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Enhanced Activity of Flavin-containing Monooxygenase in Human Subjects with High Body Mass Index and in Obese Mice Fed a High-fat Diet

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Abstract

The effect of obesity on the drug-metabolizing enzymes remains an important issue for clinician since obesity is a world wide epidemic problem. However, little is known about the effects of obesity on flavin-containing monooxygenase (FMO) production and activity. We show here for the first time that *in vivo* FMO activity determined by urinary ranitidine (RA) metabolites ratio in human, was higher in subjects with a high body mass index (BMI, kg/m^2, 21.97-30.32) than in those with an intermediate BMI (range 19.38-21.83). Moreover, there was a significant correlation between FMO activity and BMI in 209 subjects. In high fat diet-induced obese mice, we also observed that the hepatic expression of FMO (225% of lean mice) and the activity measured by the RA N-oxidation rate (513±58.1 vs. 349±66.0 pmol/hr per mg protein) were significantly higher than in lean mice fed a control diet. Unknown factors rather than leptin or insulin appeared to regulate the hepatic FMO production. Thus, FMO activity may be increased in obese or overweight individuals. Moreover, the regulation of FMO activity in subjects with morbid obesity, with or without complications and its clinical implications, should be investigated further.

Keywords: FMO, Obesity, Body mass index, Ranitidine, High-fat diet

The microsomal flavin-containing monooxygenases (FMOs; EC 1.14.13.8) comprise a family of enzymes involved in the oxidation of nucleophilic nitrogen, sulfur, phosphorus, and selenium atomic centers present in a number of compounds[1]. FMOs have been relatively neglected, although xenobiotics containing nucleophilic centers are often substrates for both cytochrome P450 and FMO. In animal models and in humans, FMOs might be regulated by physiological factors, such as age, hormones, and nutrients[2-5]. In addition, hepatic FMO activity is increased both in insulin-dependent and independent diabetic animal models[2,6,7]. The effect of obesity on the metabolism of drugs remains an important issue for public health, as obesity is now a major world wide epidemic problem. However, studies on the effect of obesity on drug metabolizing enzymes, particularly FMOs, remain noticeably lacking from the literature. In general, hormonal changes, hepatic fatty infiltration, and increases in cytokine levels characterize the physiological changes in most obese individuals, and the degree to which this occurs appears to be proportional to the extent of obesity. However, there has been no characterization of the effects of obesity on FMO activity or on the relationship between adiposity and FMO activity in animals and human subjects.

In the present study, we evaluated the relationship between the body mass or obesity and FMO activity both in human and animal model. Here, we show that the *in vivo* hepatic FMO activity measured by the urinary ranitidine (RA) metabolic ratio of subjects with relatively higher body mass index (BMI) was significantly higher than that of subjects with intermediate BMI. In addition, we present evidence that hepatic production and activity of FMO1 in male mice is increased by fatty diet-induced obesity.

Relationship between FMO Activity and BMI in Human Subjects

The BMI of all participants ranged from 16.53 to

30.32 kg/m^2 (mean 21.6±2.7). We found a marginal but significant correlation (Pearson r=0.16; P= 0.0191) between FMO activity and BMI. As shown in Figure 1, we also observed that FMO activities of subjects with higher (fourth and fifth quintile; 21.97-30.32 kg/m^2) BMI values were significantly higher than among those with intermediate BMIs (second and third quintiles; 19.38-21.83 kg/m^2). BMIs of all our volunteers except for one person (BMI=30.32 kg/m^2) were less than 30 kg/m^2, which is not obese

