Heat Shock Treatment with Mild Electrical Stimulation Safely Reduced Inflammatory Markers in Healthy Male Subjects

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Summary

Objective: Obesity induces chronic inflammation, which contributes to the development and progression of insulin resistance, diabetes and atherosclerosis.

We have recently shown that induction of heat shock protein 72 by mild electric current and thermo (MET) treatment in mouse model of type 2 diabetes ameliorated glucose homeostasis and insulin resistance accompanied by reduced adiposity. For clinical application of MET, we confirmed its safety in healthy subjects.

Methods: MET was applied for 10 healthy Japanese male (12V, 55 pulses per second, 30 min at 42^{o}C) twice a week for 8 weeks. Fat volume was measured by CT scan and several parameters were investigated.

Results: MET did not induce any adverse effects nor muscle contraction/pain. There were no significant alterations in glucose homeostasis or insulin resistance. Visceral and subcutaneous fat volume showed a trend of decrease without significant difference (- 3.9% and - 4.3%, respectively), which were restored 8 weeks after withdrawal of MET. Interestingly, serum tumor necrosis factor-α (TNF-α: 0.91 ± 0.05 v.s. 0.67 ± 0.06 pg/mL; p =0.006) and high sensitivity-C reactive protein (hs-CRP: 521.9 ± 73.9 v.s. 270.8 ± 43.7 ng/mL; p=0.023) levels , both of which are associated with chronic inflammation, were significantly decreased.

Conclusion: MET may be beneficial for the reduction of an inflammatory response observed in diabetes and metabolic syndrome.

KEYWORDS: obesity; heat shock protein; inflammation; mild electrical stimulation; diabetes

Introduction

Type 2 diabetes as well as obesity and metabolic syndrome are considered to be important risk factors in the development of atherosclerosis [1]. These metabolic disorders are mainly caused by excessive accumulation of visceral fat, which is associated with insulin resistance, the precursor to type 2 diabetes. Increased visceral fat also creates an atherogenic inflammatory milieu, characterized by increased tumor necrosis factor (TNF)-α, high sensitivity of C-reactive protein (hs-CRP) and other inflammatory markers [2].

Systemic inflammation accelerates the development of atherosclerosis as well as the induction of insulin resistance. Increased concentration of TNF-α found in acute and chronic inflammatory conditions (e.g., trauma, sepsis, infection, rheumatoid arthritis), is closely associated with both pro-atherogenic condition and impaired glucose tolerance [3]. Emerging experimental and epidemiologic data also link inflammation and hs-CRP to insulin resistance in that hs-CRP levels have been associated with impaired insulin sensitivity and the development of metabolic syndrome and type 2 diabetes [4]. Hs-CRP levels in the low-normal range have been shown to have predictive value for subsequent clinical events among patients with cardiovascular disease [5].

Recent studies suggest that several cellular stresses such as endoplasmic reticulum (ER) stress and oxidative stress due to metabolic disorders increase chronic inflammation and accelerate atherogenesis [6]. In animal models, ER stress signal impairs insulin signaling and pancreatic β-cell function [7, 8]. Molecular chaperones, which aid appropriate protein folding and prevents abnormal protein aggregation, are up-regulated during ER stress condition to adapt cells toward these stresses. Therefore, induction of molecular chaperone could be one of the novel therapeutic approaches to attenuate cellular stresses and to cure metabolic disorders as well as to prevent atherosclerosis [9].

We have recently demonstrated that the induction of heat shock protein (Hsp) 72, which is

one of the most important molecular chaperones, using a device which simultaneously produces both heat shock and mild electrical stimulation (MES), ameliorates glucose homeostasis and insulin resistance in mouse models of type 2 diabetes, that is accompanied by significant reduction of visceral fat mass [10, 11]. Recently, it has also been shown that induction of Hsp72 by several means (heat shock or transgenic animal or pharmacological agent) improved glucose homeostasis and insulin resistance [12, 13]. However, it is still unknown whether induction of Hsp72 in humans can prevent the accumulation of visceral adiposity and/or improve glucose homeostasis.

