ORIGINAL

Long-term treatment with hyperbaric air improves hyperlipidemia of db/db mice

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Abstract : Hyperbaric air (HBA) is used to improve healing of wounds including diabetic ulcer. The aim of this study was to clarify the effects of HBA exposure on lipid and glucose metabolism in db/db mice. HBA did not influence the weight of db/db mice. Serum levels of free fatty acid and triglyceride, but not glucose and insulin, were significantly decreased after 6 weeks of treatment with HBA. The mRNA expressions of CPT-1, PPAR*α* and PGC-1*α* genes, which are related to lipid metabolism, were significantly up-regulated in the muscle and liver. Increases in TNF*α* and MCP1 mRNA, which impaired lipid metabolism, were also attenuated by HBA treatment. These results suggest that exposure of HBA could have beneficial effects on lipid metabolism in patients with type 2 diabetes mellitus. J. Med. Invest. 57 : 224-231, August, 2010

Keywords **:** *HBA, hyperlipidemia, TNFα, MCP1*

INTRODUCTION

Type 2 diabetes mellitus is characterized by a chronic hyperglycemic state due to decreased insulin sensitivity in target tissues, including skeletal muscle, adipocytes and the liver, and/or due to the impairment of insulin secretion (1, 2). Obesity is a robustly pandemic and pathological disease and is responsible for type 2 diabetes mellitus, hyperlipidemia and hypertension (3). Increased serum levels of free fatty acid (FFA) or triglyceride (TG) deteriorate hyperglycemia through peripheral insulin resistance, finally resulting in cerebral infarction and cardiovascular disease (4, 5). Thus, in obese type 2 diabetes patients, treatment of hyperlipidemia is clinically important to prevent these commorbidities.

Hyperbaric oxygen (HBO) therapy is a therapeutic procedure that provides tissues with hyperoxygenation by inhalation of high oxygen density at a pressure of more than one atmosphere in a hyperbaric chamber (6). HBO has been utilized for the treatment of various diseases, including gas poisoning (7, 8) and autism (9). In diabetic patients, HBO

Abbreviations used
GPO : glycerol-phosphate oxidase, DAOS : sodium n-ethyl-n-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, ACS : acyl-CoA synthetase, ACOD : acyl coenzyme A oxidase, CPT-1 : carnitine palmitoyltransferase-1, PPARα : peroxisome proliferator-activated receptor α, PGC-1α : peroxisome proliferatorγ coactivator-1α, TNFα : tumor necrosis factor-1α, MCP-1 : monocyte chemoattractant protein-1, RT-PCR : reverse transcription polymerase chain reaction, C/EBP : CCAAT/enhancer-binding protein. UCPs : Uncoupling proteins. FBS : Fasting blood sugar.

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is also utilized for therapy of gangrene (10) and retinopathy (11). It has been reported that levels of blood glucose in patients with hypertension and type 2 diabetes were significantly lowered by exposure to HBO (12). In animal experiments, HBO treatment prevented an increase in blood glucose level during growth and changed the muscle type to slow twitch subtype (13-15). On the other hand, HBO treatment significantly increased blood glucose levels in type 1 diabetic rats compared with the levels in non-diabetic controls (16). However, there has been no investigation of the effects of HBO on lipid metabolism except for decreased oxidized low-density lipoprotein (17). In contrast to its various beneficial effects, HBO treatment has been shown to have serious adverse effects, including oxidative stress and oxygen poisoning, because of high oxygen concentrations (18-20).

Hyperbaric air (HBA) therapy is a therapeutic method for exposing patients to a pressure that exceeds one atmosphere while maintaining oxygen density at a normal level. It is thought that HBA treatment has less adverse effects than those of HBO treatment. Recently, an HBA chamber has been used commercial for athletes to recover from muscle fatigue. However, there have been no beneficial effects of HBA therapy for diseases such as diabetes or hyperlipidemina. In this study, using obese diabetic mice, db/db mice, we examined the effects of HBA on diabetes and hyperlipidemia.

