ORIGINAL RESEARCH COMMUNICATION

Biphasic Response of Mitochondrial Biogenesis to Oxidative Stress in Visceral Fat of Diet-Induced Obesity Mice

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Abstract

Aims: Studies in skeletal muscle demonstrate a strong association of mitochondrial dysfunction with insulin resistance (IR). However, there is still a paucity of knowledge regarding the alteration of mitochondria in adipose tissue (AT) in the pathogenesis of IR in obesity. We investigated the mitochondrial biogenesis in visceral fat (VF) and subcutaneous fat (SF) in C57BL/6J mice fed a high-fat high-sucrose diet for 12 months. **Results:** Impairment of glucose tolerance and insulin sensitivity developed after 1 month of the diet and was associated with a prompt increase of VF. The VF adipocytes were larger than those in the SF and had increased expressions of HIF-1 α and p-NF κ B p65. However, the alteration of mitochondrial biogenesis did not occur in the early stage when increased intracellular reactive oxygen species (ROS), mitochondrial oxygen consumption rate, and mitochondrial ROS emerged at the 1st, 2nd and 2nd month, respectively. Until the 6th month, the VF had markedly increased mitochondrial DNA content and expression of PGC-1 α , Tfam, ATP5A, and MnSOD. This increase of mitochondrial biogenesis was followed by a generalized decrease at the 12th month and the mitochondrial morphology altered markedly. In the late stage, although mitochondrial ROS decreased, the increased expression of 8-OHdG in VF continued. **Innovation and Conclusion:** These data suggest that IR and ROS production occur before the biphasic changes of mitochondrial biogenesis in AT, and the VF plays a more crucial role. *Antioxid. Redox Signal.* 20, 2572–2588.

Introduction

DIABETES AND ALL TRAITS of metabolic syndrome (MtS) are risk factors for cardiovascular diseases (CVD) (17, 20, 41, 53). Although the original conceptualization of this syndrome was based on insulin resistance (IR), the pathogenesis is not clear (41).

Studies in skeletal muscle have demonstrated a strong association of mitochondrial dysfunction with IR and type 2 diabetes (T2DM) (16, 35, 36). The increased intramyocellular lipid

content in insulin-resistant individuals has been attributable to an inherited defect in mitochondrial oxidative phosphorylation (36). It has been demonstrated that there are coordinated reductions of genes of oxidative metabolism in muscle biopsies from humans with IR/T2DM (35). These studies suggest that mitochondrial dysfunction may be a cause of IR. However, recent animal studies have demonstrated that mitochondrial alterations do not precede the onset of IR but result from increased reactive oxygen species (ROS) production in muscle in diet-induced diabetic mice (5, 19). Furthermore, mitochondrial

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Innovation

The conceptualization of metabolic syndrome (MtS) was based on insulin resistance (IR). Visceral fat (VF) excess, a trait of MtS, correlates with cardiovascular risk and mortality. Mitochondria are the power plant and also the source of oxidative stress in the cells. A strong association of mitochondrial dysfunction with IR has been demonstrated in skeletal muscle. However, there is a paucity of knowledge regarding mitochondria alteration in adipose tissue (AT) in the pathogenesis of IR. This study suggests that IR and oxidative stress occur before the biphasic changes of mitochondria in AT, and the VF plays a more crucial role.

ROS production is a common feature of many cellular models of IR. When exposed to agents that reduce ROS production, IR in these cellular models is rapidly reversible (19). In our study of mitochondrial DNA (mtDNA) copy number of peripheral leukocytes in people with glucose dysregulation, the mtDNA content is correlated with degree of hyperglycemia, but not IR (58), suggesting the changes of mtDNA biogenesis were secondary to the emergence of hyperglycemia. These findings call into question the causal relationship between IR and mitochondrial metabolism.

However, there remains still a paucity of knowledge regarding the alteration of mitochondria in adipose tissue (AT) in the pathogenesis of IR in obesity. Accumulating evidence suggest that deranged adipocyte metabolism and altered body fat distribution are important determinants of IR (3, 12, 40, 45, 49). Visceral fat (VF) excess correlates with cardiovascular risk factors (13, 28, 51) and is independently associated with mortality (24). On the contrary, peripheral fat may even have a protective effect (39, 62). Fat accumulation in an insulin-sensitive tissue is an important determinant of its sensitivity to insulin (16, 22, 49). Fatty liver or hepatic steatosis is no longer considered to be a benign manifestation (9, 29, 33, 46). The molecular events linking excessive fat to IR have focused on the oxidative stress and chronic inflammation in accumulated AT (11, 14, 15, 21, 38, 50, 57). Furthermore, localized AT hypoxia has been suggested to be a unified cellular mechanism for the variety of metabolic disturbance (60). Abdominally obese patients with an excess of visceral AT have elevated plasma CRP concentrations and reduced adiponectin concentrations (4, 10, 27, 37, 54). Moreover, there is evidence of macrophage infiltration in AT, which could contribute to the inflammation in obese patients (56).

The purpose of this study was to observe the mitochondrial alterations in visceral and subcutaneous AT in C57BL/6J mice fed high-fat high-sucrose diets (HFHSD) during different stages of development of IR and diabetes. The results of this study may shed light on the association and a possible causal relationship between mitochondria, IR, and deranged fat metabolism.

Results

Metabolic characteristics of mice under HFHSD and chow diet feeding

The metabolic characteristics of the mice are summarized in Table 1. There was a significant increase in body weight and

fasting blood sugar and impaired glucose tolerance in the HFHSD group starting 4 weeks into the diets. At the same time, there was impaired insulin sensitivity, as demonstrated by the intraperitoneal insulin tolerance tests (IPITT).

