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ABSTRACT

Whether obesity is a disease or a risk factor of chronic diseases including diabetes and fatty liver remains debating. We report here that a high-fat diet (HFD) alone or HFD and intramuscular injection of mice with a high dose (1.2 mg/kg) of lipopolysaccharide (LPS) induces the peripheral noninflammatory obesity. In contrast, HFD and intraperitoneal injection of mice with a low dose (0.25 mg/kg) of LPS induces the visceral low-grade inflammatory obesity. While the noninsulin dependent diabetes mellitus (NIDDM)- and nonalcoholic fatty liver disease (NAFLD)-related genes are globally upregulated in HFD+low-dose LPS mice, NIDDM and NAFLD genes are not extensively upregulated in HFD+high-dose LPS mice. The mitochondrial uncoupler 2,4-dinitrophenol (DNP) was found to exert a weight-reducing effect in obese mice by downregulating NF-κB-primed inflammatory response accompanying with NIDDM and NAFLD genes, thereby abrogating inflammatory hepatic injury. In conclusion, visceral low-grade inflammatory obesity that predisposes NIDDM and NAFLD can be ameliorated by DNP via anti-inflammation.

INTRODUCTION

Although the American Medical Association (AMA) declared obesity being a disease (http://www.ama-assn.org), Katz (2014) argued that obesity is not a disease, but a risk factor of other chronic diseases, such as the noninsulin dependent diabetes mellitus (NIDDM) and nonalcoholic fatty liver disease (NAFLD). He insisted that not only can chronic diseases develop in the absence of obesity, but not every obese person develops any such conditions. Clinically, some obese people without inflammation are sensitive to insulin and do not develop diabetes, but

others with inflammation are resistant to insulin and can develop diabetes (Hawkesworth *et al.* 2013). Therefore, it seems that the noninflammatory obesity or "healthy obesity" does not precede the chronic diseases, whereas the inflammatory obesity or "unhealthy obesity" predisposes the chronic diseases (Jais *et al*. 2014).

It is currently much discrepant about the origin of inflammation in obesity. For example, some authors suggested that increased adipocyte O_2 consumption induces hypoxia inducible factor 1α (HIF-1α), causing inflammation and insulin resistance (Lee *et al*. 2014), but others supposed that hepatic and macrophage heme oxygenase-1 (HO-1) drives the metaflammation and insulin resistance (Jais *et al*. 2014). The inflammation in obesity might be attributed to either an infectious origin or a noninfectious origin although some authors underscored a dual origin, in which the bacterial endotoxin lipopolysaccharide (LPS) and free fatty acids (FFAs) were thought to equally activate NF-κB, thereby upregulating the proinflammatory cytokines that induce the insulin resistance (Heinrichsdorff & Olefsky 2012).

LPS binding to Toll-like receptor 4 (TLR4) via the co-receptor CD14 has been previously confirmed, but a detailed mechanism by which FFAs binding to TLR4 has been poorly understood for a long time. Upon the recent finding that the liver secretory protein Fetuin A (Fet A) serves as an adaptor for FFAs binding to TLR4 (Pal *et al*. 2012), it is now believed that FFAs deem to elicit the inflammatory response in the high-fat diet (HFD)-induced obesity. It has been concluded that saturated FFAs exert the dominant proinflammatory effects, whereas ù-3 polyunsaturated FFAs (PUFAs) exhibit the potent anti-inflammatory roles (Glass & Olefsky 2012).

A putative association of gut microbiota with obesity has currently evoked much enthusiasm in recent years. Ding *et al.* (2010) indicated that intestinal inflammation precedes and correlates with HFD-induced obesity, adiposity and insulin resistance, but the absence of gut microbiota in germ-free mice blunts the upregulation of primary inflammatory indicators. Kim *et al*. (2012) also revealed a relevance of HFD with gut dysbiosis by showing the overgrowth of *Enterobecteriaceae*, which exacerbates inflammation and obesity in mice. A recent work further indicated that adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling, in which visceral fat is depoted for filtering the gut-derived LPS leakage (Asterholm *et al*. 2014), seemingly addressing a link between gut bacterial dysbiosis and visceral adipose storage.

Mounting evidence has emerged that Gram negative *Bacteroides*-enhanced mucus degradation is responsible for reduced gut lining integrity, thereby leading to leakage of LPS from the gastrointestinal tract into the blood stream (Qin *et al*. 2012; Le Chatelier *et al*. 2013). Given that

LPS is an initiator of inflammatory responses, it is anticipated that the exogenous LPS injected into the skeletal muscle should mimic the endogenous LPS leaked from the gut to upregulate the proinflammatory cytokines. In our preliminary tests, however, we found with surprise that either HFD alone or HFD combined with intramuscularly injected 1.2 mg/kg LPS can even downregulate the majority of cytokines/chemokines, including the proinflammatory cytokines, TNFα, IL-1β, and INFγ. Otherwise, Imajo *et al.* (2012) demonstrated that a single intraperitoneal injection of mice with 0.25 mg/kg LPS increases the infiltration of inflammatory cells into the hepatic tissue of HFD-fed mice.

We thought that intraperitoneal injection with low-dose LPS could simulate the trace-amount LPS leakage capable of inducing the chronic inflammation, eventually leading to the inflammatory obesity. In contrast, intramuscular injection with high-dose LPS should resemble the large-scale bacterial infection to initiate the acute inflammation, thereby developing the noninflammatory obesity. In human, approximately 40–50% of obese adults do not develop fatty liver, and the level of inflammatory biomarkers is higher in obese subjects with fatty liver compared to BMI-matched subjects without fatty liver (Zhao *et al*. 2015).

So we compared the expression profiles of inflammatory response genes, noninsulin dependent diabetes mellitus (NIDDM)-involved genes, and nonalcoholic fatty liver disease (NAFLD)-related genes in HFD+high-dose LPS mice with those in HFD+low-dose LPS. As results, we found that HFD+high-dose LPS induces the peripheral noninflammatory obesity with hepatic high-grade inflammation, whereas HFD+low-dose HFD+LPS induces the hepatic low-grade inflammatory obesity with adipose high-grade inflammation. Low-grade inflammation has been defined as a two- to threefold increase in the systemic concentrations of proinflammatory cytokines, such as tumor necrosis factor á (TNFá), interleukin 6 (IL6), and C-reactive protein (CRP) (Petersen & Pedersen 2005).

Based on the obese mouse model with the visceral low-grade inflammation established by HFD+0.25 mg/kg LPS, we examined whether the classic weight-reducing drug 2,4-dinitrophenol (DNP) would exert a weight-reducing effect through an alternative mechanism in addition to mitochondrial uncoupling, from which DNP-mediated anti-inflammation was elucidated for the first time. Our data available in the present study have not only verified the presence of dose-dependent LPS-driven inflammatory and noninflammatory obesity, but also disclosed the anti-inflammatory response as a mechanism underlying DNP for weight loss.

MATERIAL AND METHODS

Animals and experimental procedures

Kunming (KM) mice, belonging to an outbred population originated from SWISS mice, were used. All mice were housed on a 12-h light: 12-h dark cycle at 25°C, and fed with either HFD (60% basic feed-stuff + 20% lard + 10% sucrose + 10% yolk) or *ad libitum* chow (AL). HFD+1.2 mg/kg LPS mice were first fed with AL for two weeks, and then fed with HFD for two months, during which 1.2 mg/kg LPS was injected into the hind-leg muscle (Islam & Pestka 2006) one day before sampling. HFD+0.25 mg/kg LPS mice were first fed with AL for two weeks, and then fed with HFD for 1.5 months, during which 0.25 mg/kg LPS was injected into peritoneal (Imajo *et al*. 2012) from the 5th week of HFD feeding with the regimen of injection on every two days for two weeks. For drug treatment, mice were intraperitoneally injected daily with 16 mg/kg DNP for two weeks. Animal procedures were in accordance with the animal care committee at the Guangzhou University of Chinese Medicine, Guangzhou, China. The protocol was approved by the Animal Care Welfare Committee of Guangzhou University of Chinese Medicine (Permit Number: SPF-2011007).

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted by a Trizol method. The target gene *NF-κB* was amplified with a forward primer, CGACAACATCTCCTTGGCTGGCT, and a reverse primer, GGGTCTGCTCCTGCTGCTTTG. The house-keeping gene *GAPDH* was amplified with a forward primer, GGAGAAACCTGCCAAGTATGATGAC, and a reverse primer, GAGACAACCTGGTCCTCAGTGTA. The copy numbers of amplified genes were estimated by $2-\Delta\Delta Ct$, in which $\Delta\Delta Ct$ = [target gene (treatment group) / target gene (control group)] / [housekeeping gene (treatment group) / house-keeping gene (control group)].

