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Abstract—Coronary risk factors, including age, hypertension, diabetes mellitus, hyperlipidemia, and smoking, are associated with enhanced oxidative stress, which is implicated as a potential mechanism for atherogenesis and atherosclerotic cardiovascular diseases. Male sex is one of the well-known cardiovascular risk factors. We tested the hypothesis that oxidative stress is greater in men than in women. Plasma thiobarbituric acid–reactive substances (TBARS) and urinary 8-isoprostaglandin $F_{2\alpha}$ (8-iso-PGF2α) were measured in 52 young men and 51 age-matched women. The subjects were healthy, were not smokers, and were not taking any medications or vitamins. Age, blood pressure, plasma cholesterol, and glucose did not differ between the groups. Baseline TBARS (2.32 ± 0.11 [men] versus 1.87 ± 0.09 [women] nmol/mL, $P<0.01$) and 8-iso-PGF2α (292 ± 56 [men] versus 164 ± 25 [women] pg/mg creatinine, $P<0.05$) were higher in men than in women. Supplementation of antioxidant vitamins for 4 weeks in men produced a significant reduction in TBARS and 8-iso-PGF2α by 34% ($P<0.01$) and 48% ($P<0.05$), respectively. Plasma superoxide dismutase, catalase, and vitamin E levels were comparable between the groups. Enhanced oxidative stress in men may provide a biochemical link between male sex and atherosclerotic diseases related to oxidative stress. (*Arterioscler Thromb Vasc Biol*. 2002;22:438-442.)

Key Words: oxidative stress ■ sex difference ■ cardiovascular disease ■ antioxidants

Oxidative stress, an imbalance between oxidant production and antioxidant defenses in favor of the former, has been shown to be involved in the process of atherogenesis.[1] A common approach to estimate oxidative stress in vivo is to measure the end products of lipid peroxidation. The most widely used index is plasma malondialdehyde, which is measured by the thiobarbituric acid–reacting substances (TBARS) assay.[2] Recently, one of the prostaglandin F_2 (PGF2)-like compounds (F_2-isoprostanes), 8-isoprostaglandin $F_{2\alpha}$ (8-iso-PGF2α, also referred to as iPF2α-III), has been shown to be easily and reliably measured in plasma and urine.[3] Urinary excretion of 8-iso-PGF2α is a sensitive in vivo marker of oxidative stress and is elevated in patients at risk for future cardiovascular events.[4] Specifically, it is increased in subjects with advanced age,[5] hypercholesterolemia,[6] diabetes mellitus,[7] and cigarette smoking.[8,9] Furthermore, this compound has been demonstrated to induce vasoconstriction and to modulate platelet function.[4] Thus, quantification of these indices may provide a valuable approach to assess oxidative stress in humans. In patients with hypercholesterolemia and diabetes mellitus,[6,7] supplementation of vitamin E could normalize enhanced lipid peroxidation. Similar findings were also demonstrated in apoE-deficient mice.[10]

There is a well-established association of male sex with the development of atherosclerosis and with the occurrence of atherosclerotic cardiovascular diseases, including stroke and coronary artery disease.[11] In contrast, premenopausal women are at lower risk for developing such diseases. On the basis of these observations, it is conceivable to hypothesize that oxidative stress is enhanced in men compared with women. However, no previous studies have specifically addressed the presence of sex difference in oxidative stress.

In the present study, we investigated whether plasma TBARS and urinary 8-iso-PGF2α are increased in healthy young men compared with age-matched premenopausal women and whether they are correlated with the endogenous level of circulating enzymatic antioxidants and/or estrogen. Furthermore, we examined the effects of combined supplementation of vitamin E (α-tocopherol) and vitamin C on these indices of oxidative stress.

