic syndrome in mice. We also show that IAP supplementation was able to reverse HFD-induced metabolic syndrome and improves the lipid profile in mice fed a standard low-fat chow diet (LFD). Taken together, these findings suggest that oral IAP supplementation may represent a unique therapeutic approach for the prevention or treatment of metabolic syndrome in humans.

Results

IAP Prevents Endotoxia. Given that IAP detoxifies LPS and other bacterial toxins, we predicted that IAP knockout (KO) mice (18) would suffer from metabolic endotoxia and also metabolic syndrome. Indeed, we found that IAP-KO mice suffer from endotoxia (Fig. 1) as well as overexpression of the proinflammatory cytokines TNF-α (Fig. 1B) and interleukin-1β (IL-1β) (Fig. S1A) IAP-KO mice also had greatly increased gut permeability based on enhanced absorption of orally administered dextran-FITC (Fig. 1C). As expected, this increased gut permeability caused enhanced LPS translocation in IAP-KO mice compared with WT mice (Fig. 1D).


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IAP prevents endotoxemia. Groups of 4- to 17-wk-old C57BL/6 male IAP-KO (Akp3<sup>−/−</sup>) mice and their WT littermates (n = 5–10 each group) were used in these experiments unless otherwise indicated (SI Materials and Methods). Mice received a low-fat diet (LFD, 14% kcal from fat) unless otherwise indicated. (A) Serum endotoxin levels. (B) Serum TNF-α levels. (C) Intestinal permeability as determined by quantifying the amount of FITC-dextran (70 kDa) levels in the serum after its oral gavage. (D) Change in serum endotoxin levels after oral LPS gavage. (E) Blood glucose levels during glucose tolerance test (GTT). (F) Serum insulin levels during the first 30 min of GTT. (G) Blood glucose levels during insulin tolerance test (ITT). (H) Body weight gain (g) by mice on LFD. (I) White adipose tissue (WAT) distribution. (J) Blood glucose levels during GTT in mice receiving LFD (LF group) or high-fat diet (HFD, 45% kcal from fat) (HF group) treated with L-phenylalanine (Phe, 10 mM in the drinking water), a specific inhibitor of IAP. (K) Inhibition of corn-oil–induced endotoxemia in CD-1 mice by IAP in a dose-dependent manner. (L) Inhibition of corn-oil–induced endotoxemia in WT and IAP-KO mice. (M) Inhibition of corn-oil–plus LPS-induced endotoxemia in CD-1 mice by IAP in a dose-dependent manner. Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student’s t test. For multiple comparisons, analysis of variance with Tukey was used. * or ** or ## p < 0.05; *** or ## or *** or *** p < 0.01; *** and ### p < 0.001. The number sign (#) refers to the HF vs. HF + Phe comparison.

The above data established that the IAP-KO mice display evidence of metabolic endotoxemia. We next investigated whether these mice also display features of metabolic syndrome. We observed that IAP-KO mice suffer from glucose intolerance (Fig. 1E and Fig. S1B) and hyperinsulinemia (Fig. 1F). An insulin tolerance test (ITT) showed that IAP-KO mice had significantly higher glucose levels at multiple time points, indicating a state of insulin resistance in these animals (Fig. 1G and Fig. S1C). We also observed metabolic syndrome–associated obesity in IAP-KO mice (Fig. 1H) and found that these mice accumulated higher body fat (white adipose tissue, WAT) compared with WT (Fig. 1I), including much more intraabdominal fat (Fig. S1D). These data indicate that endogenous IAP plays a critical role in preventing metabolic endotoxemia and the resultant metabolic syndrome in mice.

The amino acid Phe specifically inhibits the enzymatic activity of IAP. Mice receiving an HFD and Phe (HF + Phe group) had impaired glucose tolerance compared with mice receiving an HFD alone (HF group), indicating the preventive role of endogenous IAP (Fig. J and Fig. S1E). In addition, the HF + Phe group had significantly higher serum endotoxin levels (Fig. S1F). Interestingly, the related groups consumed the same amount of food and had similar weight gain (Fig. S1 G and H). Furthermore, IAP-KO mice receiving an HFD also suffered from type 2 diabetes as evidenced by higher fasting glucose levels and abnormal glucose tolerance tests (Fig. S1 I and J).

An HFD is associated with enhanced translocation of LPS from the gut to the systemic circulation through chylomicrons. We found higher serum LPS concentrations in WT mice fed corn oil alone compared with corn oil plus calf IAP, and these inhibitory effects of IAP were found to be dose dependent (Fig. 1K). We also observed higher serum LPS concentrations in IAP-KO mice fed with corn oil compared with WT mice (Fig. 1L). Oral IAP supplementation prevented corn-oil–induced endotoxemia in both groups of mice (Fig. 1L) and in a dose-dependent manner was able to reduce endotoxemia when excess LPS was administered along with the corn oil (Fig. 1M).