Before applying this technique in subjects with diabetes or metabolic syndrome, we have investigated the safety and efficacy of heat shock with MES treatment in healthy human subjects in this study

Materials and Methods

Study subjects. A total of 10 healthy Japanese male subjects who had a yearly medical checkup at Kumamoto University Hospital were recruited for the present study. This research was approved by the Ethics Review Committee in Kumamoto University (No. 154). This clinical trial was registered with an approved ICMJE clinical trial registry, UMIN (ID UMIN000001336, www.umin.ac.jp). No subjects were receiving any medical treatments. Background and characteristics of the subjects are shown in Table 1.

Mild Electrical stimulation and Thermo (MET) treatment. The devices which produce Mild Electrical stimulation + Thermo (MET) were provided by Tsuchiya Rubber Co. Ltd. (Kumamoto, Japan; Fig. 1A). Mild electrical stimulation was delivered from abdominal (positive electrode) to lower back surfaces (negative electrode) on subjects' body using a pair of 10 × 30 cm sized electro-conductive and thermo-generative rubber electrodes (Fig. 1B), which were padded with soft cotton cloth (Fig. 1B). The electrodes were connected to a MET generator that delivered 12V (55 pulses per second) of direct current with individual duration of 0.1 millisecond (Fig. 1A). Temperature at the surface of the electrode was adjusted to 42^{o}C. Ten healthy male subjects were treated with MET twice a week for 8 weeks. Each treatment with MET was carried out for 30 min. This treatment protocol in this study was designed according to our previous reports [10, 11], which detailed that Hsp72 induction was most prominent and prolonged with this optimized protocol *in vivo*.

Biochemical analysis. All the biochemical analysis of blood samples was analyzed at the clinical laboratory of the SRL, Inc (Tokyo, Japan). Plasma hs-CRP was measured by a latex-enhanced immunonephelometric assay on a BN II analyzer (Dade Behring, Hamburg,

Germany) [14]. TNF-α was measured by ELISA (Enzyme Linked ImmunoSorbent Assay).

The medical history and information on current cigarette-smoking habits were obtained using a self-administered questionnaire. Blood pressure was measured twice using a sphygmomanometer with the subject in a sitting position, and the averaged value was used in the analyses. The homeostasis model assessment of insulin resistance (HOMA-IR), an index of insulin resistance, was calculated using the method of Matthews *et al* [15]. Standard 75g-oral glucose tolerance test with measurements of glucose and insulin at baseline and 30 min intervals for 2 hours was performed. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the ethics committee of Kumamoto University (No. 154), and written informed consent was obtained from each subject.

Visceral and subcutaneous fat quantification by computed tomography (CT). The amount of visceral and subcutaneous fat were determined three times by CT scans, before the study, after the end of 8 weeks MET treatment, and another 8 weeks after MET withdrawal. The volume of visceral and subcutaneous fat was calculated using Advantage Workstation AW4.3-05 (GEMS, Tokyo, Japan).

Statistical analysis. All data are expressed as mean ± S.D. values. Statistical analyses were performed using Student's *t*-test. Probability values of $p < 0.05$ were considered indicative of statistical significance.

Results

Ten healthy Japanese male subjects were recruited for this study. The subjects' pre- and post-clinical and biological characteristics are summarized in Table. 1.

No adverse effects using MET. After 8 weeks of MET treatment, body parameters such as body weight, body mass index, % body fat were not changed significantly (Table. 1). Vital signs such as systolic blood pressure, diastolic blood pressure and heart rates were also not changed during the study (Table. 1). Therefore, no physiological adverse effects using MET were detected during and after the study. Complete blood count and major blood chemistry data also did not show any significant alterations during and after the study (Table. 1). Because only mild electrical current was used, no perceptible muscle contraction or muscle pain was generated in the subjects.