Methods

Study Subjects

The protocol was approved by the ethics committee of Kyushu University, and written informed consent was obtained from all participating subjects. The study subjects consisted of 103 healthy

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volunteers (52 men and 51 women aged 20 to 39 years). All participants were normotensive, normocholesterolemic, and nondiabetic, were receiving no medication (including vitamin supplements), and were nonsmokers. We also excluded individuals with a history of alcohol-related liver diseases and alcoholism by an interview with the participants and by the baseline liver function test. Furthermore, to exclude the acute effects of alcohol intake on lipid peroxidation, the study subjects were prohibited from taking alcoholic drinks 24 hours before the study. The women reported regular menstrual cycles (every 26 to 32 days) before the study, and none of them were taking oral contraceptives. The women were studied between the 7th and 14th days of their menstrual cycles.

Plasma TBARS Measurements

A cross-sectional comparison of plasma TBARS was performed between men and women. All subjects were studied after a 12-hour fast. The formation of TBARS in peripheral blood samples was measured by a fluorometric assay, as described previously.[12] In brief, the plasma was mixed with 0.1 mol/L H_2SO_4 and 1% phosphotungstic acid, and the mixture was centrifuged. The sediment was suspended in distilled water, 1% thiobarbituric acid, and 0.1% butylated hydroxytoluene. The reaction mixture was then heated at 100°C for 60 minutes in an oil bath. After the mixture was cooled with tap water, it was extracted with *n*-butanol and centrifuged at 1600*g* for 15 minutes. The fluorescence intensity of the organic phase was measured by use of a spectrofluorometer with a wave length of 515-nm excitation and 553-nm emission. Malondialdehyde standards (Sigma Chemical Co) were included with each assay batch, and plasma TBARS were expressed as nanomoles per milliliter of plasma in reference to these standards.

Effects of Antioxidant Vitamin Supplementation

To determine the effects of antioxidant supplementation on plasma TBARS and urinary 8-iso-PGF2α, after a baseline measurement, a randomly selected group of study subjects received 300 mg of vitamin E and 600 mg of vitamin C daily for 4 weeks before a second measurement was taken. Furthermore, to evaluate the intrasubject reproducibility, another measurement was taken in a different group of study subjects not taking vitamin E and vitamin C after 4 weeks. No subjects experienced an adverse reaction or dropped out during the study period.

For the measurement of urinary 8-iso-PGF2α, urine samples were supplemented with 1 mmol/L of the antioxidant 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy (4-hydroxy-TEMPO, Sigma) and kept refrigerated during the collection period, after which they were immediately centrifuged, divided into aliquots, and stored at −70°C until extraction. Urinary 8-iso-PGF2α was measured by a liquid chromatography–electrospray ionization/mass spectrometry (LC-ESI/MS) assay as described previously.[13] In brief, urine samples (1 mL) were spiked with 5 ng of [3,3,4,4-2H$_4$]-8-iso-PGF2α, mixed well, and loaded onto the disk cartridge (Empore, 3M), which was preconditioned with methanol and HCl (1 mmol/L). The cartridge was washed with 1 mmol/L HCl and then with heptane. The sample was eluted with ethyl acetate containing 1% (vol/vol) methanol, evaporated under a stream of N_2, and dissolved in the mobile phase of 0.1% CH_3COOH (pH 3) and acetonitrile. An extraction was performed with high-performance liquid chromatography (HPLC, model HP1100 system, Hewlett Packard) with a Symmetry C8 column (3.9×150 mm, 5 μm, Waters). Then the solution was filtered with a membrane filter (0.45 μm), and the filtrate was used for LC-ESI/MS analysis (4-Sector type MStation 700 tandem mass spectrometer, JEOL) equipped with an ESI source. The mass spectrometer was operated in the negative ion–selected ion-monitoring mode. Ions monitored were of mass-to-charge ratio 353.24 and 357.26 for 8-iso-PFG2α and [3,3,4,4-2H$_4$]-8-iso-PGF2α, respectively. Our assay of 8-iso-PGF2α in the human urine samples was linear (r^2=0.99, $P<0.01$) and highly reproducible, with an interassay and intra-assay variability <9%.[13] An aliquot was stored for creatinine measurement, and all measurements were corrected for creatinine excretion.