MATERIALS AND METHODS

Animals and treatments

Six-week-old male db/db diabetic mice (n=12) and db/+m non-diabetic mice (n=12) (Japan Charles River, Kanagawa, Japan) were randomly assigned to HBA groups (n=6) and control groups (n=6), respectively. Mice in the HBA groups were exposed to 1.3 atmospheric pressure by a commercially available hyperbaric chamber (Oasis O_2, Nihon Light Service, Inc., Tokyo, Japan) for 6 hours (10 : 00-16 : 00) per day, and mice in the control groups were kept in an environment similar to that for mice in the HBA groups but at normal atmospheric pressure. Food intake and body weight were measured, and blood samples were collected from the tip of the tail vein weekly in each group before HBA exposure at 10 : 00. Blood samples were immediately centrifuged to collect serum supernatant. Serum samples were stored at -80°C until use for measurement of metabolic parameters. Mice were sacrificed 8 weeks later to obtain tissue samples of the liver, soleus muscle and epididymal fat. The tissues were immediately frozen in liquid nitrogen and stored at -80°C until used for RNA preparation. The mice were housed at a constant room temperature of 23±2°C with a 12-h light/dark cycle and were fed a normal chow diet (Oriental Yeast, Tokyo, Japan) with water *ad libitum*. This study was approved by the Ethics Committee of the University of Tokushima for Animal Studies.

Measurement of lipid parameters

Plasma TG and FFA concentrations were measured by the GPO-DAOS method and ACS-ACOD method (Wako Pure Chemical Industries, Osaka, Japan), respectively.

Quantitative real-time RT-PCR

Total RNA was extracted from the liver, soleus muscle and epididymal fat by using an RNeasy kit (Qiagen, Valencia, CA), and then total RNAs were reverse-transcribed using a Takara PrimeScript RT reagent kit (Takara, Kyoto, Japan). Quantitative real-time PCR was performed with the LightCycler system (Roche Diagnostics, Switzerland) using Takara SYBR Premix Ex Taq II (Takara, Kyoto, Japan). The following gene-specific primers were used : CPT-1a (sense : 5'-cttccatgactcggctcttc-3' ; antisense : 5'-agcttgaacctctgctctgc-3'), CPT-1b (sense : 5'-cccatgtgctcctaccagat-3' ; antisense : 5'-ccttgaagaagcgacctttg-3'), PPARα (sense : 5'-agaccctcggggaacttaga-3' ; antisense : 5'-cagagcgctaagctgtgatg-3'), PGC-1α (sense : 5'-tcacaccaaacccacagaaa-3' ; antisense : 5'-tctggggtcagaggaagaga-3'), TNF-α (sense : 5'-atggcctccctctcatcagtt-3' ; antisense : 5'-acaggcttgtcactcgaattttg-3'), MCP-1 (sense : 5'-cccaatgagtaggctggaga-3' ; antisense : 5'-tctggacccattccttcttg-3') and 18S ribosomal RNA (sense : 5'-aaacggctaccacatccaag-3' ; antisense : 5'-ggcctcgaaagagtcctgta-3'). After the PCR reaction, each PCR product was confirmed for its single amplification by analyzing a melting curve of the PCR products.

Statistical analysis

Data are expressed as means± SEM. Data were analyzed by *ANOVA* or unpaired Student's t-test. A p-value < 0.05 was accepted as statistically significant.

RESULTS

Serum FFA and TG concentrations were decreased in db/db mice after HBA treatment but not in db/+m mice.

To determine the effects of HBA on lipid and glucose metabolism in obese diabetic mice, db/db mice were exposed to HBA for 6 hours, which is the same duration as that used in a previous study in which diabetic rats were exposed to HBO (14). The food intake in the db/db mice groups was much higher than that in the db/+m mice groups. Change in body weight during a period of 8 weeks was not altered by HBA exposure in either the db/db mice groups or db/+m groups (Figure 1A). The food intake, however, was significantly increased by HBA exposure in the db/db mice but not in the db/+m mice (Figure 1B).