Significant reduction of TNF-α and hs-CRP. Several cytokines and inflammatory markers such as leptin, adiponectin, interleukin-6 (IL-6), TNF-α and hs-CRP were measured before and after the treatment period (Table. 1). Leptin, adiponectin and IL-6 levels after the treatment were comparable to baseline (Fig. 2A, B, C, respectively). Interestingly, the markers of chronic inflammation, TNF-α (0.91±0.05 v.s. 0.67±0.06 pg/mL, p=0.006, Fig. 2D) and hs-CRP (521.9±73.9 v.s. 270.8±43.7 ng/mL, p=0.023, Fig. 2E) were significantly decreased after MET treatment.

No evident alterations in glucose homeostasis. Fasting plasma glucose, fasting insulin and HOMA-IR were not altered during the study (Table. 1). Upon 75 g-oral glucose tolerance test (OGTT), changes in several insulin sensitivity indices such as blood glucose AUC on OGTT,

insulin AUC on OGTT (Table. 1), quantitative insulin sensitivity check index (QUICKI: 0.36 ± 0.01 v.s. 0.38 ± 0.02, p=0.52), composite whole body insulin sensitivity index (WBISI: 7.37 ± 1.22 v.s. 9.33 ± 2.57, p=0.50) failed to reach statistical significance. Insulin secretion capability after MET treatment evaluated by HOMA-β and insulinogenic index upon OGTT were also comparable to baseline (Table. 1).

A trend of reduction in visceral and subcutaneous fat mass. Linear regression analysis identified a significant correlation between waist circumference and the volume of visceral fat (Fig. 1C). 85 cm of waist size was consistent with approximately 3,700 cm^3 of visceral fat volume (Fig. 1C). Waist circumference was decreased by 1.1 cm (from 82.7 ± 10.2 to 81.6 ± 10.3 cm) after 2 months of MET treatment, but the reduction was not statistically significant. Visceral fat mass, which was detected by CT and three-dimensionally calculated, was monitored pre-, post- and after the withdrawal of MET. Individual distributions and changes were plotted (Fig. 1D). The amount of visceral fat was decreased from 3,400.5 ± 443.1 to 3,268.1 ± 459.4 cm^3. The rate of reduction was 3.9%, but was not significant (Fig. 1E). Eight weeks after withdrawal of treatment, visceral fat mass was increased to nearly the initial value of 3,425.6 ± 523.2 cm^3. Subcutaneous fat mass was also decreased from 4,464.5 ± 744.2 to 4,271.3 ± 737.1 cm^3. The rate of reduction was 4.3%, but was not statistically significant (Fig. 1F). After withdrawal, subcutaneous fat mass was also restored to near the initial value of 4,422.8 ± 782.5 cm^3. Total amount of abdominal fat mass also showed similar changes (7,865.1 ± 1,166.8 to 7,539.4 ± 1,179.7 (4.1% reduction), to 7,848.4 ± 1,287.1 cm^3), although the changes were not statistically significant.

Discussion

Accumulation of visceral fat induces cellular stresses and chronic inflammation that is implicated in many clinically important disorders, including obesity, insulin resistance, metabolic syndrome, type 2 diabetes and atherosclerosis. It now appears that, in most obese subjects, obesity is associated with a low-grade inflammation of white adipose tissue resulting from chronic activation of the innate immune system, and which can subsequently lead to insulin resistance, impaired glucose tolerance and even diabetes [16]. Thus, the association of metabolic disorders, cellular stresses and chronic inflammation has become the focus of scientific attention.

We undertook the current study to investigate the safety and efficacy of the device, which simultaneously produces heat shock and MES in healthy humans. This combination therapy showed a trend of body fat reduction and significant decreases of serum TNF-α and hs-CRP levels with no adverse effects.

Any physiological or biochemical adverse effects using MET were not detected. Since no side effects were observed either in high-fat-fed C57/BL6 mice, db/db mice [10] or normal dieted lean C57/BL6 mice (data not shown), it is suggested that this method is considerably safe in both rodents and humans.

