

Rapid and Weight-Independent Improvement of Glucose Tolerance Induced by a Peptide Designed to Elicit Apoptosis in Adipose Tissue Endothelium

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A peptide designed to induce apoptosis of endothelium in white adipose tissue (WAT) decreases adiposity. The goal of this work is to determine whether targeting of WAT endothelium results in impaired glucose regulation as a result of impaired WAT function. Glucose tolerance tests were performed on days 2 and 3 of treatment with vehicle (HF-V) or proapoptotic peptide (HF-PP) and mice pair-fed to HF-PP (HF-PF) in obese mice on a high-fat diet (HFD). Serum metabolic variables, including lipid profile, adipokines, individual fatty acids, and acylcarnitines, were measured. Microarray analysis was performed in epididymal fat of lean or obese mice treated with vehicle or proapoptotic peptide (PP). PP rapidly and potently improved glucose tolerance of obese mice in a weight- and food intake-independent manner. Serum insulin and triglycerides were decreased in HF-PP relative to HF-V. Levels of fatty acids and acylcarnitines were distinctive in HF-PP compared with HF-V or HF-PF. Microarray analysis in AT revealed that pathways involved in mitochondrial dysfunction, oxidative phosphorylation, and branched-chain amino acid degradation were changed by exposure to HFD and were reversed by PP administration. These studies suggest a novel role of the AT vasculature in glucose homeostasis and lipid metabolism. *Diabetes* 61:2299–2310, 2012

The increased prevalence of obesity is accompanied by comorbidities such as diabetes and cardiovascular disease (1). Given that sustained weight loss is difficult (2,3), it is crucial that new weight-loss therapies be developed. One proposed strategy is to starve adipose tissue (AT) by inhibiting angiogenesis (4–7). White AT (WAT) is highly vascularized, and AT expansion is highly dependent on its vasculature (6,8). Using phage display, a group of investigators identified a peptide sequence that bound uniquely to the endothelium of WAT. When this homing sequence was coupled to a sequence that produces apoptosis, the result was profound weight

loss (5), which we found was primarily the result of anorexia that was not the result of visceral illness (7).

Despite the impressive weight loss resulting from this proapoptotic peptide (PP) treatment, interfering with WAT function may have deleterious effects on other aspects of metabolic regulation. For example, reduced fat mass in the context of lipodystrophy causes severe insulin resistance due to ectopic fat accumulation in liver and skeletal muscle (9,10). Similarly, when adipocytes are targeted for apoptosis, the result is a rapid impairment in glucose tolerance (11).

The primary goal of these studies is to directly examine the effect of the PP on key aspects of metabolic regulation. Given that even small weight loss can greatly improve glucose homeostasis, we examined the effects of PP after relatively short-time periods and compared with mice that were pair-fed to the reduced food intake of PP-treated mice. In addition, we sought to identify potential mechanisms underlying changes in glucose regulation by examining changes in WAT morphology, gene expression, and circulating metabolites.

RESEARCH DESIGN AND METHODS

Animals and treatment. Male C57Bl/6 mice purchased from The Jackson Laboratory at 8 weeks of age were housed in single cages with a 12-h light/dark cycle. For glucose and insulin tolerance tests, three cohorts of comparable C57Bl/6 mice were prepared by being fed a high-fat diet (HFD) with 40% calories as fat (D03082706 or D012451; Research Diet) or a low-fat diet 4% (LFD; D03082705; Research Diet) for 3 or 4 months. Obese mice were treated with either 3 mg/kg of the PP [CKGGRKDC-GG- γ (KLAKLAK)₂] or vehicle (HF-V or mice pair-fed to HF-PP [HF-PF]) subcutaneously daily before dark for 2 or 3 days. For the longer-term study, obese mice were administered either PP in 0.5% DMSO/saline or the same dose of the control peptide (CKGGRKDC), and lean mice were injected with 0.5% DMSO/saline (LF-V) for 27 days after 8 weeks on the HFD (D03082706; Research Diet) or LFD (5,7). All animal protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Lipid profiling. Total nonesterified fatty acids (NEFA), phospholipids, total ketone bodies, and β -OH-butyrate were measured using the kits from Wako. Cholesterol was measured using infinity total cholesterol kit (Fisher Diagnostics). Triglyceride was measured using RANDOX kit (RANDOX Laboratories Ltd.).

Metabolomics. Individual NEFAs were measured by a targeted gas chromatography/mass spectrometry approach (12–14). Serum acylcarnitines and amino acids were analyzed by tandem mass spectrometry as described previously (13,14).

Plasma hormone measurements. Glucose was measured by a glucose analyzer or glucose strips. Insulin was measured by a radioimmunoassay kit (Linco). Adiponectin was determined using a mouse/rat adiponectin ELISA kit (B-Bridge International). Resistin was measured using a mouse resistin ELISA kit (Millipore).

Microarray analysis. A total of 20 ng of total RNA was isolated from epididymal fat and hybridized to Affymetrix Mouse Genome 430 2.0 microarrays (Affymetrix) (7). Analysis was performed using R statistical software and the limma Bioconductor package (15). All steps of data preprocessing were performed using robust multiarray analysis. Estimated fold changes were calculated using ANOVA, and resulting *t*-statistics from each comparison were modified using an intensity-based empirical Bayes method (16). We

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identified canonical pathways enriched for differentially expressed genes in the comparisons using the Ingenuity Pathway Analysis software (Ingenuity Systems).

WAT gene expression and morphometry. Total RNA was isolated, and cDNA was synthesized using iScript (7). The expression of adipose genes was measured using either iCycler (Bio-Rad) or 7900HT Fast Real-Time PCR

system (Applied Biosystems) with primers (Supplementary Table 1). Ribosomal protein L32 was used as an endogenous control. Determination of adipocyte size, distribution of adipocyte size, and the number of crown-like structures was modified from previous studies (17).

Glucose and insulin tolerance tests. Mice on an LFD or HFD were given 1.5 or 2 mg/g intraperitoneally of glucose for glucose tolerance tests after an

