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2,4-Dinitrophenol Downregulates Genes for Diabetes and Fatty Liver in Obese Mice

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Abstract

Whether obesity is a disease or a risk factor of metabolic diseases including type 2 diabetes and fatty liver remains debating, we report here that a high-fat diet (HFD) alone or HFD-combined intramuscular injection with a high dose (1.2 mg/kg) of lipopolysaccharide (LPS) induces mouse peripheral noninflammatory obesity. In contrast, HFD-combined intraperitoneal injection with a low dose (0.25 mg/kg) of LPS induces mouse 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) in the dosage of 16 mg/kg was found to exert a weight-reducing effect in obese mice by compromising NF-κB-primed inflammatory responses, thereby down regulating NIDDM and NAFLD genes. Conclusively, mouse visceral low-grade inflammatory obesity that predisposes NIDDM and NAFLD can be ameliorated by DNP via anti-inflammation.

Keywords

Obesity, Metabolic Disease, Low-Grade Inflammation, 2,4-Dinitrophenol, Anti-Inflammation

1. Introduction

It has been much discrepant about the origin of inflammation in obesity. Some authors suggested that increased adipocyte O_2 consumption induces hypoxia inducible factor 1α (HIF-1α), causing inflammation and insulin resistance [1], but others supposed that hepatic and macrophage heme oxygenase-1(HO-1) drives meta-flammation and insulin resistance [2]. Although inflammation in obesity might be attributed to either an infectious or a noninfectious origin, 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 insulin resistance [3].

A putative association of gut microbiota with obesity has currently evoked much enthusiasmin recent years. It has been 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 [4]. It has been also revealed a relevance of HFD with gut dysbiosis by showing the overgrowth of *Enterobecteriaceae*, which exacerbates inflammation and obesity in mice [5]. A recent work fur-

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ther indicated that adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling, in which visceral fat is deposited for filtering the gut-derived LPS leakage [6], seemingly addressing a link between gut bacterial dysbiosis and visceral adipose depot.

We thought that intraperitoneal low-dose LPS injection could simulate trace-amount LPS leakage capable of inducing chronic inflammation, eventually leading to inflammatory obesity. In contrast, intramuscular high-dose LPS injection should resemble large-scale bacterial infection to initiate acute inflammation, thereby developing noninflammatory obesity. In human, approximately 40% - 50% of obese adults do not develop fatty liver, and the levels of inflammatory biomarkers are higher in obese subjects with fatty liver compared to BMI-matched subjects without fatty liver [7].

Based on the obese mouse models with 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 [8]. Our data available from the present study not only verified the presence of dose-dependent LPS-driven inflammatory obesity and non-inflammatory obesity, but also disclosed the anti-inflammatory response as a novel mechanism underlying DNP application to weight loss.

2. Material and Methods

2.1. 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/12h 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 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 from the 5^{th} week of HFD feeding with 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).

2.2. Quantitative Polymerase Chain Reaction (qPCR)

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

2.3. Reverse Transcription (RT)-PCR Array

The RT^2 Profiler™ PCR Array Mouse Fatty Liver (PAMM-157Z) was purchased from SAB ioscience Qiagen, Hilden, Germany, and Quantibody® Mouse Cytokine Antibody Array 4000 was purchased from Ray Biotech, Inc, Norcross, GA, USA. The experiments were performed respectively by Kangchen Biotechnology Co, Ltd, Shanghai, China, and Ray Biotech, Inc. Guangzhou, China.

2.4. Cytokine Antibody Array

Protein extraction by Cell & Tissue Protein Extraction Reagent (Kang Chen 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.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The target protein NF-κB and the reference protein GAPDH were immunoquantified by the ELISA kits manu-

factured by Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China according to manufacture's manuals.

2.6. Statistical Analysis

Statistical analyses were conducted by the one-way ANOVA method using SPSS version17.0 for Windows. All data were represented as mean ± SEM unless otherwise stated. The XY graphs and column graphs were plotted and depicted using Graph Pad Prismversion 4.0.