The mechanism of beneficial effects exerted by heat shock and MES is considered to be, at least in part, due to the induction of molecular chaperone, Hsp72 [9, 12]. Hsp72 is a principal heat shock protein, which assists other proteins to fold, refold and translocate across membranes [17]. Although Hsp72 is up-regulated upon cellular stresses, Hsp72 mRNA [18, 19] as well as protein levels [12] in skeletal muscles of insulin resistant diabetic patients were decreased. In other aspects, Hsp72 also has the ability to protect cells from cell death against myocardial re-perfusion [20] and brain ischemia [21]. In this context, proper induction of Hsp72 may be a novel therapeutic strategy to treat such diseases including diabetes and

metabolic syndrome. Indeed, Hsp72 induction by several means can improve insulin resistance and glucose homeostasis in mouse model of type 2 diabetes [10, 12, 13]. We also detected significant reduction of visceral and subcutaneous fat mass by heat shock and MES in high-fat diet mice as well as in db/db mice [10]. MES attenuated the degradation of Hsp72 by inhibiting the proteosome-ubiquitin system, resulting in enhanced Hsp72 induction [11].

The major role of Hsp72 in these beneficial effects may be explained by suppression of c-Jun N-terminal kinase (JNK) through direct binding of Hsp72 to JNK and de-phosphorylation of JNK itself [12, 22]. Activation of JNK in insulin target tissues due to such cellular stresses results in phosphorylation of insulin receptor substrates (IRSs) at serine and threonine residues that inhibit insulin signaling. Deletion of JNK1 protects mice from high-fat-diet induced insulin resistance, in part through decreased adiposity [23]. In this regard, inhibition of JNK could also be a therapeutic target to prevent visceral adiposity and chronic inflammation. Indeed, suppression of the JNK pathway in diabetic mice improves insulin resistance and ameliorates glucose tolerance [24, 25].

In this study, although we have limited number of subjects, we observed significant reduction of TNF-α and hs-CRP in healthy humans after MET treatment. Increased concentration of TNF-α is found in acute and chronic inflammatory conditions (e.g., trauma, sepsis, infection, rheumatoid arthritis), and is associated with impaired insulin sensitivity. It is proposed that induction of Hsp72 can decrease TNF-α levels by suppression of NF-κB activation [20], although this was not confirmed in our MET treatment yet. TNF-α has been shown to play a key role in atherogenic processes and thus its reduction might be a potential therapeutic target. TNF-α blockade may improve insulin resistance and lipid profiles in patients with chronic inflammatory diseases [3].

Recent data also link inflammation and hs-CRP to insulin resistance in that hs-CRP levels have been associated with impaired insulin sensitivity, and the development of metabolic

syndrome and type 2 diabetes. In large prospective studies, hs-CRP adds prognostic information about cardiovascular risk beyond that provided by the metabolic syndrome [4]. Moreover, baseline levels of CRP predict the risk of future myocardial infarction, stroke, and peripheral atherosclerosis among apparently healthy middle-aged men after adjusting for other known cardiovascular risk factors. As well as having a critical role in risk prediction, recent evidence implicates that CRP directly accelerates atherogenesis. CRP has been found in human atherosclerotic plaque and has been shown to cause endothelial cell dysfunction, oxidative stress and intimal hypertrophy in experimental models [26].

The synthesis of CRP is regulated at the transcriptional level and a critical transcriptional regulatory element on the CRP promoter contains a NF-κB p50 binding site. Therefore, reduction of inflammatory cytokines in this study appeared to be achieved by NF-κB inactivation upon Hsp72 induction. Indeed, Hsp72 blocks NF-κB activation and expression of several downstream inflammatory genes by interacting with the NF-κB:IκB complex and by preventing the phosphorylation of IκB [21]. Therefore, appropriate induction of Hsp72 by MET could decrease inflammatory markers, such as TNF-α and CRP, through suppression of NF-κB activation, because NF-κB may regulate TNF-α and CRP in transcriptional level.

