Abstract

Many studies have reported the causes of obese metabolic syndrome (MS); however, the causes of nonobese MS (NMS) remain unknown. In this study, we demonstrated that inflamed dysfunctional adipose tissue plays a crucial role in cholesterol-induced NMS. Control (C), high cholesterol (HC) and HC with 10% fructose in drinking water (HCF) diets were fed to Sprague–Dawley rats for 12 weeks. After 12 weeks, the body weights of the C- and HC-fed rats were comparable, but the weights of the HCF-fed rats were relatively low. Cholesterol caused metabolic problems such as high blood pressure, hypercholesterolemia and hypoinsulinemia. The HCF-fed rats exhibited whole-body insulin resistance with low circulating high-density lipoprotein levels. Increases in the tumor necrosis factor-α level in the plasma, the number of CD68+ macrophages and the free nuclear factor-κB level in gonadal white adipose tissue (gWAT) resulted in local inflammation, which appeared as inflamed dysfunctional gWAT. Reduced superoxide dismutases (SODs) deteriorate natural antioxidant defense systems and induce reactive oxygen species in gWAT. Dysregulation of plasma levels of catecholamine, adipokines (leptin and adiponectin), hormone-sensitive lipase and perilipin in cholesterol-induced inflamed adipose tissue contributed to increased lipolysis and increased circulating nonesterified fatty acids. Cholesterol activated inflammation, lipolysis and cell death in 3T3-L1 adipocytes. Moreover, Chol-3T3-CM reduced the population of M2-type Raw264.7 macrophages, indicating that the macrophage polarization is mediated by cholesterol. Together, our findings indicate that inflamed dysfunctional adipocytes are critical in NMS, supporting the development of anti-inflammatory agents as potential therapeutic drugs for treating NMS.
Introduction

Obesity is associated with several complications, including diabetes, hypertension and cardiovascular disease (CVD) (Malnick & Knobler 2006, Poirier et al. 2006, Nguyen & El-Sera 2010). Chronic low-grade inflammation and increased macrophage infiltration are the major characteristics of hypertrophic adipose tissues, which result in obese insulin-resistant patients (Wellen & Hotamisligil 2003, Murano et al. 2008, Guerra et al. 2011). However, a proportion of obese patients are considered metabolically healthy, whereas some nonobese patients are considered metabolically unhealthy. Until now, increasing evidence has revealed that inflammation plays an important role in the pathogenesis of insulin resistance (de Luca & Olefsky 2008, Kramer et al. 2013, Phillips & Perry 2013).

White adipose tissue (WAT) is composed of lipids with a high ability to preserve triglycerides (TGs) during feeding and to release free fatty acids (FFAs) and glycerol during fasting (Guilherme et al. 2008). Therefore, the balance between stored and released lipids is exquisitely controlled by adipose tissue. Dysfunctional adipose tissue loses the function of a metabolic homeostasis regulator and promotes lipolysis. Increased circulating FFAs and ectopic lipid accumulation in the heart, skeletal muscle and liver have been reported to play a role in the development of metabolic diseases, type 2 diabetes mellitus and CVD (Guilherme et al. 2008, Hajer et al. 2008). Therefore, functional adipocytes with well-controlled lipid homeostasis and adipokine secretion are crucial in the regulation of whole-body metabolism to maintain an ideal body weight.

Obese patients have been reported to show chronic low-grade inflammation and negative effects of inflammation on adipose tissue (Tilg & Moschen 2006, Gustafson et al. 2007). However, the metabolic disturbances and inflammation status in patients with nonobese MS (NMS) remain unclear. Recently, cohort studies have reported that inflammation deteriorates the health of both obese and nonobese patients (Pajunen et al. 2011, Phillips & Perry 2013). Therefore, we suggest that inflamed dysfunctional adipocytes deteriorate metabolic homeostasis in nonobese patients.

Recent studies and our previous study have shown that dietary cholesterol promotes macrophage accumulation and inflammation in visceral adipose tissue, independent of body weight gain (Deng et al. 2008, Subramanian & Chait 2009, Pajunen et al. 2011, Chung et al. 2014). Moreover, the occurrence of hypercholesterolemia in underweight and normal-weight unhealthy individuals suggests that the cholesterol level is a crucial factor that distinguishes metabolically healthy and unhealthy conditions (Goday et al. 2016). Therefore, animals that are fed a cholesterol-rich diet provide an appropriate animal model for the molecular study of NMS. In our study, we demonstrated that inflamed dysfunctional adipocytes are critical in the pathogenesis of cholesterol-induced NMS.

Materials and methods

Ethical approval

The animal care and experimental procedures used in this study conformed to the recommendations of the Guide for Care and Use of Laboratory Animals (Eighth Edition), published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Chang Gung University in Taiwan (Approval no. CGU12-166 and CGU13-006 to Li-Man Hung).