The HFHSD group had significantly greater VF and subcutaneous fat (SF), as compared with the chow diet (CD) group, since 4 and 8 weeks of the diet, respectively. The ratio of VF to SF was stable in the CD group during the 12-month experiment. However, the ratio in the HFHSD group was significantly higher in the early stage (1st and 2nd month) of the disease development, indicating a more prompt response of the VF to nutrition excess.

Characteristics of adipocytes in VF and SF

The detailed cell sizes of the visceral and subcutaneous adipocytes are shown in Figure 1.

Figure 1A shows hematoxylin and eosin (H&E) staining of the AT. Figure 1B compares the cell size of SF and VF adipocytes between CD and HFHSD mice during the experiment. The cell size of both VF and SF adipocytes in the HFHSD group became larger than that in the CD group starting 2 months into the diet. Within the HFHSD group, the cell size of VF adipocytes was found to be larger than that of SF adipocytes starting 2 months of the diet. Within the CD group, VF adipocytes were found to be larger than SF adipocytes at the 12th month of the diet. Figure 1C summarizes the changes of cell size in different AT over the course of the experiment. In the CD group, the cell size of both SF and VF adipocytes did not increase until the 12th month of the diet. In the HFHSD group, the cell size of both VF and SF adipocytes increased starting 2 months into the diet. Besides the alteration of cell sizes of the adipocytes, AT hypoxia and the resulting chronic inflammation in SF and VF were compared. Figure 2A shows the immunofluorescence expression of hypoxia-inducible factors 1 α (HIF-1 α) and p-NF κ B p65 in AT. Figure 2B shows the western blot analysis of the protein levels of HIF-1 α and p-NF κ B p65 over the course of the experiment. Figure 2C compares the expression of HIF-1 α in VF and SF between CD and HFHSD mice. Figure 2D compares the expression of p-NF κ B p65/NF κ B ratio in VF and SF between CD and HFHSD mice during the course of the experiment. Expression of HIF-1 α was more evident in the VF of HFHSD mice than CD mice at 2 and 6 months of the diet. Expression of HIF-1 α was more evident in the SF of HFHSD mice than CD mice at 6 months of the diet. Similarly, a markedly increased expression of p-NF κ B p65 was detected in the VF of HFHSD mice at the 2nd and 6th month and in the SF at the 6th month of the experiment.

Changes of intracellular and mitochondrial ROS in AT

Figure 3A shows the fluorescence expression of dichlorofluorescein (DCF) in frozen sections of AT under laser confocal microscope. Figure 3B compares the intracellular steady-state level of ROS in VF and SF between CD and HFHSD mice. The intracellular steady-state level of ROS in both VF and SF increased significantly in HFHSD mice, compared to CD mice, starting 2 months into the diets. Within the HFHSD group, there was higher intracellular steady-state level of ROS in VF than in SF starting as early as in the 1st month. Within the CD group, there was no difference of intracellular steady-state level of ROS between VF and SF

TABLE 1. METABOLIC CHARACTERISTICS OF MICE UNDER HIGH-FAT HIGH-SUCROSE DIET AND CHOW DIET FEEDING

Animal body weight (g)																
	1 month			2 month			6 month			12 month						
CD	24.3±0.6			25±1.1			32.6±2.0			45.6±1.6						
HFHSD	28.7±2.8			32.8±3.9			46.7±5.6			61±6.2						
p-value	0.00			0.00			0.00			0.00						
Animal body fat (volume measured by micro-CT, mm ³)																
	1 month			2 month			6 month			12 month						
	VF	SF	VF/SF	VF	SF	VF/SF	VF	SF	VF/SF	VF	SF	VF/SF				
CD	831±96	747±73	1.1±0.0	962±13	819±75	1.2±0.1	1995±249	1864±287	1.1±0.0	5905±489	5029±573	1.2±0.1				
HFHSD	1370±157	961±103	1.4±0.1	2948±587	1885±234	1.5±0.1	6758±1301	6689±965	1.1±0.1	8440±386	7433±363	1.1±0.0				
p-value	0.04	0.19	0.01	0.03	0.01	0.05	0.00	0.00	0.05	0.00	0.00	0.40				
IPGTT (blood glucose measured by the glucose meter, mg/dl)																
	1 month			2 month			6 month			12 month						
	0 min	60 min	120 min	0 min	60 min	120 min	0 min	60 min	120 min	0 min	60 min	120 min				
CD	86±11	360±30	186±8.2	95±5.5	411±44	241±19	130±26	330±37	228±33	169±42	339±107	362±145				
HFHSD	102±3.5	377±25	243±21	137±25	502±143	333±88	178±30	409±76	352±107	217±40	679±186	561±216				
p-value	0.03	0.45	0.00	0.02	0.19	0.04	0.00	0.01	0.01	0.04	0.03	0.15				
IPITT (mean percent of baseline blood glucose)																
	1 month (%)				2 month (%)				6 month (%)				12 month (%)			
	0 min	30 min	45 min	60 min	0 min	30 min	45 min	60 min	0 min	30 min	45 min	60 min	0 min	30 min	45 min	60 min
CD	100	59±21	51±18	53±28	100	58±11	42±11	34±06	100	63±21	55±24	43±15	100	69±27	46±14	32±10
HFHSD	100	86±11	80±04	72±03	100	68±07	51±08	43±05	100	95±20	92±34	83±26	100	84±14	66±13	64±16
p-value	ND	0.06	0.02	0.22	ND	0.04	0.05	0.00	ND	0.00	0.08	0.02	ND	0.21	0.03	0.00

Data are expressed as mean±SD of 5–10 mice per group.