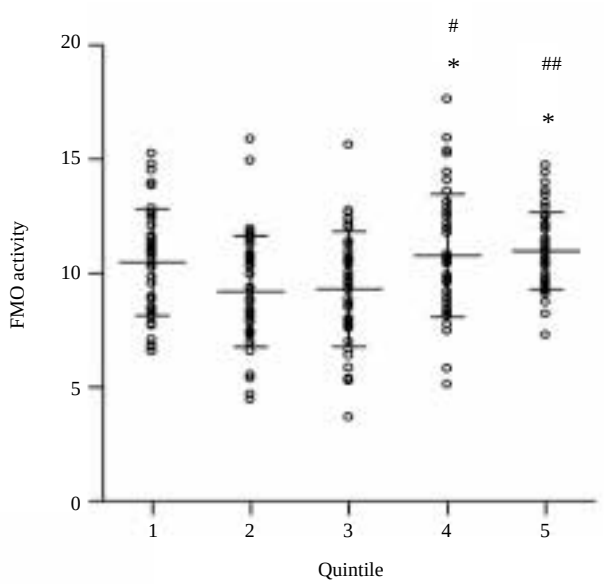

Figure 1. Aligned dot plot of FMO activity determined by urinary RA metabolite ratio according to the BMI quintiles in 209 Korean subjects. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. 2nd quintile; $^{*}P<0.05$ vs. 3rd quintile.

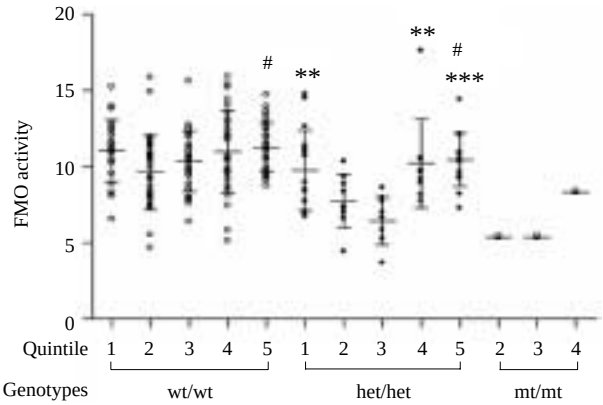

Figure 2. Aligned dot plot of FMO activity according to the major genotypes for G472A and A923G alleles (wt/wt, *FMO3*^{472}*G/G* and *FMO3*^{923}*A/A*; het/het, *FMO3*^{472}*G/A* and *FMO3*^{923}*A/G*; mt/mt, *FMO3*^{472}*A/A* and *FMO3*^{923}*G/G*) and BMI quintiles. $^{\#}P<0.05$ vs. 2nd quintile; $^{**}P<0.01$, $^{***}P<0.001$ vs. 3rd quintile within each genotype. Horizontal lines indicate the means (long) and standard deviation (short).