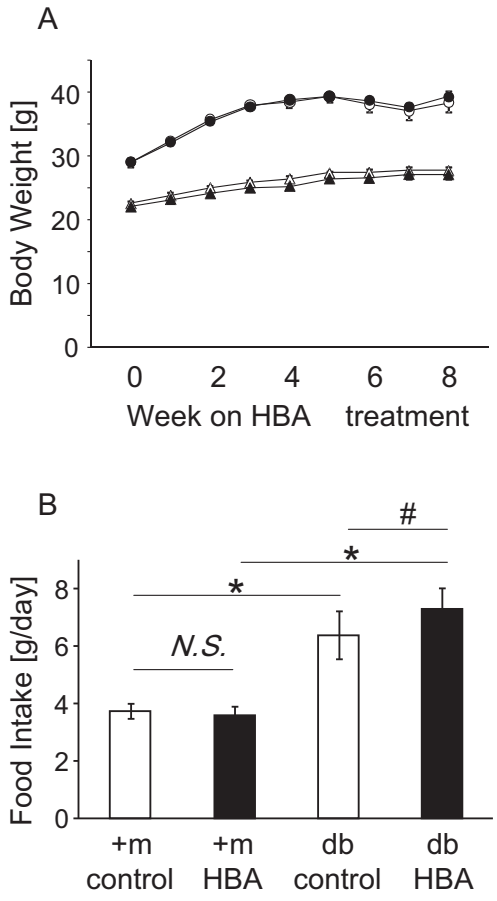

Figure 1. Body weights and food intakes of control groups or HBA groups.
The body weight (A) of db/db mice was greater than that of db/+m mice, and HBA treatment did not alter the body weight during a period of 8 weeks. Food intake (B) of db/db mice was greater than db/+m mice, and it was increased after HBA treatment. ○ control group of db mice, • HBA group of db mice, △ control group of +m mice, ▲ HBA group of +m mice. Data are means ± SEM (n=6). ** : $p < 0.05$, \# : $P < 0.01$. N.S. : no significant difference*.

The weights of the slow twitch muscle : soleus muscle, liver and fat tissues were not significantly altered by HBA exposure either in the db/db or db/+m mice (not shown). The concentration of fasting blood glucose and insulin sensitivity assessed by an oral glucose tolerance test and insulin tolerance test, respectively, were not altered significantly by HBA exposure either in the db/db or db/+m mice (Figure 2A, 2B and not shown). Interestingly, the concentrations of serum FFA and TG were significantly decreased by HBA exposure in the db/db mice but not in the db/+m mice (Figure 2C, 2D).

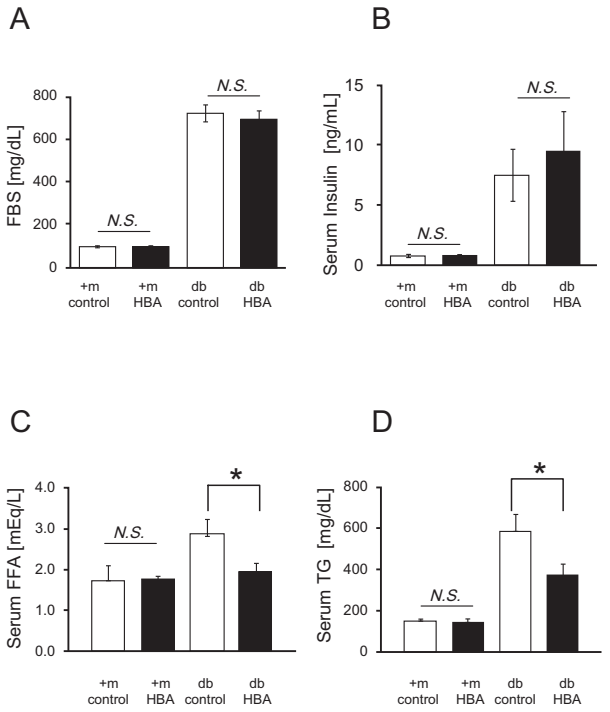

Figure 2. Serum levels FBS, Insulin, FFA and TG after HBA treatment.
Serum concentrations of FBS (A), Insulin (B), FFA (C) and TG (D) of db/db mice were greater than that of db/+m mice and these values were decreased by HBA treatment for 8 weeks. Data are means ± SEM (n=6). ** : $p < 0.05$. N.S. : no significant difference*.