CD, chow diet; HFHSD, high-fat high-sucrose diet; SF, subcutaneous fat; VF, visceral fat; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test.

throughout the 12-month study. The data showed that a more prompt (in the 1st month) and greater oxidative stress occurred in VF of HFHSD mice. Figure 3C summarizes the changes of intracellular ROS in different AT over the course of the experiment. There was a trend of progressive increase of intracellular ROS in the SF of HFHSD mice, whereas an early abrupt increase of intracellular ROS was observed in the VF, reaching plateau at the 6th month and declined thereafter.

Figure 3D shows the fluorescence expression of MitoSox Red in frozen section of AT under laser confocal microscope. Figure 3E compares the mitochondrial steady-state level of ROS in VF and SF between CD and HFHSD mice. Mitochondrial steady-state level of ROS in both VF and SF increased significantly in HFHSD mice, compared to CD mice, at 2–6 months of the diets. Within the HFHSD group, higher mitochondrial steady-state level of ROS in VF than in SF was noted at the 2nd month. Within the CD group, there was no difference of mitochondrial steady-state level of ROS between VF and SF throughout the 12-month study. Figure 3F summarizes the changes of mitochondrial ROS in different AT over the course of the experiment. There was only significant increase of mitochondrial ROS in the AT of HFHSD mice. The increase of ROS in VF was earlier and greater than that in the SF in HFHSD mice.

Alterations of mitochondrial bioenergetics in AT

Alterations of mitochondrial oxygen consumption rate. Figure 4A compares the fresh AT mitochondrial oxygen consumption rate (OCR) in VF and SF between CD and HFHSD mice during 1–6 months of the experiment. The OCR in VF increased significantly in HFHSD mice, compared to CD mice, at 2, 4, and 6 months of the diets. The OCR in SF increased transiently in HFHSD mice, compared to CD mice, at the 2nd month. Figure 4B summarizes the changes of mitochondrial OCR in different AT during 1–6 months of the experiment. Within the CD group, there was no change of OCR in VF and SF. Within the HFHSD group, there was rapid and stable elevated OCR in VF during 2–6 months of the diet, whereas only a short-term increase at the 2nd month was detected in the SF.

Alterations of mitochondrial ATP content. Figure 4C compares the mitochondrial ATP content in VF and SF between CD and HFHSD mice. The ATP content in VF increased significantly in HFHSD mice, compared to CD mice, at 4 and 6 months of the diets. Within the HFHSD mice, there was more ATP content in VF than in SF at 6 month of the diet. Figure 4D summarizes the changes of mitochondrial ATP

FIG. 1. Characteristics of adipocytes in the VF and SF over the course of the 12-month experiment. (A) Microscopy image (400 $\times$) of adipose tissue in H&E stain. **(B)** Comparison of the cell size in SF and VF adipocytes between CD and HFHSD mice. **(C)** The changes of cell size in different adipose tissue over the course of the experiment. Cell size of adipocytes is analyzed by the ImageJ software. Four hundred to 500 cells are randomly selected for each treatment group. Results were obtained from three independent experiments and are expressed as mean $\pm$ SE. CD, chow diet; H&E, hematoxylin and eosin; HFHSD, high-fat high-sucrose diet; SF, subcutaneous fat; VF, visceral fat.

content in different AT over the course of the experiment. There had been increased ATP levels since the 4th month, reaching peak at the 6th month and decreased at the 12th month in the VF of the HFHSD group. There was no significant change of ATP levels in the SF and VF of CD mice and the SF of HFHSD mice.

Oxidative damage of AT

We compared the extent of oxidative damage of protein and nuclear DNA between VF and SF in both the groups. We

found the expression of protein carbonylation to be greater in VF at 2 and 6 months, and in SF at 6 months of the diet in HFHSD mice, compared to CD mice (Fig. 5A, B).

The expression of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was greater in VF at 1st, 6th and 12th months in HFHSD mice, compared to VF of CD mice. Within the HFHSD group, more DNA damage in VF than SF was detected in later stage of experiment at 6 and 12 months of the diet. Within the CD group, there was no difference in 8-OHdG expression between VF and SF throughout the 12-month study period (Fig. 5C).

FIG. 2. Expression of HIF-1 α and p-NF κ B p65 in the VF and SF over the course of the 12-month experiment. (A) Immunofluorescence staining reveals the expression of HIF-1 α (upper panel, green fluorescence), p-NF κ B p65 (middle panel, red fluorescence), and merged image of HIF-1 α , p-NF κ B p65, and cell nuclei (lower panel) in adipose tissue, represent three repeats (1000 $\times$). (B) Western blot analysis of HIF-1 α and p-NF κ B p65 during the course of the experiment. (C) Comparison of HIF-1 α in VF and SF between CD and HFHSD mice. The data are normalized to the CD group = 1 in each time point. (D) Comparison of p-NF κ B p65/NF κ B in VF and SF between CD and HFHSD mice. The data are normalized to CD group = 1 in each time point. Results were obtained from three independent experiments and are expressed as mean $\pm$ SE. HIF-1 α , hypoxia-inducible factors 1 α ; p-NF κ B p65, p-transcription factor NF κ B p65.

FIG. 3. Changes of intracellular and mitochondrial ROS in frozen sections of adipose tissue. (A) Merged image of DCF (green fluorescence) and cell nuclei (blue fluorescence) under laser confocal microscope, represent three repeats (2000 $\times$). **(B)** Comparison of intracellular steady-state level of ROS in VF and SF between CD and HFHS mice. **(C)** The changes of intracellular steady-state level of ROS in different adipose tissue over the course of the experiment. **(D)** Merged image of MitoSox Red (red fluorescence) and cell nuclei (blue fluorescence) under confocal microscope, represent three repeats (2000 $\times$). **(E)** Comparison of mitochondrial steady-state level of ROS in VF and SF between CD and HFHS mice. **(F)** The changes of mitochondrial steady-state level of ROS in different adipose tissue over the course of the experiment. Data are calculated from 100 to 200 cells randomly selected for each treatment group. Relative quantification of intensity of fluorescence is determined with the use of the ImageJ software. Data were obtained from three independent experiments, and the results are expressed as mean $\pm$ SE. ROS, reactive oxygen species.