IAP Prevents Chronic High-Fat-Diet–Induced Metabolic Syndrome. We next assessed whether over a prolonged period, oral supplementation with IAP could prevent metabolic syndrome. We exposed 15-wk-old male C57BL/6 mice to an HFD (45% kcal from fat) ± IAP (100 units/mL drinking water) for 11 wk. Control mice (low fat, LF group) consumed a standard chow LFD (14% kcal from fat) only. As expected, the mice receiving the HFD + IAP (HF + IAP group) exhibited better glucose tolerance (Fig. 2A) and less postprandial hyperinsulinemia (Fig. 2B) and insulin resistance (Fig. 2C) than mice receiving the HFD alone (HF group). Fasting blood glucose levels were higher in the HF compared with LF mice, whereas there were no differences between LF and HF + IAP groups (Fig. 2A and D and Fig. S2A and B). We observed higher body weight in the HF group, however no significant difference between LF and HF + IAP groups (Fig. S2C). The HF and HF + IAP groups had nearly equal energy intake (Fig. S2D); however, the feed efficiency [FE = weight gained (g)/kcal consumed × 100] was lower, although insignificant, in the HF + IAP group (Fig. S2E). We also found that IAP prevented HFD-induced decrease in pancreatic weight (Fig. 2F). Compared with
HF+IAP

**Fig. S3**

HFD-induced increase in liver weight (Fig. S3) and also protected the cacy of IAP in preventing HFD-induced liver injury. IAP prevented compared with the HF mice, the IAP-treated animals had lower levels of aminotransferase (AST), gamma-glutamyl transferase (GGT), and alanine aminotransferase (ALT) (Fig. 3A–C). In addition, compared with the HF mice, the IAP-treated animals had lower levels of total liver lipids (Fig. 3D) and triglycerides (TG) (Fig. 3E). Finally, histological analyses showed that HF mice had accumulated much higher amounts of hepatic fat, and these changes were not seen in the HF + IAP animals (Fig. 3F and G).

**Fig. 2.** IAP prevents chronic HFD-induced glucose intolerance and insulin resistance. Groups of 15-wk-old WT male C57BL/6 mice (n = 5 for each group) were fed an HFD (45% kcal from fat) or IAP (100 units/mL in drinking water) for 11 wk. A control group of mice received LFD (14% kcal from fat). (A) Blood glucose levels during GTT in mice receiving LFD (LF group), HFD (HF group), and HFD + IAP (HF + IAP group). (B) Serum insulin levels during GTT. (C) Insulin resistance index (homeostasis model assessment of insulin resistance, HOMA-IR). (D) Blood glucose levels during ITT. (E) Weight of pancreas. (F) White adipose tissue (WAT) expressed as percentage of body weight (BW). (G) Adiposity index. (H) Energy intake by different groups. Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student’s t test. For multiple comparisons, analysis of variance with Tukey was used. * P < 0.05; ** P < 0.01; *** P < 0.001. The asterisk (*) refers to the LF vs. HF comparison and the number sign (#) refers to the HF vs. HF + IAP comparison.

The HF group, mice in the HF + IAP group also showed an overall reduction in body fat, most dramatically seen in the case of s.c. fat (Fig. 2F). As expected, the adiposity index (sum of the weights of adipose depots expressed as the percentage of total body weight) was also significantly decreased in the HF + IAP group compared with the HF group (Fig. 2G), despite the fact that the two groups consumed nearly equal amounts of food (Fig. 2H). We noticed that even after just 6 wk of an HFD, mice developed glucose intolerance that was prevented in the IAP-treated group (Fig. S2F–I).

**IAP Prevents HFD-Induced Liver Injury.** We next examined the efficacy of IAP in preventing HFD-induced liver injury. IAP prevented HFD-induced increase in liver weight (Fig. S3) and also protected mice from HFD-induced increase in the liver enzymes, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and alanine aminotransferase (ALT) (Fig. 3A–C). In addition, compared with the HF mice, the IAP-treated animals had lower levels of total liver lipids (Fig. 3D) and triglycerides (TG) (Fig. 3E). Finally, histological analyses showed that HF mice had accumulated much higher amounts of hepatic fat, and these changes were not seen in the HF + IAP animals (Fig. 3F and G).

**IAP Prevents HFD-Induced Dyslipidemia.** Lipid profile analyses showed that IAP prevents HFD-induced dyslipidemia (Fig. 4A–E).

Most notably, the HDL-C levels markedly increased by ~73% in the HF + IAP group compared with the HF group (Fig. 4D). The HF group receiving IAP supplementation exhibited a great reduction in atherogenic propensity including the important cholesterol ratios and the atherogenic index (Fig. S4A–F).