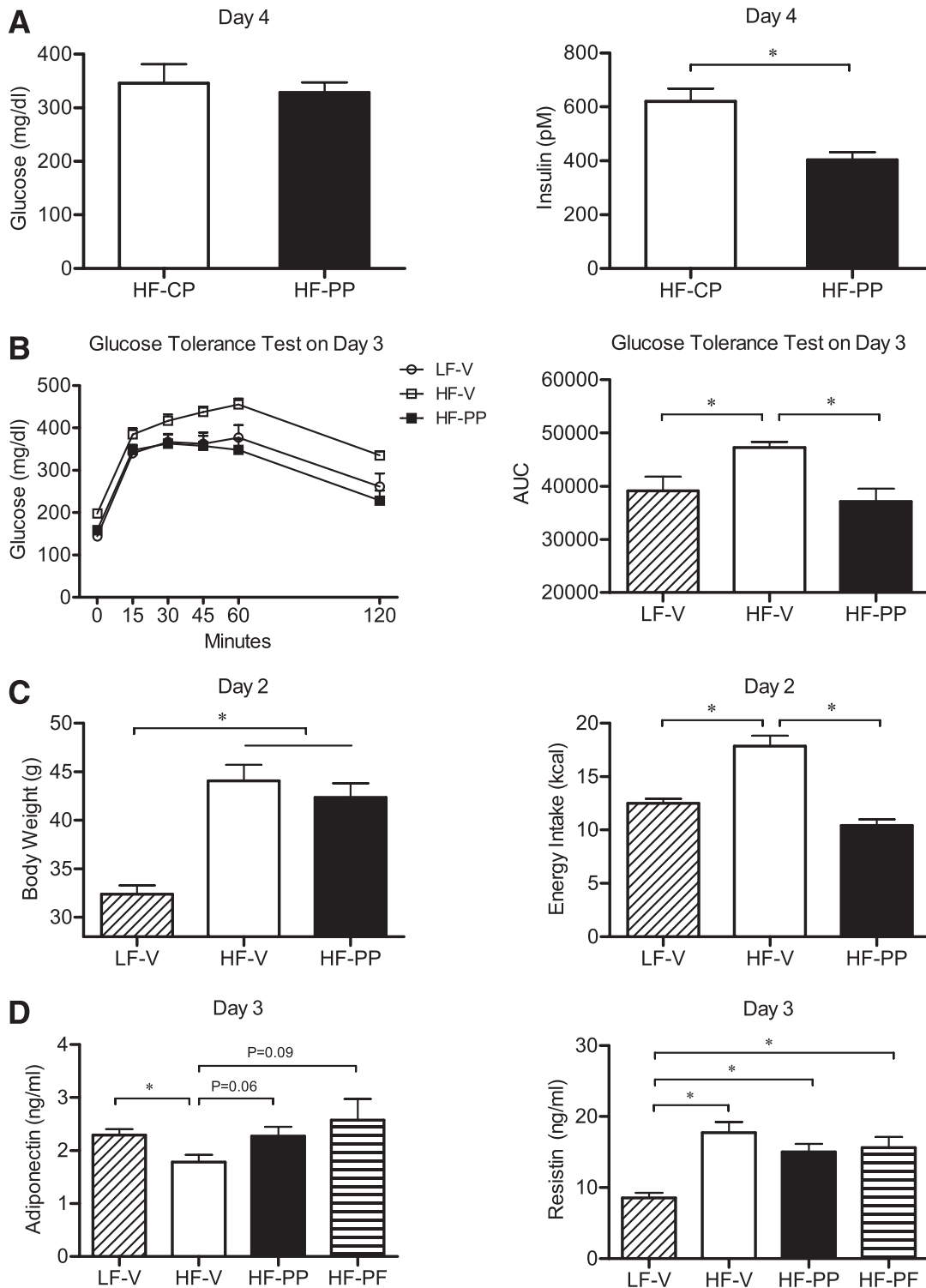


FIG. 1. Effect of PP administration on glucose tolerance. **A:** Change in serum levels of glucose and insulin in obese C57Bl/6 mice on an HFD after 4 days of treatment with PP or vehicle. **B:** Effect of the PP on glucose tolerance of mice on an HFD. Change of glucose levels after intraperitoneal injection of glucose (1.5 mg/g), AUC of glucose levels on day 3. **C:** Body weight and energy intake on day 2 of treatment in LF-V, HF-V, and HF-PP. Glucose tolerance test was performed on day 3 after an overnight fast. **D:** Effect of the PP on adiponectin and resistin levels in obese C57Bl/6 mice on an HFD in a fast state after 3 days of treatment. All data are represented as mean \pm SEM. LF-V, HF-V, HF-CP, HF-PP, and HF-PF, $n = 8$. * $P < 0.05$.

overnight fast. HF-PP were given the same amount of an HFD every 12 h. For the insulin tolerance test, the mice on an LFD or HFD were given 1 mU/g i.p. of bovine insulin (Novolin R) after a 3-h fast.

Statistics. All data are expressed as mean \pm SEM. Data from two groups or three groups were analyzed by Student *t* test or ANOVA followed by Tukey post hoc test.

RESULTS

PP and glucose tolerance. To determine whether the PP rapidly affects glucose homeostasis in obese mice on an HFD, we measured serum glucose and insulin levels after a 4-h fast on day 4. Although the glucose level was not changed by the PP, HF-PP had lower insulin levels relative to control peptide-injected mice on an HFD (HF-CP) (Fig. 1A). To confirm the rapid change of glucose homeostasis, a glucose tolerance test was performed on day 3 after an overnight fast in LF-V, HF-V, and HF-PP. The PP potently improved glucose tolerance in obese mice, whereas there was no difference in body weight (Fig. 1B and C).

In previous studies, long-term treatment with angiogenesis inhibitors improved glucose tolerance (4,5). However, those studies did not discriminate whether the effect of inhibiting angiogenesis on glucose tolerance was secondary to weight loss. To directly address this, we ran the additional control group of mice on an HFD pair-fed to energy intake of HF-PP (HF-PF) every 12 h to better match the pattern of intake between groups in a separate experiment. In this experiment, mice were less obese than in the previous experiment (43.6 ± 1.1 vs. 32.5 ± 1.0 g). In a glucose tolerance test performed on day 3, the area under the curve (AUC) of glucose levels of both HF-CP and HF-PF was greater than that of LF-V ($P < 0.05$), whereas the AUC of HF-PP was not different from that of LF-V. The AUC of HF-PP was significantly reduced relative to HF-CP, and there was a strong trend toward a decrease in HF-PP compared with HF-PF ($P = 0.06$). The body weight of HF-PP was comparable to that of HF-PF and HF-CP. Glucose levels were significantly higher in HF-CP and HF-PF relative to LF-V throughout the test after injection of glucose; however, the absolute glucose level was significantly different only at 60 min between HF-PP and LF-V. On day 10, an insulin tolerance test revealed a similar pattern of improvement as seen in the glucose tolerance test. HF-PP had a reduced AUC for glucose following insulin treatment after a 4-h fast compared with HF-CP. Additionally, there was a trend toward improvement compared with HF-PF. During the insulin tolerance test, the

overall slope of HF-PP was significantly steeper relative to that of both HF-CP and HF-PF, whereas there was no difference between HF-CP and HF-PF, indicating increased glucose removal independent of body weight and/or food intake (Supplementary Fig. 1).

To clarify the direct effect of the PP on glucose tolerance, we prepared another cohort of obese mice (initial body weight: 43.2 ± 0.8 g) for the comparison of glucose tolerance between HF-PP and HF-PF. Both HF-PP and HF-PF were fed the same amount of the HFD on days 1 and 2 every 12 h, and a glucose tolerance test was performed on day 2 before change of food intake occurred. On day 2, there was no significant difference in body weight or change of body weight. The AUC of HF-PP was significantly smaller than that of HF-PF, and the absolute glucose levels of HF-PP were significantly lower at 45, 60, and 120 min (Fig. 2). These data strongly support the idea that the PP improves glucose tolerance in obese mice by a mechanism that is independent of the loss of body weight or anorexia.

