Comparison of Serum Ferritin and Oxidative Stress Biomarkers between Japanese Workers with and without Metabolic Syndrome

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hydrogen peroxide (H2O2);
metabolic syndrome;

Summary

Objective: Metabolic syndrome (MS) is closely associated to life-style and is characterized by central obesity causing severe diseases such as diabetes mellitus (DM) or atherosclerosis. This study investigates the role of oxidative stress and inflammation in MS.

Subjects: Total of 685 workers stratified by gender (293 men and 392 women) with a mean age of 41.2 ±10.4 in different offices in a city in Japan.

Methods: Fasting blood and urine tests for MS, oxidative and/or inflammatory biomarker analysis and blood pressure (BP) measurement were performed. MS was defined on the basis of the Japanese criterion.

Results: Serum ferritin and urinary hydrogen peroxide (H_2O_2) levels were significantly higher in subjects with MS than those without. Ferritin was positively correlated with 8-hydroxy-2'-deoxyguanosine (8-OHdG) in all subjects and it was negatively correlated with 8-isoprostane and H_2O_2 in men. In addition, there was a significant positive correlation between ferritin and homeostasis model assessment (HOMA-R) in men. By using multiple regression analysis, ferritin was closely correlated with HOMA-R, γ-GT, 8-OHdG, smoking value and amount of alcohol ingestion in men, and it was correlated with 8-OHdG, γ-GT, HOMA-R in women under 50 years old.

Conclusions: Ferritin is a useful marker of MS including insulin resistance, reflecting the importance of oxidative stress as a cause of MS, especially in men.

Introduction

Metabolic syndrome (MS) is a condition related to life-style and characterized by central obesity leading to pathological conditions such as diabetes mellitus (DM) or atherosclerosis. Oxidative stress appears to be involved in this process and we have investigated several oxidative stress biomarkers in Japanese workers with and without MS and have shown that the urinary concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-isoprostane are useful prospective biomarkers of lifestyle-related disease risks [1]. We have also reported that 3-nitrotyrosine is related to exercise habits among healthy Japanese [2], and high-sensitivity C-reactive protein (hs-CRP) appears to mediate the smokers' renal dysfunction [3].

In addition to the above mentioned nitric oxide system for 8-isoprostane formation as well as the inflammatory process causing hs-CRP elevation, iron is also involved in oxidative stress by forming superoxide anion from hydrogen peroxide (H_2O_2) and Fe^{++} by the Fenton reaction. Serum ferritin level represents the amount of stored body iron and is regarded as one of the oxidative stress marker by forming Fe^{++} for the Fenton reaction. Total iron-binding capacity (TIBC) in serum is in reciprocal relationship with ferritin level and its increase represents the reduction in stored body iron. Serum iron level and its change are marker of chronic inflammation independent of serum ferritin or TIBC. Therefore, it is interesting to evaluate these markers of iron metabolism together with other oxidative stress markers and hs-CRP in MS in considering the effect of oxidative stress on life-related diseases.

Oxidative stress is defined as a situation in which an increased level of reactive oxygen species, such as superoxide anion and H_2O_2, overwhelms the antioxidative defense capacity, resulting in oxidative damage to lipids, DNA and proteins [4]. Thus, the determination of reactive oxygen species or the above mentioned oxidative stress markers in subjects with MS provide useful strategy in public health field [5].

In the present study, biomarkers of oxidative stress, including urinary 8-OHdG, 8-isoprostane, H_2O_2, hs-CRP, and serum ferritin were determined and compared among

Japanese workers with and without MS.

Methods

Subjects

Data were obtained from a worksite lifestyle intervention study in six offices in a city in Japan in which 847 individuals were participated. For the purpose of this study, we excluded 162 subjects who had any history of cancer, stroke, DM, ischaemic heart disease or asthma, and who were taking any medicines. Seven subjects were also excluded because their urinary concentrations of oxidative stress biomarkers and serum ferritin were under the limit of detection. Therefore, we finally used data for 685 subjects (293 men and 392 women). All subjects had been instructed to overnight-fast and not to consume any beverage and food except plain water before the sample collection.

The ethics committee of Okayama University approved the study, and written informed consent was obtained from all the subjects.