**IAP Prevents HFD-Induced Endotoxemia and Intestinal Permeability.** We next sought to assess some of the factors known to underlie metabolic syndrome. Compared with the LF mice, serum endotoxin levels were much higher in the HF group (Fig. 4F), whereas these levels were reduced in the HF + IAP group. In addition, compared with the HF group, the endotoxin levels were also greatly reduced in the cecal contents of the IAP-treated group (Fig. S4G). To further explore the mechanism by which IAP prevents endotoxemia, we sought to determine whether oral IAP detoxifies LPS within the intestinal lumen or, alternatively, if IAP enters the systemic circulation to detoxify circulating LPS. Accordingly, we treated separate groups of mice with oral or i.p. IAP and oral or i.p. LPS. Oral IAP supplementation blocked the luminal LPS but was unable to prevent endotoxemia induced by two different doses of i.p. LPS (Fig. 4G and Fig. S4H). In contrast, the i.p. IAP was able to detoxify the i.p. LPS. These results support the concept that oral IAP prevents endotoxemia by detoxifying LPS within the intestinal lumen.

**Fig. 3.** IAP prevents HFD-induced liver injury. (A) Serum aspartate aminotransferase (AST) levels (see Fig. 2 for description of mice). (B) Serum gamma-glutamyl transferase (GGT) levels. (C) Serum alanine aminotransferase (ALT) levels. (D) Total liver lipids. (E) Liver triglyceride levels. (F) Liver steatosis score. (G) Oil Red O staining of frozen liver sections (10x objective). Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student’s t test. For multiple comparisons, analysis of variance with Tukey was used. * P < 0.05; ** P < 0.01; *** P < 0.001.
We found that IAP prevented HFD-induced increased levels of serum TNF-α and IL-1β (Fig. 4H and Fig. S4J, respectively). To further assess the local inflammatory status within the intestine, we measured TNF-α levels in the cecal contents and found approximately threefold higher levels of TNF-α in the HF compared with the HF + IAP mice (Fig. 4I). We then directly determined the proinflammatory status of the cecal contents by exposing mouse leukaemic monocyte macrophage RAW264.7 cells to cecal contents and found that the fluid from the HF + IAP mice was less inflammatory (Fig. S4J). In addition, we found that the IAP-treated animals were protected from the increase in intestinal permeability that occurs in response to an HFD (Fig. 4J). We also studied the acute (10 d) and chronic (16 wk) effects of oral IAP supplementation on the expression of IAP (mouse intestinal alkaline phosphatase gene Akp3) mRNA (Fig. 4K and Fig. S4K), as well as the activity of the endogenous IAP enzyme (Fig. S4L and M) and observed no significant differences in IAP expression or activity among the groups.

Based on the known activity of the IAP enzyme and our data on luminal LPS and endotoxemia, it appears that IAP may prevent metabolic syndrome by inhibiting ligands derived from luminal bacteria, such as LPS.

**Fig. 4.** IAP prevents HFD-induced dyslipidemia and endotoxemia. (A) Serum total cholesterol levels (see Fig. 2 for description of mice). (B) Levels of serum triglycerides. (C) Total HDL-C and LDL-C levels. (D) Serum endotoxin levels after i.p. injection of LPS (different groups of mice, see SI Materials and Methods). (E) Serum endotoxin levels after i.p. injection of LPS (different groups of mice, see SI Materials and Methods). (F) Serum endotoxin levels after i.p. injection of LPS (different groups of mice, see SI Materials and Methods). (G) Serum endotoxin levels after i.p. injection of LPS (different groups of mice, see SI Materials and Methods). (H) Serum tumor necrosis factor-alpha (TNF-α) activity among the groups.

**IAP Exerts Beneficial Effects in Treating HFD-Associated Metabolic Syndrome.** We next investigated whether IAP supplementation would be able to reverse any features of metabolic syndrome. We exposed mice to an HFD for 14 wk to induce metabolic syndrome (Fig. S5 A–D) and then treated them with calf IAP supplementation (100 units/mL drinking water) for 6 wk. The IAP-treated group showed a significant reduction in glucose intolerance and postglucose hyperinsulinemia (Fig. 5A and B and Fig. S5E). Fig. 5C shows weekly glucose levels during GTT at the 15-min time point, indicating the beneficial effects of IAP. Glucose levels were also significantly reduced in the IAP-treated group during ITT (Fig. 5D and Fig. S5F). As expected, we observed lower endotoxin levels in the cecal contents (Fig. 5E) as well as in serum (Fig. 5F) of the HF + IAP group. We found that the HF + IAP group had lower levels of serum TNF-α and IL-1β (Fig. 5G and H) and their insulin resistance index was slightly reduced, but not significantly (Fig. S5G). During this treatment period, we observed nearly equal energy intake by the HF and HF + IAP mice and IAP treatment did not have any effect on body weight (Fig. 5H and I). We saw slight improvement in the dyslipidemia and extent of liver injury, but in this short experiment these differences did not reach statistical significance.