Experimental animals and diets

Male, 8-week-old Sprague–Dawley (SD) rats (weight, 250–300 g) were purchased from BioLASCO (Taipei, Taiwan). The animals were maintained in a climate-controlled facility under a 12-h light–darkness cycle with water and food ad libitum. The animals were randomly divided into three groups and fed either a regular chow diet (C, Labdiet 5001), high-cholesterol diet (Harlan Teklad 2018 plus with 5% coconut oil, 4% cholesterol and 1% cholic acid) or an HC diet with 10% fructose in drinking water (HCF) for 12 weeks. The primary compositions and energy contents of the C and HC diets are listed in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Plasma biochemistry

After 8 h of fasting, blood samples were collected from the animals by tail amputation and gently mixed with heparin. The samples were subsequently centrifuged at 9300 g for 10 min. Total cholesterol, triglycerides (TGs), high-density lipoprotein (HDL) and nonesterified fatty acids (NEFAs) were measured using commercially available kits (Randox reagent kits; Randox Laboratories, Antrim, UK). The insulin level was measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia; Uppsala, Sweden). Blood glucose was measured using One
Touch Sure Step strips (Life Scan, Johnson and Johnson, Milpitas, CA, USA). Plasma leptin and adiponectin levels were measured using ELISA, according to the General Sandwich ELISA protocol (ThermoFisher; [https://www.thermofisher.com/tw/zt/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/general-elisa-protocol.html](https://www.thermofisher.com/tw/zt/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/general-elisa-protocol.html)). The following antibodies and recombinant proteins were used: recombinant rat adiponectin (4903-25, BioVision), rabbit antiadiponectin antibody (AB3269P, Merck Millipore), biotinylated antiadiponectin/Acrp30 antibody (BAF3100, R&D Systems), recombinant rat leptin (400-21, PeproTech, Rocky Hill, NJ 08553 USA), chicken antileptin antibody (AB3521, Merck Millipore) and biotinylated antileptin antibody (500-P185GBt, PeproTech). The circulating tumor necrosis factor (TNF) α level was measured using a commercial kit (rat TNFα ELISA kit, R&D Systems, RTA00).

**Mean blood pressure measurement**

The animals were anesthetized with inactin (100 mg/kg plus urethane, 0.3 g/kg, i.p.). The core temperature was maintained at 37°C by using heating pads (TC-1000 temperature controller; CWE). A microtip pressure-volume catheter (SPR-838; Millar Instruments, Houston, TX, USA) was inserted into the right common carotid artery for measuring the mean blood pressure (MBP).

**Euglycemic–hyperinsulinemic clamp (EHC)**

At the end of the study, the rats were anesthetized with pentobarbital (65 mg/kg, i.p.) and subjected to an euglycemic–hyperinsulinemic clamp (EHC) with tracer dilution, as described previously ([DeFronzo et al. 1979, Meszaros et al. 1987, Smith et al. 1987, Hsieh 2004, Deng et al. 2008]).

**Histological analysis and CD68+ macrophage staining**

Gonadal white adipose tissue (gWAT) was fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), dehydrated, embedded in paraffin and sectioned (6–8 μm). The slides were subjected to hematoxylin and eosin (H&E) and CD68+ macrophage staining by using a standard protocol with deparaffinized, rehydrated antigen retrieval and quenching by xylene, ethanol, potassium PBS (KBP; KH₂PO₄ : 0.45 g, K₂HPO₄ : 3.81 g, and NaCl : 9 g in 1L of ddH₂O, pH 7.4), citrate buffer (10 mM C₆H₇O₂₋, 0.05% Tween 20, pH 6.0) and 0.3% H₂O₂. After H₂O₂ quenching, the slides were washed three times with KPBS and incubated with primary mouse antirat CD68 antibody (MCA341GA, AbD Serotec, NC, USA) for 24 h at 4°C. Secondary biotinylated antimouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) was subsequently added and incubated for 1 h. For visualization, avidin–biotin peroxidase complexes (Vector Laboratories) were added with 3.3-diaminobenzidine tetrachloride (Vector Laboratories).

**Western blotting**

HNTG buffer was used to extract the gWAT lysate, according to a previously published protocol (HNTG buffer: HEPES, 50 mM; NaCl, 150 mM; glycerol, 10%; Triton X-100, 1%; pH 7.4). Na₂VO₄ (2 mM), NaF (20 mM) and phenylmethanesulfonyl fluoride (1 mM) were added in the buffer as phosphatase and protease inhibitors before use ([Cao et al. 2002, Inoue et al. 2006]). The crude homogenate was transferred to an Eppendorf tube and rotated at 4°C for 2 h for further protein extraction. The samples were centrifuged at 15,700 g at 4°C for 20 min. The fat cake was removed from the top, and the supernatant was transferred to a new Eppendorf tube. The gWAT lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20, washed and blotted with antibodies. Information on the antibodies is listed in [Supplementary Table 2](#). The membrane was then incubated with horse radish peroxidase-conjugated secondary antibody before chemiluminescence detection (Pierce). ImageJ (RRID:SCR_003070, NIH, Bethesda, MD, USA) was used to quantify the blotting results.