RT-PCR array

The RT² Profiler™ PCR Array Mouse Fatty Liver (PAMM-157Z) was purchased from SABioscience Qiagen, Hilden, Germany, and Quantibody® Mouse Cytokine Antibody Array 4000 was purchased from RayBiotech, Inc, Norcross, GA, USA. The experiments were performed respectively by Kangchen Biotechnology Co, Ltd, Shanghai, China, and RayBiotech, Inc. Guangzhou, China.

Cytokine antibody array

Protein extraction from blood cells by Cell & Tissue Protein Extraction Reagent (KangChen KC-415) was conducted according to the manufacturer's instruction. Cytokine antibody array was carried out by Kangchen Bio-Tech, Shanghai, China using RayBio® Mouse Cytokine Antibody

Array.

Enzyme-linked immunosorbent assay (ELISA)

The target proteins including insulin, leptin, and NF-κB as compared to the reference protein GAPDH were immunoquantified by ELISA kits manufactured by Shanghai Yuanye Bio-Technology Co., Ltd, Shanghai, China according to manufacture's manuals.

Serological tests

Triglycerides, total cholesterol, aspartate transaminase (AST), and alanine tranaminase (ALT) were determined by ECHO Automatic Chemistry Analyzer, I.S.E. S.r.l., Via delle Driadi, Roma, Italy. The reagent kits were purchased from Shanghai Kehua Laboratory System Co, Ltd, Shanghai, China.

Histopathological analysis

A piece of the tissue was fixed by 10% formaldehyde followed by paraffin embedding and haematoxylin-eosin (HE) staining. The degenerative scores of inflammatory lesions were recorded as: 1-2 points - mild/severe hydropic degeneration; 3-4 points - mild/severe adipose degeneration; 5 points - necrosis.

Selective bacterial culture

The selective cultural media of EMB agar powders for *Escherichia coli*, LBS agar powders for *Lactobacillus*, and TPY agar powders for *Bifidobacterium* were purchased from Qingdao Hope Bio-Technology Co. Qingdao, China. Selective bacterial culture was performed in the anaerobic jar and AnaeroGen paper sachets from OXOID, UK, according to the manufacture's instructions.

Determination of lactic acid, NO and 3NT levels

Lactic acid (LA) and NO levels were determined using the reagent kits manufactured by Jiancheng Biotechnology Institute, Nanjing, China. The LA level (mM) = (OD test − OD blank)/(OD standard − OD blank) • standard LA concentration (3 mM) • sample dilution folds. The NO level (μM) = (OD test − OD blank)/(OD standard − OD blank) • standard nitrate concentration (20 μM). The 3NT content was measured by the reagent kit manufactured by Shanghai Westang Bio-Tech Co, Ltd, Shanghai, China. The 3NT content (μg/mg) = 3NT concentration (μg/mL)/protein concentration (mg/mL), in which 3NT concentration was calculated from the regression formula: y=(0.372x - 0.016, R^2=1.000).

Statistical analysis

Statistical analyses were conducted by the one-way ANOVA method using SPSS version 17.0 for Windows. All data were represented as mean ± SEM unless otherwise stated. The XY graphs and column graphs were plotted and depicted using GraphPad Prism version 4.0.

RESULTS

Peripheral noninflammation in HFD-fed obese mice with intramuscular injection of 1.2 mg/kg LPS

To decipher the etiological cause of inflammation in obesity, we assumed that HFD alone is sufficient to induce gut dysbiosis, LPS leakage, and inflammatory obesity, during which challenges with the exogenous LPS should accelerate such a process. So we firstly intended to reveal whether HFD would lead to gut dysbiosis by enhancing the overgrowth of specific bacteria. By collecting the fecal samples of HFD-fed mice and selectively culturing the commensal gut microbiota, including *E. coli*, *Lactobacillus*, and *Bifidobacterium*, we confirmed that the colony numbers of *E. coli* overwhelm those of *Lactobacillus* and *Bifidobacterium*, but there is no significant difference in colony numbers of *Lactobacillus* from those of *Bifidobacterium* (Figure 1A). Furthermore, we also noticed that intramuscular injection of mice with LPS or LPS-containing complete Freund's adjuvant (CFA) can potently induce the serum high-level NO, whereas live yeast feeding does not elevate the serum NO level (Figure 1B), implying that *E. coli*-produced LPS can initiate the inflammatory response and trigger NO burst.

To ensure how many times of LPS challenges are suitable to potentiate the inflammatory responses, we simply monitored the serum NO production and the muscular 3NT formation after multiple intramuscular injections of mice with 1.2 mg/kg LPS. Consequently, a gradual reduction of the NO level was observed after injection of mice with 1.2 mg/kg LPS in the 14-day durations and four-time injections (Figure 1C), suggesting a gradual compromise of the inflammatory response to LPS. Similarly, we also noticed a significant decrease of the 3NT content after the prime-boost LPS inoculation (Figure 1D). So a singular injection other than multiple injections should be preferred to prompt the progression to LPS-driven inflammatory obesity.

As illustrated in Figure 1E, both HFD mice and HFD+1.2 mg/kg LPS mice become obese with the heavier body weight and higher body adipose percentage after fed with HFD for 1.5-2 months (Figure 1E and 1F). Logically, we anticipated that the proinflammatory cytokines should be upregulated in both HFD-fed obese mice and LPS-injected HFD obese mice. Surprisingly, we found that the expression levels of all examined 40 cytokines and chemokines in the skeletal

muscle of either HFD mice or HFD+1.2 mg/kg LPS mice are totally unchanged or even lower than those in AL mice (Figure 1G, see also Table S1). Among the most common proinflammatory cytokines, IL-1β in HFD mice maintains an equivalent level with AL mice (0.95-fold changes), whereas it is much lower in HFD+1.2 mg/kg LPS mice than in AL mice (0.16-fold downregulation). While TNFα is almost unchanged in HFD mice or slightly downregulated in HFD+1.2 mg/kg LPS mice (0.95- and 0.85-fold changes), IFNγ is dramatically downregulated in both obese mice (0.11- and 0.05-fold downregulation).

The possible reasons on the downregulation of proinflammatory cytokines in HFD mice or HFD+1.2 mg/kg LPS mice might be attributed to antibody neutralization or immune suppression. In fact, it was previously found that a single optimal immunogenic dose of LPS can trigger an antibody response (Hiernaux *et al.* 1982), and anti-LPS antibodies can reduce the plasma LPS titers in humans (Wells *et al.* 1990). Recently, a high dose of LPS was validated to be an immunosuppressor (Kelly *et al.* 2012). Therefore, either HFD alone or HFD+1.2 mg/kg LPS can only induce the peripheral/subcutaneous noninflammatory obesity in mice.

Visceral high-grade inflammation in HFD-fed obese mice with intramuscular injection of 1.2 mg/kg LPS

To investigate whether HFD+1.2 mg/kg LPS would cause the visceral inflammation, we quantified the hepatic inflammatory response transcripts and NIDDM-involved transcripts among 84 NAFLD-related transcripts in HFD mice and HFD+1.2 mg/kg LPS mice (Table 1, see also Table S2).

From the data listed in Table 1, it can be clearly noticed that HFD+1.2 mg/kg LPS can stimulate a potent hepatic inflammatory response, in which the most common proinflammatory cytokine transcripts, including *Il1b* mRNA (94.24 folds), *Il6* mRNA (12.54 folds), and *Tnf* mRNA (10.24 folds), are extremely upregulated compared with AL mice. However, HFD differentially allows the proinflammatory cytokines to be unchanged (*Tnf* mRNA for 1.15 folds), mildly upregulated (*Il1b* mRNA for 6.46 folds), or slightly downregulated (*Ifng* mRNA for -5.21 folds). In HFD+1.2 mg/kg LPS mice, the anti-inflammatory cytokine transcript *Il10* mRNA is dramatically upregulated for 50.20 folds, and the dual anti-/proinflammatory cytokine *Il6* mRNA is also considerably upregulated for 12.54 folds. On the other hand, *Il10* mRNA exhibits 4.32-fold upregulation and *Il6* mRNA shows unchanged expression (1.51 folds) in HFD mice.

In regard to the NIDDM transcripts, HFD+1.2 mg/kg LPS mice have two kinds of mRNAs

(*Socs3* and *Xbp1* mRNAs) being higher than AL mice for over two folds, whereas HFD mice have only one kind of mRNA (*Xbp1* mRNA) being higher than AL mice for over two folds. It has been demonstrated that SOCS3 (suppressor of cytokine signaling-3) is responsible for the specific inhibition of Janus kinases (JAKs), suggesting an implication in the suppression of cytokine signaling (Babon *et al.* 2012). XBP1 (X-box binding protein 1) was also proved to be implicated in the compromise of stress from endoplasmic reticulum (ER) and mitigation of susceptibility to inflammatory processes (Casus-Tinto *et al*. 2011).