3. Results and Discussion

3.1. Peripheral Noninflammation in HFD-Fed Mice with Intramuscular Injection of 1.2 mg/kg LPS

As compared with AL mice, both HFD mice and HFD+ 1.2 mg/kg LPS mice become obese with the heavier body weight (50 - 60 g vs 35 g) and higher body adipose percentage (8% - 10% vs 3%) after 1.5 - 2 months. Logically, we anticipated that the systemic proinflammatory cytokines should be upregulated in obese mice. Surprisingly, we found that the expression levels of 40 cytokines and chemokines examined in obese mice are totally unchanged or even lower than those in AL mice (**Figure 1**). 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 for 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 [9], and anti-LPS antibodies can reduce the plasma LPS titers in humans [10]. A high dose of LPS was validated to be an immunosuppressor [11]. Interestingly, we have previously observed that live bacterial feeding can dramatically downregulate the proinflammatory cytokines [12].

3.2. Visceral Low-Grade Inflammation in HFD-Fed Mice with Intraperitoneal Injection of 0.25 mg/kg LPS

To avoid the possible immune depletion of LPS, we replaced the high-dose and short-term LPS exposure by the

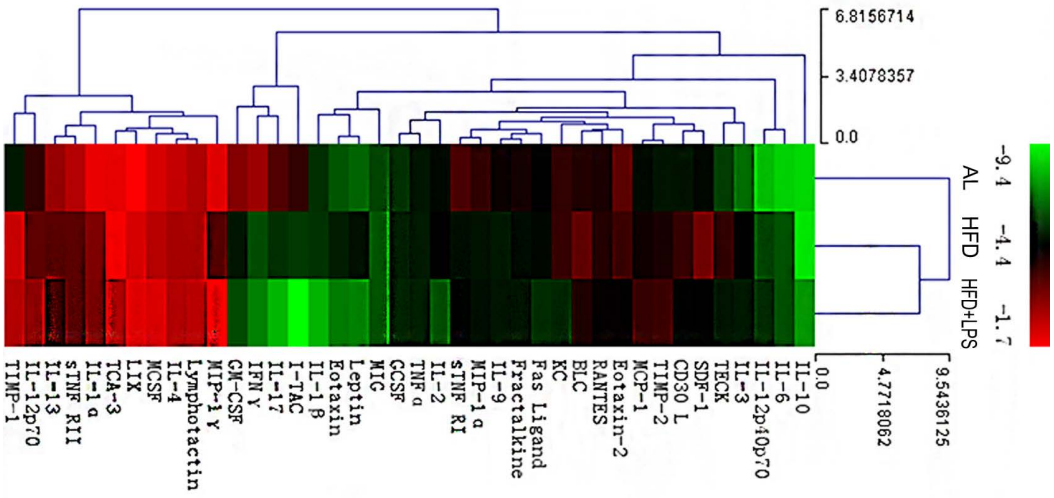

Figure 1. A schematic diagram of hierarchical clustering for the up/downregulation of 40 cytokines/chemokines in the muscular tissue of AL mice, HFD mice and HFD+ 1.2 mg/kg LPS mice by cytokine antibody array. The red color represents upregulation as compared with AL, and the green color represents downregulation 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.

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 [13].

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 (about 45 g) than AL mice (about 36 g). The hepatic inflammatory response transcripts in those obese mice exhibit the restricted upregulation for only 2 - 4 folds, which are well coincided with the definition of low-grade inflammation [14]. Importantly, there are also as many as six transcripts responsible for the noninsulin-dependent diabetes mellitus (NIDDM) showing upregulation for 2 - 4 folds in the hepatic tissue of HFD+ 0.25 mg/kg LPS mice (**Table 1**).

3.3. DNP Reduces Adipose Deposits with Downregulated NF-κB

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 relatively lower level similar with AL mice, but the body liver percentage was not significantly affected. NF-κB, a transcript factor initiating global inflammatory responses in all types of cells, can be activated by numerous external signals [15]. In our experiments, we observed a tremendous elevation of *NF-κB* mRNA in untreated mice, but an extremely decline of

Table 1. Fold changes of hepatic inflammatory responses and NIDDM transcripts in HFD+ 0.25 mg/kg LPS mice compared with AL mice[a].