**gWAT superoxide anion measurement**

The level of superoxide anions in gWAT was measured through modified lucigenin-enhanced chemiluminescence. The chemical specificity of this light-yielding reaction for superoxide anions was determined as previously described ([Hung et al. 2002]).

**Reverse transcription polymerase chain reaction**

The total RNA was extracted from rat gWAT with TRIzol (Life Technologies) according to the manufacturer’s protocol. The target gene primers are as follows:
Leptin: Forward-GATGACACAAAAACCCTCAGT, Reverse-GCCACACCTCCTGTGGAGTAG.
Adiponectin: Forward-TGTTCTCCTTAATCCTGCCCC, Reverse-CCACAACCTGCACAGTTCCCT.
Actin: Forward-TGGAATCCTGTGGCATCCATGAAAC, Reverse-TAAAACCGAGCTCAAGTACCGTG.
Expected size: leptin, 353bp; adiponectin, 104bp; and actin, 349bp.

3T3-L1 cell culture

3T3-L1 preadipocytes were purchased from FIRDI (Hsinchu, Taiwan) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine calf serum in an atmosphere containing 5% CO₂ at 37°C according to the ATCC-recommended protocol. The cells (P15 to P17) were cultured in MDI medium (high glucose DMEM containing 10% fetal bovine serum (FBS), 0.5 mM iso-butylmethylxanthine (I7018, Sigma), 1 μM dexamethasone (D4902, Sigma) and 10 μg/mL insulin (Novo Nordisk A/S, Actrapid)) for 2 days. The culture medium was subsequently replaced with insulin medium (high glucose DMEM containing 10% FBS and 10 μg/mL insulin) for another 6 days. All media contained 1% penicillin–streptomycin (100 U/mL). Cholesterol (C3045, Sigma) stock solutions were prepared with 100% ethanol (0.1, 0.3, 1, 3, 10 and 30 mg/mL). The cells were then cultured with cholesterol-containing medium (1% ethanol in the final culture medium) for 72h. After 72h, the medium was collected and labeled as 3T3-L1 conditioned medium (Chol-3T3-CM) for a Raw264.7 cell polarization test. The cell viability was assessed using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay.

Oil red staining

The lipid content of the 3T3-L1 cells was determined using oil red staining. Briefly, the cells were fixed in 4% formaldehyde and incubated for 1 h in a 0.7% oil red solution (in 1,2-propanediol) with gentle agitation. The cells were subsequently washed with 85% 1,2-propanediol and PBS. The stained oil red was extracted with isopropanol and measured spectrophotometrically at 490 nm by using a microplate spectrophotometer (BioTek Instruments).

Real-time quantitative PCR

Total RNA was extracted from the 3T3-L1 cells after supplying different doses of cholesterol using the TRI reagent (Sigma, T9424). To avoid genomic DNA contamination, the RNA samples were treated with DNase I (Invitrogen, 18068-015). Complementary DNA was synthesized using the ToolScript MMLV RT kit. The mRNA levels were quantitatively assessed using SYBR-green-based RT-qPCR with gene-specific primers (Table 1) in a 7500 Fast Real-Time PCR System (Applied Biosystems). As an internal normalization control, 18s ribosomal RNA was used.

Inflammatory cytokine and glycerol levels in the 3T3-L1 culture medium

The levels of MCP-1, TNF-α, IL-1β, IL-6 and glycerol were measured in the 3T3-L1 culture medium after treatment with different doses of cholesterol for 3 days by using commercial glycerol assay (GY105, Randox Laboratories, Antrim, UK) and ELISA kits (Boster Bio, mouse MCP-1 Picokine ELISA kit EK0568; mouse TNF-α Picokine ELISA kit EK0567; mouse IL-1β Picokine ELISA kit EK0567; mouse IL-1β Picokine ELISA kit EK0567; mouse IL-1β Picokine ELISA kit EK0567; mouse IL-1β Picokine ELISA kit EK0567) according to the manufacturers’ instructions.