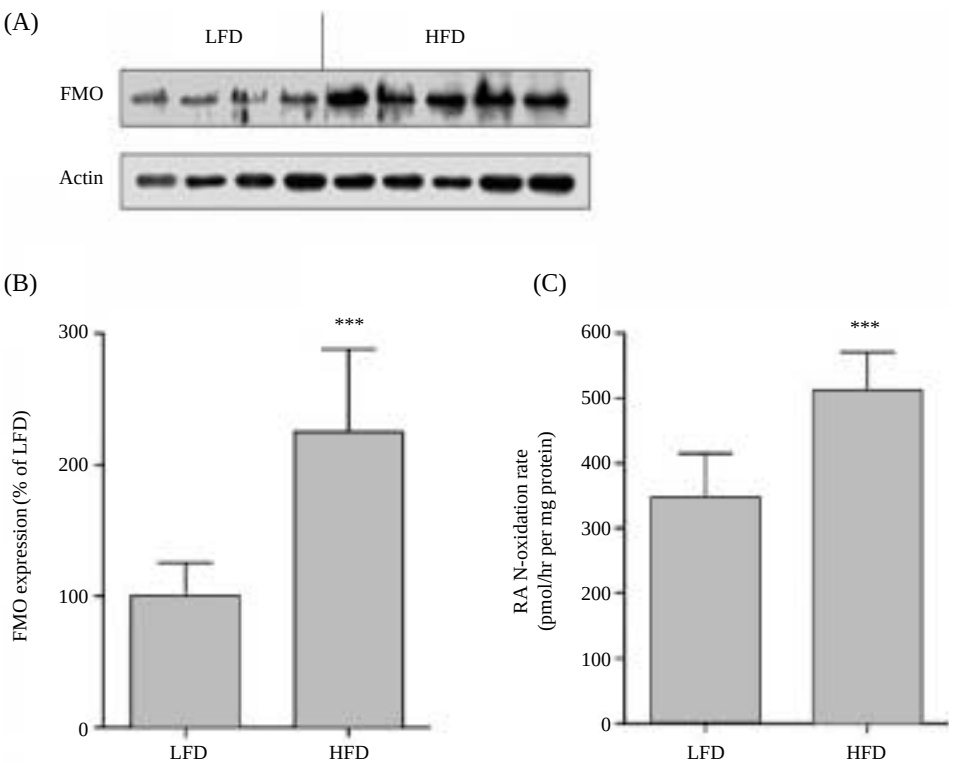

Figure 3. Hepatic microsomal expression and activity of FMO1 in mice fed with a high-fat diet (HFD) or low-fat control diet (LFD) for three months. (A) A representative western blot of hepatic FMO1 production. (B) The relative expression of FMO1 in obese mice (n=9) compared with lean mice (n=8). (C) The mean hepatic FMO activity (RA N-oxidation rate, pmol/hr per mg protein) in mice (n=8-9). $^{***}P<0.001$ vs. LFD.

according to the WHO classification[8,9]. Nevertheless, there is a significant difference of mean FMO activity between groups with upper BMI values (approximately >22.0 kg/m^2) and groups with intermediate BMIs (second and third quintiles). Although it was not significant statistically, the mean FMO activity levels of subjects with the lowest BMI (16.53-19.27 kg/m^2) were higher than among those with intermediate BMI

In our previous studies[10], we presented a clear relationship between genotypes and the activity of FMO3 in human subjects. In addition, for the most part, women were in the lower (first and second quintile) BMI groups. When we performed multiple regression analysis including demographic data and the *FMO3* genotypes G472A and A923G, we found that genotypes ($P<0.0001$) and BMI ($P=0.031$) were significant variables influencing FMO activity. In consistent with this correlation, mean FMO activities of subjects with intermediate BMI (second or third quintile) were still lower than those of subjects with higher (fourth or fifth quintile) BMI both in wild (*FMO3*^{472}*G/G* and *FMO3*^{923}*A/A*) and heterozygous genotypes (*FMO3*^{472}*G/A* and *FMO3*^{923}*A/G*) (Figure 2). There was no significant difference in the mean FMO activity between men and women (data not shown).

Effects of High-fat Diet on Hepatic FMO1 Expression and Activity in Mice

To test whether diet-induced obesity might increases the production of hepatic FMO in laboratory animals, we used obese mice fed with a HF diet for three months. As might be expected, body weight (49.1 ± 1.6 g; $P<0.0001$) and plasma leptin levels (6.34 ± 0.81 ng/mL; $P=0.0002$) of obese mice were significantly higher than those of the lean control mice (30.6 ± 1.9 g and 1.43 ± 0.63 ng/mL, respectively). The hepatic FMO1 protein level of obese mice was 2.2 fold higher than that of lean mice (Figure 3A and B). To measure the increase of hepatic FMO activity, we used RA as a substrate. This is mainly metabolized by FMO1 and FMO3 to RA N-oxide[11]. As shown in Figure 3C, the mean hepatic microsomal FMO activity (RA N-oxidation rate) of HF diet-induced obese mice was significantly higher (513 ± 58.1 pmol/hr per mg protein) than that of lean mice (349 ± 66.0 pmol/hr per mg protein, $P=0.0003$) in accordance with the protein expression. In the present study, the plasma insulin concentration of obese mice was also significantly higher than that of lean mice (3.02 vs. 0.86 ng/mL, respectively; $P=0.0003$) in addition to the hyperleptinemia in the obese mice.

Discussion

The effect of obesity on the drug-metabolizing enzymes remains an important issue for clinician since obesity is a world wide epidemic problem. However little is known about the effects of obesity on FMO production and activity. Here, we reported for the first time that FMO activity determined by urinary RA metabolites ratio in human with relatively higher BMI (>22 kg/m^2) was significantly higher than that in subjects with intermediate BMI. Furthermore, a marginal but significant correlation between the FMO activities of subjects and their BMIs was found. As a measure of relative weight, BMI is easy to measure and the WHO BMI classification is an internationally acceptable proxy for body fatness reflecting the risk for Type 2 diabetes and cardiovascular diseases. However, recent studies suggested that the BMI cutoff value for assessing the disease risk of Asian populations, who have a higher percentage of body fat at a low BMI, should be lowered[12-14]. Generally, for the same level of body fat, the BMI of Asians is approximately 3 kg/m^2 lower than Caucasians[12]. In that case, our subjects with higher BMI (>22 kg/m^2) can correspond to overweight BMI. Therefore, we reclassified the BMI to assess the body fatness by lowering the cutoff values by 3 kg/m^2. The increase of FMO activity in groups with high BMI values was still observed after this reclassification (Table 1, right