TABLE 1. Baseline Characteristics of Study Subjects

	Men (n=52)	Women (n=51)
Age, y	26.1±0.7	25.3±0.6
Blood pressure, mm Hg		
Systolic	122±2	117±2
Diastolic	65±1	63±1
Total cholesterol, mg/dL	176±4	169±3
Triglycerides, mg/dL	87±5	77±7
Fasting blood glucose, mg/dL	98±2	94±1
17β-Estradiol, pg/mL	37±2	94±10*

Values are mean±SEM.
*$P<0.01$ vs corresponding value for men.

Biochemical Measurements

Plasma glucose and total cholesterol levels were determined by an enzymatic method. Plasma concentration of 17β-estradiol was measured by solid-phase radioimmunoassay (Diagnostic Products Co). Plasma polyunsaturated fatty acids (PUFAs), including linoleic acid, linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, were measured by using gas chromatography. Plasma antioxidant capacity was determined by measuring the activity of scavenger enzymes, including superoxide dismutase (SOD),[14] catalase,[15] and glutathione peroxidase (GSHPx).[16] Plasma vitamin E levels were determined by using HPLC with an electrochemical detector.[17]

Statistical Analysis

All values are expressed as mean±SEM. Unpaired Student *t* tests were used to compare means between men and women. For the comparison of baseline values and those after vitamin supplementation and the analysis of the intrasubject reproducibility data, paired *t* tests were used to analyze changes for men and women separately. The Pearson coefficient (*r*) was calculated to quantify the direction and magnitude of correlation between variables. A value of $P<0.05$ was considered to be statistically significant.

Results

Characteristics of Study Subjects

The baseline characteristics of the study subjects are summarized in Table 1. No significant differences were noted in age and blood pressure between men and women. There were no apparent differences in surrogate nutritional indices, including glucose, total cholesterol, and triglyceride levels. However, plasma 17β-estradiol levels were significantly higher in women.

Plasma TBARS and Urinary 8-Iso-PGF2α

Plasma TBARS were significantly higher ($P<0.01$) in men (2.32±0.11 nmol/mL) than in age-matched women (1.87±0.09 nmol/mL, Figure 1). Consistent with plasma TBARS, urinary 8-iso-PGF2α excretion was significantly higher in men than in women at baseline (292±56 versus 164±25 pg/mg creatinine, respectively; Figure 2).

Plasma PUFAs did not differ between men and women (1048±26 versus 1101±24 ng/mL, respectively; P=NS). Plasma TBARS formation was significantly higher in men than in women even after normalization by plasma PUFAs (0.51±0.12 versus 0.46±0.11 nmol/ng, respectively; $P<0.05$). Similarly, normalized urinary 8-iso-PGF2α excre-

tion was significantly higher in men than in women
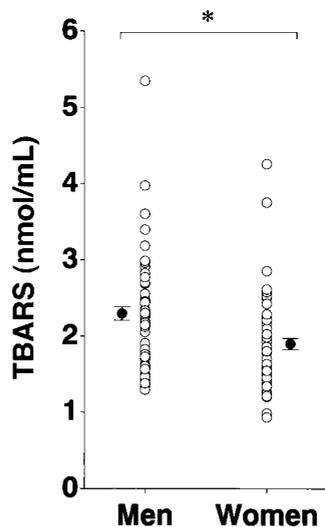
Figure 1. Plasma TBARS. Open circles represent individual measurements. Closed circles and vertical bars represent mean values and SE for each group, respectively. *$P<0.01$ for difference between men and women.

(2.37±0.55 versus 1.14±0.20 pg · mL per mg creatinine · ng, $P<0.05$).