Flow cytometric analysis of Raw264.7 M1-type and M2-type surface markers

Polarization of Raw264.7 macrophages by Chol-3T3-CM was performed through flow cytometry. In brief, Raw264.7 cells were purchased from FIRDI (Hsinchu, Taiwan), seeded at a density of 2 × 10⁴/dish in a 6-cm dish and cultured in 25% Chol-3T3-CM and 75% DMEM (containing 10% FBS) in an atmosphere containing 5% CO₂ at 37°C for 3 days.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
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<tr>
<td>Rn18s</td>
<td>NR_003278.3</td>
<td>CGA GCC GCC TGG ATA CC</td>
<td>CTC AGT TAT TTT TGA AACA</td>
<td>76</td>
</tr>
<tr>
<td>Mcp1</td>
<td>NM_011333.3</td>
<td>CAG CCA GAT GCA GAT AAT AC</td>
<td>GGC TCA CAC TCA GAT CAT CTT</td>
<td>73</td>
</tr>
<tr>
<td>Tnfα</td>
<td>NM_013693.3</td>
<td>GCC TCA CAC TCA GAT CAT CTT</td>
<td>CCA TCA GAT CAT CTT</td>
<td>61</td>
</tr>
<tr>
<td>Il1b</td>
<td>NM_008361.4</td>
<td>ACT CAT TGT GGC TGT GA GA</td>
<td>TTG TTC ATC TCG GAG CCT GT</td>
<td>199</td>
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<td>Il6</td>
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<td>TTG TGC TCT AGC CAC TCC TTC</td>
<td>76</td>
</tr>
<tr>
<td>Casp3</td>
<td>NM_001284499.1</td>
<td>CAG CCA ACC TCA GAG AGA CA</td>
<td>ACA GCC CCA TTT GTC CCA TA</td>
<td>190</td>
</tr>
</tbody>
</table>
The cells were harvested and washed two times with ice-cold PBS. The cells were then stained using antimouse F4/80-FITC (eBioscience, 11-4801-82, as a pan-macrophage marker), antimouse CD80-APC (eBioscience, 17-0801-82, as a M1-macrophage marker) and antimouse CD206-PE-Cy7 (eBioscience, 25-2061-82, as a M2-macrophage marker) antibodies in ice-cold PBS containing 2% FBS and 0.09% sodium azide for 1 h on ice. After staining, the cells were washed two times with ice-cold PBS containing 2% FBS and analyzed using the Attune NxT Acoustic Focusing flow cytometer (Thermo Fisher Scientific). Unstained Raw264.7 cells were used as the negative control.

Statistical analysis

The sample size of experiments were determined empirically based on previous studies and listed in the respective figure captions. Data are expressed as means±S.E.M. Significance was analyzed using Student’s unpaired t-test or one-way analysis of variance (ANOVA) followed by Tukey’s test. Two-tailed $P<0.05$ was considered significant.

Results

General metabolic characteristics of rats after HC or HCF feeding

Eight-week-old male SD rats were fed C, HC or HCF diets for 12 weeks. The body weights of HC- and C-fed rats were comparable. However, the body weights of HCF-fed rats had decreased (Table 2). The food intake of the average HC-fed rat was lower than that of the average C-fed rat; however, the calorie intakes were similar for all three groups because of the increased fructose water intake of HCF-fed rats compensated for their lower food intake. The MBP was elevated in both HC- and HCF-fed rats. Both HC and HCF diets had no effect on blood glucose but resulted in hypoinsulinemia after 12 weeks, particularly in HCF-fed rats. Elevated plasma cholesterol and reduced plasma TG were observed in cholesterol-rich diet groups. However, plasma HDL had declined in HCF-fed rats, indicating severe metabolic disturbance and higher CVD risk because of the cholesterol–fructose combination. Altogether, these data indicated that rats fed a cholesterol-rich diet constitute an ideal animal model for studying the pathogenesis of NMS.

Table 2  General metabolic characteristics of rats after HC or HCF feeding for 12 weeks.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>HC</th>
<th>HCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>544±13</td>
<td>531±13</td>
<td>506±13**</td>
</tr>
<tr>
<td>Food intake (g/rat/day)</td>
<td>23.4±1.2</td>
<td>20.5±1.1</td>
<td>16±0.7***</td>
</tr>
<tr>
<td>Calorie intake (Kcal/rat/day)</td>
<td>79±4</td>
<td>83±3.3</td>
<td>76±4</td>
</tr>
<tr>
<td>Water intake (ml/rat/day)</td>
<td>47.6±3.1</td>
<td>42.2±2</td>
<td>61.3±5.6*</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>105±5</td>
<td>122±5*</td>
<td>127±5*</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>75±1.7</td>
<td>80±1.6</td>
<td>79±1.6</td>
</tr>
<tr>
<td>Insulin (µg/L)</td>
<td>0.65±0.13</td>
<td>0.41±0.03</td>
<td>0.3±0.04**</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>92±3.3</td>
<td>204±10.1**</td>
<td>198±13.5**</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>65±5.9</td>
<td>45±3.3*</td>
<td>50±5.3**</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>39.5±4.7</td>
<td>35.2±7.6</td>
<td>17.4±3.5**</td>
</tr>
</tbody>
</table>

Data are expressed as means±S.E.M. (n=8–10 rats per each group) Significant differences between subject and C is given as *, $P<0.05$; **, $P<0.01$, ***$P<0.001$. HDL, high-density lipoprotein; MBP, mean blood pressure; TG, triglyceride.
the key mediators of the insulin-dependent anabolic metabolism of cell growth and differentiation (Fig. 1D and E and Supplementary Fig. 1A and B). However, the p-p38 level seemed similar after cholesterol feeding. Notably, although the adipocyte size was similar in cholesterol-fed rats, a crown-like structure, the hallmark of adipose tissue inflammation, was observed compared with the control rats (Fig. 1C). Therefore, we suggest that cholesterol-induced macrophage infiltration plays a crucial role in the development of local inflammation, which may further block insulin sensitivity and impair adipocyte function.