PP and metabolic parameters. To identify potential mechanisms for the improved glucose tolerance, we measured circulating levels of key adipokines linked to glucose regulation. Serum adiponectin levels were not significantly different in the fed state in HF-V and HF-PP on day 3 (Supplementary Fig. 2) (7). Adiponectin levels in both groups were significantly lower relative to those in LF-V. However, serum adiponectin levels in HF-V tended to increase in the fasted state of HF-PP and HF-PF (Fig. 1D). Serum resistin levels were slightly decreased ($P = 0.08$) on day 3 in the fed state of HF-PP relative to HF-V (Supplementary Fig. 2), consistent with the significant decrease on day 4 in a previous study (7). However, there was no difference in serum resistin level between HF-PP and HF-PF in the fasted state (Fig. 1D). Given the beneficial effect of adiponectin in glucose tolerance (18) and the detrimental effect of resistin (19,20), the lack of change in serum adiponectin and resistin induced by PP relative to HF-PF strongly suggests that they are not the key mediators for the potent and rapid effect of the PP to improve glucose homeostasis.

The effect of the PP on metabolites. In addition to measuring the effect on glucose regulation, we also examined changes in various serum lipid parameters after PP administration. Given the rapid and robust reduction in WAT, it was possible that we would observe increases in serum lipids. However, serum triglycerides were

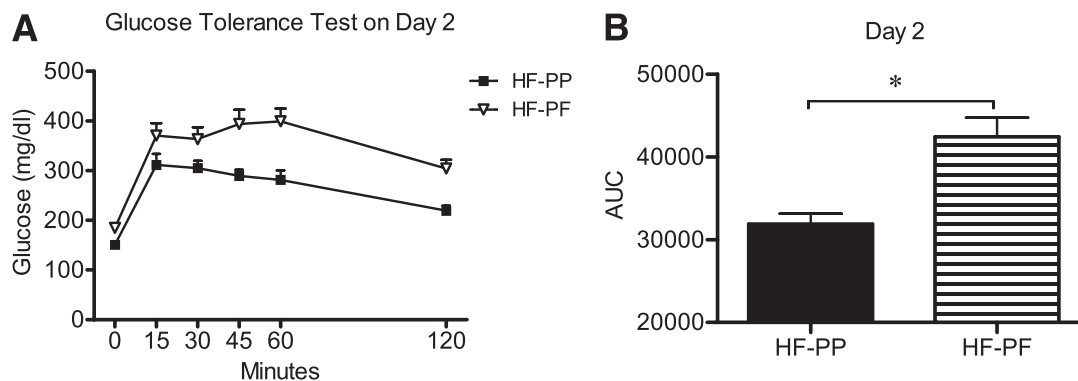


FIG. 2. Rapid improvement of glucose tolerance induced by PP. Glucose tolerance test was performed on day 2 after an overnight fast with obese C57Bl/6 mice on an HFD. Change of glucose levels after intraperitoneal injection of glucose (2 mg/g) (A), and AUC of glucose levels (B) on day 2. Body weight and food intake were similar between HF-PP and HF-PF on day 1. HF-PP and HF-PF, $n = 8$. * $P < 0.05$.

significantly lower in HF-PP in the fed state relative to HF-V on day 3. Other lipids such as NEFA, cholesterol, and phospholipid were not significantly different in either the fasted or fed states of these mice (Fig. 3A and Supplementary Fig. 3). These data suggest that the rapid and robust reduction of WAT due to inhibition of angiogenesis does not produce an increase in serum NEFA or triglycerides, which may subsequently cause hepatic steatosis or lipid accumulation in muscle as observed in lipodystrophy models (10,11,21,22).

Total ketones and β -OH-butyrate were not different between HF-V and HF-PP, although they were significantly higher in HF-PP in a fed state than in HF-V (Fig. 3B). Thus, ketogenesis was not increased in HF-PP as much as in HF-PF when food intake of both mice was restricted to the same degree. These data are consistent with the lack of change in serum NEFA in HF-PP compared with HF-V, given that ketogenesis in the liver is dependent on increases in circulating free fatty acids released from WAT. The difference in ketogenesis among groups was not evident after fasting (Fig. 3B).

A variety of data link specific fatty acids such as C16:1n7-palmitoleate to the regulation of glucose homeostasis (23). Thus, we sought to describe changes in circulating individual fatty acids that might mediate the metabolic improvements caused by PP. We found the blunted increase of serum NEFAs and some individual NEFAs, such as C16:0, C18:1, and C18:0 in obese mice in response to overnight fasting or pair-feeding that is often referred to as metabolic inflexibility (Supplementary Fig. 4) (13,24). There was a significant decrease in individual NEFAs in HF-PP including C14:0, C16:1, C18:2, C18:3, and C18:1 relative to HF-V. Interestingly, there was a significant decrease in serum levels of C14:0 and C16:1 only in HF-PP, whereas there was no difference between HF-V and HF-PF. Additionally, C18:0 was not decreased even though it was greatly reduced in HF-PF. These data indicate that there are unique metabolic changes in NEFA metabolism caused by targeting WAT vasculature that are not secondary to the changes in intake and weight (Fig. 3C).

Serum acylcarnitines provide a window on mitochondrial metabolism of metabolic fuels, particularly fatty acids