Recently, induction of Hsp27 (human homologue of rodent Hsp25) prevents pancreatic b-cell apoptosis upon cytokine- or streptozotocin-stimuli via regulation of (I kappa kinase γ) IKKγ - NF-κB - TNF-α cascade [27]. Using our modality, it is possible that upregulation of Hsp25/27 could lead to TNF-α reduction through suppression of NF-κB activation. This possibility should be elucidated in mouse and cell systems in near future.

We also observed a tendency of visceral and subcutaneous adiposity reduction with this treatment, which was almost restored after an 8 weeks of withdrawal period. Although the precise mechanisms of this phenomenon remain to be elucidated, these beneficial effects could be associated with JNK inactivation and mitochondrial activation [12]. As Hsp72

inhibits JNK activation, inactivated JNK function could lead to reduced adiposity, because an absence of JNK in mice results in decreased adiposity [23]. It is also reported that Hsp72 can enhance mitochondrial capacity and function [28]. Mild reduction of adiposity in this study may also contribute to lower inflammatory cytokines to some extent. Therefore, mild electrical current and thermo therapy can be a novel and promising interventional technology to treat metabolic disorders. Of course, we cannot simply expect these beneficial results of heat with MES on healthy subjects into the patients with type 2 diabetes and metabolic syndrome. Therefore, application of this method to type 2 diabetics and metabolic syndromes is now being operated in clinical trials to confirm the effects of MET. Indeed, the study of MET on metabolic syndrome subjects had just been finished, and the results were preferable and quite similar to those in rodents and healthy humans (in preparation).

In summary, we have shown that treatment with heat and MES in healthy humans decreases serum TNF-α and hs-CRP levels with no adverse effects. The treatment also showed a trend of reduced adiposity. Therefore, this combination therapy may provide a novel treatment of metabolic disorders such as type 2 diabetes and metabolic syndrome.

Conflict of interest

We declare that we have no conflict of interest regarding this project.

Acknowledgements

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Figure legends

Fig. 1. The device used for this study and the changes in abdominal adiposity.

(A) MET generator that delivered 12V (55 pps) of direct current with individual duration of 0.1 ms. (B) Electro-conductive and thermo-generative (42 ^{o}C) rubber electrodes, which are padded with soft cotton cloth. The volume of visceral fat (E) and subcutaneous fat (F) during the study were determined by CT and three-dimensionally calculation using Advantage Workstation AW4.3-05. Individual distributions and variations were plotted (D). % reduction upon MET treatment were shown in numbers, and standard errors of mean were also indicated.

Fig. 2. Serum cytokines and inflammatory markers during the study.

Individual distributions and variations of serum cytokines and inflammatory markers such as leptin (A), adiponectin (B), IL-6 (C), TNF-α (D) and hs-CRP (E) before (Pre) and after (Post) the study are shown. Mean values and individual distributions were plotted.