Expression of energy-regulatory proteins
Cholesterol-rich diets have no effects on adiposity in adipose tissue expansion and adipocyte enlargement; however, the expression of energy-regulatory proteins was examined to understand energy homeostasis after cholesterol alone or with fructose feeding (Fig. 1F and Supplementary Fig. 1C). ACC1 and FAS are key lipogenic enzymes in the regulation of de novo lipogenesis. HC slightly increased ACC1 protein expression but markedly reduced the FAS level. By contrast, HCF upregulated both

Figure 1
Cholesterol-rich diets induced whole-body insulin resistance and impaired insulin signaling. Insulin sensitivity index, GIR/insulin (GIR, glucose infusion rate) (A). Liver and fat pad (gWAT, gonadal white adipose tissue; sub fat, subcutaneous fat; B fat, brown fat) weight (B). Appearance of the liver and hematoxylin and eosin (H&E) staining of the liver (magnification: 400×) and gWAT (magnification: 200×). Arrows indicate crown-like structure (CLS) (C). Expression of insulin-related downstream proteins: p-AKT–Ser473, total AKT (D); p-ERK–Thr202/Tyr204, ERK, p-JNK–Thr183/Tyr185, JNK, p-p38–Thr180/Tyr182 and p38 (E) in gWAT. Expression of energy-regulatory proteins: ACC1, FAS, GLUT4, p-AMPK–Thr172 and AMPK in gWAT (actin was used as a normalization control) (F). The representative image is from one mouse from each treatment. ImageJ was used to quantify the Western blotting results, and all the data are expressed as the mean ± s.e.m., n = 5 or 6 per group (*P<0.05 vs control; #P<0.05 vs HC).
ACC1 and FAS, clearly indicating the lipogenic potency of dietary fructose. The blood glucose levels were normal in the three groups; however, the plasma insulin levels observed in the HC- and HCF-fed rats were lower than those observed in the C-fed rats. The GLUT4 expression levels were decreased in the adipose tissues of HC- and HCF-fed rats, showing that a reduced GLUT4 level might be involved in the development of dysfunctional adipose tissues. AMPK and its activated form, p-AMPK-Thr172, regulators of the energy status responsible for maintaining cellular energy homeostasis, were reduced in cholesterol-fed rats. The excessive plasma levels of NEFAs in the HC- and HCF-fed rats (Table 3) were consistent with our hypothesis that cholesterol-induced dysfunction of adipose tissues is involved in the pathogenesis of NMS.

Epinephrine and norepinephrine play a role in cholesterol-induced lipolysis

The expression of PPARγ, the key activator of adipocyte differentiation and adipogenesis (Sewter et al. 2002), was investigated after HC and HCF feeding. The PPARγ expression levels were comparable in the gWAT in the three groups, indicating that the presence of cholesterol alone or with fructose does not affect adipogenesis (Fig. 2B, and Supplementary Fig. 1D), which is consistent with the similarity in adipocyte size in the three groups. However, a previous study reported that cholesterol may trigger lipolysis (Bhattacharyya et al. 1989), and catecholamines have been reported to enhance lipolysis after exercise and fasting (McMurray & Hackney 2005, Duncan et al. 2007). Therefore, we next examined whether catecholamines are involved in cholesterol-mediated lipolysis. We found higher levels of plasma catecholamines in the HC- and HCF-fed rats than those in the C-fed rats (epinephrine in HCF-fed rats and norepinephrine in HC- and HCF-fed rats) (Fig. 2A), demonstrating that catecholamines play a role in cholesterol-mediated lipolysis. Furthermore, the expression level of HSL, a regulator of the initial step of triglyceride hydrolysis (Kershaw et al. 2007), was upregulated in the HC- and HCF-fed rats (Fig. 2B) compared with the C-fed rats; this might explain the excessive release of FFAs. Contrastingly, the expression of perilipin, the lipid droplet-associated protein, was reduced in the gWAT of HC- and HCF-fed rats (Fig. 2B).