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
	*Il*10		−1.70
	*Il*1*b*	2.64	
	*Il*6	2.80	
	Rxra	2.45	
	Tnf		−1.39
NIDDM	*Gck*	4.38	
	Insr	2.01	
	*Irs*1		1.70
	*Mapk*1 (*Erk*2)		1.02
	*Mapk*8 (*Jnk*1)		1.14
	Mtor	2.71	
	*Pik*3*r*1 (*Pi*3*k*, *P*85*a*)		−1.12
	*Slc*2*a*2	2.44	
	*Slc*2*a*4 (*Glut*4)		−1.25
	*Socs*3	3.76	
	*Xbp*1	3.10	

[a]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.

NF-κB mRNA in DNP-treated mice. In parallel, a high level and a lower level of NF-κB were also measured in untreated and DNP-treated obese mice, respectively (**Figure 2**).

It has been noted that NO can inhibit NF-κB [16], suggesting an anti-inflammatory effect. We have recently reported that the NO donor sodium nitroprusside and the NO precursor *L*-arginine can serve as the activators of adenosine monophosphate-activated kinase (AMPK) to triggers NO-mediated mitochondrial biogenesis [17]. 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. It should be reasonable that DNP can play a weight loss role because DNP was previously known to induce the expression of AMPK and phosphorylation of p38 MAPK [18]. Aspirin has been validated to ameliorate type 2 diabetesas anactivator of AMPK [19]. Metformin and salicylate have shown to activate liver AMPK to inhibit lipogenesis and improve insulin sensitivity [20].

3.4. DNP Downregulates Adipose Proinflammatory Cytokine Genes and Hepatic NAFLD and NIDDM Genes

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 2**.