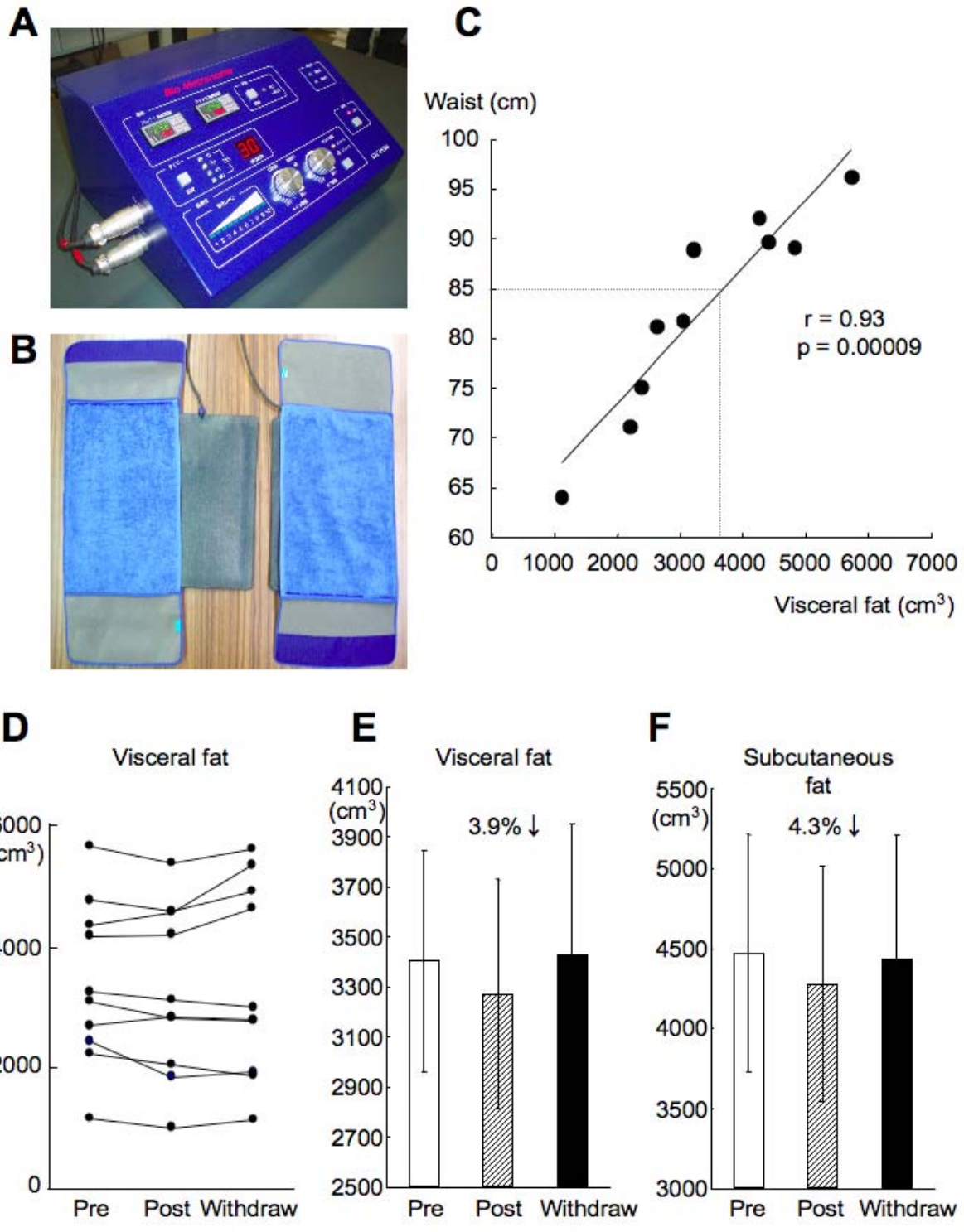
A
B
C
Waist (cm)
100
95
90
85
80
75
70
65
60
0
1000
2000
3000
4000
5000
6000
7000
Visceral fat (cm3)
r = 0.93
p = 0.00009
D
Visceral fat
6000
(cm3)
4000
2000
0
Pre
Post
Withdraw
E
Visceral fat
4100
(cm3)
3900
3700
3500
3300
3100
2900
2700
2500
3.9% ↓
Pre
Post
Withdraw
F
Subcutaneous
fat
5500
(cm3)
5000
4500
4000
3500
3000
4.3% ↓
Pre
Post
Withdraw