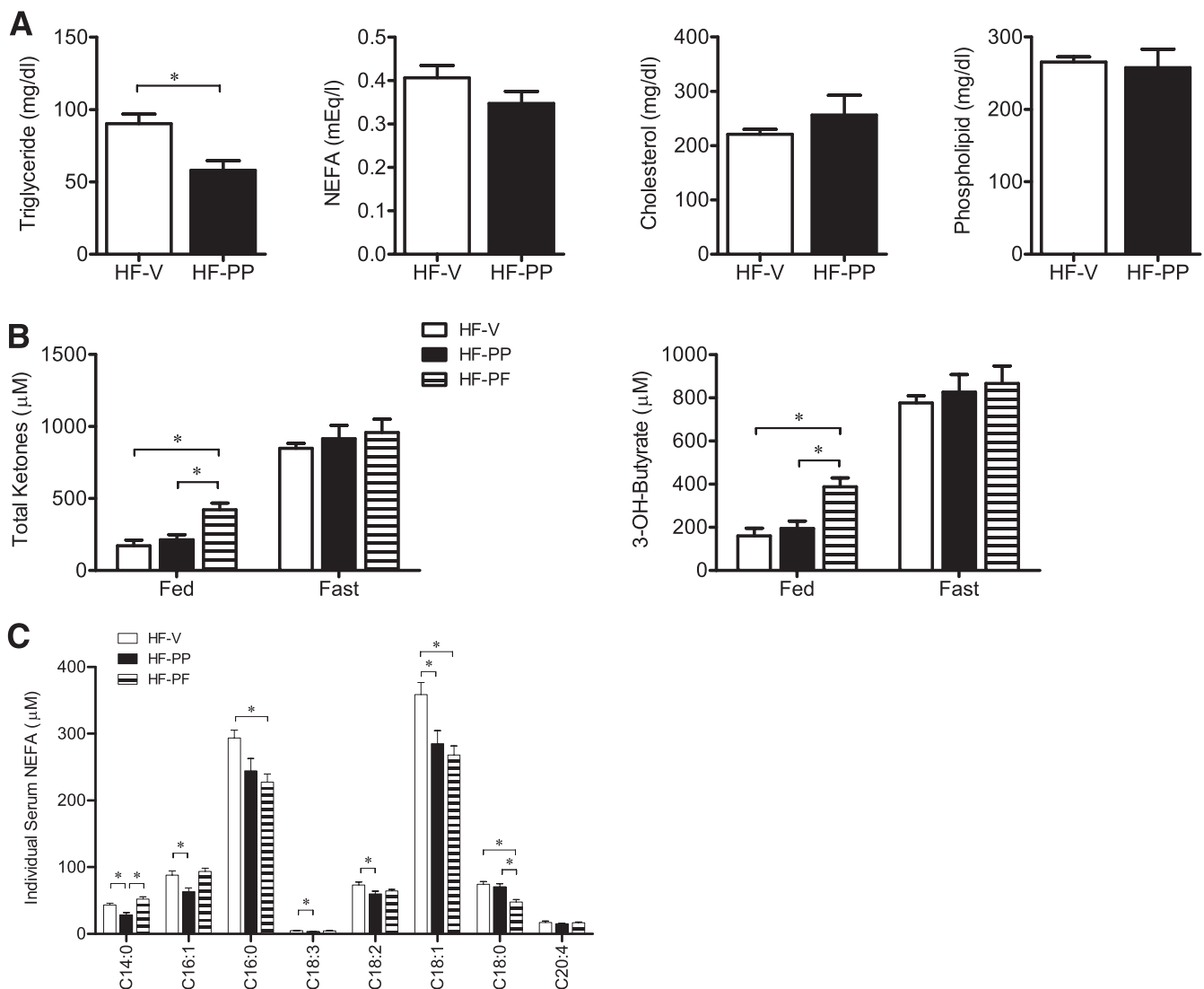


FIG. 3. Effect of PP on serum lipid profiles in obese C57B/6 mice on an HFD after 3 days of treatment. **A:** Serum triglyceride, NEFA, cholesterol, and phospholipid in a fed state. **B:** Serum total ketones and 3-OH-butyrate in a fed or fast state. **C:** Serum individual NEFA profile in a fed state. * $P < 0.05$. HF-V, HF-PP, and HF-PF, $n = 8$. C14:0, myristic acid; C16:1, palmitoleic acid; C16:0, palmitic acid; C18:3, α -linolenic acid; C18:2, linoleic acid; C18:1, oleic acid; C18:0, stearic acid; C20:4, arachidonic acid.

and amino acids (13). Multiple even-chain acylcarnitines derived from β -oxidation of fatty acid increased in the fasted state in HF-V relative to the fed state, whereas those derived principally from amino acid catabolism such as C3 and C5 acylcarnitines decreased, consistent with a previous study (13). Similar changes were observed in C2, C3, C4, and C5 acylcarnitines in the fed state in HF-V versus HF-PF, given that the restriction of intake in HF-PF was similar to an overnight fast on the last day of treatments (Supplementary Fig. 5). PP administration significantly decreased short- or medium-chain acylcarnitines, end products of key catabolic pathways. PP decreased circulating levels of total ketones, β -hydroxybutyrate, and β -hydroxybutyryl carnitine (C4-OH), a ketone-related metabolite in PP-treated animals, all of which serve as indices of decreased fatty acid oxidation. Several medium chain acylcarnitines of even carbon numbers (C8, C10, and C12), which are generated during the process of β -oxidation, are also lowered by PP treatment. Similarly, C3 and C5 acylcarnitines, primarily derived from oxidation of branched

chain and other amino acids, are decreased in HF-PP relative to the HF-V. Finally, C2 (acetyl) carnitine, which indicates the mitochondrial pool of acetyl-CoA, a universal end product of fatty acid, amino acid, and glucose oxidation, is also down in the HF-PP compared with either the HF-V or HF-PF. These data suggest that treatment of obese mice with the PP causes a decrease in mitochondrial oxidation of fatty acids and amino acids. Interestingly, long-chain acylcarnitines such as C18, C18:1, and C20 were increased in the fed state in HF-PP, suggesting that the peptide has directly or indirectly reduced entry of the long-chain species into the β -oxidative pathway (Fig. 4). We propose that PP reduces fatty acid and amino acid oxidation, favoring glucose oxidation for their energetic requirements and thereby improving glucose homeostasis in the HF-PP.

Gene expression changes in metabolic and signaling pathways in WAT induced by long-term treatment with PP. To identify possible changes in the characteristics of WAT resulting from the PP, some of which may contribute to the changes in energy intake or glucose

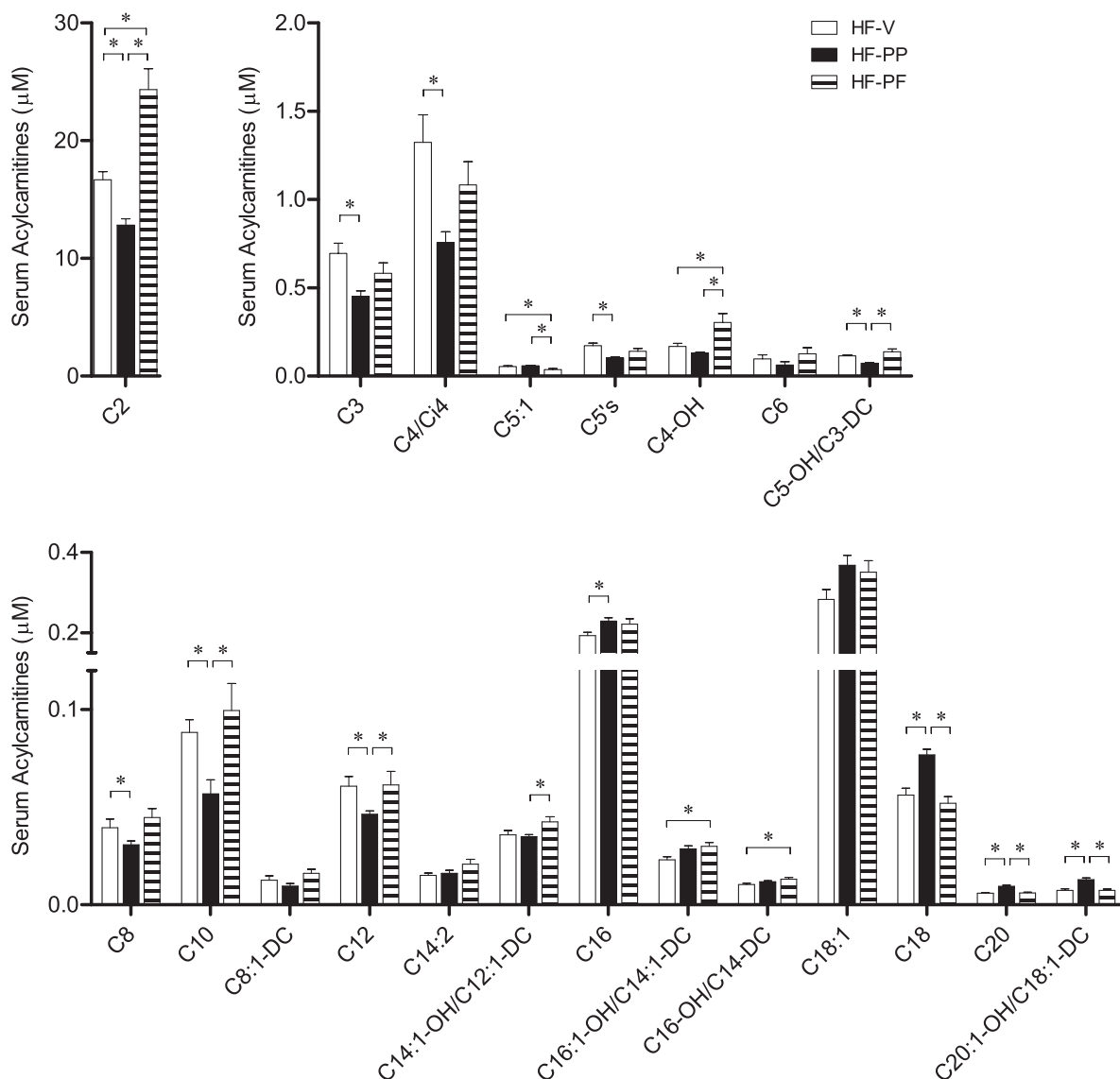


FIG. 4. Effect of PP on serum acylcarnitines in obese C57Bl/6 mice on an HFD in a fed state after 3 days of treatment. A total of 45 acylcarnitines were measured in serum of HF-V, HF-PP, and HF-PF in a fed or fast state by tandem mass spectrometry. * $P < 0.05$. HF-V, HF-PP, and HF-PF, $n = 8$.