It can be briefly summarized that HFD+1.2 mg/kg LPS induce the hepatic high-grade inflammation because their expression levels of proinflammatory cytokines are highly elevated for more than 2-3 folds. Meanwhile, HFD+1.2 mg/kg LPS also upregulates some NIDDM genes beneficial for anti-inflammation. Taken together, these results demonstrated that HFD+1.2 mg/kg LPS can induce obesity with the peripheral noninflammation but visceral high-grade inflammation, which might resemble the acute pathogenic infection and would eventually lead to the immune deprival of LPS in the blood circulation.

Visceral low-grade inflammation in HFD-fed obese mice with intraperitoneal injection of 0.25 mg/kg LPS

To avoid the immune depletion of LPS, we replaced the high-dose and short-term LPS exposure by the low-dose and long-term LPS exposure, i.e., the injection dose was changed from 1.2 mg/kg LPS to 0.25 mg/kg LPS, the injection procedure was changed from the intramuscular injection to the intraperitoneal injection, and the injection frequency was changed from only one injection to multiple injections. Other authors have reported that the infiltration of inflammatory cells are enhanced in the liver tissue of HFD-fed mice after treatment by 0.25 mg/kg LPS (Imajo *et al.* 2012).

After multiple LPS injections on every two days for two months from the 5th week of HFD feeding, HFD+0.25 mg/kg LPS mice become obese than AL mice (Figure 2A). The LA level that indicates a hypoxic state is as expected higher in HFD+0.25 mg/kg LPS mice than that in AL mice (Figure 2B). Accordingly, HFD+0.25 mg/kg LPS mice show the extremely increased adipose expression levels of as many as 200 cytokines/chemokines, among which those with the upregulated levels above 100 folds compared with AL mice were illustrated (Figure 2C). For example, IL-1β mRNA is elevated for 3477 folds, and TNFα mRNA is increased for 546 folds. While AL mice does not exhibit the acute hepatic injury (Figure 2D), hepatic inflammatory pathogenesis could be seen in HFD+0.25 mg/kg LPS mice albeit with only one-point

inflammatory score (Figure 2E).

Intriguingly, the hepatic inflammatory response transcripts in those obese mice exhibit the restricted upregulation for only 2- to 3-folds, which are well coincided with the definition of low-grade inflammation (Petersen & Pedersen 2005). Importantly, there are also as many as six NIDDM transcripts showing the similarly restricted upregulation for 2-4 folds in the hepatic tissue of HFD+0.25 mg/kg LPS mice (Table 2, see also Table S3).

From above results, we can draw a conclusion that HFD+0.25 mg/kg LPS can extensively induce the inflammatory response genes and NIDDM genes, in which the fold changes of proinflammatory cytokines are extremely upregulated in the adipose tissue, but restrictively upregulated in the hepatic tissue. So it was understandable that HFD+0.25 mg/kg LPS through multiple intraperitoneal injections can dually induce the hepatic low-grade inflammation and adipose high-grade inflammation in obese mice. From the minimal hepatic injury, it seems that the adipose high-grade inflammation might have been abrogated as soon as possible, like the acute pathogenic infection.

DNP reduces adipose deposits without elevated transaminase activity and detectable acute hepatic injury

By the multiple intraperitoneal injections of HFD+0.25 mg/kg mice with 16 mg/kg DNP for two weeks, we observed that the body adipose percentage is dramatically decreased to a lower level similar with AL mice (Figure 3A). However, DNP was not found to affect the body liver percentage of HFD+0.25 mg/kg mice (Figure 3B). The concentrations of triglycerides and total cholesterols were not noticed to be changed by DNP although an elevated level of the total cholesterol was seen in HFD+0.25 mg/kg mice (Figure 3C and 3D). Noticeably, DNP does not significantly alter the serum levels of AST and ALT (Figure 3E and 3F). Compared with AL mice without hepatic injury (Figure 3G) and HFD+0.25 mg/kg mice with 3-point scores indicating focal necrosis (Figure 3H), DNP-treated HFD+0.25 mg/kg mice does not cause any detectable hepatic inflammatory lesions (Figure 3I), suggesting that DNP might possess an anti-inflammatory effect but not exert any hepatic cytotoxicity.

DNP downregulates insulin but upregulates leptin, accompanying with downregulated NF-κB and CD14

Accompanying with adipose reduction, it was observed that insulin (INS) and leptin (LEP) are synchronously modulated by DNP, in which the concentration of INS equal to that in AL mice was noticed in the weight-reducing mice after injection with DNP (Figure 4A). On the other hand, the concentration of LEP higher than that in AL mice was observed in the weight-reducing mice upon treated by DNP (Figure 4B). Those results indicated the blood sugar sensing is increased and the satiety to normal is recovered in the weight-reducing mice.

NF-κB, a transcript factor initiating global inflammatory responses in all types of cells, is known to be activated by numerous external signals through the interaction of a specific ligand with a corresponding receptor. For example, TLR4 is such a receptor for binding LPS or FFA, during which the co-receptor CD14 or the adapter FetA is necessary for initiating NF-κB-activated proinflammatory responses (Heinrichsdorff & Olefsky 2012). In our experiments, a tremendous elevation and a extremely decline of *NF-κB* mRNA were notably observed in untreated and DNP-treated obese mice, respectively (Figure 4C). In parallel, a high level and a lower level of NF-κB were also measured in untreated and DNP-treated obese mice, respectively (Figure 4D). Those results suggest that NF-κB might be highly inducible.

To investigate whether CD14 and FetA are modulated by LPS and FFA or affected by DNP, we quantified the levels of CD14 and FetA in obese mice prior to and post treatment by DNP. As results, CD14 is downregulated in untreated obese mice, but upregulated in DNP-treated obese mice (Figure 4E), suggesting high-level LPS in untreated mice and low-level LPS in treated obese mice. On the other hand, we found that FetA is nearly unchanged in both untreated and treated obese mice (Figure 4F), indicating a minor regulatory role on the induction of NF-κB exerted by FFA in the present study.

DNP downregulates adipose proinflammatory cytokine genes and hepatic NAFLD and NIDDM genes

As compared with the adipose tissue of untreated obese mice, DNP maximally decreases the proinflammatory cytokine transcripts in the adipose tissue of treated obese mice. Notably, IL-1β mRNA and TNFα mRNA were unable to detectable after treatment by DNP, in which IL-1β mRNA is declined for more than 3000 folds, and TNFα mRNA is declined for more than 500 folds. Among 200 examined adipose cytokines/chemokines mRNAs, DNP downregulates almost all of them, in which the declined levels over 500 folds were depicted in Figure 5A. It is currently obscure why DNP can exert an anti-inflammatory role although a recent report has indicated that no effect of DNP was found on the plasma markers of inflammation (Perry *et al.*

2015). However, it seems understandable that DNP might downregulate the proinflammatory cytokines by downregulating TNFα because NF-κB can be activated by TNFα via TNFR1 (Baker *et al.* 2011).

Interestingly, DNP also downregulates a majority of NAFLD genes in the hepatic tissue of obese mice, in which those transcripts with twofold changes were illustrated in Figure 5B. It was predominantly that *Lepr* mRNA encoding leptin receptor and *Igfbp1* mRNA encoding insulin-like growth factor binding protein 1 are downregulated for 17.93 and 10.83 fold, respectively, implying an increased sensitivity to leptin and IGF. As to *CD36* mRNA encoding a fatty acid translocase that determines the taste of fatty acids, its upregulation (17.01 folds) might represent an increase of taste sensitivity. CD36 was found to be downregulated in the taste bud cells of obese sand rats compared to lean controls (Abdoul-Azize *et al.* 2013).

To clearly exhibit the improvement of NIDDM gene expression profile in obese mice treated by DNP, the up/downregulation of NIDDM transcripts in the hepatic tissue of DNP-treated obese mice, untreated obese mice, and AL mice were listed as Table 3 (see also Table S4).

From the fold changes of expression levels, it was unambiguously indicated that the most of NIDDM transcripts are downregulated to reach or below the levels seen in AL mice. For example, *Gck* mRNA is less abundant in DNP-treated obese mice than untreated obese mice for 4.29 folds, and DNP-treated obese mice have a similar *Gck* mRNA level (1.02 folds) with AL mice. Additionally, *Slc2a4 (Glut4)* mRNA encoding glucose transporter 4 and *Tnf* mRNA implicated in the inflammatory response are also declined in comparison of DNP-treated mice with untreated obese mice and AL mice, implying a declined blood sugar level and attenuated inflammatory response.