The effects of antioxidant vitamin E and C supplementation for 4 weeks on plasma TBARS and urinary 8-iso-PGF2α excretion were examined in a randomly selected group of subjects (23 men and 30 women). As shown in Table 2, baseline level of plasma vitamin E was comparable between men and women. Vitamin E supplementation produced a statistically significant (≈2-fold) increase ($P<0.01$) in its plasma levels for men and women. However, it did not influence the plasma concentration of total cholesterol (Table 2). Baseline TBARS formation and urinary 8-iso-PGF2α excretion were again increased in men (Figure 2). After 4 weeks of vitamin supplementation, plasma TBARS in men were reduced by 34% ($P<0.01$, Figure 2A). Similarly, urinary 8-iso-PGF2α was significantly reduced in men by 48%. Individual values of TBARS and 8-iso-PGF2α in men treated with vitamins fell within the range of values for women. In contrast, there were no significant changes in plasma TBARS or urinary 8-iso-PGF2α in women after vitamin supplementation.
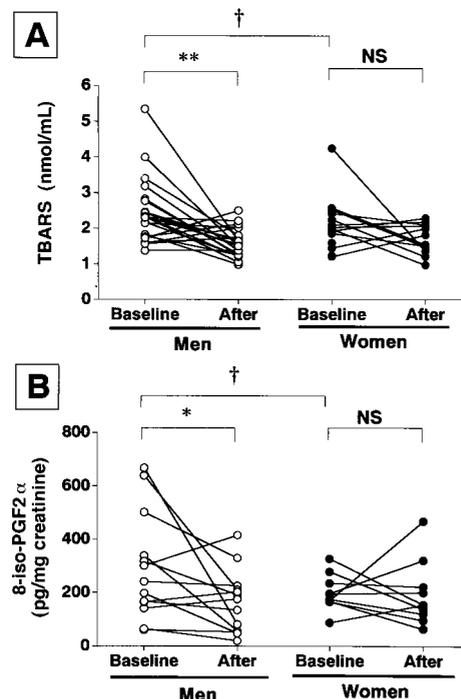
To assess the intrasubject reproducibility of plasma vitamin E, plasma TBARS, and urinary 8-iso-PGF2α concentra-

Figure 2. Plasma TBARS (A) and urinary excretion of 8-iso-PGF2α (B) at baseline and after the supplementation of antioxidant vitamin E and C for 4 weeks. Open and closed circles represent individual measurements. *$P<0.05$ and **$P<0.01$ for difference from each baseline value. †$P<0.05$ for difference from the value for men at baseline.

tions, 2 paired samples were obtained over a 4-week period of follow-up without any interventions. Vitamin E did not differ between baseline and after 4 weeks (1248±63 versus 1241±84 µg/dL, respectively [$P=NS$]; n=50). Similarly, for baseline values versus values after 4 weeks, TBARS (2.05±0.11 versus 2.09±0.09 nmol/mL, respectively [$P=NS$]; n=50) and 8-iso-PGF2α (285±42 versus 261±35 pg/mg creatinine, respectively [$P=NS$]; n=36) were comparable. Intrasubject coefficients of variation for vitamin E, TBARS, and 8-iso-PGF2α were 19±2%, 20±3%, and 10±10%, respectively.

Endogenous Antioxidants

Plasma levels of total SOD and catalase did not differ significantly between men and women (Table 3). In contrast, GSHPx was significantly higher in men than in women. Even though plasma 17β-estradiol levels were higher in women (Table 1), TBARS and estrogen levels showed no statistically significant inverse correlation ($r=0.07$, $P=0.54$; n=77). Similarly, there was no significant correlation between urinary 8-iso-PGF2α and plasma estrogen levels ($r=0.13$, $P=0.90$; n=42).

TABLE 2. Effects of Antioxidant Vitamin E and C Supplementation on Plasma Concentrations of Vitamin E and Total Cholesterol

	Men (n=23)	Women (n=30)
Vitamin E, µg/dL		
Baseline	920±40	1010±60
After supplementation	1903±121*	1908±114*
Total cholesterol, mg/dL		
Baseline	163±5	163±4
After supplementation	170±5	164±5

Values are mean±SEM.

*$P<0.01$ vs corresponding baseline value.