**Low-Fat Diet Supplemented with Oral IAP Improves Lipid Profile.** We next sought to determine whether oral supplementation with IAP would have any beneficial effects in mice exposed to an LFD. We fed 5-wk-old female mice a LFD ± IAP (100 units/mL drinking water) for 7 wk. Mice receiving the LFD + IAP appeared to show slight improvement in glucose tolerance and insulin sensitivity, but these differences were not significant. On the other hand, we found dramatic beneficial effects in the case of the lipid profile (Fig. 6 A–E). The IAP-treated mice had higher levels of HDL-C (Fig. 6D) and greatly increased HDL-C.
LDL-C ratio (Fig. 6E). We also calculated the various atherogenic indices and found that the LF + IAP group had a great reduction in the atherogenesis risk (Fig. S6A–F).

Discussion

Metabolic syndrome is one of the most important modern global health problems. The etiology of HFD-induced metabolic syndrome is thought to be related to metabolic endotoxemia (19). An HFD has also been associated with an imbalance in the normal composition and number of microbes in the gut (dysbiosis), resulting in barrier dysfunction followed by LPS translocation to the systemic circulation (20).

The present study was undertaken based upon work in our laboratory and others that points to the role for IAP in protecting the host from bacterial toxins (16). The present data in mice provide a “proof of principle” that IAP could be an effective oral supplement against endotoxemia, thus protecting the host from metabolic syndrome. We have shown that IAP reduces corn-oil-induced endotoxemia and prevents the inflammation and intestinal permeability changes that occur in response to an HFD. Interestingly, the excess secretion of IAP seen in rats fed an HFD (21) could represent a physiological response of the body to protect against HFD-associated systemic inflammation. IAP is primarily expressed in the enterocytes of the proximal small intestine and is bidirectionally secreted into the intestinal lumen as well as the systemic circulation (22). IAP is strictly conserved among species (23) and exists within a microbial environment of bacterial proinflammatory factors as well as the prevention of dysbiosis (16, 24). Various isozymes of alkaline phosphatases (APs) exist, namely IAP, placental AP, tissue nonspecific (liver/bone/kidney/neutrophils) AP, and germ cell AP (23). These various AP enzymes share significant structural homology as well as functional similarities. It will be interesting in future studies to determine whether these other AP isozymes also have the capability to protect the host from metabolic syndrome.

IAP exists within an environment that contains a wide range of bacterially derived proinflammatory factors and we have previously demonstrated that in addition to LPS, other targets for the IAP enzyme include flagellin and CpG DNA (16). In regard to metabolic syndrome, although LPS has been the most intensively studied gut-derived inflammatory mediator, it is likely that it is not the only one because the other mediators also activate the NF-κB pathway and ultimately increase the levels of proinflammatory cytokines (25). As such, we speculate that the beneficial effects of IAP may be due to its detoxification of a number of proinflammatory factors, not just LPS. Our results using antibiotics to decrease the luminal bacterial content before the HFD ± IAP add support to the idea that the mechanism of IAP action largely involves its ability to block luminal, bacterially derived inflammatory mediators. Of course, we are unable to completely rule out the possibility that IAP may also be exerting some impact on metabolic syndrome through a mechanism that is independent of luminal bacterial products.

In regard to the gut microbiota, we have previously reported that IAP-KO mice display an overall decrease in the number of intestinal bacteria and oral supplementation with IAP in WT mice was able to rapidly restore the normal gut flora in mice exposed to antibiotics (24). The prevention of HFD-induced metabolic endotoxemia in this study could be due in part to the role of IAP in preventing HFD-induced gut dysbiosis, although our acute experiments with corn oil and LPS show an inhibitory impact of IAP that is independent of any chronic changes in the flora. The critical role for IAP at the interface between the host and the intestinal lumen is supported by studies in zebrafish (26), who found that IAP expression was induced only when the fish were exposed to bacteria, whereas the enzyme was absent under germ-free conditions. Interestingly, Jang et al. (27) showed that
endogenous IAP levels in rats decrease with age, indicating a possible role of “loss of IAP” as a precipitating cause of metabolic syndrome that is known to be common with aging (28).

In a therapeutic context, Lukas et al. (29) reported on a single arm study in humans with severe ulcerative colitis and showed that enterally administered bovine IAP is extremely safe. We have previously shown that oral IAP supplementation in mice increases IAP concentrations in stools in a dose-dependent manner (24), suggesting that oral IAP dose could be easily adjusted to achieve an effective therapeutic level.

In summary, we have demonstrated that oral IAP supplementation both prevents an HFD-induced metabolic syndrome and reverses the changes associated with an HFD-induced metabolic syndrome. Furthermore, IAP can also improve the lipid profile during an LFD feeding. Although caution needs to be taken in extrapolating the findings in mouse models of inflammation to human diseases (30), taken together, the present findings suggest that oral IAP supplementation in humans could represent a safe, effective, and unique approach to the prevention or treatment of metabolic syndrome.


Calf intestinal alkaline phosphatase (IAP), ±100f8 = ELISA kits were purchased from eBioscience. C57BL/6 IAP-KO (mouse intestinal alkaline phosphatase IAP (50 units/mL) groups were euthanized by the Animal Veterinary Medical Association (AVMA)-approved protocol of i.p. injection of pentobarbital (200 mg/kg).