The mRNA expression levels of factors involved in lipid homeostasis were increased after HBA treatment.

To clarify the mechanism underlying the effect of HBA on lipid metabolism, mRNA expression of CPT-1, a rate-limiting enzyme for β-oxidation mainly in the soleus muscle and liver, was quantified by real-time RT-PCR. As shown in Figures 3A and 3D, the mRNA expression of CPT-1 (a of liver type and b of skeletal muscle type), but not that of CPT-2

(not shown), was increased significantly by HBA exposure both in the soleus muscle and liver of db/db mice. CPT-1 mRNA expression in the soleus muscle and liver was not altered by HBA exposure in db/+m mice.

The mRNA expressions of the transcription factors PPARα and PGC-1α were examined since the former was reported to control lipid metabolism (21, 22) and the latter was reported to increase β-oxidation in brown adipocytes (23) or in skeletal muscle with enhanced mitochondria function coordinated with exercise (24), even though PGC-1α usually has roles in glucose metabolism to attribute a gluconeogenesis and mitochondria biosynthesis (25). Moreover, it has been shown that PGC-1α can cooperate with PPARα to express the genes of mitochondrial fatty acid oxidation enzymes such as CPT-1 in a hepatoma cell line (26). In the soleous muscle, mRNA expression of PPARα and PGC-1α in db/db mice was decreased significantly compared to that in db/+m mice. The mRNA expression of PPARα was increased after HBA treatment in the skeletal muscle of both db/db and db/m mice (Figure 3B). In the liver, however, the mRNA expression of PPARα was increased after HBA treatment only in db/db mice (Figure 3E). HBA treatment enhanced the mRNA expression of PGC-1α in db/+m and db/db mice (Figure 3C). On the other hand, the mRNA expression of PGC-1α was significantly greater in the liver of db/db mice than in the liver of db/+m mice. Exposure to HBA significantly enhanced the mRNA expression of PGC-1α only in db/db mice (Figure 3F).

mRNA expression levels of TNFα and MCP-1 were decreased after HBA treatment.

In adipocytes, lipolysis from fat droplets rather than β-oxidation contributes to the development of hyperlipidemia. On the other hand, adipocytes become larger by accumulating TG and become smaller by lipolysis *via* output of FFA. In this study, however, the weight of adipose tissue with HBA