TABLE 3. Plasma Activity of Antioxidant Enzymes

	Men (n=51)	Women (n=45)
SOD, U/mL	2.60±0.31	3.00±0.40
Catalase, U/L	1.06±0.11	1.13±0.12
GSHPx, U/L	386±5	356±7*

Values are mean±SEM.

*$P<0.05$ vs corresponding value for men.

Discussion

The present study demonstrated that the in vivo biomarkers of oxidative stress were greater in healthy young men than in age-matched women. Chronic administration of antioxidant vitamins resulted in significant reduction in oxidative stress in men. This sex difference for oxidative stress was not mediated by any changes in endogenous antioxidant enzymes, vitamin E, or estrogen in the plasma. This may be a partial explanation for the greater susceptibility of men to atherosclerotic cardiovascular diseases that are linked to the increased generation of reactive oxygen species (ROS).

Direct demonstration of enhanced ROS production in humans is difficult because of the technical limitations. ROS interact with membrane lipids, resulting in the generation of aldehydes such as TBARS, which have yielded a conventional index of oxidative stress.[2,18] 8-Iso-PGF2α, one of the free radical–catalyzed products of arachidonic acid, is another useful marker, which has been shown to be more sensitive and specific.[4] In fact, elevated levels of 8-iso-PGF2α have been demonstrated in urine samples from humans under a wide variety of conditions of oxidative stress.[5–9] The present study has used urinary 8-iso-PGF2α measurement based on the highly specific LC-ESI/MS assay, which has been well validated in our recent study.[13] In the healthy volunteers participating in the present study, the urinary excretion of 8-iso-PGF2α averaged 228 pg/mg creatinine, which is consistent with the results of Davi and colleagues[6,7] in healthy subjects ($\approx$200 pg/mg creatinine).

By using plasma and urinary indices of oxidative stress, the present study demonstrated that oxidative stress was increased in men compared with women. This finding was further strengthened by consistent results; ie, after vitamin E and C supplementation in men, these increased indices fell significantly and reached levels comparable to those in women. Sex differences in oxidative stress have been also reported in the generation of superoxide radicals in rat aortic tissue.[19]

Previous studies have shown that the urinary excretion of 8-iso-PGF2α is enhanced under conditions such as advanced age,[5] hypercholesterolemia,[6] diabetes mellitus,[7] cigarette smoking,[8,9] and liver diseases.[20] Thus, we excluded the contribution of advanced age by assessing healthy young volunteers and the contribution of cigarette smoking by recruiting only nonsmokers into the present study. Furthermore, we carefully excluded subjects with hypertension, hypercholesterolemia, diabetes mellitus, and liver diseases from the present study.

Recently, oxidative stress has captured considerable attention as a potential mechanism for atherosclerotic vascular diseases.[21] Major cardiovascular risk factors can induce oxidative stress and atherogenesis.[4] Thus, an intimate link between coronary risk factors and cardiovascular diseases has been proposed to involve ROS. The contribution of increased ROS in atherosclerosis has been recently established for humans in which 8-iso-PGF2α is immunolocalized in atherosclerotic plaque[22] and for apoE-deficient mice, a model of atherosclerosis, in which urinary extraction of 8-iso-PGF2α is increased.[10] ROS are produced in the endothelial cells and/or vascular smooth muscle cells and may contribute to LDL oxidation, local monocyte chemotaxis, smooth muscle prolifera-

tion, upregulation of adhesion molecules and macrophage recruitment, endothelial dysfunction, and eventual plaque rupture.[23] Therefore, ROS can be harmful not only by the direct damaging effects on the vascular wall but also by modulating the biological signals in atherosclerosis. The association between male sex and oxidative stress has led to a renewed interest in an individual's sex as a potential risk factor for atherosclerotic diseases. Furthermore, our results have potential clinical implications for increased cardiovascular risk in postmenopausal women.[11]