Fig.1

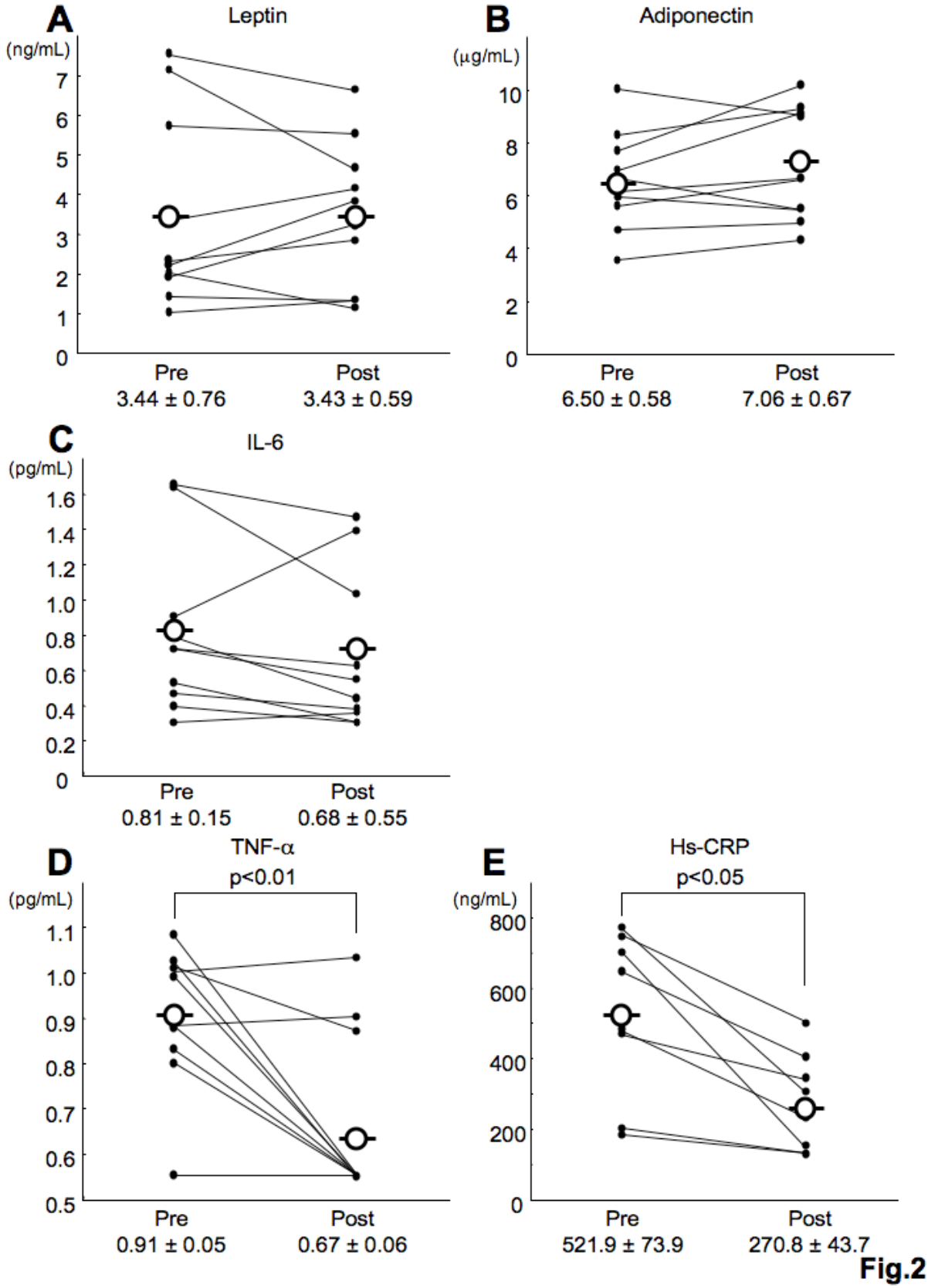
A
Leptin
(ng/mL)
7
6
5
4
3
2
1
0
Pre
3.44 ± 0.76
Post
3.43 ± 0.59
B
Adiponectin
(μg/mL)
10
8
6
4
2
0
Pre
6.50 ± 0.58
Post
7.06 ± 0.67
C
IL-6
(pg/mL)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Pre
0.81 ± 0.15
Post
0.68 ± 0.55
D
TNF-α
p<0.01
(pg/mL)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
Pre
0.91 ± 0.05
Post
0.67 ± 0.06
E
Hs-CRP
p<0.05
(ng/mL)
800
600
400
200
0
Pre
521.9 ± 73.9
Post
270.8 ± 43.7

Fig.2

Table. 1

Background and post-treatment characteristics of the subjects (n=10)