Table 1. FMO activity in 209 Koreans according to their BMI. Each value represents the mean ± S.D. Subjects were divided by quintile BMI (left column) or by graded unit (right column) according to the references[12,14].

Quintile (BMI range)	n (M : F)	FMO activity* (range)	BMI range	n (M : F)	FMO activity# (range)
1 (16.53-19.27)	41 (19 : 22)	10.49 ± 2.33 (6.56-15.24)^{a,b}	BMI <18.5	22 (8 : 14)	10.98 ± 2.37 (6.74-15.24)^{b}
2 (19.38-20.59)	42 (33 : 9)	9.21 ± 2.44 (4.44-15.87)^{a}	$18.5\le$ BMI <22	105 (84 : 21)	9.37 ± 2.42 (3.68-15.87)^{a}
3 (20.62-21.83)	42 (38 : 4)	9.31 ± 2.52 (3.68-15.62)^{a}	$22\le$ BMI <27	73 (71 : 2)	10.83 ± 2.31 (5.11-17.63)^{b}
4 (21.97-23.84)	42 (41 : 1)	10.78 ± 2.69 (5.11-17.63)^{b}	BMI ≥27	10 (10 : 0)	11.65 ± 1.19 (9.59-13.08)^{b}
5 (23.94-30.32)	42 (41 : 1)	10.99 ± 1.69 (7.27-14.73)^{b}			
Total	209 (172 : 37)	10.16 ± 2.46 (3.68-17.63)	Total	209 (172 : 37)	10.16 ± 2.46 (3.68-17.63)

*$P=0.0005$, #$P<0.0001$: One-way ANOVA with Tukey's multiple comparison test. Data with the different superscript are significantly different.

column). Interestingly, when we reclassified the BMI, the FMO activity of group with lowest BMI (BMI < 18.5 kg/m^2) was significantly higher than that of group with intermediate BMI ($18.5 \leq$ BMI < 22 kg/m^2). Although we could not explain the mechanism of this result, a previous animal study also reported that hepatic activities of FMO in guinea pigs with food restriction (10-15% weight loss) were higher than those in guinea pigs fed *ad libitum* in consistent with our result[15]. However, further study for the effect of undernutrition or underweight on the FMO activity should be conducted to elucidate the mechanisms, since the regulation systems of rodent and human FMO are quite different.

Previously, we presented a significant correlation between genotypes and the activity of FMO3 (urinary RA metabolite ratio) in human subjects[10]. Under adjustment of genotypes of FMO3, we still observed a positive correlation between FMO activity and BMI (Figure 2, and $P=0.031$ for BMI in multiple regression analysis). Therefore, we suggest that BMI is a meaningful endogenous factor affecting individual FMO activity, in addition to the most important endogenous factor, *FMO3* genotypes. Our results implicated that further characterization of the effects of human morbid obesity on FMO activity is necessary, since we could not recruit a population with morbid obesity.

To supporting the human data, we also examined the FMO expression and activity in HF diet-induced obese mice. In accordance with human data, FMO expression and activity of HFD group were significantly higher than those of LFD control group (Figure 3). Plasma leptin concentration is a biochemical indicator of adipose tissue mass. Previously, Rouer *et al*. reported that hepatic mRNA expression and FMO enzyme activity were higher in genetically (leptin deficient and hyperinsulinemic) or chemically-induced (insulin deficient) diabetes animals[6,7]. Hepatic FMO activity is also increased by streptozotocin-induced hyperglycemia and nearly normalized by insulin supplementation, resulting in only partial improvement of hyperglycemia[2]. In our present study, plasma leptin and insulin level of HFD group were significantly higher than those of LFD group. Taken together, the effects of hyperleptinemia and hyperinsulinemia on hepatic FMO activity may be minimal. Unfortunately, because we did not measure the plasma leptin concentration of the human subjects, the effect of leptin or adiposity on FMO activity in human should be investigated further.