Besides, *Pik3r1* mRNA that codes for phosphatidylinositol 3-kinase is upregulated in DNP-treated obese mice. Phosphatidylinositol 3-kinase has been noted to play a role in the metabolic action of insulin, and a mutation in this gene is associated with insulin resistance (Kapeller *et al.* 1995). So DNP-mediated upregulation of *Pik3r1* mRNA would benefit to overcome insulin resistance.

DISCUSSION

A logical relevance of HFD to inflammation is likely that HFD allows the overgrowth of *Bacteroides* that enable the secretion of mucin-degrading sulfatase, which reduces gut integrity, enhance LPS leakage, and elicit inflammatory responses (Qin *et al.* 2012; Le Chatelier *et al.* 2013). In the present study, however, we found that proinflammatory cytokines examined are

unchanged in the skeletal muscle of HFD-fed obese mice although LPS-releasing *E.coli* is overloaded in the gut of HFD mice. The explanation to this peripheral noninflammatory obesity seems that the gut lining of obese mice typically remains intact and LPS is hardly leaked into the blood stream. To mimic LPS leakage, therefore, we injected the skeletal muscle of HFD mice with 1.2 mg/kg LPS. Surprisingly, we did not note any downregulation of the proinflammatory cytokines tested in the skeletal muscular tissue of HFD+1.2 mg/kg LPS mice. It might be able to decipher such a phenomenon by antibody neutralization (Hiernaux *et al.* 1982; Wells *et al.* 1990) and/or immune suppression (Kelly *et al.* 2012) because consecutive challenges with 1.2 mg/kg LPS can gradually mitigate NO burst and 3NT formation triggered by inflammatory responses. Therefore, HFD or HFD+1.2 mg/kg LPS only induces the obesity without peripheral inflammation in mice.

Nevertheless, almost all quantified transcripts of the proinflammatory cytokines exhibit the extreme elevation higher than five folds in the hepatic tissue of HFD+1.2 mg/kg LPS mice, suggesting a simulation of the acute inflammation, which can be considered the high-grade inflammation (Petersen & Pedersen 2005). As described earlier, we observed that live *E. coli* feeding can dramatically downregulate the proinflammatory cytokines and sharply decline the NO and LA levels (Bao *et al*. 2012). From the present study, we observed the gradual declines of NO and 3NT levels in the peripheral tissue during the prime-boost challenges with 1.2 mg/kg LPS, so we believed that the acute inflammatory presentation should be eventually attenuated in the LPS-challenged subjects. This anticipation should be real because no extensive upregulation of NIDDM and NALFD genes were detected, and no severe hepatic inflammatory injury was observed in HFD+1.2 mg/kg LPS mice.

Although the high-grade inflammation in the adipose tissue of HFD+0.25 mg/kg LPS mice should not be maintained like the acute inflammation, the low-grade inflammation must remain to result in a mild but sustained hepatic inflammatory lesion. It was noticeably from the large-scale upregulation of NIDDM and NALFD genes. In particularly, the inflammatory response genes are upregulated for only 2-3 folds, which can be classified as the low-grade inflammation (Petersen & Pedersen 2005). So HFD+0.25 mg/kg LPS can induce visceral low-grade inflammation obesity in mice.

We did not observed the upregulation of Fet A responsible for binding of FFAs to TRL4 in HFD+0.25 mg/kg LPS mice, implying irrelevance of HFD-derived FFAs to Fet A. Interestingly, a recent report has delineated that the disruption of Fat A expression renders animals more susceptible to endotoxinemia, whereas supplementation with Fet A confers protection against lethal endotoxinemia (Li *et al*. 2011), suggesting an elevation of Fet A due to LPS accumulation.

Alternatively, saturated FFAs have been confirmed to stimulate adenine nucleotide translocase 2 (ANT2), an inner mitochondrial membrane protein, which leads to an uncoupled respiratory state (Lee *et al.* 2014). Additionally, palmitic acid has been validated to be responsible for the downregulation of vascular endothelial growth factor (VEGF) in obesity (Shimizu *et al*. 2014). Therefore, it remains uncertain whether HFD triggers inflammation via FFAs in addition to LPS.

It has been previously demonstrated that the chronic blockade of iNOS by the *L*-arginine analogue inhibitor *L*-NG-monomethylarginine (*L*-NMMA) reduces adiposity and improves insulin resistance in HFD-induced obese mice (Tsuchiya *et al.* 2007). When iNOS is upregulated by inflammation, eNOS should be downregulated due to an unfavorable competition with a limited supply of the substrate *L*-arginine, and a deficiency of eNOS-derived NO should in turn block mitochondrial biogenesis (Wang *et al*. 2015), thereby resulting in enhanced adipose whitening and reduced energy expenditure, eventually leading to overweight/obesity. This is because the brown adipose tissue (BAT) are converted to the white adipose tissue (WAT), which usually emerges in the adipose tissue due to the progressive decrease of mitochondria (Shimizu *et al*. 2014).

The classic anti-inflammatory agent salicylate, a degraded product of aspirin, has been reported to reduce the circulating lipids in obese rats and improves the insulin sensitivity (Yuan *et al*. 2001). Similarly, nitro-aspirin has also therapeutic potential for NAFLD (Ibrahim *et al.* 2011). Mechanically, aspirin has validated to ameliorate type 2 diabetes as an activator of adenosine monophosphate-activated kinase (AMPK) (Hawley *et al*. 2012). It has been currently shown that metformin and salicylate can synergistically activate liver AMPK to inhibit lipogenesis and improve insulin sensitivity (Ford *et al.* 2015). DNP was previously known to induce the expression of AMPK and phosphorylation of p38 MAPK (Pelletier & Coderre 2007), so it should be reasonable that DNP can play a weight loss role.

Interestingly, we have recently reported that the NO donor sodium nitroprusside and the NO precursor *L*-arginine can serve as the activators of AMPK, which in turn upregulates eNOS for triggering mitochondrial biogenesis (Wang *et al.* 2015). We therefore suggested that DNP might exert a weight-reducing effect via a dually tuned mechanism underlying upregulating AMPK for mitochondrial biogenesis and downregulating NF-κB for anti-inflammation, as illustrated in Figure 6.

The healthy obesity can be classified as the adiposity with a high body mass index (BMI) but without the low-grade inflammation and insulin resistance, whereas the unhealthy obesity is categorized as the adiposity with a high BMI, accompanying with the low-grade inflammation, and probably exhibiting insulin resistance or type 2 diabetes. It has been suggested that the

elevation of inflammation-induced HIF-1α (Lee *et al*. 2014) or HO-1 (Jais *et al.* 2014) can be considered a hallmark in human discriminating healthy obesity with insulin sensitivity from unhealthy obesity with insulin resistance. The chronic inflammation is most likely derived from an interaction of HFD with Gram negative bacteria, during which HFD-elicited gut dysbiosis might lead to decreased gut lining integrity and increased LPS leakage and even endotoxinemia. Upon the upregulation of proinflammatory cytokines via activating NF-κB, LPS can induce the expression of iNOS, leading to potent NO burst, metabolic hypoxia, and adipogenesis.

Activation of iNOS predisposes inactivation of eNOS due to a restriction of the common substrate *L*-arginine. Consequently, deficiency of eNOS-derived NO would suppress the mitochondrial biogenesis and accelerate the adipose whitening, in which BAT is gradually converted to WAT. DNP can exert the anti-obese effects by inactivating iNOS and decreasing the high-level NO towards adipogenesis, and synchronously activating eNOS and increasing the low-level NO for mitochondrial biogenesis and adipose browning, during which WAT can be recovered to BAT. Additionally, eNOS-derived NO can inhibit NF-κB (Clancy *et al*. 2004), thereby downregulating proinflammatory cytokines and compromising inflammatory responses.

Despite DNP was earlier used as a weight-reducing drug, it was later discarded due to intractable toxicity. The LD_{50} of DNP in mice, rats, or cats is shown to be 30, 45, or 75 mg/kg, but the lowest-observed-adverse-effect level (LOAEL) in human to be 2 mg/kg (http://www.epa.gov/ttnatw01/hlthef/dinitrop.html). It has been reported that a very low intracellular concentration of DNP, 75-fold lower than a toxic DNP level (a dose of 25 mg/kg or a peak plasma concentration of 380 μM), is sufficient to achieve significant weight-reducing effects without any systemic toxicities (Perry *et al*. 2013).

To minimize the toxicity of DNP, we would further evaluate the weight-reducing effects of low doses of nontoxic DNP in the future.

Conclusively, we have successfully established an obese mouse model with visceral low-grade inflammation using HFD+0.25 mg/kg LPS, and also revealed for the first time that the well-known mitochondrial uncoupler DNP can exert an anti-inflammatory effect for downregulation of genes responsible for NIDDM and NAFLD during weight loss.