FIG. 4. Alterations of mitochondrial bioenergetics in adipose tissue. (A) Comparison of mitochondrial oxygen consumption rate (OCR) in VF and SF between CD and HFHSD mice. (B) The changes of mitochondrial OCR in different adipose tissue over the course of the experiment. (C) Comparison of mitochondrial ATP content in VF and SF between CD and HFHSD mice. (D) The changes of mitochondrial ATP content in different adipose tissue over the course of the experiment. Data were obtained from three independent experiments and are expressed as mean $\pm$ SE.

Alterations of mitochondrial biogenesis in AT

Alterations of mtDNA content. Figure 6A compares the mtDNA content in SF and VF between CD and HFHSD mice. A marked difference of mtDNA content in the VF had been detected in the late stage of experiment. An increase of mtDNA content at 6th month, whereas a decrease of mtDNA at 12th month was observed in HFHSD mice, compared to CD mice. Within the HFHSD group, VF had more mtDNA content than SF at 2nd and 6th month but decreased mtDNA content at the 12th month. Figure 6B summarizes the changes of mtDNA content in different AT over the course of the experiment. Alteration of mtDNA content did not occur until the 6th month of the diet. There was a biphasic response of mtDNA copy number in VF of HFHSD mice—a marked increase at the 6th month followed by a decrease at the 12th month. In the VF of CD mice, only a decrease of mtDNA copy number at the 12th month was observed.

Alterations of mitochondrial biogenesis. As shown in Figure 7A and B, until the 6th month, there was a markedly increased expression of the mitochondrial biogenesis regulators, PPAR γ coactivator 1 α (PGC-1 α) and transcription factor A, mitochondrial (Tfam), and the component of the electron transport chain, ATP5A and the mitochondrial antioxidant MnSOD in the VF of the HFHSD group. This increase in mitochondrial biogenesis, however, was followed by a generalized decrease of PGC-1 α , Tfam, ATP5A, and MnSOD in the VF at the 12th month of the experiment. This biphasic response of mitochondrial biogenesis was observed mainly in

the VF; only a transient increase of Tfam at 6th month in the SF was observed in the HFHSD group.

Alteration of mitochondrial ultrastructure in AT

Figure 8 compares the alterations in the mitochondrial structure in SF and VF between CD and HFHSD mice during the course of the experiment. There were no obvious changes of mitochondrial ultrastructure in the SF and VF of CD mice throughout the experiment. In the SF and VF of baseline, the portion of cytoplasm that is near to the fat cell nucleus contains many round profiles of mitochondria with cristal cristae (Fig. 8A). Some lipid droplets were observed within the cytoplasm. In the cells of SF and VF of CD mice at 6 month of age (Fig. 8B), the ultrastructure of mitochondria and cytoplasm was similar to baseline. In the cells of SF and VF of HFHSD mice at 6 month of age, the number of membranous vesicles near-by the mitochondria appeared to be increasing (Fig. 8B). The transverse diameters of mitochondria became larger in the VF of HFHSD mice but do not reach statistical significance at 6 months of age (Fig. 8C). At 12 month of age, however, the diameter of mitochondria of VF of HFHSD mice was significantly larger than that of VF of CD mice and larger than that of SF of HFHSD mice ($p < 0.01$). In some fat cells of mice at 12 month of age, the number of small lipid droplets also increased. The degenerative changes were noted in the fat cells of VF of HFHSD mice at 12th month. Marked swelling and distortion of the mitochondria were detected (upper figure of Fig. 8D). Apoptotic body-like structures resembling disrupted nuclear material (bottom

FIG. 5. Changes of oxidative damage in adipose tissue. (A) Representative images of expression of protein carbonylation in adipose tissue. (B) Representative bar chart of oxidative protein damage determined with the use of the ImageJ software. The data are normalized to the CD group = 1 in each time point. Results were obtained from three independent experiments and are expressed as mean $\pm$ SE. (C) Comparison of oxidative DNA damage by labeling index of 8-OHdG in 500–800 adipocytes in each treatment group. Results were obtained from three independent experiments and are expressed as mean $\pm$ SE. 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

figure of Fig. 8D) and disrupted cytoplasm were frequently found associated with the inner surface of cytoplasm in many fat cells. Fragmentation of cytoplasm was also found in some fat cells (Fig. 8D).

Discussion

AT can respond rapidly and dynamically to alterations in nutrient excess and deprivation by AT remodeling (32, 52). In our diet-induced obesity mouse model, calorie overfeeding in C57BL/6J male mice rapidly resulted in increased body fat, plasma glucose levels, and IR (Table 1), suggesting an acute modulation of tissue insulin sensitivity associated with the expansion of AT. In the AT of HFHSD mice, there was higher steady-state ROS level (Fig. 3) and tissue oxidative damage (Fig. 5) compared to AT of the CD group. Furthermore, we observed a difference between SF and VF in the HFHSD group. VF expanded more rapidly than the SF, as shown by a greater VF/SF ratio in early stage of experiment (Table 1) and produced ROS earlier (Fig. 3) in the HFHSD group. Theoretically, the possible mechanism of prompt response of VF to nutrition excess may be attributed to the hypothesis of ectopic fat storage syndrome (40). The relative inability of SF to act as a protective metabolic sink and therefore a limited capacity to accommodate an increased energy influx and resulting in flow of lipid to other areas. Alternatively, VF was shown to have significantly higher rates of fractional glucose uptake per volume than SF in a

recent study using dynamic PET imaging uptake of ^{18}F -FDG at a steady-state insulin infusion (61).