tolerance, we performed microarray analysis in WAT of LF-V, HF-CP, and HF-PP after 27 days of treatment. The body weights of HF-PP and LF-V were comparable, whereas HF-CP was clearly heavier at the time of the experiment. Pathways involved in mitochondrial dysfunction, oxidative phosphorylation, branched-chain amino acid (BCAA) degradation, citric acid cycle, and ubiquinone biosynthesis were altered in WAT of HF-PP compared with HF-CP (Table 1). In turn, these same pathways were also modified in HF-CP compared with LF-V, meaning that the genes in these pathways were changed by consumption of the HFD, consistent with previous findings (25–27). Seventy percent of genes in that analysis differentially expressed in HF-CP versus LF-V were also changed in HF-PP versus HF-CP, but the expression pattern for common genes was reversed (96, 86, and 100% of genes, respectively). In other words, the effect of HFD to alter the expression of the majority of the genes in each of these pathways is reversed by the PP even though mice are maintained on HFD (Table 2, Supplementary Table 3, and Fig. 5B and C). Interestingly, proinflammatory cytokine genes such as *Tnf* and *Ccl3* and macrophage markers such as *Emr1* and *Cd68* were not changed in HF-PP compared with HF-CP (28,29). However, HF-PP had a significant decrease in *Ccr2* with a trend toward a decrease in *Ccl2* and *Itgax*, genes important for macrophage recruitment (Supplementary Table 2).

Rapid change of gene expression related to mitochondrial function in WAT induced by PP. Given the improved expression of mitochondrial genes by the long-term treatment with the PP relative to HF-CP, we hypothesized that this enhanced expression of mitochondrial genes in WAT may be involved in the rapid change of glucose tolerance. However, the expression of genes related to mitochondrial complexes was significantly suppressed in both HF-PP and HF-PF relative to HF-V (Fig. 6E), indicating that these genes in WAT are unlikely to be directly involved in the rapid improvement of glucose tolerance.

Acute change in WAT morphology by PP. To investigate how PP rapidly affects WAT morphology, we compared adipocyte size on day 3 among HF-V, HF-PP, and HF-PF. There was no significant difference of adipocytes size among the groups ($P = 0.16$) (Fig. 6B). Interestingly, relatively

TABLE 1
Canonical pathways most affected by the PP in HFD-fed obese mice

Canonical pathway	P value
Mitochondrial dysfunction	2.80E-17
Oxidative phosphorylation	1.01E-14
Ubiquinone biosynthesis	4.18E-10
Valine, leucine, and isoleucine degradation	3.74E-08
Axonal guidance signaling	1.08E-07
Citrate cycle	4.85E-07

Extracted total RNA from epididymal fat of single mouse was applied to each Affymetrix Mouse Genome 430 2.0 microarray chip (Affymetrix). Top list of the pathways was identified by the Ingenuity Systems Pathway Analysis Software (Ingenuity Systems). A total of 45,101 genes were analyzed by using $P < 0.05$ as the cutoff level.

smaller adipocytes ($<4,000 \mu\text{m}^2$) tended to be increased in HF-PP relative to both HF-V and HF-PF (Fig. 6C). However, there was no significant difference of genes expression regarding lipolysis such as *Lipe*, *Pnpla2*, and *Adbr3* (Fig. 6F).

Rapid change of macrophage and inflammation in WAT induced by PP. To determine whether the change in macrophage level and inflammation in WAT contributes to the rapid improvement of glucose tolerance, we compared the macrophages and inflammatory markers in WAT by counting the crown-like structures and measuring gene expression. Interestingly, the crown-like structures and *Cd68* trended to increase in both HF-PP and HF-PF relative to HF-V. *Emr1* and *Itgax* were significantly increased in HF-PP relative to HF-PF (Fig. 6D). The expression of *Ccl2* was significantly decreased in HF-PF relative to HF-V, whereas there was no significant decrease in HF-PP. Proinflammatory cytokines such as tumor necrosis factor- α and *IL-6* were not different among the groups. This indicates that the rapid improvement of glucose tolerance is not associated with the change of WAT macrophages and inflammation.

DISCUSSION

The aim of these studies is to determine whether a peptide designed to cause apoptosis of endothelium only in WAT and therefore selectively inhibits angiogenesis can improve

TABLE 2
Genes altered in their expression by exposure to the HFD and subsequently reversed by the PP

Genes involved in mitochondrial dysfunction					
(-) <i>Aifm1</i>	(+) <i>App</i>	(-) <i>Atp5a1</i>	(-) <i>Atp5b</i>	(-) <i>Atp5c1</i>	(-) <i>Cox6c</i>
(+) <i>Cpt1a</i>	(+) <i>Cyb5r3</i>	(-) <i>Cyc1</i>	(-) <i>Gpd2</i>	(-) <i>Gsr</i>	(-) <i>Hsd17b10</i>
(+) <i>Maoa</i>	(-) <i>Maob</i>	(-) <i>Ndufa10</i>	(-) <i>Ndufb8</i>	(-) <i>Ndufb11</i>	(-) <i>Ndufb10</i>
(-) <i>Ndufs1</i>	(-) <i>Ndufs2</i>	(-) <i>Ndufs3</i>	(-) <i>Park7</i>	(-) <i>Pdha1</i>	(-) <i>Prdx3</i>
(-) <i>Sdha</i>	(-) <i>Sdhb</i>	(-) <i>Txn2</i>	(-) <i>Uqcrc1</i>	(-) <i>Uqcrc2</i>	(-) <i>Uqcrcs1</i>
Genes involved in branched amino acid degradation					
(-) <i>Acad8</i>	(-) <i>Acadvl</i>	(-) <i>Acat1</i>	(-) <i>Aldh2</i>	(+) <i>Bcat1</i>	(-) <i>Bcat2</i>
(-) <i>Bckdhb</i>	(-) <i>Dbt</i>	(-) <i>Echs1</i>	(-) <i>Hadh</i>	(-) <i>Hibadh</i>	(-) <i>Hibch</i>
(+) <i>Hmgcs1</i>	(-) <i>Hsd17b10</i>	(-) <i>Ivd</i>	(-) <i>Mccc1</i>	(-) <i>Mccc2</i>	(-) <i>Mut</i>
(-) <i>Pccb</i>					
Genes involved in citrate cycle					
(-) <i>Aco2</i>	(-) <i>Cs</i>	(-) <i>Fh</i>	(-) <i>Idh3a</i>	(-) <i>Idh3b</i>	(-) <i>Idh3 g</i>
(-) <i>Mdh1</i>	(-) <i>Mdh2</i>	(-) <i>Pck1</i>	(-) <i>Sdha</i>	(-) <i>Sdhb</i>	(-) <i>Suclg2</i>