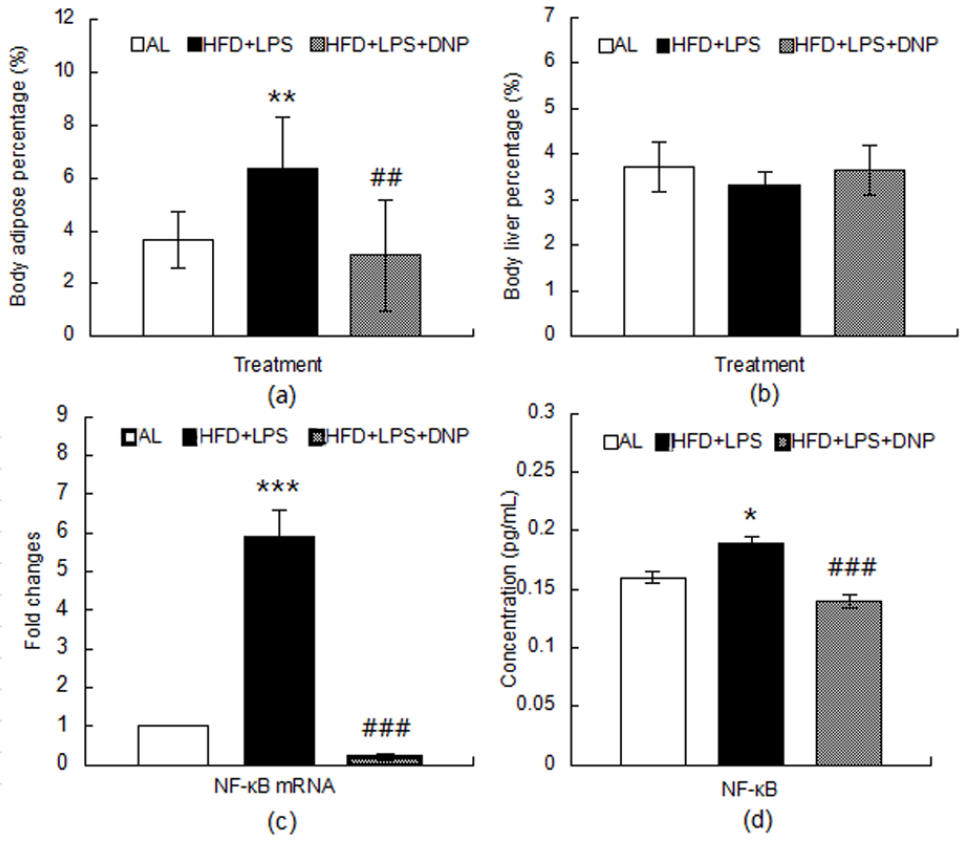

Figure 2. The phenotypic and expressional identification in HFD+ 0.25 mg/kg LPS-induced obese mice (n = 3) after treatment by 1.6 mg/kg DNP. (a) and (b) The body adipose and body liver percentages; (c) The fold changes of hepatic *NF-κB* mRNA quantified by qPCR; (d) The hepatic NF-κB concentration determined 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 5th week on every two days for two weeks. The obese mice were injected into peritoneal daily by 16 mg/kg DNP for two weeks. The asterisks (*, **, or ***) represent significant difference from AL mice at the level of $P < 0.05$, $P < 0.01$, or $P< 0.001$; and the pound signs (## or ###) represent significant difference from HFD+ 0.25 mg/kg LPS mice at the level of $P < 0.01$ or $P < 0.001$.

Table 2. Comparison of DNP effects on the expression of NIDDM transcripts among HFD+ 0.25 mg/kg LPS+ 16 mg/kg DNP mice, HFD+ 0.25 mg/kg LPS mice, and AL mice[a].

NIDDM transcript	HFD + LPS + DNP vs HFD + LPS	HFD + LPS + DNP vs AL
Gck	−4.29	1.02
Insr	−1.52	1.36
*Irs*1	−1.25	1.31
*Mapk*1 (*Erk*2)	−1.01	1.00
*Mapk*8 (*Jnk*1)	−1.47	−1.32
Mtor	−1.19	2.27
*Pik*3*ca* (*p*110*A*)	1.04	1.23
*Pik*3*r*1 (*Pi*3*k p*85*α*)	3.40	3.08
Pklr	−1.58	−1.06
*Slc*2*a*4 (*Glut*4)	−3.62	−4.52
*Slc*2*a*2	−1.81	1.33
*Socs*3	−2.06	1.87
Tnf	−2.17	−3.08
*Xbp*1	−2.38	1.30

[a]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.

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 in AL mice. For example, *Gck* mRNA is less abundant in DNP-treated obese mice than untreated obese mice, and DNP-treated obese mice have a similar *Gck* mRNA level with AL mice. Additionally, *Slc*2*a*4 (*Glut*4) 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. Besides, *Pik*3*r*1 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 [21]. So DNP-mediated upregulation of *Pik*3*r*1 mRNA would benefit to overcome insulin resistance.

It has been previously demonstrated that the chronic blockade of inducible nitric oxide synthase (iNOS) by the inhibitor *L*-NG-monomethylarginine (*L*-NMMA) reduces adiposity and improves insulin resistance in HFD-induced obese mice [22]. When iNOS is activated by inflammation, endothelial NOS (eNOS) should be inactivated due to a limited supply of the substrate *L*-arginine, and a deficiency of eNOS-derived NO should in turn block mitochondrial biogenesis [16], thereby resulting in enhanced adipose whitening and reduced energy expenditure, eventually leading to overweight/obesity. The brown adipose tissue (BAT) would be converted to the white adipose tissue (WAT) when the adipose tissue experiences progressive mitochondrial decreases [23].

Conclusively, we have successfully established an obese mouse model with visceral low-grade inflammation using HFD+ 0.25 mg/kg LPS. We also revealed for the first time that the well-known mitochondrial uncoupler DNP can exert the effects of anti-inflammation for weight loss except for the function of mitochondrial uncoupling.

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