**Animals.** C57BL/6 IAP-KO (mouse intestinal alkaline phosphatase gene Akp350mMKCl.10MmTri500a55) unless otherwise specified. Genotype was confirmed by PCR analysis (1). CD-1 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 Animal Resources, National Research Council [Department of Health, Education and Human Services, Publication 85e23 (National Institutes of Health), revised 1985]. All animal protocols were reviewed and approved by the IACUC at MGH. Animals were euthanized by carbon dioxide anesthesia test (ITT), and lipid profile analyses were performed.

**Animal Experiments.** Mice were housed in a biosafety level 2 (BL2) room in hard top cages with three or two mice per cage. Mice 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 ad libitum. Male C57BL/6 mice, which were bred at the MGH animal facility, were used and the number of mice per group was five (n = 5) unless otherwise specified. Mice were fasted for 6 h during the light phase period and blood was collected from the facial vein unless otherwise specified.

i) Determining the role of endogenous IAP in preventing endotoxemia. Basal serum LPS concentrations were measured in fasted 8-wk-old WT and IAP-KO mice fed a standard chow diet. An LPS absorption test was performed as follows: Fasted, 10-wk-old, WT, and IAP-KO mice were gavaged with LPS (1 g/kg) diluted in saline or saline only; blood was collected before and 45 min after gavage. To investigate metabolic syndrome in IAP-KO mice, immediately after weaning, WT and IAP-KO mice were fed LFD for 17 wk at which point glucose tolerance test (GTT), insulin tolerance test (ITT), and lipid profile analyses were performed. Thereafter, mice were killed and fat pads weighed. GTT analyses were also performed in WT and IAP-KO mice fed an HFD for 8 wk from weaning. To determine the effects of inhibiting endogenous IAP activity, 12-wk-old WT mice on LFD or HFD were allowed to drink autoclaved water alone or water containing 10 mM 1-phenylalanine. Serum LPS quantification and GTT were then performed in fasted mice. For acute corn oil experiments, fasted, 12-wk-old CD-1 mice were gavaged with 500 μL corn oil ± 1 mg LPS ± IAP (10-500 units). In addition, 12-wk-old WT control and IAP-KO mice were gavaged 500 μL of corn oil ± 500 units of IAP. Blood was collected before and 45 min after gavage, and serum LPS was measured.

ii) Studying the role of oral IAP supplementation in preventing metabolic syndrome. Groups of 15-wk-old mice were fed LFD or HFD with or without 100 units/mL calf IAP in drinking water for 11 wk. Control mice received an equal volume of “vehicle for IAP” [50 mM KCl, 10 Mm Tris-HCl (pH 8.2), 1 mM MgCl2, 0.1 mM ZnCl2, and 50% (vol/ vol) glycerol] in the drinking water. Pretreatment body weight, blood glucose level, and lipid profile were determined for individual animals; a GTT was done after 6 and 11 wk and an ITT was done after 11 wk. After collecting blood for serum lipids, mice were fasted for 6 h, killed, and fat pad wet weights were measured. Liver and cecal contents were immediately harvested and stored at −80 °C for further analyses.

iii) Evaluating oral IAP supplementation to treat established metabolic syndrome. Groups of mice were fed a HFD for 14 wk. After mice on an HFD exhibited obesity and diabetes mellitus (DM) type 2, as measured by fasting blood glucose and abnormal GTT and ITT, mice were then divided to receive continuing HFD, plus IAP or vehicle for an additional 6 wk. Animals were then subjected to weekly GTT and ITT (after 6 wk). After collecting blood, mice were fasted for 6 h, killed, and cecal contents were immediately stored at −80 °C for further analyses.

iv) Evaluation of the effects of oral IAP supplementation in mice on a LFD. Groups of 5-wk-old female mice were fed a LFD plus vehicle or LFD plus IAP (100 units/mL drinking water) for 7 wk, at which point GTT, ITT, liver enzymes, and serum lipid profile analyses were performed.

v) Intestinal permeability measurement. Intestinal permeability was assessed in 17-wk-old WT and IAP-KO mice receiving a LFD. For determining HFD-induced intestinal permeability in WT mice, groups of 5-wk-old mice were fed a LFD (LF group) or HFD for the next 22 wk, and HFD mice were then divided into two groups, one group received only HFD (HF group) and the other group received HFD plus IAP (100 units/mL drinking water) (HF + IAP group) for an additional 12 d and then permeability assay was performed (see below).

vi) Studying the role of antibiotics in preventing metabolic syndrome. Five-week-old male WT mice (n = 10) were given broad spectrum antibiotics [ampicillin (1 g/L) plus norfloxacin (1 g/L), Sigma] in the drinking water for 3 wk (2) and another five mice were simultaneously kept as controls without any antibiotics. After 3 wk, mice were tested to assess changes in the glucose tolerance because of antibiotic treatment. Stool microbes were quantified for aerobic and anaerobic bacteria (3). The mice divided into HFD ± IAP (50 units/mL) groups and then fed HFD for 4 wk. After 4 wk of HFD feeding, glucose tolerance was tested by intraperitoneal (IP) GTT.

vii) Defining the role of enteral IAP on the detoxification of parenteral endotoxin. Groups of 8-wk-old IAP-KO mice (n = 5) were on IAP (200 units/mL drinking water) for more than 48 h, and while they continued to receive IAP, the...
mice then received LPS (25 μg) as an oral gavage or as an i.p. injection. After 2 h, mice were euthanized and serum LPS levels were determined (see below). In a separate experiment a lower dose of LPS (100 ng, i.p.) was used.