Canonical pathways enriched for genes affected by HFD (i.e., differentially expressed in HF-CP vs. LF-V) or for genes affected by the PP (i.e., differentially expressed in HF-PP vs. HF-CP) were identified using the Ingenuity Systems Pathway Analysis software (Ingenuity Systems). The expression profiles of the differentially expressed genes in the affected pathways are provided in Supplementary Table 3. Positive or negative indicates increased or decreased level of gene expression in HF-CP vs. LF-V.

glucose tolerance. In previous studies, we showed that the PP greatly decreases body weight and adiposity by reducing food intake without illness (7). Because WAT is affected by the peptide, a potential concern of targeting WAT vasculature is that by undermining the function of WAT, metabolic regulation may actually worsen as occurs in lipodystrophic syndromes (10,11,21).

To the contrary, PP administration reduced insulin levels of animals maintained on the HFD and improved glucose tolerance after only 3 or 4 days compared with both HF-CP and the pair-fed controls at a time when there was no difference of body weight among groups. Furthermore, the improvement of glucose tolerance caused by the PP was more prominent in more obese mice (Fig. 1B and Supplementary Fig. 1A). Moreover, this ability of PP was more obvious in a third group of obese mice compared with a group of mice receiving vehicle but pair-fed to the reduced intake produced by the PP as soon as day 2 (Fig. 2). Given that weight loss is well-documented to improve insulin sensitivity (30–32), the rapid improvement of glucose tolerance observed in HF-PP without change in that of HF-PP after 2 or 3 days of treatment and decreased serum insulin levels suggests the possibility that targeting the vasculature in WAT produces changes that increase systemic insulin sensitivity independent of reduced intake. The key question then becomes, what can account for the rapid and potent effect of the PP to improve glucose regulation? WAT makes various factors known to regulate glucose levels including adiponectin and resistin (18,20). Consistent with the improved glucose tolerance, adiponectin levels are higher in HF-PP in a fasted state on day 3 (Fig. 1D), and resistin levels are lower in HF-PP in a fed state on day 4 (7). The picture that emerges from these data is consistent with the hypothesis that WAT function is improved rather than undermined with PP treatment.

One potential explanation for the beneficial effect of the PP would be to reverse detrimental effects of the HFD on WAT function. Recent evidence indicates that mitochondrial dysfunction occurs in WAT of various obese mice (27,33) and that these can be reversed by rosiglitazone via increased mitochondrial biogenesis and activity in WAT (26). Our microarray data suggest that this occurs during PP treatment as well (Table 2 and Fig. 5B and C) (27,33). Reversal of the suppressed canonical pathways in WAT of obese mice by the PP makes an important point. Causing apoptosis in the WAT vasculature does not result in dysfunctional WAT. Rather, it appears to improve WAT function in a way similar to what occurs with thiazolidinediones (26,34,35) even when adipose mass is reduced. These results support the hypothesis that improved WAT function may contribute to the improved glucose regulation observed with longer-term treatments with PP.

Other aspects of the metabolic changes observed after PP also may contribute to the improved glucose regulation. Plasma levels of BCAAs are elevated in obese humans and rodents (25,36–38), which have been linked to insulin resistance and obesity (12,39–42). Moreover, changes in BCAA metabolism in WAT are thought to contribute to the increased levels seen in obesity (25). Consistent with these findings, our observation that HFD feeding downregulates genes in the BCAA degradation pathway supports this hypothesis. Interestingly, this downregulation is largely reversed by PP (Table 2 and Supplementary Table 2) as also occurs in response to thiazolidinediones (43).

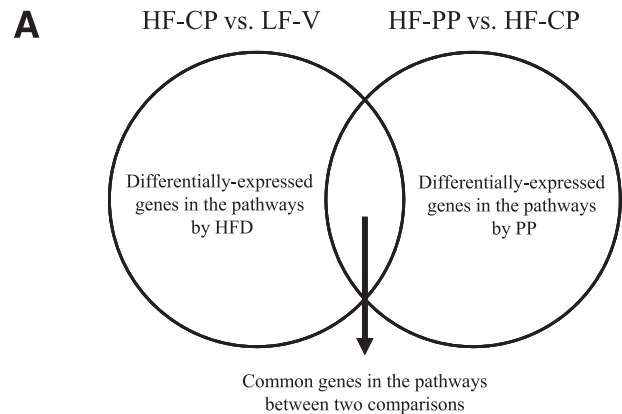
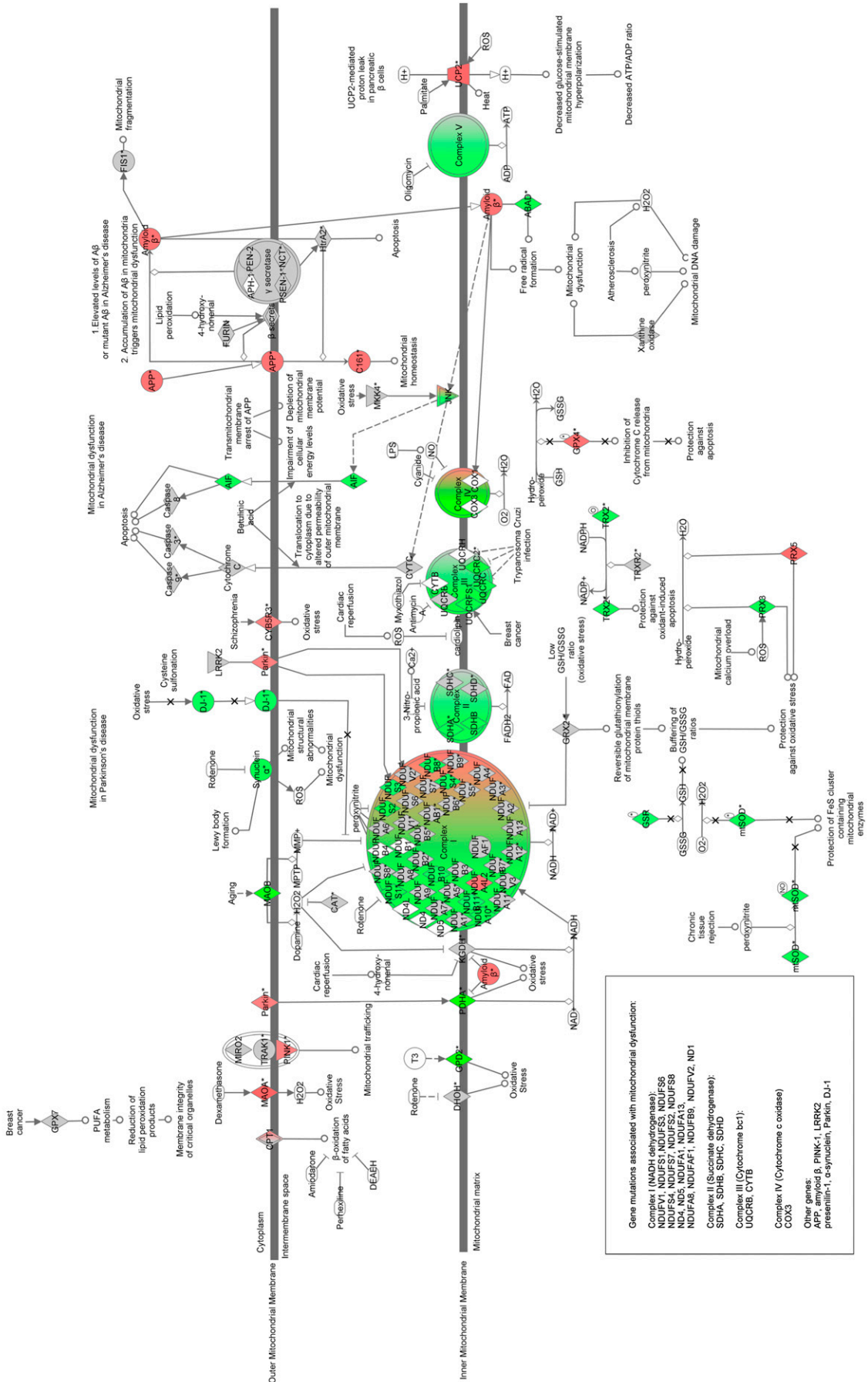