Health assessment

Health assessment was performed from September to December, 2007 by collecting blood samples after overnight fasting for at least 10 hours. Serum and plasma samples were preserved at 4°C for the measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GT), triglycerides (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), fasting glucose, fasting insulin, hemoglobin A1c of NGSP (HbA1c), and hs-CRP. In addition, ferritin, Fe and unsaturated iron-binding capacity (UIBC) were determined in serum samples stored at –80°C until analyses, because there was a time delay until measuring these markers. Serum ferritin was measured by iatro ferritin kit (Mitsubishi chemical medience corporation; MBC, Tokyo, Japan) using automated analyzer

(HITACHI 7700). Serum Fe was analyzed by colorimetric assay using iatro Fe Ⅱ kit (MBC, Tokyo, Japan), and UIBC was measured by colorimetric assay using iatro LQ UIBC Ⅱ kit (MBC, Tokyo, Japan). So, TIBC was calculated as TIBC = UIBC + Fe, and the homeostasis model assessment (HOMA-R) levels were calculated as fasting insulin (μU/mL) × fasting glucose (mg/dl)/405 [6].

Anthropometric measurements were performed according to a standard protocol. Blood pressure (BP) was measured in the morning after 10 min of rest in the sitting position. Abdominal circumference was measured horizontally at the umbilical level at the end of normal expiration by trained nurses. Body mass index (BMI) was calculated by body weight (kg)/height $(m)^2$ and BMI over 25 was diagnosed as obesity according to the criteria for Japanese [7].

Information on lifestyles including cigarette smoking, alcohol consumption was obtained by self-reported questionnaires. The amount of alcohol consumption was calculated by assuming one unit was equivalent to 9–12 g of ethanol [8]. The habit of alcohol intake was expressed by the number of units per week, and over 20 units of alcohol consumption per week represented excessive drinking.

Definition of metabolic syndrome

MS was diagnosed using the Japanese criteria [9]. In Japan, MS was defined as having a abdominal circumference in excess of 85cm for men and a abdominal circumference in excess of 90cm for women [10], having 2 or more components among the followings: (1) dyslipidemia: TG $\geq$ 150 mg/dl and/or HDL-C < 40 mg/dl, (2) high BP: BP $\geq$ 130/85 mmHg, and (3) impaired glucose metabolism: fasting plasma glucose $\geq$ 110 mg/dl.

Analysis of oxidative stress biomarkers

We measured urinary 8-OHdG, 8-isoprostane, and H_2O_2, as oxidative stress biomarkers.

Urinary oxidative stress biomarkers were determined in spot urine samples stored at –80°C until analysis, because Helmersson and Basu reported that urinary F_2-isoprostane isomer levels in spot urines showed no significant variation from those levels measured by radioimmunoassay in 24-h urine samples in the same healthy individuals [11]. Urinary H_2O_2 was analyzed by the ferrous ion oxidation xylenol orange version-1 (FOX-1) assay [12, 13], and the intra-assay and inter-assay coefficients of variation (CV) were 4.3% and 9.7%, respectively. Measurement of 8-OHdG was carried out with an enzyme-linked immunosorbent assay (ELISA) kit from the Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan [14], and the intra-assay and inter-assay CV were 5.2% and 8.1%, respectively. Møller and Loft indicated that the correlation coefficient of 8-OHdG measurements by ELISA between spot and 24-h urine sample was 0.87 [15]. Urinary 8-isoprostane was analyzed using commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI, USA) [16-18], and the intra-assay and inter-assay CV were 5.4% and 11.0%, respectively. Values for 8-OHdG, 8-isoprostane and H_2O_2 were normalized by per milligram of creatinine measured in urine determined with creatinine test kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data are presented as mean ± standard deviation (S.D.). Log-transformed data of fasting glucose, insulin, HbA1c, HOMA-R, TG, AST, ALT, γ-GT, hs-CRP, ferritin, urinary 8-OHdG, 8-isoprostane, and H_2O_2 were used in all analyses because of their skewed distributions.

Unpaired *t*-test was used to test the differences in health assessment of MS data and oxidative stress biomarkers, and $\boldsymbol{x}^2$-test was used to test the differences in MS with respect to sex, smoking habit and alcohol consumption. In addition, unpaired *t*-test was used to assess the difference in serum ferritin, hs-CRP, H_2O_2 with respect to the MS of

Japanese criteria (abdominal circumference, impaired glucose tolerance, dyslipidemia, BP), and analysis of covariance (ANCOVA) was used to test these parameters by the Japanese criteria of MS for age adjustment.

Pearson's correlation analysis was performed to examine the relation between serum ferritin and the variables related to oxidative stress biomarkers. One-way analysis of variance (ANOVA) was used to determined differences in concentrations of serum ferritin and oxidative stress biomarkers in women's different age groups. A stepwise multiple regression analysis was performed to examine the relationship between ferritin and the variables that have potential association with MS.