We also measured the cross-sectional volume of the adipocytes in VF and SF, and VF adipocytes to be larger in the HFHSD group starting at the 2nd month (Fig. 1A, B). There was an earlier and more prominent expression of HIF-1 α and p-NF κ B p65 in VF adipocytes in HFHSD mice (Fig. 2). These results support the hypothesis that unhealthy AT expansion induces massive enlargement of existing adipocytes along with limited angiogenesis and ensuing microhypoxia (32, 52). The proinflammatory responses of NF κ B in VF may be implicated to the ultimate development of systemic IR (47, 59). Our data suggest that VF plays a more crucial role in the initiation of the modulation of metabolic response and support the clinical observation that VF excess correlates with cardiovascular risk factors (13, 28, 51) and is independently associated with mortality (24).

The early instigator of obesity-associated MtS has been proposed to be increased oxidative stress in accumulated fat, rather than liver or skeletal muscle in an animal experiment (15). In our study, increased intracellular ROS production in AT occurred immediately following the expansion of AT in the 1st month and systemic IR increased concurrently with intracellular ROS overproduction. Both phenomena are early events in this time course experiment. Following the increased intracellular ROS, mitochondrial OCR and ROS in HFHSD mice increased at the 2nd month when increased expression of HIF-1 α was detected at the same time. These findings were

FIG. 6. Alterations of mtDNA copy number in adipose tissue. **(A)** Comparison of mtDNA content in SF and VF between CD and HFHSD mice. **(B)** The changes of mtDNA content in different adipose tissue over the course of the experiment. mtDNA copy number is calculated by the threshold cycle number values of the nuclear *Tert* gene and the mitochondrial *ND1* gene in the same quantitative PCR run. The data were obtained from three independent experiments and are expressed as mean $\pm$ SE of the logarithmic transformed value of the relative mtDNA copy number. mtDNA, mitochondrial DNA.

not consistent with the results in cell suspensions of fibroblasts exposure to 24 h of hypoxia, in which HIF-1 down-regulated mitochondrial oxygen consumption (34). However, in a study of hypoxic osteoclasts, hypoxia (24 h, 2% O_2) increased ATP production and mitochondrial electron transport chain activity (30). This atypical HIF-driven pathway was proposed to be an adaptive mechanism of osteoclasts to permit rapid bone resorption. From the literature reviewed, the interaction between mitochondria and HIF-1 α may be complex and interdependent; the stabilization of HIF-1 α expression requires high rates of the TCA cycle and ROS production (18, 23, 48). In our study, an early increase of ROS may ensure HIF expression, which was, however, accompanied by increased OCR in AT. Whether it is a cell-type difference of adaptive mechanism to rapid cellular metabolic change or possibly HIF derangements to increase O_2 consumption needs further investigation.

In this study, we observed a time difference between the increase of intracellular and mitochondrial ROS. Under physiological condition, mitochondria have been proved to be the major source of cellular ROS. However, there is an independent mechanism responsible for the generation of ROS. A cell membrane-associated enzyme NADPH oxidase, activated through the PKC-dependent pathway, may contribute to oxidative stress (31). There is a recent study indicating that

NADPH oxidase-mediated triggering of inflammasome activation plays an important role in the pathogenesis of mouse glomerular injury induced by hyperhomocystinemia (1). Under stress conditions, ROS may be generated from different intracellular sources, such as NADPH oxidase. In our experiment, increase of intracellular ROS marker measured by DCFH preceded the increase of mitochondrial ROS marker (MitoSox Red). Therefore, it is rational to suggest that mitochondrial oxygen consumption and ROS production (at the 2nd month) could response slower after the initiation of NADPH oxidase-dependent generation of ROS (at the 1st month).

Changes of mitochondrial biogenesis (mtDNA content, PGC-1 α , Tfam, ATP5A, MnSOD) were late events. Although increase of intracellular and mitochondrial ROS production and AT oxidative damage was observed early in the 1st and 2nd month, the mice showed no evidence of alteration of mitochondrial biogenesis at this time. An extended HFHSD diet intervention till the 6th month, under a sustained ROS overproduction, induced a marked increase of mitochondrial biogenesis in AT. The response of increase of mitochondrial biogenesis was more obvious in VF. However, a further extension of HFHSD diet intervention until the 12th month resulted in a generalized decrease of mitochondrial biogenesis (mtDNA content, PGC-1 α , Tfam, ATP5A, MnSOD) in the VF

FIG. 7. Alterations of mitochondrial biogenesis in adipose tissue over the 12-month experiment. (A) Representative western blots of expression of PGC-1 α (92 kDa), Tfam (24 kDa), ATP5A (60 kDa), MnSOD (24 kDa), catalase (60 kDa), and β -actin protein (43 kDa). (B–F) Representative bar chart of PGC-1 α , Tfam, ATP5A, MnSOD, and catalase protein expression. The amount of detected protein is quantified by the ImageJ software and is expressed as the ratio to β -actin protein. The data are normalized to the CD group = 1 in each time point. The results are shown as mean $\pm$ SE and obtained from three independent experiments. MnSOD, superoxide dismutase 2, mitochondrial; PGC-1 α , PPAR γ coactivator 1 α ; Tfam, transcription factor A, mitochondrial.