FIG. 5. Representative figures of change in mitochondrial dysfunction pathway after 27 days of treatment with PP. **A:** Illustration showing how we analyzed the microarray data for comparison of gene expression patterns. Canonical pathways in each comparison were identified using Ingenuity Systems Pathway Analysis software (Ingenuity Systems). A total of 45,101 genes were analyzed, and we used a *P* value threshold of 0.05 to identify pathways enriched with differentially expressed genes. The expression profiles of the differentially expressed genes in the pathways in HF-CP versus LF-V were further compared with those in HF-PP versus HF-CP. **B:** Differential expression of genes in mitochondrial dysfunction pathway in AT induced by long-term exposure to an HFD (HF-CP vs. LF-V). **C:** Differential expression of genes in mitochondrial dysfunction pathway in AT changed by the PP (HF-PP vs. HF-CP). Green color indicates downregulation of genes, and red indicates upregulation of genes in the comparison. LF-V, HF-CP, and HF-PP, *n* = 8. (Continued on pp. 2306 and 2307.)

The PP was designed to initiate apoptosis of WAT endothelium, thus potentially compromising the route by which macrophages are recruited (44). Recent evidence suggests that chronic WAT inflammation is related to obesity and that macrophage recruitment in WAT may contribute to insulin resistance (28,29,45). Interestingly, expression of proinflammatory cytokines (tumor necrosis factor- α and macrophage inflammatory protein 1 α) and macrophage markers (*CD68* and *EMRI*) were not significantly changed between HF-PP and HF-CP despite the reduced adiposity and improved glucose tolerance, whereas they were significantly increased in HF-CP compared with LF-V consistent with other previous findings (28,29,46,47). Thus, PP did not reverse these markers of inflammation. However, there was a significant decrease in *CCR2* with a strong trend toward a decrease in monocyte chemoattractant protein 1 and *Itgax* (*CD11c*) in HF-PP compared with HF-CP, indicating a possibility of a phenotypic switch of macrophages (45,48).

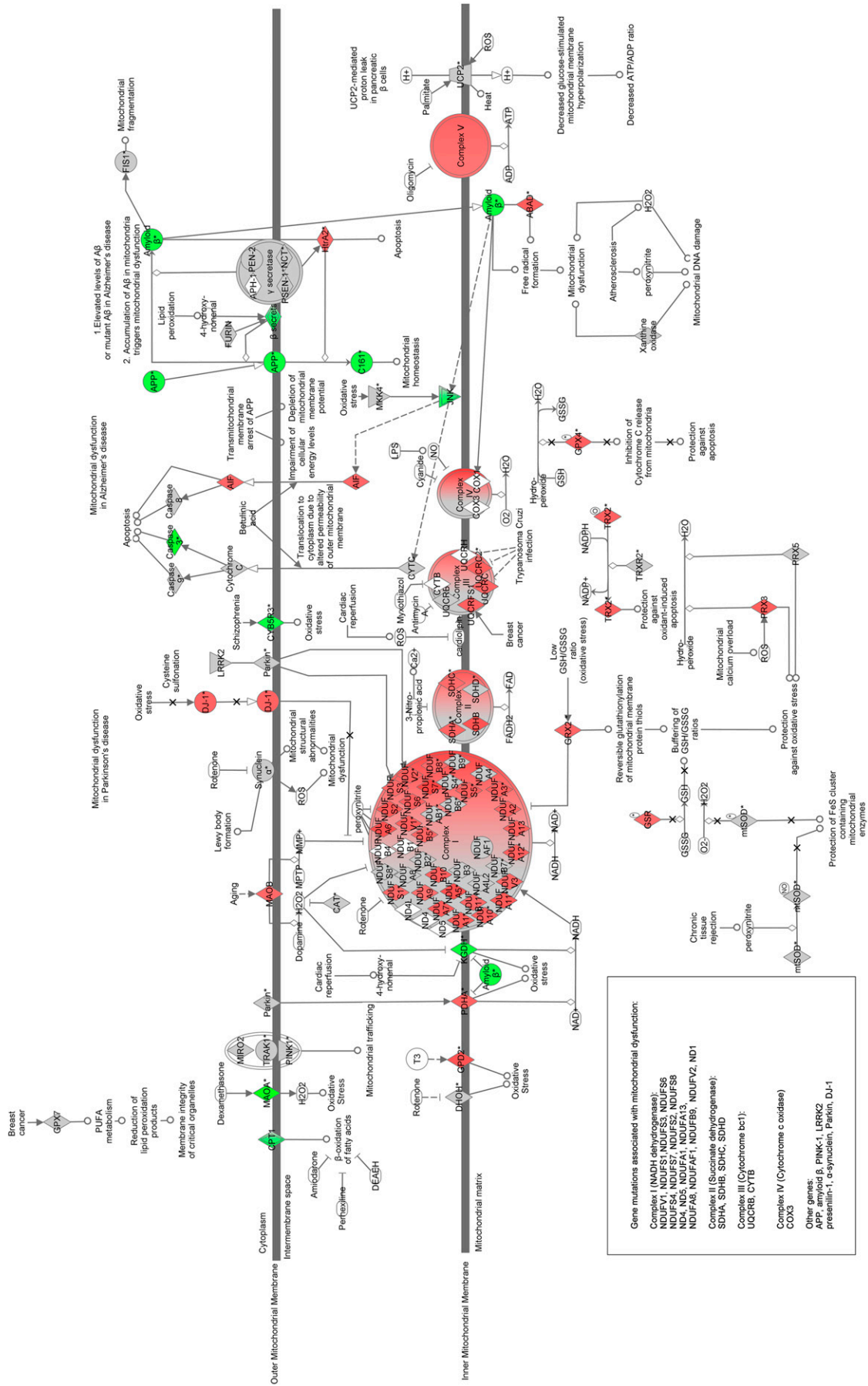
This change of inflammation in WAT after long-term treatment with the peptide was different from that during short-term treatments. After 3 days, there was a trend toward increased macrophages in WAT in both HF-PP and HF-PP. This is consistent with recent reports in which short-term negative energy balance caused an initial increase in macrophage recruitment to WAT even though such manipulations are associated with improved glucose regulation (49). What cannot be ruled out by these data, however, is the possibility of changes in key characteristics of the macrophages without changing their overall numbers (45,49).