A probability value of $p<0.05$ was considered to be statistically significant. All analyses were performed using Statistical Package of SPSS 12.0J for Windows.

Results

Characteristics of subjects

The characteristics of the subjects studied are presented in Table 1. The group of MS was 6.9% (n=47, 40 men and 7 women). The proportion of excessive drinkers (over the 20 units per week) was 3.9% (n=27, 22 men and 5 women).

Health examination variables based on metabolic syndrome

Results of health parameter analysis by MS are shown in Table 2. Statistically significant differences between subjects with or without MS were present for all the parameters related to MS such as age, abdominal circumference, HOMA-R, hs-CRP and so on, and only for ferritin and H_2O_2 as oxidative stress biomarkers. By using x^2-test, there was a significant difference in alcoholic habits between subjects with and without MS; namely, a higher percentage of alcoholics in the MS group, There was no difference in smoking

habits between subjects with or without MS.

Parameters directly involved in oxidative stress were compared between subjects with and without MS, and the results are summarized in Tables 3-1 (all subjects), 3-2 (men) and 3-3 (women). Ferritin level was significantly higher in subjects with MS and its components than those without even after adjusting for age. Hs-CRP was significantly higher in subjects with MS, abdominal obesity, dyslipidemia, high BP than that without such components in all subjects. However, there was no significant difference in hs-CRP between subjects with and without impaired glucose tolerance.

In women consuming alcohol less than 20 units per week (non-drinkers), the significant difference in ferritin level was only noted in subjects with and without MS after adjusting for age. On the other hand, hs-CRP was significantly higher in subjects with all parameters except impaired glucose tolerance level than that in subjects without. H_2O_2 failed to show any significant difference between those with and without MS.

Relationship between serum ferritin and health examination variables

The results of simple correlation analysis between serum ferritin and health assessment data are presented in Table 4. Age was significantly positively correlated with serum ferritin level in women; however, no correlation between serum ferritin level and age was observed in men. As for oxidative stress biomarkers, serum ferritin significantly negatively correlated with H_2O_2 and 8-isoprostane, and significantly positively correlated with 8-OHdG in men. However, serum ferritin was significantly positively correlated with only 8-OHdG in women.

On the other hand, HOMA-R in men was significantly positively correlated with serum ferritin, but not in women (Table 4, Figure 1). Figure 1 shows the results of simple correlation analysis between serum ferritin and hs-CRP (r=0.194, p=0.001) and HOMA-R (r=0.314, p<0.001) in men. In addition, hs-CRP was significantly and positively correlated with HOMA-R (r=0.404, p<0.001) in men (data not shown).

Serum ferritin and oxidative stress biomarkers in different age groups among women

The results of the difference in serum ferritin and oxidative stress biomarkers between two different age groups (<50 years and ≥ 50 years) in women are shown in Table 5 in considering that 50 years old of age in women stand for the age of menopause. Serum ferritin, hs-CRP and H_2O_2 in women over 50 were significantly higher than those in women under 50.

Figure 2 shows comparison of serum ferritin and urinary 8-OHdG by ANOVA classified by different age groups in women (n=392). The levels of urinary 8-OHdG and serum ferritin in younger groups of age 20's, 30's and 40's were significantly lower than those in older groups of age 50's and 60's.

Stepwise Multiple regression analysis for serum ferritin

The results of stepwise multiple regression analysis for serum ferritin are shown in Table 6. The model for men demonstrated that positive HOMA-R, γ-GT, 8-OHdG and alcohol intake, negative smoking value were important determinant factors of ferritin (24.9%) after adjustment for age, abdominal circumference, LDL-C, hs-CRP, 8-Isoprostane, H_2O_2 and systolic BP. On the other hand, the model for women under 50 revealed that positive 8-OHdG, γ-GT, and HOMA-R were important determinant factors of ferritin (33.4%) after adjusting for abdominal circumference, LDL-C, hs-CRP, alcohol intake, smoking value, 8-Isoprostane, H_2O_2 and systolic BP. In addition, the model for women of over 50 revealed that only positive 8-OHdG and negative H_2O_2 were important determinant factors of ferritin (26.0%). In other words, the effect of age on ferritin level in women is very large, resulting in a large difference between the groups of age below and above 50.

Discussion

Health parameters and oxidative stress markers were compared between subjects with and without MS. As a result, most of the health parameters such as LDL-C, HbA1c, HOMA-R, AST, ALT or others were significantly higher in subjects with MS as compared with those without MS, except for Fe, TIBC and smoking habits. According to the metabolic parameters related to oxidative stress, hs-CRP, ferritin and H_2O_2 were significantly higher in subjects with MS than those in subjects without MS, except for 8-OHdG and 8-isoprostane. Among iron related markers, only serum ferritin was high in subjects with MS but serum iron was not.