Cholesterol triggers inflamed dysfunctional gWAT

Macrophage infiltration induces proinflammatory cytokines, and chronic inflammation is widely considered as a causative mediator of insulin resistance and lipolysis. Therefore, we analyzed the CD68+ cell content, which is a specific marker of macrophages, to validate whether macrophages are involved in cholesterol-mediated inflammation. As shown in Fig. 3A, several CD68+ cells were recruited in the gWAT of HC- and HCF-fed rats. Furthermore, the plasma levels of the proinflammatory cytokine TNF-α was markedly increased in the HCF-fed rats (HC, P = 0.09 with an increasing trend, Fig. 3B). To further evaluate the inflammatory status in gWAT, we measured NFκB-related signal expression, the most important factor for inflammation in gWAT. NFκB and p-IκB proteins (p-IκB–Ser32/36) were highly expressed in gWAT, whereas IκB expression was reduced in cholesterol-fed rats (Fig. 3C, and Supplementary Fig. 1E). These results revealed that freely activated NFκB may translocate into nuclei and initiate inflammatory responses in gWAT. In addition, cholesterol reduced Cu/ZnSOD and MnSOD, antioxidant enzymes that prevent attack from reactive oxygen species (ROS) (Fig. 3D, and Supplementary Fig. 1F), which further resulted in the elevation of ROS because of the loss of the antioxidant ability (Fig. 3E). Altogether, macrophage infiltration, NFκB signaling activation and impaired SOD expression all contribute to the inflamed condition of gWAT. Adipose tissue is the primary site of energy storage and serves as an endocrine organ. It secretes adipokines, such as leptin and adiponectin, to regulate metabolic homeostasis. Dysfunctional adipose tissue impairs adipokine production. As shown in Table 3, cholesterol-rich diets, particularly HCF, upregulated plasma leptin but downregulated plasma adiponectin. Abnormal levels

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Plasma NEFA, leptin and adiponectin after HC or HCF feeding for 12 weeks.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.78±0.04</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>5±1.56</td>
</tr>
<tr>
<td>Adiponectin (μg/mL)</td>
<td>5.5±0.9</td>
</tr>
</tbody>
</table>

Data are expressed as means±S.E.M. (n=8–10 rats per each group) Significant differences between subject and C is given as *P<0.05; **, P<0.01, ****, P<0.001. NEFA, non-esterified fatty acid.
Pathogenesis of nonobese metabolic syndrome

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Printed in Great Britain
Published by Bioscientifica Ltd.

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of adipokines in gWAT were confirmed through RT-PCR (Fig. 4A and B, and Supplementary Fig. 2A and B). Collectively, the results suggest that cholesterol-rich diets cause inflammatory dysfunction of gWAT in NMS.

**Figure 3**
Cholesterol-triggered inflamed gWAT and loss of SOD-mediated ROS defense ability. Immunohistochemical staining of CD68-positive macrophages in gWAT under 200×; arrows indicate CD68-positive cells (A). Plasma TNF-α level (B). NFκB, p- IκB–Ser32/36, and IκB (C); CuZnSOD and MnSOD protein expression (D). The representative image is from one mouse from each treatment. Actin was used as a normalization control, and ImageJ was used to quantify the Western blotting results. Gonadal WAT (gWAT) ROS level (E). Values are expressed as mean ± s.e.m., n=5–6 per group (*P<0.05 vs control).

**Cholesterol increases lipolysis in differentiated 3T3-L1 adipocytes**

Our previous results demonstrated that cholesterol causes inflammatory dysfunction of gWAT in SD rats. Thus, we verified whether cholesterol can directly impair the function of 3T3-L1 adipocytes by measuring their lipid content. Well-differentiated 3T3-L1 cells (with lipid droplets) were treated with various doses of cholesterol for 3 days (Fig. 5A). As shown in Fig. 5B, cholesterol
treatment directly reduced the lipid droplet number and size, suggesting that cholesterol has a lipolytic effect. To further investigate whether cholesterol could trigger lipolysis, oil red staining and a glycerol assay were performed. (Glycerol is a lipolytic product, and the glycerol assay is the most direct method for assessing the extent of lipolysis.) As shown in Fig. 5C, D and E, cholesterol treatment reduced the lipid content of adipocytes and simultaneously increased the glycerol concentration in the 3T3-L1 culture medium, providing strong evidence of cholesterol-mediated lipolysis in 3T3-L1 adipocytes.

Cholesterol triggers inflammation and cell death in 3T3-L1 adipocytes

Inflammation plays a key role in lipolysis in adipocytes; thus, the expression levels of inflammatory cytokine genes were determined using RT-qPCR for evaluating the inflammatory status of the cells after cholesterol treatment. As shown in Fig. 6A, the mRNA levels of inflammatory cytokines (MCP-1, TNF-α, IL-1β and IL-6) increased after cholesterol treatment. The level of the apoptosis trigger caspase 3 mRNA was also upregulated. These results revealed that inflammation caused by cholesterol plays a critical role in triggering lipolysis, which is independent of macrophage infiltration. Moreover, the augmentation of inflammatory cytokines (MCP-1, TNF-α and IL-6) due to cholesterol in the 3T3-L1 culture medium (Fig. 6B, C, D and E) may exert a deleterious effect on the cells through a paracrine or an endocrine action. The viability of cells was evaluated after cholesterol treatment by using the MTT assay. Cholesterol dose-dependently triggered 3T3-L1 cell death (Fig. 6F and G), demonstrating the lethal effects of cholesterol on 3T3-L1 adipocytes.

Figure 4
Cholesterol dysregulates the mRNA expression of leptin and adiponectin in inflamed gWAT. Increased leptin (A) and decreased adiponectin mRNA expression (B) in gWAT after cholesterol treatment for 3 days (n=5 per group). Data are expressed as mean ± s.e.m. (*P<0.05 vs control).