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1

Image of obese modeling

Figure 1 The modeling of obese mice without muscular inflammation by HFD or HFD+1.2 mg/kg LPS. (A) Colony counts from fecal dilutions of AL (*n*=3) and HFD mice (*n*=3) containing *E. coli*, *Lactobacillus*, and *Bifidobacterium* strains on the plates of selective EMB, LBS, and TPY media after cultured for 24 h, 72 h, and 72 h, respectively. For HFD modeling, mice were first fed with AL for two weeks and then fed with HFD for 1.5 months. (B) The serum NO level in mice fed with overnight cultured live yeast (*n*=3), or injected with 1.2 mg/kg LPS (*n*=3) or 5 mg/kg CFA (*n*=3) into hind-leg muscles. The determination was conducted on the next day after feeding or injection. (C) and (D) The serum NO level and the muscular 3NT content in AL mice and LPS-injected mice. 1.2 mg/kg LPS was injected into hind-leg muscles on the 1st, 3rd, 6th, and 12th days. The serum NO level and muscular 3NT content were determined on the 3rd, 5th, 8th, and 14th days. (E) The body weight of AL mice (*n*=3), HFD mice (*n*=4) and HFD+1.2 mg/kg LPS mice (*n*=5); (F) The body adipose percentage of AL mice (*n*=4), HFD mice (*n*=5) and HFD+1.2 mg/kg LPS mice (*n*=6). (G) A schematic diagram of hierarchical clustering for the up/down-regulation of 40 cytokines/chemokines in the muscular tissue of AL mice (*n*=1), HFD mice (*n*=1) and HFD+1.2 mg/kg LPS mice (*n*=1) by the cytokine antibody array. The red color represents upregulation as compared with AL, and the green color represents downregulation as compared with AL. HFD mice were first fed with AL for two weeks and then fed with HFD for two months. HFD+1.2 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for two months, during which 1.2 mg/kg LPS was injected into hind-leg muscles one day before sampling.

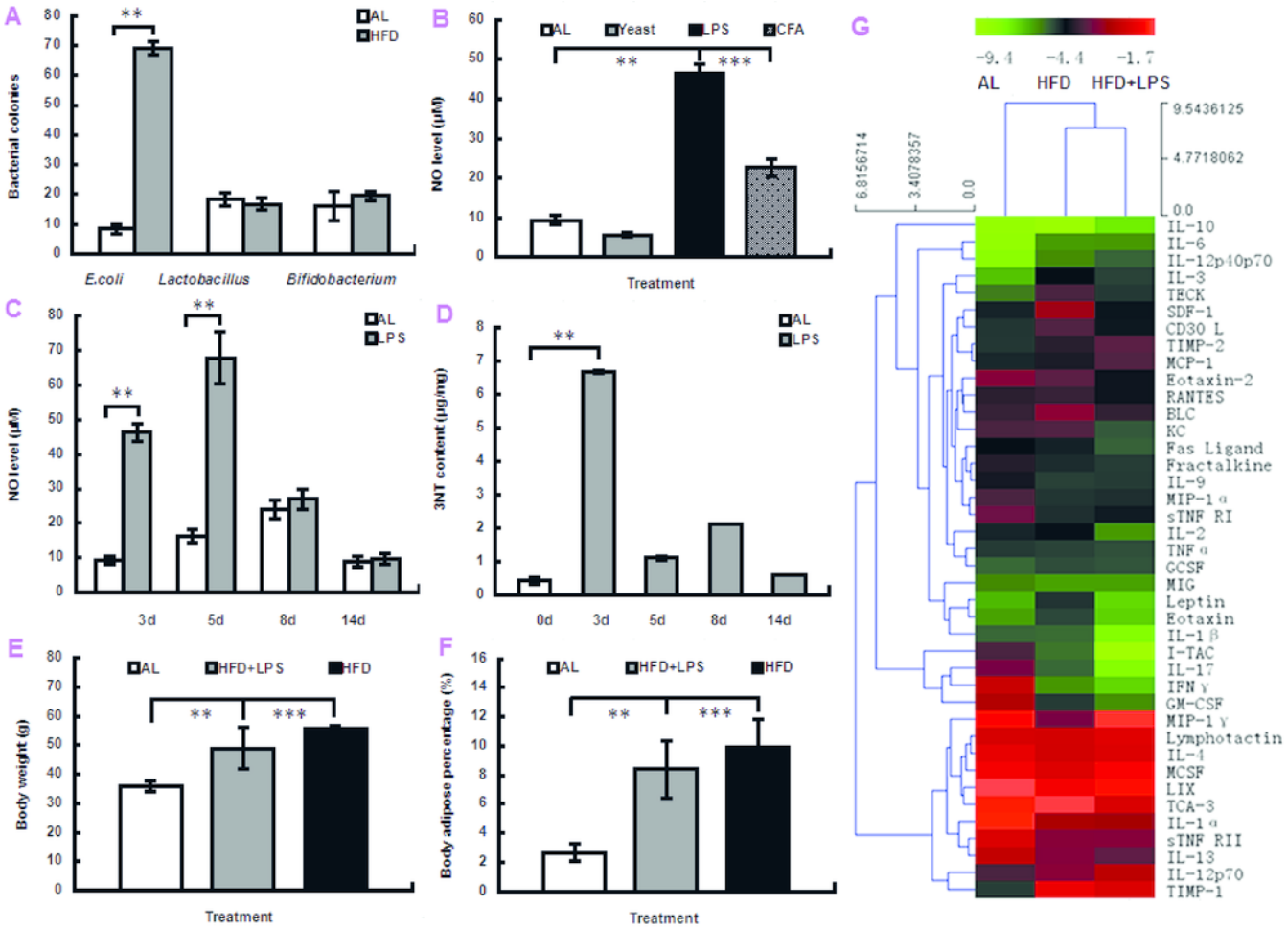

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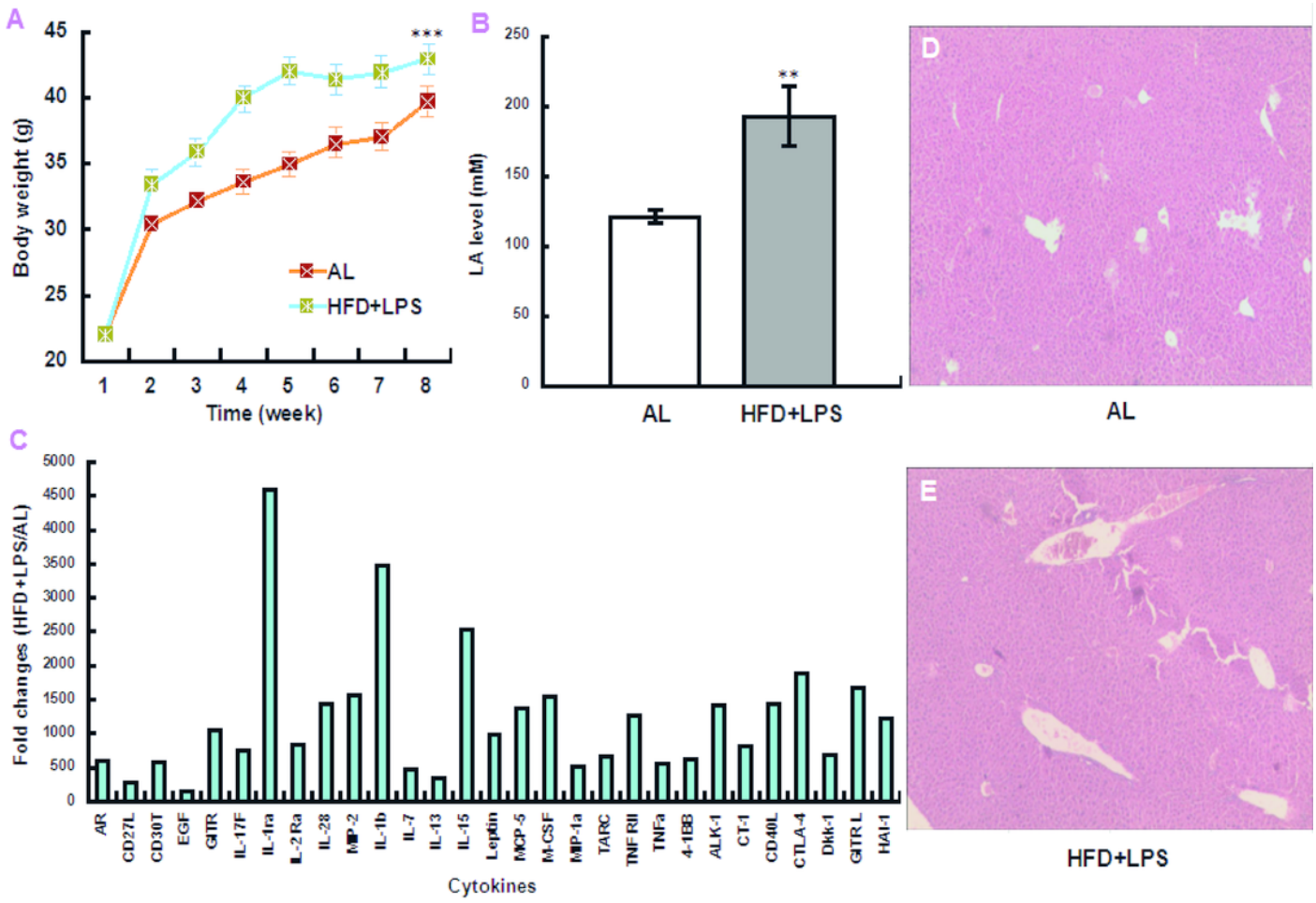
Image of inflammatory modeling