FIG. 8. Transmission electron microscopy images of changes in mitochondrial ultrastructure in the white adipose tissue. The fat of white fat cell (fc) occupies the bulk of cell. The thin cytoplasm of fat cells envelopes the whole surface of fat. The outer surface of cytoplasm, facing the connective tissue, is covered with a thin layer of basal lamina (arrows). (A) Baseline fat cells at the beginning of experiment. Many round profiles of mitochondria (m) with cristal cristae and some lipid droplets (l) are observed within the cytoplasm. (B) Fat cells at 6 and 12 months of age. There were no obvious changes of mitochondrial ultrastructure in the SF and VF of CD mice. Progressive swelling and degenerative changes of mitochondria were detected in HFHSD mice. (C) Statistical analysis of mitochondrial diameter. (D) Signs of degenerative changes in fat cells of VF of HFHSD mice at 12th month. Marked swelling and distortion of the mitochondria are detected (upper figure). Apoptotic body-like structures (ap) are frequently found in many fat cells. Fragmentation of cytoplasm, marked by asterisks, is found in some fat cells.

despite sustained intracellular ROS production. The mitochondrial ROS and HIF-1 α overproduction disappeared along with the decreased mitochondrial biogenesis at the 12th month. These changes were supported by the alterations of mitochondrial ultrastructure under transmission electron microscope. In the VF of HFHSD mice, marked swelling and distortion of the mitochondria associated with disarrayed cristae, disrupted membrane, and apoptotic bodies were detected at 12th month of the experiment.

Our 12-month time course experiment revealed a biphasic response of alterations of mitochondrial biogenesis during the pathogenesis of nutrient-induced obesity. The changes in mitochondrial biogenesis are probably related to the oxidative stress—an initial compensatory increase to overcome the oxidative damage and followed by exhaustion once the oxidative damage progresses beyond the threshold of self-repair (25, 26, 55). Aging is not a factor to affect mitochondrial biogenesis in this experiment since the lifespan for C57BL/6J male mice is around 800 days (44). Superoxide is a fleeting molecule with a half-life that lasts no longer than minutes. However, the cellular superoxide targets with a long half-life, once modified by reactive species, can exert an altered cellular function over a prolonged time. Clinically, ROS overproduction by mitochondria has been proposed to cause the onset and progression of diabetes and its complications (6, 8, 43). In our experiment, the mitochondrial ROS overproduction decreased at the 12th month, but the increased expression of 8-OHdG in VF of HFHSD mice persisted. Therefore, metabolic memory can last for a long time after the disappearance of ROS overproduction (7). At the end of this experiment, although the increased mitochondrial ROS production is no longer detectable, the mitochondrial damage is irreversible.

Whether there is a tissue-specific difference to response to ROS between skeletal muscle and AT is not known. Bonnard *et al.* (5), studying the skeletal muscles of diet-induced obese C57BL/6J mice, found decreased mitochondrial biogenesis and mitochondrial number, and an alteration of mitochondrial structure in mice fed the HFHSD for 16 weeks. In their experiment, the decrease of mitochondrial biogenesis in skeletal muscle occurred much earlier than that in AT in our experiment. Their mice did not demonstrate an initial increase of mitochondrial biogenesis before the 16th week. However, both experiments reported that mitochondrial alterations did not precede the onset of IR and both showed that mitochondrial alterations resulted from increased ROS production in muscle and AT, in mice with diet-induced diabetes.

Furthermore, our observation that mitochondrial alterations do not precede the onset of IR is limited to the development of obesity and IR within a species with the same genetic background. A causal relationship may exist when different species or different individuals are compared. In a hepatic steatosis experiment using Otsuka Long-Evans Tokushima Fatty rats, which are a commonly studied model of obesity and T2DM, Rector *et al.* demonstrated that reduced hepatic fatty acid oxidation and mitochondrial enzyme activity preceded fatty liver development and IR (42). A higher susceptibility to IR is likely in individuals who have low mitochondrial capacity.

In conclusion, this study found that in diet-induced obese mice, IR and ROS production occur before the biphasic changes of mitochondrial biogenesis, an occurrence that may be related to oxidative stress in VF.

Materials and Methods

Animals

C57BL/6J male mice were housed five per cage in a temperature-controlled room with a 12-h light–dark cycle. They were allowed access to water and diet *ad libitum*. The experimental group was fed a HFHSD (60% kcal fat) and the control group a CD (10% kcal fat). Diet was purchased from TestDiet[®] Division of LabDiet[®]. It contained about 20% protein and met the American Institute of Nutrition requirements for mice with regard to mineral and vitamin content. Animals were maintained on these diets for 12 months.

C57BL/6J mice fed HFHSD were compared with those fed CD at 1, 2, 6, and 12 months during disease development. Blood tests for plasma sugar, plasma insulin levels, and IR index by intraperitoneal glucose tolerance tests (IPGTT) and IPITT, and body fat measurement categorized into VF and SF were checked. We compared cell size, expression of hypoxia, oxidative stress and antioxidants capacity, and mitochondrial biogenesis in adipocytes from VF and SF.

The animal protocol was approved by the Institutional Animal Care and Use Committee at the Kaohsiung Chang Gung Memorial Hospital.

Animal glucose and insulin tolerance tests

IPGTT and IPITT were performed at 4, 8, 24, and 48 weeks. All animals fasted 16 h before experiments. Mice were placed in restrainers, and blood samples were obtained by tail bleeding and analyzed by glucose meter (Abbott; Optium Xceed XCN 289-2337) immediately before and at 5, 15, 30, 60, 90, and 120 min after an IP glucose (2g/kg in 0.9% NaCl) or insulin (0.75 U/kg) injection.

Assessment of VF and SF by microcomputed tomography

Mice were scanned by rotation X-ray (50 kV, 200 μ A, and 316 ms) using a small animal imaging system (www.skyscan.be) (Skyscan-1076; Skyscan). Briefly, serial radiographic images were performed on anesthetized mice from L1 to sacrum-caudal to measure VF and SF. To capture the adipose depot, the computed tomography density range was set at the upper thresholds of 3–4 and lower thresholds of 8–10 from the 0 to 256 gray scales. VF was determined by mesenteric and retroperitoneal adipose depots. SF was determined by abdominal subcutaneous, inguinal subcutaneous, and epididymal adipose depots.