	Pre	Post	*p* value
Male/females	10 / 0	10 / 0	---
Age (years)	34.0 ± 4.7	34.3 ± 4.7	---
Body mass index (kg/m^2)	23.7 ± 2.9	23.6 ± 3.1	0.95
% Body fat	22.1 ± 4.6	22.4 ± 5.7	0.89
Waist (cm)	82.7 ± 10.2	81.6 ± 10.3	0.81
Systolic blood pressure (mmHg)	122.7 ± 9.2	120.2 ± 12.3	0.61
Diastolic blood pressure (mmHg)	72.5 ± 8.7	74.4 ± 10.3	0.66
Heart rate (beats/min)	76.8 ± 13.4	71.2 ± 12.6	0.35
Current smoking (yes/no)	3 / 7	3 / 7	---
Fasting plasma glucose (mg/dL)	92.1 ± 6.0	96.1 ± 10.1	0.30
Fasting insulin (μIU/mL)	7.5 ± 3.3	7.8 ± 4.3	0.88
HOMA-R	1.72 ± 0.78	1.88 ± 1.05	0.72
Insulinogenic index	0.96 ± 0.64	1.03 ± 0.74	0.81
HOMA-β	93.5 ± 44.1	85.4 ± 66.6	0.76
Blood glucose AUC on OGTT (0-2h) (mg/h/dL)	236.7 ± 7.6	243.4 ± 12.3	0.48
Insulin AUC on OGTT (0-2h) (IU/min/mL)	4820.5 ± 523.7	3889.0 ± 541.7	0.23
LDL-cholesterol (mg/dL)	128.2 ± 27.7	115.4 ± 19.2	0.25
HDL-cholesterol (mg/dL)	59.4 ± 9.0	57.0 ± 12.8	0.63
Triglyceride (mg/dL)	78.7 ± 40.4	84.9 ± 30.3	0.70
WBC (/μL)	5220 ± 542.8	5340 ± 527.3	0.88
RBC (10^4/μL)	511.9 ± 28.4	511.1 ± 25.4	0.95
Hb (g/dL)	15.7 ± 0.9	16.0 ± 1.3	0.18
Plt (10^4/μL)	22.6 ± 1.5	19.3 ± 1.6	0.16
BUN (mg/dl)	14.0 ± 3.7	12.9 ± 2.4	0.44
Creatinine (mg/dL)	0.87 ± 0.07	0.82 ± 0.09	0.16
AST (GOT) (IU/L)	20.8 ± 7.0	24.1 ± 9.1	0.38
ALT (GPT) (IU/L)	23.7 ± 11.3	23.6 ± 13.2	0.99
LDH (IU/L)	177.1 ± 28.2	207.7 ± 78.4	0.27
Adiponectin (μg/mL)	6.50 ± 0.58	7.06 ± 0.67	0.55
Leptin (ng/mL)	3.44 ± 0.76	3.43 ± 1.86	0.99
Interleukin-6 (pg/mL)	0.81 ± 0.15	0.68 ± 0.14	0.55
Tumor necrosis factor-α (pg/mL)	0.91 ± 0.05	0.67 ± 0.06	0.006**
High sensitivity C-reactive protein (ng/mL)	521.9 ± 73.9	270.8 ± 43.7	0.023*

The subjects' pre- and post-clinical and biological characteristics indicated that TNF-α and hs-CRP were significantly decreased by MET treatment. Values are expressed as mean ± S.D. or numbers of subjects.

HOMA-R=the homeostasis model assessment of insulin resistance. HOMA-β=the homeostasis model assessment of β-cell function. LDL=low-density lipoprotein. HDL= high-density lipoprotein. WBC=white blood cells. RBC=red blood cells. Hb=hemoglobin. Plt=platelets. BUN=blood urea nitrogen. AST=aspartic aminotransferase. GOT=glutamic oxaloacetic transaminase. ALT=alanine aminotransferase. GPT=glutamic pyruvate transaminase. LDH=lactate dehydrogenase.. *p<0.05, **p<0.01