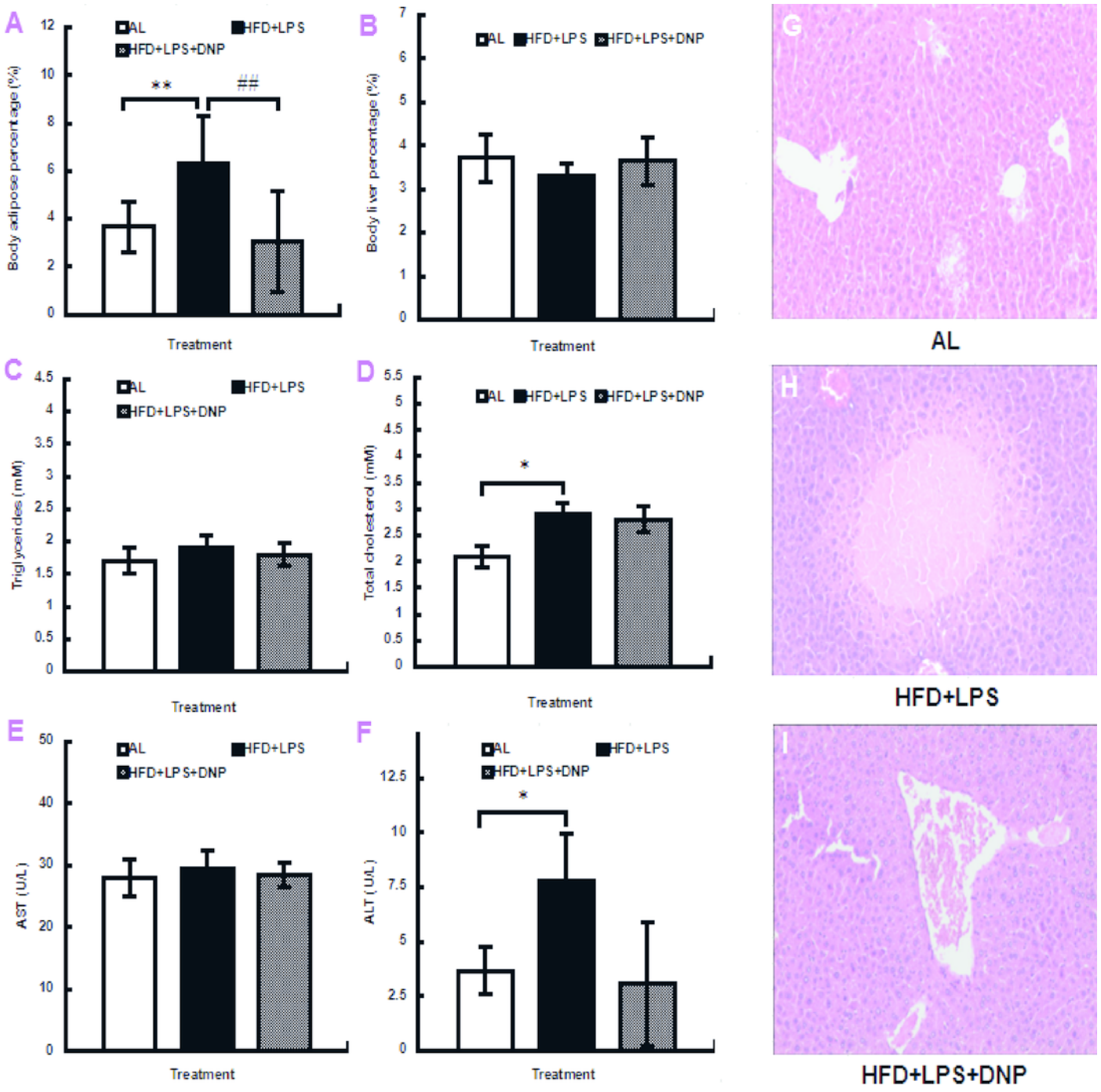
Figure 2 The modeling of obese mice with hepatic low-grade inflammation by HFD+low-dose LPS. (A) A body weight curve of AL mice (*n*=16) and HFD+0.25 mg/kg LPS mice (*n*=14). (B) The LA levels in the serum of AL mice (*n*=3) and HFD+0.25 mg/kg LPS mice (*n*=3). (C) The fold changes of cytokines/chemokines with above 100-fold upregulation in the adipose tissue of HFD+0.25 mg/kg LPS mice (*n*=1) compared with AL mice (*n*=1). (D) and (E) The histochemical staining of hepatic inflammatory pathogenesis (4×10) in AL mice and HFD+0.25 mg/kg LPS mice. HFD+0.25 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for 1.5 months, during which 0.25 mg/kg LPS was injected into peritoneal from the 5th week on every two days for two weeks. All measurements and analysis were carried out after the completion of modeling for eight weeks.



3

Image of identification

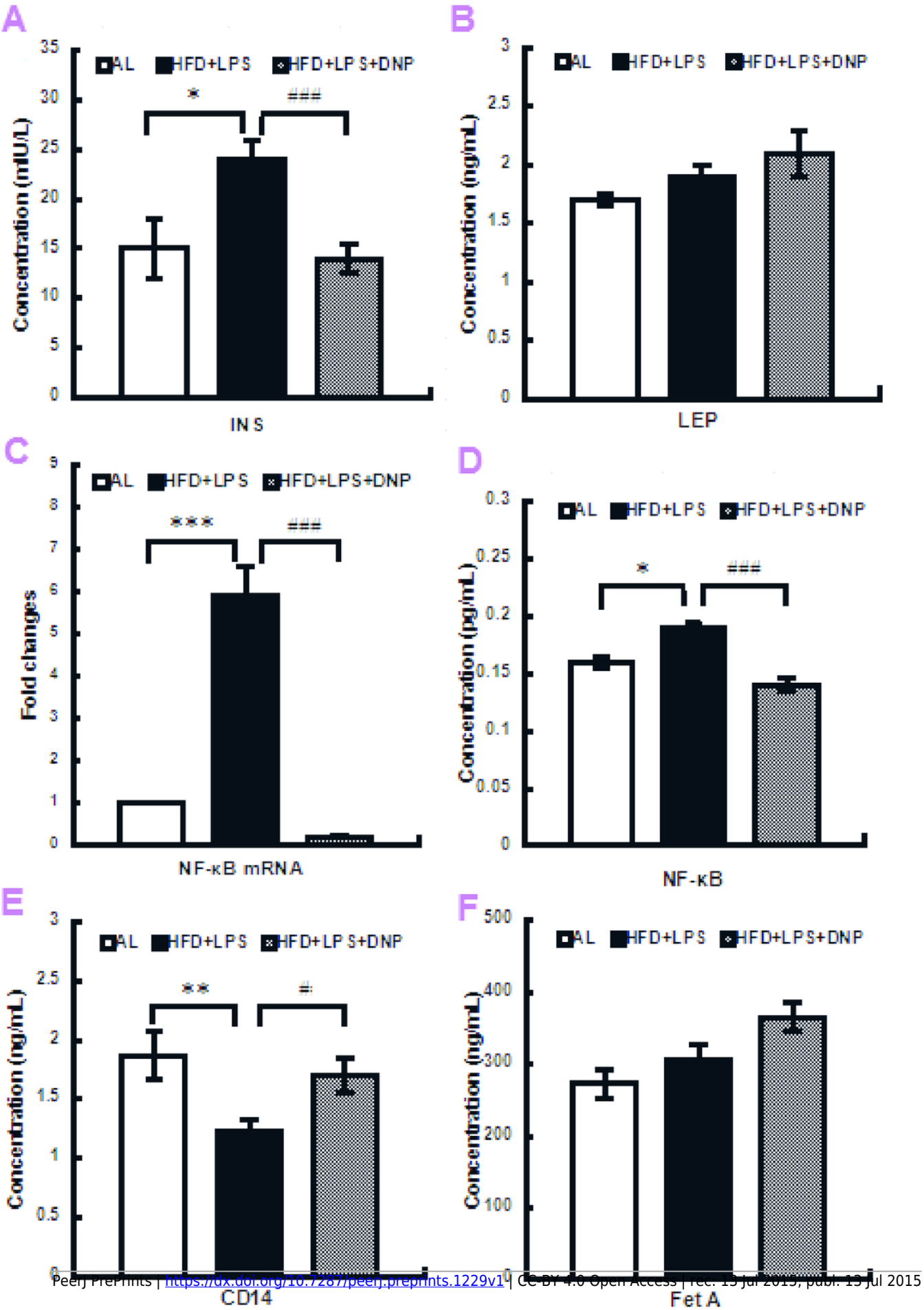
Figure 3 The phenotypic, serological, and histochemical identification in HFD+0.25 mg/kg LPS-induced obese mice after treatment by 1.6 mg/kg DNP. (A) and (B) The body adipose and body liver percentages; (C) and (D) The serum triglycerides and total cholesterol concentrations; (E) and (F) The serum AST and ALT titers; (G)-(I) HE staining of the hepatic tissue (10×10) in AL, HFD+0.25 mg/kg LPS, and HFD+0.25 mg/kg LPS+1.6 mg/kg DNP mice. HFD+0.25 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for 1.5 months, during which 0.25 mg/kg LPS was intraperitoneally injected from the 5^{th} week on every two days for two weeks. The obese mice were injected into peritoneal daily by 16 mg/kg DNP for two weeks.