Figure 5
Cholesterol reduces the lipid content of 3T3-L1 adipocytes. Well-differentiated 3T3-L1 cells were treated with various concentrations of cholesterol (control, vehicle, 1, 3, 10, 30, 100, and 300 µg/mL). Before the cholesterol treatment, the lipoid droplet size and number were similar in all groups (A). After the treatment for 3 days, the lipoid droplet size and number decreased dose-dependently (B). Oil red staining of cholesterol-treated 3T3-L1 cells (C). Lipid content was quantified at 490 nm through spectrophotometry (D), n=4 per group. Glycerol, the lipolytic product concentration in the 3T3-L1 cell culture medium, n=8 per group (E). Values are expressed as the mean ± s.e.m. (*P<0.05 vs vehicle).
Chol-3T3-CM reduces the polarization of Raw264.7 to M2-type macrophages

Macrophage polarization affects the inflammatory status during metabolic disorders. Therefore, the extent of Raw264.7 macrophage polarization was assessed through flow cytometry after Chol-3T3-CM incubation (pan-macrophage marker: F4/80; M1 marker: CD80; and M2 marker: CD206). Chol-3T3-CM-treated Raw264.7 cells (R1 population) exhibited an increase in cell size and granularity (R2 population), especially in the 100 and 300 μg/mL groups (Supplementary Fig. 4), demonstrating the effect of Chol-3T3-CM on cell differentiation/polarization. Therefore, two types of double-positive (F4/80+/CD80+ and F4/80+/CD206+) macrophage subpopulations (R1 and R2) were further analyzed. Chol-3T3-CM slightly reduced the percentage of M1-type (F4/80+/CD80+) macrophages in the R1 subpopulation (V-3T3-CM: 94.6% vs 300-3T3-CM: 80.9%), whereas it showed no effect on the R2 subpopulation (V-3T3-CM: 99.7% vs 300-3T3-CM: 98.1%) (Fig. 7A and B). However, Chol-3T3-CM considerably reduced the percentage of M2-type (F4/80+/CD206+) macrophages.
Figure 7
Cholesterol reduces the polarization of Raw264.7 to M2-type macrophages. Raw264.7 macrophages were cultured in 25% Chol-3T3-CM (control, vehicle, 1, 3, 10, 30, 100 and 300 µg/mL) and 75% DMEM (containing 10% FBS). The cells were then analyzed through flow cytometry to detect the polarization of Raw264.7 macrophages. Percentages of F4/80+CD80+ macrophages in the R1 (A) and R2 subpopulations (B). Percentages of F4/80+CD206+ macrophages in the R1 (C) and R2 subpopulations (D). A schematic that illustrates the pathogenesis of how cholesterol-rich diet triggers NMS. TNFR, TNF-α receptor; βAR, β adrenergic receptor (E).
macrophages in the R1 (V-3T3-CM: 35.3% vs 300-3T3-CM: 7.9%) and R2 subpopulations (V-3T3-CM: 87.5% vs 300-3T3-CM: 31.2%). The Chol-3T3-CM-induced reduction in M2-type macrophage polarization might contribute to the abrogation of the regulation of inflammation and exacerbate metabolic disorders.

**Discussion**

Studies have described that inflammation may cause metabolic disease in both obese and nonobese patients (Kramer et al. 2013, Phillips & Perry 2013). Therefore, it is important to understand that either obesity or inflammation can be critical in the regulation of insulin resistance and MS. In the present study, we elucidated the role of inflamed dysfunctional adipose tissue in cholesterol-induced NMS. According to MS criteria, HCF-fed rats exhibited MS with insulin resistance, elevated blood pressure and low HDL. However, HC and HCF diets reduced plasma TG, consistent with studies that reported lower plasma TG after dietary cholesterol intervention in wild-type and ApoE-null mice (Wouters et al. 2008, Obama et al. 2011). Adipokine levels were also dysregulated in HC- and HCF-fed rats.

Insulin/AKT/mitogen-activated protein kinase (MAPK) signaling regulates insulin sensitivity. Defective activations of AKT and MAPK/ERK are potential contributors to insulin resistance (Zhang et al. 2011, Ruiz-Alcaraz et al. 2013). The cholesterol treatment considerably reduced the expression of p-AKT and p-ERK in the gWAT of nonobese rats (Fig. 1D and E). This result was similar to that in the gWAT of obese rats. Excessive leptin from inflamed dysfunctional gWAT may play a role in the inhibition of pre-proinsulin gene transcription and insulin secretion (Kieffer & Habener 2000). Moreover, cholesterol-triggered pancreatic β-cell death might directly reduce plasma insulin levels after consumption of a high-cholesterol diet (Zhao et al. 2010). Therefore, we speculate that, in our study, cholesterol simultaneously reduced insulin sensitivity and insulin secretion. A high-fat diet enhances adiposity in individuals with hypertrophic adipocytes and hyperinsulinemia (Huang et al. 2017). By contrast, the HC and HCF diets demonstrated catabolic reaction characteristics with lipolysis and hypoinsulinemia. Excessive levels of plasma catecholamines play a role in cholesterol-mediated lipolysis. Moreover, AMPK and p-AMPK downregulation might be involved in the lipolysis process. Downregulation of AMPK and p-AMPK expression reduced the suppression of HSL expression, enhanced lipolysis in adipose tissue and increased the rate of FFA release into the circulation. Excess circulating metabolic substrates (e.g., glucose and FFAs) have been reported to impair AMPK activity (Kraegen et al. 2006, Saha et al. 2010). Therefore, increased levels of plasma FFAs might abrogate AMPK activity, initiating a vicious cycle.