Greater oxidative stress in men is due to an increased generation of ROS and/or reduced activity of antioxidants. Under healthy conditions, cellular respiration in the mitochondria is the dominant source of ROS. Therefore, a higher baseline metabolic rate in men than in women[24] might contribute to a higher level of oxidative stress in men in the present study. No previous reports have shown sex differences of known sources of ROS, such as NADPH oxidase and xanthine oxidase. Another possible mechanism is that women have a source of antioxidant protection that is lesser or absent in men. However, we could not find any increase in plasma antioxidant enzymes, including SOD and catalase, in women (Table 3), and GSHPx activity was even higher in men. Even though it is not clear in the present study how these changes in antioxidant levels contribute to ROS generation, increased GSHPx activity may be a compensatory response to increased oxidative stress in men.[25] Antioxidant properties of estrogen may also contribute to lower oxidative stress in women.[26] However, there was no significant correlation between plasma 17β-estradiol and TBARS or 8-iso-PGF2α levels. Furthermore, we cannot exclude the possibility that the differences may be due to the level of daily exercise, because exercise has been shown to cause oxidant stress.[27,28]

Having established that the formation of 8-iso-PGF2α largely depends on ROS, we set out to assess the reversibility of its increase in response to vitamin E and C supplementation. The administration of 300 mg/d of vitamin E caused a 2-fold increase in its plasma level after 4 weeks of treatment. In agreement with the study by Reilly et al[9] in chronic cigarette smokers, we have shown significant depression of TBARS and 8-iso-PGF2α excretion in men by the administration of vitamin E and C. Vitamin C not only suppresses the lipid peroxidation by itself but also recycles the vitamin E radical back to vitamin E, prolonging its antioxidant effect.[29] Thus, a combination of vitamin E and C might be beneficial in lowering the risk of atherosclerotic cardiovascular diseases in patients with high risk for oxidative stress.[30] When vitamin E is used alone, it can become pro-oxidant or at least lose its efficacy because it becomes a tocopherol radical on reaction with other radicals, which might explain, at least in part, the lack of its protective effects against atherosclerotic cardiovascular diseases.[31,32]

There are several potential limitations to be acknowledged in the present study. First, a measurement of the putative 2,3-dinor-5,6-dihydro-iPF2α-III metabolite of 8-iso-PGF2α (iPF2α-III), which has been shown to be a better marker of in vivo oxidative stress than 8-iso-PGF2α,[33] was not available for our study participants. However, previous studies have shown that 8-iso-PGF2α is a well-established in vivo marker of lipid peroxidation, and this measure is well correlated with its major metabolite.[4] Moreover, 8-iso-PGF2α is metabolized

in the liver, and its measurement may be influenced in patients with liver dysfunction.[33] However, the liver function was normal in our study subjects. Thus, we consider that our measurement of 8-iso-PGF2α provides a specific in vivo marker of oxidative stress. Second, assays to detect lipid peroxidation are dependent not only on the extent of free radical activity but also on the amount of serum lipid substrate and the protective effects of serum antioxidants. Furthermore, 8-iso-PGF2α has been shown to be produced via enzyme-dependent mechanisms as well as nonenzymatic oxidative modifications of arachidonic acid.[4] Therefore, the assumption that measurements of lipid peroxidation are a reflection of free radical activity requires further validation. Third, our study subjects did not have the control diet; thus, data on the composition of PUFAs in the diet were not available in the present study. Therefore, we need to further confirm the present findings also in the setting of a controlled feeding study. Fourth, the majority of 2 groups overlapped entirely in terms of TBARS and 8-iso-PGF2α levels. Larger numbers of study subjects are clearly needed to confirm our results.

In conclusion, whole-body ROS production is higher in healthy young men than in premenopausal women under ambulatory conditions. Even though there is no evidence that this sex difference is functionally significant in terms of the risk of atherosclerotic cardiovascular disease or implies a mechanism for the differences in the propensity of men and women to develop atherosclerosis, such links between female sex and lower oxidative stress may contribute to a better understanding of the role of an individual's sex in the pathogenesis of atherosclerosis.

Acknowledgments

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