4

Image of profiling

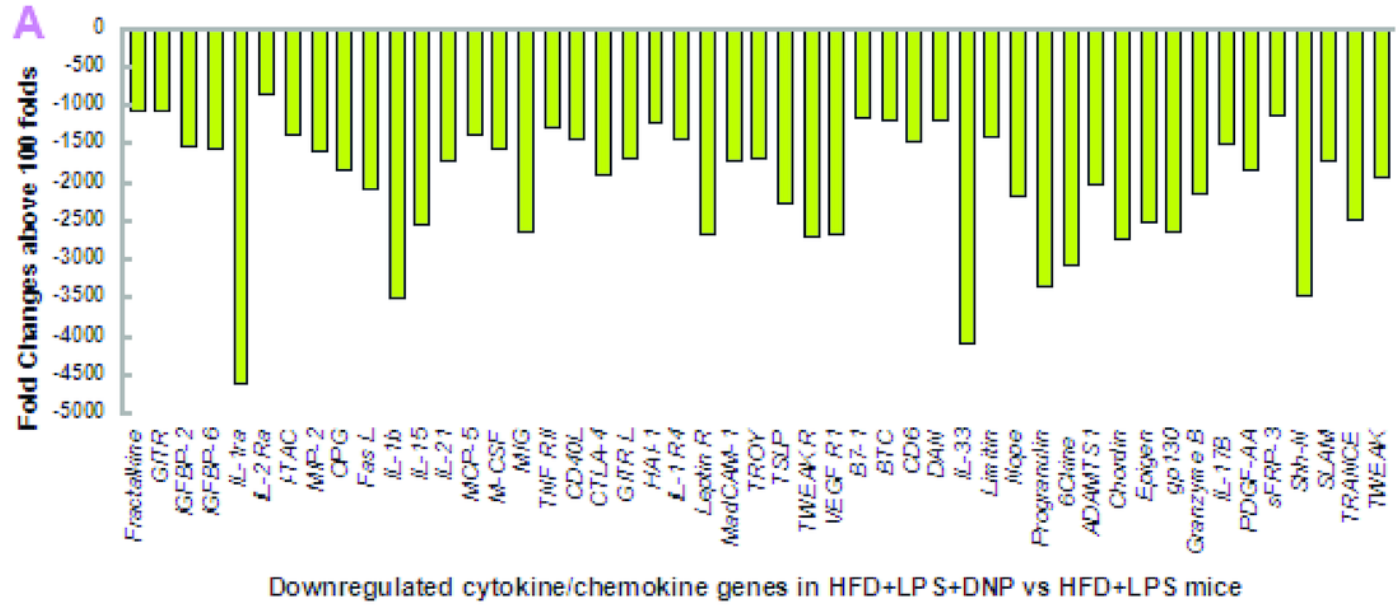
Figure 4 The expression profiling of insulin, leptin, NF-κB, CD14, and Fet A in HFD+0.25 mg/kg LPS-induced obese mice treated by 1.6 mg/kg DNP. (A) The serum INS concentration determined by ELISA; (B) The serum LEP concentration determined by ELISA; (C) The fold changes of hepatic NF-κB mRNA quantified by qPCR; (D) The hepatic NF-κB concentration determined by ELISA; (E) The hepatic CD14 concentration determined by ELISA; and (G) The hepatic Fet A concentration determined by ELISA. HFD+0.25 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for 1.5 months, during which 0.25 mg/kg LPS was intraperitoneally injected from the 5^{th} week on every two days for two weeks. The obese mice were injected into peritoneal daily by 16 mg/kg DNP for two weeks.



5

Image of downregulation

Figure 5 The fold changes of downregulated cytokines/chemokines and NALFD genes by 1.6 mg/kg DNP in HFD+0.25 mg/kg LPS-induced obese mice. (A) DNP-downregulated cytokines/chemokines in the adipose tissue of obese mice; (B) DNP-up/downregulated NALFD genes in the hepatic tissue of obese mice. HFD+0.25 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for 1.5 months, during which 0.25 mg/kg LPS was intraperitoneally injected from the 5^{th} week on every two days for two weeks. The obese mice were injected into peritoneal daily by 16 mg/kg DNP for two weeks.



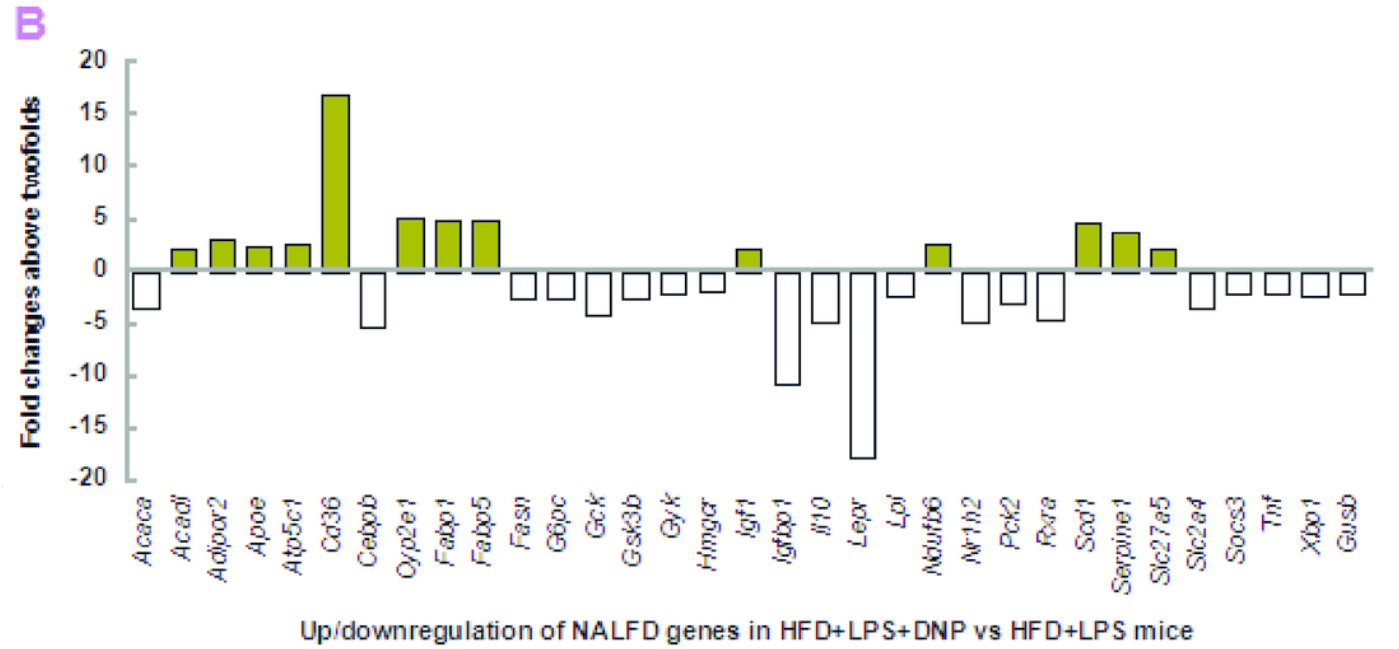


Image of mechanism

Figure 6 A putative mechanism underlying LPS-driven inflammatory obesity and DNP-exerted weight loss effects. Gut dysbiosis-induced proinflammatory cytokines can activate iNOS for high-level NO production, which results in metabolic hypoxia, adipogenesis and adipose whitening. DNP can activate both AMPK and eNOS for low-level NO release, which leads to mitochondrial biogenesis and adipose browning. The red line with the symbol + represents activation; the black line with the symbol - represents inhibition.