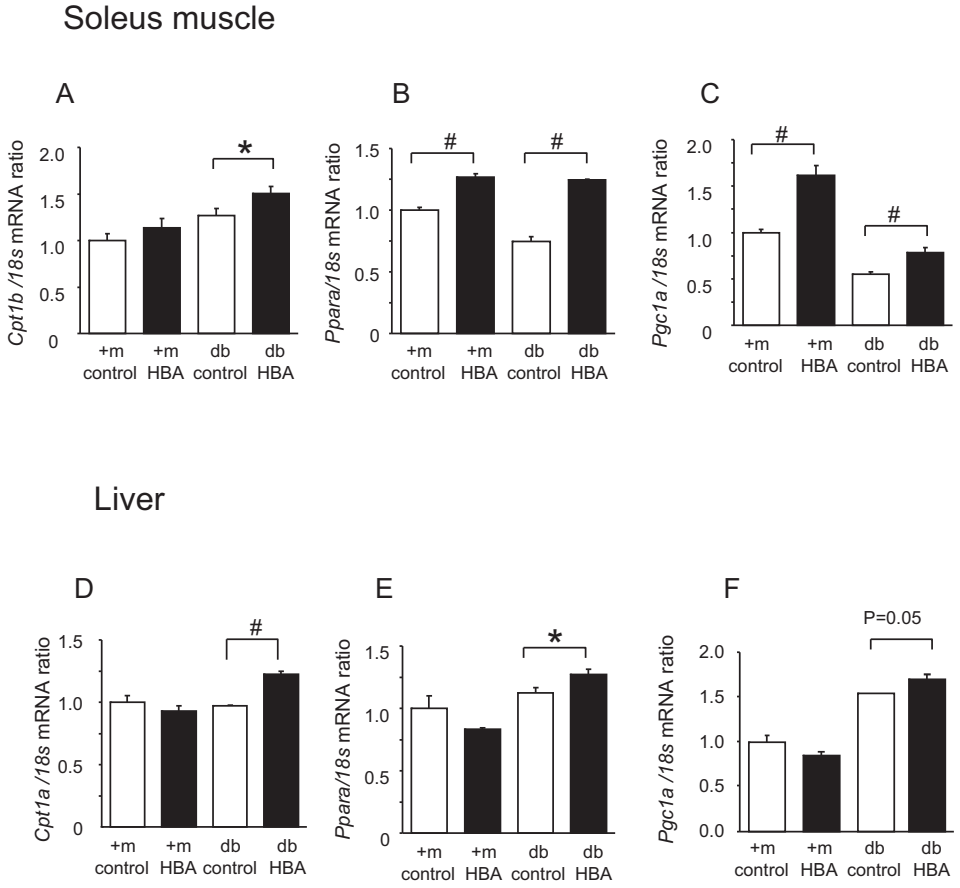

Figure 3. mRNA expression of factors involved in lipid homeostasis.
The soleus muscle and liver were obtained from db/db and db/+m mice with or without HBA exposure for 8 weeks. Total RNA isolated from these tissues was subjected to quantitative real-time RT-PCR with primers specific for CPT-1a/b (A, D) PPARγ (B, E) and PGC-1α (C, F) as described in the *Materials and Methods* section. Data were normalized by 18S ribosomal RNA (* ***: P< 0.05*** and # ***: P< 0.01***). Data are means ± SEM (n=6).

exposure, as mentioned previously, did not differ from that without HBA exposure in db/db mice (not shown). Recently, it has been reported that adipocyte inflammation in obesity causes insulin resistance and subsequently type 2 diabetes or hyperlipidemia (27, 28). HBO treatment decreases lipopolysaccharide-induced production of proinflammatory adipokines production such as TNFα and IL6 (29) without changing body weight. Therefore, we studied the mRNA expression of adipokines. As shown in Figure 4, the mRNA expression levels of TNFα and MCP-1 were significantly decreased after HBA exposure in db/db mice. The mRNA expression level of adiponectin tended to decrease after HBA exposure in db/db mice, although it did not reach a level of statistical significance (not shown).

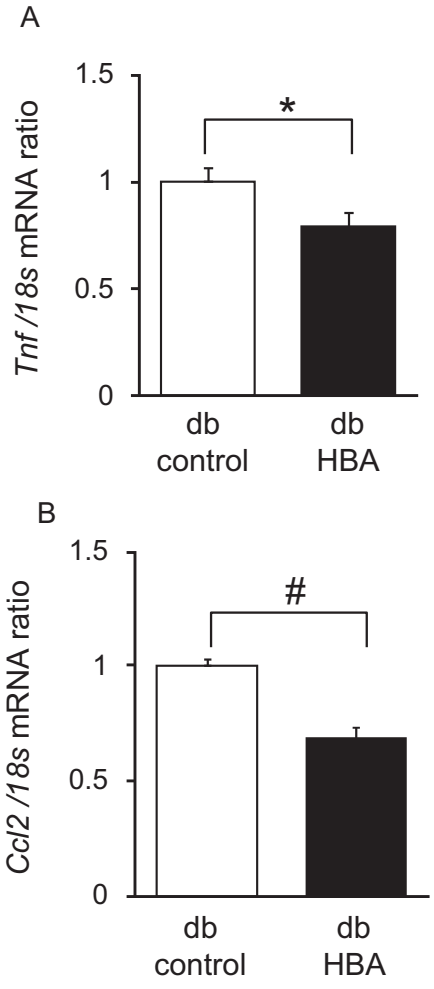

Figure 4. *mRNA expression of TNFα and MCP-1 after HBA treatment.*
Epididymal fat was obtained from db/db mice with or without HBA exposure for 8 weeks. Total RNA isolated from these tissues was subjected to quantitative real-time RT-PCR with primers specific for TNFα (A) and MCP-1 (B) as described in the *Materials and Methods* section. Data were normalized by 18S ribosomal RNA (* ***: P< 0.05*** and # ***: P< 0.01***). Data are means ± SEM (n=6).