To our knowledge, this regulation of FMO activity by diet-induced obesity has not been observed previously. This study for the first time has shown that HF diet-induced obesity increases the expression and activity of hepatic FMO. Moreover, our retrospective analysis of the relationship between BMI and *in vivo* FMO activity in human subjects could support the animal model results. Our results imply that the alteration of FMO-mediated drug metabolism in obese and/or overweight individuals should be considered in addition to their *FMO3* genotypes. Moreover, the clinical implications of the possible alteration of FMO activity in individuals with morbid obesity should be investigated further.

Methods

Subjects and Measurement of *in vivo* FMO Activity

We reported previously that FMO activity measured by urinary metabolite ratio of RA is affected by two common genetic variants of the *FMO3* gene[10]. However, we overlooked the possible differences in FMO activity according to the subject's gender and BMI. We reanalyzed data already collected from 209 participants (172 men and 37 women; mean age 22.7 $\pm$2.6 years) to evaluate demographic factors including gender, age, smoking status (110 nonsmokers and 99 smokers), body weight, height (171.9$\pm$6.8 cm), and BMI on FMO activity. The study was carried out in accordance with the Declaration of Helsinki. BMI was calculated as the body weight in kilograms divided by the height in meters squared, which was measured at the time volunteers were submitting their informed consent. We divided the BMI range into quintile groups.

Animals and Diets

Eight-week-old male C57BL/6 mice (8-9 per group) were obtained from Japan SLC Inc. (Shizuoka, Japan) and were housed individually in a temperature- (22 $\pm$2°C) and humidity-controlled (50-60%) room under a 12/12 hr light/dark cycle with free access to food and water. After one week of acclimatization, mice were fed a high fat (HF) diet (modified AIN-76A diet with 40% beef tallow; Dyets, Bethlehem, PA, USA; HFD group) to induce diet-induced obesity, or with a matched control AIN-76A diet (Dyets; LFD group) for three months *ad libitum*. The liver was quickly removed with the mice under ketamine /xylazine anesthesia and kept at -80°C until analysis. Plasma leptin (R&D Systems Inc., Minneapolis, MN, USA) and insulin levels (Shibayagi Co., Gunma, Japan) were determined using commercially available kits according to each manufacturer's instructions.

Western Blotting of Hepatic FMO1

To determine the hepatic expression of FMO1 according to diet, hepatic microsomes were extracted by differential centrifugation as described by Ryu *et al*.[16]. Twenty micrograms of microsomal proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidine difluoride membranes. After blocking of membranes with Tris-buffered saline containing 5 % nonfat milk and 0.1% Tween 20, blots were probed with a polyclonal antibody specific for the FMO1 protein[16] followed by a secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Determination of Hepatic Microsomal FMO Activity

Hepatic microsomal FMO activities of lean and obese mice were determined using a RA N-oxidation assay, as described by Chung *et al*.[11]. Briefly, 1 mg of microsomal proteins was reacted with 0.1 mM potassium phosphate buffer (pH 8.4) containing 0.15 mM NADPH and 1 mM RA (Sigma Chemical Co., St Louis, MO, USA) for 1 hr. The reaction mixtures were centrifuged for 1 hr at $100,000 \times g$ to separate the supernatants. The supernatants mixed with same volume of the hydrophilic high performance liquid chromatography (HPLC) mobile phase (50 mM NaH_2PO_4 containing 10% acetonitrile) were then injected directly into an HPLC system after centrifugation for 5 min at $13,000 \times g$.

Statistical Analysis

Data are presented as the means ± standard deviation and $P < 0.05$ was accepted as statistically significant to assess the effect of each demographic factor and genotype on FMO activity, multiple regression analysis with stepwise selection was performed using SPSS software (version 12.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance with Tukey's comparison was used to compare the FMO activity among groups divided according to BMI. The Mann-Whitney nonparametric *U* test was used to compare the production and activity levels of FMO between lean and obese mice.

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