Cholesterol is an essential component of various cell types and is a precursor of all steroid hormones. It is also responsible for normal body functions (Ikonen 2008). Therefore, the physiological functions of cholesterol mainly occur inside different cells rather than in the plasma. Excessively high levels of cholesterol in the plasma are considered to cause CVD and to be a component of MS. However, the molecular-level understanding of the negative effects of cholesterol in patients with MS is limited. The ATP-binding cassette transporters (ABCA1 and ABCG1) are responsible for cholesterol and lipid efflux to the serum and HDL release from cells (Weibel et al. 2014). Low expression levels of ABCA1 increase cholesterol and triglyceride storage in adipose tissues, thereby enlarging the fat pad and increasing body weight (de Haan et al. 2014). However, ABCA1 expression is induced by cholesterol loading and decreases after cholesterol removal (Lawn et al. 1999, Santamarina-Fojo et al. 2001). Therefore, ABCA1 expression induced by high levels of cholesterol may be involved in the pathogenesis of dysfunctional adipose tissue.

The induction of impaired glucose tolerance, insulin resistance, hyperinsulinemia, hyperlipidemia and hypertension by dietary fructose has been extensively studied (Elliott et al. 2002, Basciano et al. 2005). The liver is the main organ involved in metabolizing fructose and rapidly synthesizing TG through a significantly enhanced rate of de novo lipogenesis (Basciano et al. 2005). The liver weight of the HCF-fed rats was considerably higher than that of the HC-fed rats, indicating TG accumulation in the liver after fructose intervention; eventually a disturbance in the energy balance between the liver and adipose tissue was observed. The weight of gWAT and the size of adipocytes from HC and HCF are similar. Farina et al. have demonstrated that fructose-rich diet (FRD) increases the contents of saturated fatty acids (SFAs, C16:0 and C18:0) in a hypertrophic-abdominal adipose tissue (AAT) (Farina et al. 2013). In addition, FRD also decreases mono-unsaturated (MUFA, C16:1) and polyunsaturated (C18:3 n-3 and C20:4 n-6) fatty acids (PUFAs) (Farina et al. 2013). Moreover, FRD increases fatty acids release from AAT (Farina et al.
Our previous article has also demonstrated the fructose-induced hypertrophic adipocytes (Huang et al. 2017). Therefore, the levels of SFA, MUFA and PUFA in HCF may play a role in the induction of hypertrophic adipocytes. By contrast, the lipolytic factors from cholesterol (increased oxidative stress, inflammation and elevated HSL protein level) may counteract the fructose-mediated lipogenesis and trigger the inflamed dysfunctional adipose tissue. HCF-induced NMS was reported previously (Deng et al. 2008). Rabbets fed a high-cholesterol diet exhibited pathological features of inclusion body myositis, which could result in muscular dystrophy and weakness (Chen et al. 2008). Based on growing evidence, we suggest that the inflammation from cholesterol-induced muscular dystrophy causes body weight loss in HC- and HCF-fed rats.

We observed that cholesterol intervention induces inflammation, independent of the body weight. Our animal and 3T3-L1 cell experiments are the first to address the effects of cholesterol on NMS and clearly demonstrate that inflammation, lipolysis, adipocyte death and macrophage shifting are involved in cholesterol-induced NMS, as illustrated in Fig. 7E. This study provides novel insight, indicating the potential for developing therapeutic strategies for treating NMS with cholesterol-lowering and anti-inflammatory agents.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JME-17-0194.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This study was supported by a research grants from Chang Gung Memorial Hospital (CMRPD1F0381, CMRPD1A0203 and CMRPD1C0463), Chang Gung University (EMRPD1D0881) and the Ministry of Science and Technology (MOST 104-2320-B-182-001 and MOST 104-2320-B-182-015) of Taiwan to Dr Li-Man Hung.

Author contribution statement
J P Huang designed and performed experiments and wrote and edited the manuscript. P S Hsieh and C C Chang contributed to data collection. S C Hsu and Y J J Meir provided experimental materials and instruments and contributed to the discussion. K H Chen and J K Chen contributed to the discussion. L M Hung directed the study and wrote and edited the manuscript. All authors have approved the submitted version of the manuscript.

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Received in final form 15 March 2018
Accepted 26 March 2018
Accepted Preprint published online 26 March 2018