Determining the effects of oral IAP supplementation on IAP (Akp3) expression and endogenous IAP activity. We studied the acute (10 d) and chronic (16 wk) effects of oral IAP supplementation on the expression of IAP (Akp3) mRNA as well as activity of endogenous IAP enzyme. Groups of 8-wk-old mice were fed an HF diet for 10 d (200 units/mL IAP) or 16 wk (100 units/mL IAP). The mice were then killed and the proximal intestinal (duodenum) segments were collected, opened longitudinally, and washed thoroughly with ice-cold PBS to remove the luminal contents. The intestinal segments were then used for measuring IAP expression or endogenous activity (see below).

Animal body weight and food intake were measured weekly and water consumption was measured daily in these experiments. Drinking water tubes were changed daily. Stool IAP activity was measured weekly to confirm whether the mice receiving IAP had significantly higher stool IAP activity than the group receiving only “vehicle for IAP.”

GTT. GTT was performed in nonanesthetized mice as described (4). Briefly, mice were fasted for 6 h in the day time, 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 15, 30, 60, 90, and 120 min to measure blood glucose levels. Twenty microliters of blood was collected to assess serum insulin concentration. Glucose tolerance was assessed by calculating the incremental area under the curve (AUC) of each GTT.

ITT. ITT was performed in nonanesthetized mice as described (4). Briefly, mice were fasted for 6 h in the daytime, fasting blood sugar was measured, and insulin (0.75 units/kg) was administered by an i.p. injection. Blood samples were drawn from the tip of the incised tail at 30, 60, 90, and 120 min to measure blood glucose levels. Insulin tolerance was assessed by calculating the incremental area under the curve (AUC) of each ITT. The homeostasis model assessment of insulin resistance [HOMA-IR = (fasting insulin μU/L) × (fasting glucose mg/dL)/405] was calculated as an insulin sensitivity index (5).

Liver Histopathology and Hepatic Steatosis. Frozen liver samples were stained with Oil Red and examined by an independent pathologist masked to treatment assignment. Hepatic steatosis was graded based on the number and size of stained fat droplets: 0 (none/minimal); 1+ (mild); 2+ (moderate); and 4+ (marked).

Hepatic Triglycerides Measurement. Hepatic total lipids and triglycerides (TG) were measured as described (6). Briefly, ~300 mg of liver tissue was mixed with 350 μL of chlorform:methanol solution (2:1, vol:vol), vortexed well, and incubated at room temperature for 5 min. Additional chlorform:methanol solution was then added to bring the volume to 1 mL, which was then vigorously homogenized for 3 min and then centrifuged at 2,000 × g for 10 min and then the supernatant organic layer containing chloroform and lipids was collected in a pre-weighted empty tube. Chloroform was allowed to evaporate and weight of the remaining total lipids was measured. The lipid content was expressed as milligrams of lipid per gram of liver tissue (mean ± SEM). Next, the extracted lipid was mixed with 11.2 mL of 50% (vol:vol) methanol, and 0.2 mL of this solution was moved to a new tube containing 0.215 mL 1 M MgCl2. The mixture was vortexed and then centrifuged for 5 min. The resulting supernatant was used to quantify TG based upon lipoprotein lipase-mediated release of glycerol in a colorimetric assay using a quinoneminine dye (Sigma). After 10-min incubation at 30 °C, the samples were read at 540 nm.

Limulus Amebocyte Lysate Assay. Serum and cecal LPS concentrations were measured with a commercial kit (GenScript) following the manufacturer’s instructions. Briefly, samples were diluted 1/10–1/500 with endotoxin-free water, adjusted to recommended pH, and heated for 10 min at 70 °C to minimize inhibition or enhancement by contaminating proteins. Limulus amebocyte lysate assay (LAL) reagents were added to a sample (in duplicate) and incubated at 37 °C for 45 min, and the absorbance was read at 545 nm. All samples were validated for the recovery and internal coefficient variation using known amounts of LPS.

Determination of Serum Insulin Concentration. Serum insulin was determined using an ELISA kit (Crystal Chem) as per manufacturer’s recommendations.

Lipid Profiles and Liver Function Tests. Serum total cholesterol (TC), triglycerides (TG), and alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyltransferase (GGT) were measured by the Clinical Pathology Laboratory at the Center for Comparative Medicine, MGH, Boston. Serum high-density lipoprotein cholesterol (HDL-C) was enzymatically determined using a kit from Pointe Scientific following the manufacturer’s instructions. The concentration of low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation: LDL cholesterol = total cholesterol – HDL cholesterol – TG/5 (7).