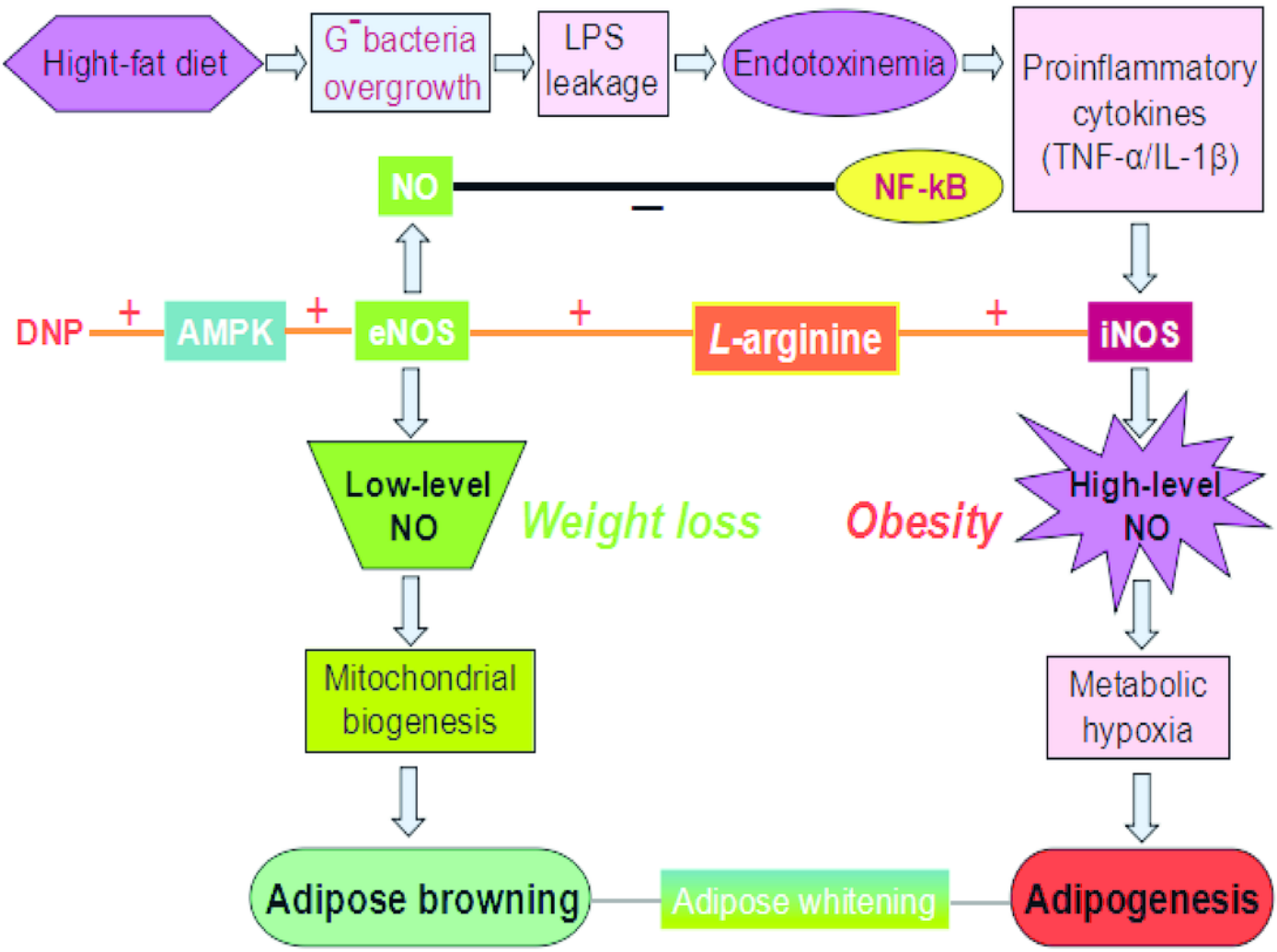


Table 1(on next page)

List of NAFLD

Table 1 Comparison of upregulated transcripts among 84 NAFLD-related pathway genes in the hepatic tissue among AL mice, HFD mice, and HFD+1.2 mg/kg LPS mice by RT-PCR array

Notes: The fold changes were calculated from the comparison of HFD+1.2 mg/kg LPS mice versus AL mice (the 1st value), the comparison of HFD mice versus AL mice (the 2nd value), and the comparison of HFD mice versus HFD+1.2 mg/kg LPS mice (the 3rd value). The 2nd values were only listed for inflammatory response transcripts and NIDDM-involved transcripts. HFD+1.2 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for two months, during which 1.2 mg/kg LPS was injected into hind-leg muscles one day before sampling. RT-PCR array was carried out after the completion of modeling.

Transcript category	Upregulated transcript	
	Gene	Fold change
Inflammatory response	*Ifng*	5.52/-5.21/-28.74
	Il1b	94.24/6.46/-14.59
	Il6	12.54/1.51/-8.33
	Il10	50.20/4.32/-11.63
	Tnf	10.24/1.15/-8.87
NIDDM-involved	*Soxs3*	7.18/1.56/-4.59,
	Xbp1	2.18/2.41/1.11
Insulin signaling pathway	*Igf1*	3.19/1.46
Adipokine signaling pathway	0	0
Metabolic pathways		
Carbohydrate metabolism	*G6pc*	3.64/72.16
	Pdk4	21.27/-5.53
	Rbp4	2.65/30.76
Fatty acid β-oxidation	*Acox1*	2.13/-1.10
	Cpt1a	2.72/-1.37
Cholesterol metabolism/transport	*Abca1*	6.48/-1.46
	Abcg1	27.10/-5.17

	Apoa1	2.83/320.07
	Apob	6.97/709.63
	Cyp2e1	34.38/24.94
	Nr1h3	3.39/-1.53
Other lipid metabolism/transport	*Acs15*	2.96/-1.36
	Acsm3	5.57/12.03
	Fabp3	2.18/-1.08
	Slc27a5	2.57/130.38
Oxidative phosphorylation	0	0
Apoptosis	*Casp3*	3.27/-1.27

1

Table 2(on next page)

List of NIDDM

Table 2 Fold changes of hepatic inflammatory response and NIDDM transcripts in HFD+low-dose LPS mice compared with AL mice

Notes: HFD+0.25 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for 1.5 months, during which 0.25 mg/kg LPS was injected into peritoneal from the 5th week on every two days for two weeks.

Transcript category	Gene	Fold change	
		Upregulated (above 2 folds)	Unchanged (-2 folds to 2 folds)
Inflammatory response	*Cebpb*		1.75
	Fas	2.00	
	Ifng		-1.78
	Il10		-1.70
	Il1b	2.64	
	Il6	2.80	
	Rxra	2.45	
	Tnf		-1.39
NIDDM	*Gck*	4.38	
	Insr	2.01	
	Irs1		1.70
	Mapk1(Erk2)		1.02
	Mapk8(Jnk1)		1.14
	Mtor	2.71	
	Pik3r1(Pi3k,P85a)		-1.12
	Slc2a2	2.44	
	Slc2a4(Glut4)		-1.25
	Socs3	3.76	
	Xbp1	3.10	

Table 3 (on next page)

List of DNP effects

Table 3 Comparison of DNP effects on the expression of NIDDM transcripts among HFD+0.25mg/kg LPS+16 mg/kg DNP mice, HFD+0.25 mg/kg LPS mice, and AL mice

Notes: HFD+0.25 mg/kg LPS mice were first fed with AL for two weeks and then fed with HFD for 1.5 months, during which 0.25 mg/kg LPS was intraperitoneally injected from the 5th week on every two days for two weeks. The obese mice were intraperitoneally injected daily by 16 mg/kg DNP for two weeks.

NIDDM transcript	HFD+LPS+DNP vs HFD+LPS	HFD+LPS+DNP vs AL
Gck	-4.29	1.02
Insr	-1.52	1.36
Irs1	-1.25	1.31
Mapk1 (Erk2)	-1.01	1.00
Mapk8(Jnk1)	-1.47	-1.32
Mtor	-1.19	2.27
Pik3ca (p110A)	1.04	1.23
Pik3r1 (Pi3k p85α)	3.4	3.08
Pklr	-1.58	-1.06
Slc2a4 (Glut4)	-3.62	-4.52
Slc2a2	-1.81	1.33
Socs3	-2.06	1.87
Tnf	-2.17	-3.08
Xbp1	-2.38	1.30

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