Tissue collection and extraction

On the day of the experiments, the animals were anesthetized with a muscle injection of ketamin. Blood samples were extracted from the heart, and the tissue samples were immediately isolated. They were frozen in liquid nitrogen (for mtDNA, mRNA, and protein studies) or fixed in 4% formalin (for immunohistochemistry).

Immunofluorescence analysis

The frozen sections (5 μ m) of AT were dried in air and then incubated in blocking buffer containing 1.5% normal goat serum and 0.2% Triton X-100 in phosphate-buffered saline (PBS). The slides were washed twice with PBS and then

incubated with the primary antibodies, including HIF-1 α (1:500; R&D Systems, Inc.) and p-NF κ B p65 antibody (1:100; Cell Signaling Technology, Inc.) at 4°C for overnight, followed by repeatedly washed with PBS, and replaced in secondary antibodies conjugated with Alexa 488 and Alexa 546 (1:500) for 1 h at room temperature. The slides were then washed, stained with DAPI, and washed again with PBS. The immunostained slides were observed and recorded under the fluorescence microscope (Olympus) and laser confocal microscope (Zeiss; LMS5).

Measurement of intracellular ROS

The intracellular ROS in AT was performed as previously described (63). Frozen fat tissue cross sections were evaluated by measuring the level of hydrogen peroxide (H₂O₂) using the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma). Briefly, fat tissue samples were harvested, placed in cold PBS, and embedded/snap-frozen in the tissue-freezing medium (TBS) for cryosectioning. Unfixed fat tissue was cut into 5- μ m-thick sections using a Leica CM3050 S cryostat. DCFH (10 μ M) was topically applied, and slides were incubated for 30 min in a light-protected chamber at 37°C. DCFH was removed and stained with DAPI, and the sections were coverslipped. Images were obtained in a darkened microscopy room with the fluorescence microscope (Olympus; 1000 $\times$) and confocal microscope (Zeiss; LMS5, 2000 $\times$). Images of several fields were taken for each treatment group, 100–200 cells were randomly selected, and the data acquisition and further fluorescence analysis were performed using the ImageJ software.

Measurement of mitochondrial ROS

The mitochondrial ROS in AT was performed as previously described (2). Frozen fat tissue cross sections from CD- and HFHSD-fed mice were evaluated by measuring the level of mitochondrial superoxide (O₂⁻) production quantified by MitoSox Red kit (Molecular probe, Invitrogen). Fat tissue samples were harvested, placed in cold PBS, and embedded/snap-frozen in the tissue-freezing medium (TBS) for cryosectioning. Unfixed fat tissues were cut into 5- μ m-thick sections using the Leica CM3050 S cryostat. Fat tissue were incubated with 5 μ M MitoSox Red in the culture medium for 15 min at 37°C, stained with DAPI, and then washed. Images were obtained in a darkened microscopy room with the fluorescence microscope (Olympus; 1000 $\times$) and confocal microscope (Zeiss; LMS5, 2000 $\times$). Data acquisition and fluorescence analysis were performed as described in the Measurement of Intracellular ROS section.

AT bioenergetics measurements

Measurement of mitochondrial OCR. The OCR of intact AT explants was measured using a Seahorse XF24 analyzer (Seahorse Bioscience). Briefly, freshly isolated AT was rinsed with the unbuffered Dulbecco's modified Eagle medium (pH 7.4). The AT was cut into sections, and 10 mg was placed in each well of an XF 24 Islet Capture Microplate (Seahorse Bioscience). The tissue was then covered with a screen, which allowed free perfusion while minimizing the tissue movement. Unbuffered Dulbecco's modified Eagle medium (500 μ l) was then added to each well. The plate was incubated at 37°C

in a non-CO₂ incubator for 1 h. The baseline OCR was closely monitored until the rates stabilized. At least three replicates from each animal were used for the assay.

Measurement of intracellular ATP. The intracellular ATP in AT was measured using an ATP Colorimetric Assay Kit (ATP Colorimetric Assay Kit; BioVision, Inc.) following the manufacturer's directions.

Measurement of cell size of adipocytes

Fat tissue were fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned into 3- μ m slices for H&E staining. The slides were observed and photographed under the microscope (Olympus). Images of several fields were taken for each treatment group. One hundred to 200 cells were randomly selected. Cell size analysis was carried out using the ImageJ software.

Transmission electron microscopy

Fresh fat tissue was cut into small pieces and fixed in 0.05 M phosphate-buffered glutaraldehyde, postfixed in buffered osmium tetroxide, dehydrated, and embedded in epoxy resin. Semithin sections were stained with methylene blue and examined under a light microscope. Ultrathin sections were double stained with uranyl acetate-lead citrate and examined with the JEM-1230 and JEM-2000 transmission electron microscopes (JEOL).