The improved glucose tolerance induced by PP extends to plasma lipids as well. Despite the rapid reduction in adiposity, PP decreases triglyceride and some individual fatty acids (Fig. 3A and C). Such data point toward reduced lipotoxicity in HF-PP, and this may also contribute to the improvement in glucose regulation.



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FIG. 5. Continued.



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FIG. 5. Continued.

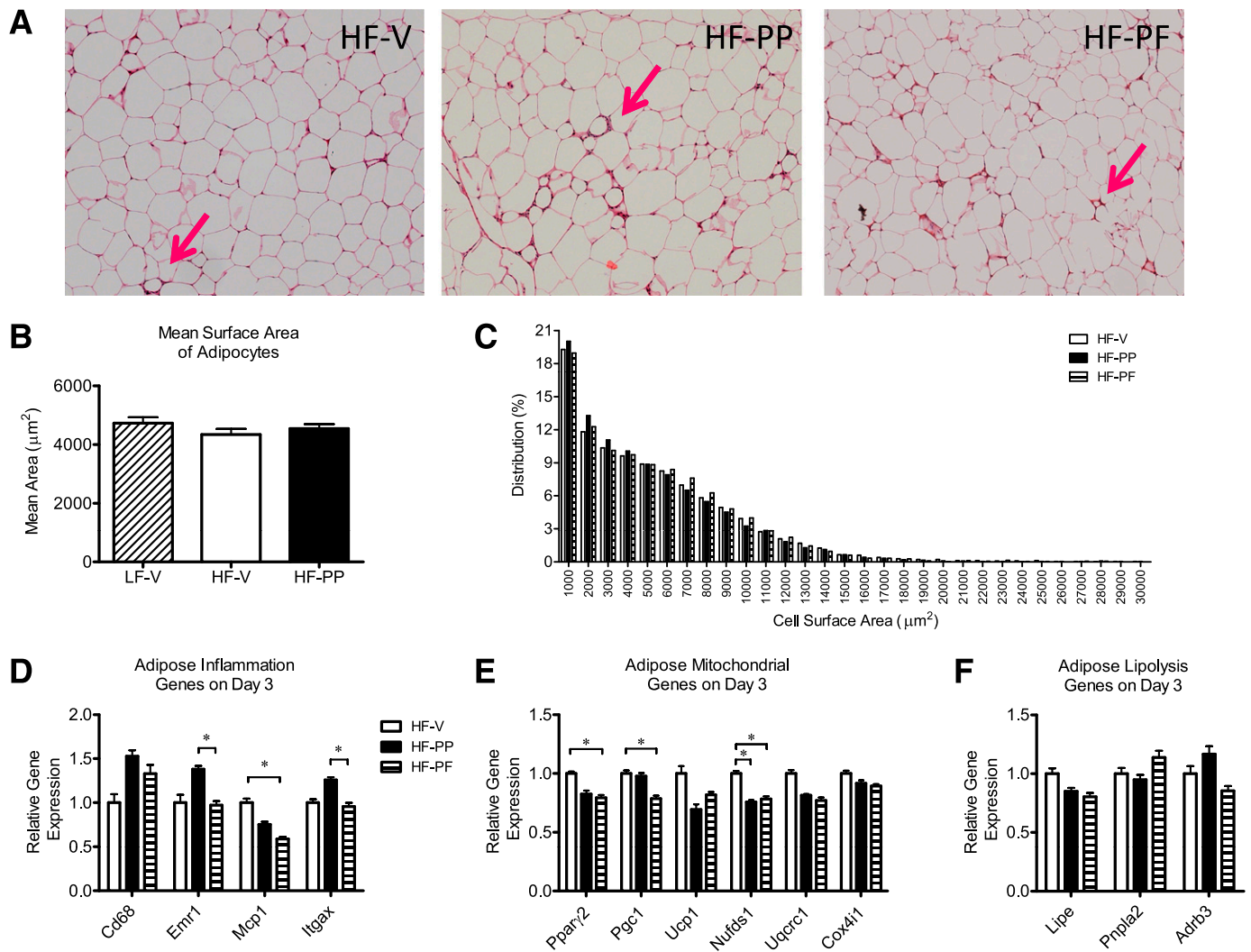


FIG. 6. Effect of PP on AT morphology and inflammation after 3 days of treatment. **A:** Representative figures of adipocytes morphology in epididymal fat of HF-V, HF-PP, and HF-PF. The arrows indicate crown-like structures of macrophages. **B:** The cross-sectional area of the adipocytes in epididymal fat. **C:** Relative distribution of adipocyte size in epididymal fat. **D:** mRNA expression of macrophage markers and proinflammatory cytokines in epididymal fat. **E:** Mitochondrial mRNA expression in epididymal fat. **F:** Expression of lipolysis-associated genes in epididymal fat. * $P < 0.05$. HF-V, $n = 8$ or 18; HF-PP, $n = 8$ or 17; HF-PF, $n = 8$ or 16. (A high-quality digital representation of this figure is available in the online issue.)

The pattern of changes in individual acylcarnitines associated with HFD-induced metabolic inflexibility was observed in this study (13). However, the pattern is quite different after PP treatment. HF-PP had much lower serum C2 carnitine, as well as other short- or medium-chain carnitines, and higher C18 carnitine and other long-chain acylcarnitines relative to HF-PF (Fig. 4). This distinctive PP-induced decrease in acylcarnitines that are known products of fatty acid and amino acid catabolism may have two explanations. First, we provide evidence of reduced availability of some mitochondrial substrates, including a decrease in selected fatty acids. Second, there may exist an indirect or direct effect of the PP on transport of long-chain acylcarnitines through the inner mitochondrial membrane or reduced conversion of acylcarnitines to acyl-CoA to undergo β -oxidation. This may lead to an overall decrease in small- to medium-chain acylcarnitines, as has also been described in obese malonyl-CoA decarboxylase knockout mice (13). In this way, PP therapy may mimic malonyl-CoA decarboxylase loss of function by reducing

lipid and amino acid substrate pressure on muscle mitochondria, thereby improving insulin action and facilitating glucose utilization, consistent with the previous study (13).

The picture that emerges from these data is that targeting of WAT vasculature via PP administration results in profound body weight and body fat loss that is largely secondary to reduced food intake. However, targeting these blood vessels would appear not to impair normal glucose regulation. On the contrary, we observe a profound and rapid improvement in glucose tolerance that cannot be attributed only to reduced food intake. The reasons for this are likely to be multifold, but PP treatment does result in improved WAT hormone secretion, plasma lipids, plasma amino acids, and WAT gene expression. Although counterintuitive, these data support the hypothesis that WAT function is improved rather than impaired by inhibiting WAT vasculature and that this contributes to the improved glucose regulation. These data open up new tools to help us understand how WAT function contributes to

various aspects of the metabolic syndrome and provide for new therapeutic strategies that would target this relationship.

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