Cytokine Assays. TNF-α and IL-1β were determined in serum and cell culture supernatant by ELISA kits following the manufacturer’s instructions (eBioscience).

Intestinal Alkaline Phosphatase Assay. The intestinal alkaline phosphatase (IAP) assay has been previously described (3). Briefly, an individual stool sample or a thoroughly washed proximal intestinal segment (duodenum) was homogenized in water (10 mg/mL) or lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Nonidet P-40, 10 mM EDTA, 0.1% SDS, including protease inhibitor mixture; Sigma), respectively, followed by incubation on ice for 30 min. Thereafter, the homogenates were centrifuged twice at 4 °C at 15,000 × g for 15 min, and the supernatants were collected to determine IAP activity as well as protein concentration. The Coomassie Blue Protein Assay (Bradford) kit from Fisher Scientific was used for protein quantitation. For IAP assay, 25 μL of supernatant was mixed with 175 μL phosphatase assay reagent containing 5 mM of p-nitrophenyl phosphate (pNPP) followed by determining optical density at 405 nm. The specific activity of the enzyme is expressed as picomoles pNPP hydrolyzed/min/μg of protein. Protein concentration in a specific sample was determined using the protein assay reagents from Fisher Scientific.

Analysis of Proinflammatory Effects of Cecal Contents on Mouse Leukemic Monocyte Macrophage RAW264.7 Cells. After separation of the cecum, the contents were collected in endotoxin-free centrifuge tubes containing 200 μL of endotoxin-free water. Each tube was weighed before and after adding the cecal contents. The sample was diluted to 10 mg/mL with endotoxin-free water, and centrifuged at 500 × g for 15 min to remove debris. The supernatant was then centrifuged for another 30 min at 13,000 × g to obtain a clear supernatant, which was then used for LPS measurement and tissue culture studies.
Mouse leukaemic monocyte macrophage RAW264.7 cells (ATCC) were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1% antibiotic-antimycotic solution (Cellgro). RAW264.7 cells were plated at a concentration of $2 \times 10^6$ cells/mL and starved for 8 h in DMEM with 0.5% FBS. Cells were then treated with 100 μL cecal supernatant. After overnight incubation, media were collected and assayed for TNF-α concentration.

**Intestinal Permeability Measured in Vivo.** Mice were fasted overnight with free access to water. In the morning, they were gavaged with a phosphate buffer saline (PBS, pH 7.2) containing FITC-dextran (70 kDa) (Sigma) at a dose of 300 mg/kg body weight. Ninety minutes later, 120 μL blood was collected from the facial vein and serum was used for the analysis of FITC-dextran concentration. Serum was diluted with an equal volume of PBS, and fluorescence intensity was measured by using a fluorospectrophotometer (Perkin-Elmer) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Serum FITC-dextran concentration was calculated from a standard curve generated by serial dilution of FITC-dextran in PBS.

**Bacterial Culture.** Standard laboratory procedures were used to grow aerobic and anaerobic bacteria (3). Individual mouse stool samples were collected in cold brain heart infusion (BHI) media and then homogenized, and if required, serial dilutions were made. Aerobic bacteria were grown overnight on BHI plates in a 37 °C incubator at ambient air. Anaerobic bacteria were plated on Brucella agar plate containing 5% horse serum (Fisher Scientific) and grown in an anaerobic bag (Fisher Scientific) in a 37 °C incubator for 72 h.

**Quantitative Real-Time PCR.** The proximal part of the small intestine was isolated, flushed with ice cold PBS, and the mesentery was trimmed off. The tissue was then processed with TRIzol (Invitrogen) to isolate total RNA. Quantitative real-time PCR (qRT-PCR) was performed with a Mastercycler realplex instrument (Eppendorf) with a two-step RT-PCR kit (Invitrogen). The following primers were used for amplifying mouse IAP (Akp3) mRNA: forward 5′-CAT GGA CCG CTT CCC ATA-3′ and reverse 5′-CTT GCA CTG TCT GGA ACC TG-3′ (product = 72 bp). The mRNA for β-actin was used as a housekeeping gene for normalization. The β-actin primers were: forward 5′-AGC GAG CAT CCC CCA AAG TT-3′ and reverse 5′-GGG CAC GGA GGC TCA TCA TT-3′ (product = 285 bp). Expression levels were calculated using the ΔΔCt method after correcting for differences in PCR efficiencies and expressed relative to control levels.

**Statistical Analysis.** Data were expressed as mean ± SE (SEM) and were analyzed using unpaired two tailed Student t test. Statistical differences between more than two test groups were evaluated by one-way analysis of variance with Tukey’s multiple comparison posttests. A significant difference was considered when $P < 0.05$. Statistical analyses were performed using SPSS software (version 20 for Mac OS).