In relation to iron over load in diabetic patients, phlebotomy improves the diabetic state [19]. Crist BL, *et al* [20] studied lipid peroxidation to see dependency on iron or superoxide anion in considering that the DNA oxidation is largely dependent on hydroxyl radicals. In the present study, a significant negative correlation was observed between urinary 8-isoprostane and ferritin only in men. The negative correlation between ferritin and H_2O_2 observed in this study may be interpreted as indicating that H_2O_2 level decreased as a result of consuming H_2O_2 to form hydroxyl radicals, although further investigation is needed to prove this hypothesis.

The levels of oxidative stress markers, such as hs-CRP, ferritin and H_2O_2 were significantly higher in subjects with MS than those in subjects without MS. Among factors related with iron metabolism, only ferritin, which represents stored body iron, was significantly increased. TIBC decreases as the ferritin level increases; however, no negative correlation with MS markers was found. The level of serum iron (Fe^{++}) is known to decrease in chronic inflammatory diseases [21] and could not serve as a positive marker of MS. As for the mechanism of the increase in serum ferritin, the release of ferritin from injured liver is possible. However, the serum levels of AST and ALT, which are the sensitive markers of liver injury, were not so high in MS. On the other hand, the

ferritin level was highly correlated with other MS markers such as HbA1c, HOMA-R and HS-CRP, which was not necessarily related with liver injury.

Since ferritin is regarded as a positive marker for anemia, it needs to be careful consideration when using ferritin as a negative marker of MS. Furthermore, ferritin level was significantly higher only in men with MS and its components than those without even after adjusting for age among all subcriterion of MS. Particularly, the significantly high correlation between ferritin and HOMA-R was noted, suggesting that ferritin is closely related to the insulin resistance. Lee KU [22] showed the highest correlation observed between plasma malondialdehyde and ferritin in the syndrome relates to insulin resistance. The enrolled subjects in the present study were not limited to such a syndrome and thus we could generalize the high ferritin level in MS and emphasized the importance of increased ferritin in subjects with MS. There are several reports showing the close correlation between ferritin and insulin resistance [23-27] or CRP [28] in patients under various treatments. Our results obtained in this study with subjects without any medications eliminate the effects of such treatments. A previous report showed positive correlation between ferritin and insulin resistance only in men [29] as the results obtained in this study. Aso Y, *et al* [30] demonstrated in type 2 diabetes, the decrease in adiponectin in MS together with the decrease in hepcidin would increase the iron absorption resulting in the increase in ferritin, and contribute further to oxidative stress in MS. Taken together, insulin resistance and its related conditions may induce an increase in levels of ferritin.

On the other hand, the levels of ferritin, hs-CRP and H_2O_2 in women over 50's were significantly higher compared with those in women under 40's as classified by age groups. The increased ferritin levels over 50 may be related to the menopause, representing the increased iron storage. Similar results have been reported on ferritin and DNA oxidation in previous studies [31-33], although no consensus has been reached on the association of urinary 8-OHdG with aging [34, 35]. In addition, during

menopause, a decreased production of estrogen may also be involved in the elevation of urinary 8-OHdG. Much attention has been focused on the antioxidant property of estrogen in relation to the suppression of cholesterol synthesis in atherosclerotic patients [34]. Therefore, the estrogen levels is as important as the body iron status while discussing the gender difference in urinary 8-OHdG and oxidative stress and also the longevity of women; however, it should be stressed that iron has a direct role in causing oxidative stress and at the same time ferritin is a controllable metabolite in the body.

Although the present findings are significant, several limitations of the study should be noted. First, causal relationships could not be determined because this study was a cross-sectional study. Second, some reporting bias may have been introduced because the information on lifestyle habits like smoking and drinking was obtained via self-reported questionnaires. Third, there was small sample size of MS in women (n=7). Further studies are required to confirm the serum ferritin in MS of women with large sample size.

In conclusion, this study shows that ferritin is related to HOMA-R as well as hs-CRP in Japanese men with MS, suggesting the importance of oxidative stress. On the other hand, ferritin level is related to subcriterion of MS, such as abdominal circumference, glucose, dyslipidemia and blood pressure, especially in men. Thus, the importance of serum ferritin as an important risk marker of MS could be concluded in summarizing the results of the present study, especially men.

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