DISCUSSION

In previous studies, HBO treatment could decrease blood glucose levels in humans (12) and rat (13, 14), but investigations with HBO were not done for hyperlipidemia. In addition, the effects of HBA on diabetes and hyperlipidemia have not been studied, either. Therefore, in the present study, obese diabetic mice, db/db mice, were used to investigate the effects of HBA on diabetes and hyperlipidemia. The results showed that HBA treatment decreased serum FFA (Figure 2C) and TG (Figure 2D) concentrations and increased mRNA expression levels of CPT-1 enzyme (Figure 3A, 3D), PPARα (Figure 3B, 3E) and PGC1-α (Figure 3C, 3F) in the liver and muscle of db/db mice. We also found that HBA treatment decreased mRNA expression levels of the proinflammatory adipokines, TNFα and MCP-1 in db/db mice (Figure 4).

The food intake was significantly increased by HBA exposure in the db/db mice (Figure 1A), but HBA had no effect of body weight in db/db mice (Figure 1B). The weight of liver, soleus muscle or epididymal fat was not changed in db/db mice with or without HBA though it was not examined the body composition of total fat or fat free mass. The mRNA of UCPs, which are important for energy expenditure, was not changed in these mice (not shown). Until now, it has been still not clear that the discrepancy of body weight and food intake.

FFA is metabolized by β-oxidation, the rate-limiting enzyme of which is CPT-1, mainly in the skeletal muscle and liver. PGC-1α with PPARα or either of them alone transcripts CPT-1 in the muscle and liver as mentioned in the results section. PPARα as a molecular target of fibrates also improves hypertriglyceridemia. Chronic adipocyte inflammation is modulated by TNFα, which increases lypolysis, finally resulting in increased level of serum FFA (28). Therefore, HBA treatment not only up-regulated mRNA of CPT-1, PGC-1α and PPARα but also decreased TNFα expression, which might consequently decrease the serum levels of FFA and TG.

HBA increases oxygen contents of the blood by about 2.5%, much less than the increase induced by HBO (30). A previous study using microarray analysis of neurons showed that HBA increases the expression levels of more genes than does normobaric oxygen (31). The genes include genes for transporters, signal transduction, growth and metabolism. Interestingly, HBA also increases the expression levels of more genes than does HBO. The

expression levels of some genes, such as C/EBP family genes, which are increased by hyperbaric air are decreased by exposure to HBO. The effects of HBA on cells are complicated and might not be the same as the effects of HBO. It is speculated that high pressure of HBA may influence the lipid metabolism. On the other hand, HBO increased parasympathetic activities in healthy volunteers (32-34) and significantly decreased cortisol levels (35). Dominance of sympathetic activities causes high FFA, because β receptor signal stimulates lypolysis. Moreover, stimulation of parasympathetic activities attenuates the increase in TNFα responded in response to inflammation (36, 37). These findings suggest that HBA increases parasympathetic activities, leading to lipid homeostasis.

Different from the results of previous studies showing that HBO had an effect on glucose metabolism (12-15), HBA treatment did not influence glucose metabolism in our experiments (Figure 2A, 2B and not shown). Tissue hypoxia (38, 39) and TNFα (40) or MCP-1 (41) induce insulin resistance, and high pressure up-regulates glycolytic genes (31). The db/db mice have a profile of severe insulin resistance with obesity unlike the GK rats used in previous studies. We speculate that HBA treatment in our experiments could not overcome the phenotype of db/db mice even though HBA might decrease insulin resistance. To clarify this possibility, effects of HBA on glucose metabolism should be tested using mice having mild phenotypes of diabetes or using a combination of antidiabetic drugs or exercise.

Taken together, the results indicate that HBA treatment might have beneficial effects on lipid metabolism in type 2 diabetes mellitus patients.

FOOTNOTE

First three authors contributed equally to this work.

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