Fig. S1. IAP prevents endotoxemia. See Fig. 1 for description of animals. (A) Serum IL-1β levels. (B) Area under curve (AUC) for glucose tolerance test (GTT) described in Fig. 1E. (C) AUC for insulin tolerance test (ITT) described in Fig. 1G. (D) Intraabdominal fat. (E) AUC for GTT (Fig. 1J). (F) Serum endotoxin levels. (G) Energy intake. (H) Body weight gain. (I) Blood glucose levels during GTT. (J) AUC for GTT described in Fig. S1J. Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student’s t test. For multiple comparisons, analysis of variance with Tukey was used. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. S2. IAP prevents chronic HFD-induced glucose intolerance and insulin resistance. See Fig. 2 for description of animals. (A) AUC for GTT described in Fig. 2A. (B) AUC for ITT described in Fig. 2B. (C) Body weight. (D) Energy intake. (E) Feed efficiency. (F) GTT after 6 wk only. (G) AUC for GTT described in Fig. S2F. (H) Serum insulin levels during GTT after 6 wk only. (I) AUC for insulin levels described in Fig. S2H. Statistics: data expressed as mean ± SEM. Analysis of variance with Tukey. * or *P < 0.05; ** or **P < 0.01; ***P < 0.001. The asterisk (*) refers to the LF vs. HF comparison and the number sign (#) refers to the HF vs. HF+IAP comparison.

Fig. S3. IAP prevents HFD-induced liver injury. Liver weight (for the description of animals see SI Materials and Methods and also Fig. 2 and Fig. S2). Statistics: data expressed as mean ± SEM. Analysis of variance with Tukey. *P < 0.05.
Fig. S4. IAP prevents HFD-induced dyslipidemia and endotoxemia. For the description of animals see SI Materials and Methods and also Fig. 2 and Fig. S2. (A) Ratio between serum total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels at 11 wk. (B) Ratio between serum triglycerides (TG) and HDL-C levels. (C) Atherogenic index of serum. (D) Serum very low-density lipoprotein cholesterol (VLDL-C) levels. (E) Non-HDL cholesterol. (F) Ratio between non-HDL-C and HDL-C levels. (G) Cecal endotoxin levels. (H) Serum endotoxin levels. (I) Serum interleukin-1beta (IL-1β) levels. (J) Tumor necrosis factor-alpha (TNF-α) levels in conditioned media (RAW264.7 murine macrophage cells treated with cecal content). (K) Relative expression of Akp3 mRNA (after 10 d of an HFD ± IAP). (L) Duodenal endogenous IAP activity (after 10 d of an HFD ± IAP). (M) Duodenal endogenous IAP activity (after 16 wk of an HFD ± IAP). (N) Number of aerobic bacteria in stool after 3 wk of oral antibiotic treatment [ampicillin (1 g/L) plus norfloxacine (1 g/L)]. (O) Number of anaerobic bacteria in stool after 3 wk of oral antibiotics treatment [ampicillin (1 g/L) plus norfloxacine (1 g/L)]. (P) Energy intake after 4 wk of HFD (preceded by 3 wk of antibiotic treatment). (Q) GTT AUC after 4 wk of HFD (preceded by 3 wk of antibiotic treatment). Statistics: data expressed as mean ± SEM. Analysis of variance with Tukey. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. S5. IAP cures high-fat diet-induced metabolic syndrome. For the description of animals see SI Materials and Methods and also Fig. S5. Groups of 5-wk-old WT male C57BL/6 mice (n = 5 for each group) were fed HFD (45% kcal from fat) for 14 wk that precipitated metabolic syndrome (Fig. S5 A–D). Mice with metabolic syndrome were then treated with or without IAP (100 units/mL drinking water) for 6 wk. (A) Blood glucose levels during GTT (pretreatment). (B) AUC for GTT described in Fig. S5A. (C) Blood glucose levels during ITT (pretreatment). (D) AUC for ITT described in Fig. S5C. (E) AUC for GTT described in Fig. 5A (posttreatment). (F) AUC for ITT described in Fig. 5D (posttreatment). (G) Insulin resistance index (posttreatment). (H) Energy intake (during 6-wk treatment period). (I) Body weight (during 6-wk treatment period). Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. S6. Low-fat diet supplemented with IAP improves lipid profile. For the description of animals see SI Materials and Methods and also Fig. 6. Groups of 5-wk-old WT female C57BL/6 mice (n = 5 for each group) were fed LFD (14% kcal from fat) ± IAP (100 units/mL drinking water) for 7 wk. (A) Ratio between TC and HDL-C levels at 7 wk. (B) Ratio between TG and HDL-C levels at 7 wk. (C) Atherogenic index of serum at 7 wk. (D) Serum VLDL-C levels at 7 wk. (E) Non-HDL cholesterol at 7 wk. (F) Ratio between non-HDL-C and HDL-C levels at 7 wk. Statistics: data expressed as mean ± SEM. Two-tailed unpaired Student’s t-test. *P < 0.05; **P < 0.01.