Quantification of mtDNA copy number by real-time PCR

mtDNA was extracted from mouse AT using QIAamp DNA Mini Kit (QIAGEN). The relative mtDNA copy numbers were measured by real-time PCR and corrected by simultaneous measurement of the nuclear DNA. The forward and reverse primers for a nuclear gene, which are complementary to the *Tert* gene, were 5'-CTAGCTCATGTGTCAAGACCCTCT-3' and 5'-GCCAGCACGTTTCTCTCGTT-3', respectively. The forward and reverse primers for mtDNA, which are complementary to the sequence of the *ND1* gene, were 5'-ACCATTTGCAGACGCCATAA-3' and 5'-TAAATTGTTTGGGCTACGG-3', respectively. The PCR was performed in a LightCycler[®] 480 System (Roche Applied Science), using the SYBR[®] Green PCR Master Mix Kit (2 $\times$) (Applied Biosystems). DNA (10 ng) was mixed with 10 μ l SYBR Green PCR Master Mix Kit containing 500 nM of forward and reverse primers to a final volume of 20 μ l. The PCR conditions were 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and primer extension at 72°C for 20 s. The melting curves analysis was performed using Dissociation Curve Software and required an additional 20 min after the real-time PCR. The amplified products were denatured and annealed at different temperatures to detect their specific melting temperatures. Samples showing primer dimers or unspecific fragments were excluded. The threshold cycle number (Ct) values of the *Tert* gene and the mitochondrial *ND1* gene were determined for each in the same quantitative PCR run. Each measurement was performed at least three times and normalized in each experiment against a serial dilution of a control DNA sample. Ct values were used as a measure of the input copy number, and Ct value differences

used to quantify mtDNA copy number relative to the *Tert* gene were calculated as follows: relative copy number (R_c) = $2^{(\Delta C_t)}$, where ΔC_t is the $C_{t\text{ }Tert} - C_{t\text{ }ND1}$. Good reproducibility was found both within and between runs. The intra-assay coefficients of variation of C_t values were around 2.1% and 3.4% for *ND1* and *Tert* gene, respectively. The interassay coefficients of variation of C_t values were around 4.5% and 5.1% for *ND1* and *Tert* gene, respectively.

Western blot analysis

AT was homogenized and the proteins were dissolved in the Protein Extraction reagent (Termo Scientific). Primary antibodies included HIF-1 α (Sigma Aldrich Co.), NF κ B (Sigma Aldrich Co.), p-NF κ B p65 (Cell Signaling Technology, Inc.), PGC-1 α , Tfam, ATP5A, MnSOD, Catalase (Santa Cruz Biotechnology, Inc.). Secondary antibodies included HRP-conjugated anti-rabbit and anti-mouse antibodies (Santa Cruz Biotechnology, Inc.). The amount of detected protein was quantified by the ImageJ software and was expressed as the ratio to β -actin protein.

Determination of tissue protein carbonylation

The protein carbonylation in AT was measured using an Oxyblot™ Protein Oxidation Detection Kit (Product #S7150; Millipore) following the manufacturer's directions. Fat tissue protein extracts were quantified by BCA assay kit. Ten micrograms of each sample was denatured using 12% sodium dodecyl sulfate (SDS) and derivatized by 15-min incubation with 2-4 dinitrophenyl hydrazine (DNPH; Sigma-Aldrich) at room temperature. After neutralization, samples were separated on a 10% SDS-polyacrylamide gel by electrophoresis and transferred onto nitrocellulose membranes (Biorad). Membranes were blocked with 5% nonfat dry milk in PBS containing 1% Tween 20 (PBST) and then probed overnight with primary antibody specific *versus* DNPH (Sigma-Aldrich) 1:5000 in PBST/5% nonfat dry milk. After washing with PBST, the membranes were incubated for 1 h in PBST containing the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000; Cell Signaling) and again washed. ECL (Pierce) was used to visualize the peroxidase-coated bands. Densitometric analysis was performed using the ImageJ analysis software.

8-OHdG analysis

The DNA base-modified product 8-OHdG is one of the most commonly used markers for the evaluation of oxidative DNA damage. Immunohistochemical staining of fat tissue was performed on formalin-fixed paraffin sections. Sections were deparaffinized and rehydrated. Sections were incubated with 0.3% H₂O₂ for 10 min to block endogenous peroxidases. Then, the slides were rinsed with Tris-Buffered Saline Tween-20 (TBST). Sections were placed in a covered humidified chamber with a primary antibody of anti-8OHdG (1:100 dilution; Trevigen) for 1 h at room temperature. Slides were rinsed with TBST and treated with secondary antibody (SuperPicTure Polymer Detection Kit; Zymed Laboratories) and DAB chromogen according to the manufacturer's directions. Sections were counterstained for nuclei with 10% methyl green. The labeling index of adipocytes was defined as the number of nuclei positive for staining among the cells counted

in five fields of view under microscope (200 $\times$). The total cell counts ranged from 500 to 800 in samples obtained from different individuals.

Statistical analysis

Results are expressed as mean $\pm$ standard deviation. Logarithmic transformation of data of the relative mtDNA copy number was used since the original values showed a nonnormal distribution. Between-group and within-group comparisons were performed using the unpaired and paired *t* tests, respectively. Multiple comparisons of variables among different time periods were performed with analysis of variance. All statistical operations were performed using the Statistical Package for Social Science program (SPSS for Windows, Version 17; SPSS). A $p < 0.05$ was considered significant.

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Author Disclosure Statement

The authors report no conflicts of interest.

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Abbreviations Used

8-OHdG = 8-hydroxy-2'-deoxyguanosine
AT = adipose tissue
CD = chow diet
CVD = cardiovascular diseases
DCF = dichlorofluorescein
DNPH = 2-4 dinitrophenyl hydrazine
H&E = hematoxylin and eosin
HFHSD = high-fat high-sucrose diets
HIF1 α = hypoxia-inducible factors 1 α
IPGTT = intraperitoneal glucose tolerance tests
IPITT = intraperitoneal insulin tolerance tests

IR = insulin resistance
MnSOD = superoxide dismutase 2, mitochondrial
mtDNA = mitochondrial DNA
MtS = metabolic syndrome
OCR = oxygen consumption rate
PBS = phosphate-buffered saline
PG-C1 α = PPAR γ coactivator 1 α
p-NF κ B p65 = p-transcription factor NF κ B p65
ROS = reactive oxygen species
SF = subcutaneous fat
T2DM = type 2 diabetes
Tfam = transcription factor A, mitochondrial
VF = visceral fat