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Association between Oxidative Stress and Bone Mineral Density

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Free radicals have been shown to be involved in bone resorption *in vitro* and in rodents. We studied the effect of oxidative stress on bone mineral density (BMD) in 48 women and 53 men from a population-based study. The levels of 8-iso-$PGF_{2\alpha}$ (a major F_2-isoprostane and a biomarker of oxidative stress) and a control, 15-keto-dihydro-$PGF_{2\alpha}$ (a biomarker of inflammatory response), were measured in urinary samples and their association with BMD and quantitative ultrasound (QUS) measurements were examined. In multivariate linear regression analyses, 8-iso-$PGF_{2\alpha}$ levels were negatively associated with both BMD and QUS. In contrast, no association was found for 15-keto-dihydro-$PGF_{2\alpha}$. Our findings establish a biochemical link between increased oxidative stress and reduced bone density and provide a rational for further studies investigating the role of pro- and antioxidants in osteoporosis. © 2001 Academic Press

Key Words: **oxidative stress; isoprostanes; prostaglandins; bone mineral density; quantitative ultrasound.**

It has previously been shown *in vitro* and in rodents that free radicals are involved in osteoclastogenesis and in bone resorption (1). The recent finding of osteopetrosis in mice lacking NF-κB (2), the discovery of receptor activator of NF-κB (RANK), the RANK ligand, and the decoy receptor osteoprotegerin (3), all demonstrated the great importance of NF-κB in osteoclastogenesis. NF-κB is an oxidative stress-responsive transcription factor (4). Thus, free radicals may increase bone resorption through activation of NF-κB. Several risk factors for osteoporosis, such as smoking (5), hypertension (6) and diabetes mellitus (7) are associated with increased oxidative stress. We have recently demonstrated that an insufficient dietary intake of antioxidants substantially increases the risk of hip fracture in current smokers, whereas current smokers with a more adequate intake seem to have a fracture risk that is similar to that seen among never smokers (8), supporting our hypothesis that oxidative stress has important effects on bone in man.

A major problem associated with the assessment of free radical-induced oxidative stress in various diseases has been the limitation in available assay methods for *in vivo* measurement of free radical generation or end products of free radical catalyzed oxidation of lipids. Isoprostanes, a family of prostaglandin derivatives generated *in vivo* by nonenzymatic free radical catalyzed oxidation of arachidonic acid (9). It has also been observed that one of the major isoprostanes, 8-iso-$PGF_{2\alpha}$ is increased in several syndromes that are associated with oxidant injury and the measurement of isoprostanes is now widely regarded as a reliable biomarker for *in vivo* measurement of lipid peroxidation (10–12). Administration of antioxidant vitamins or radical scavengers has an effect on the isoprostane formation in various experimental models (13–15).

Cyclooxygenase-2, an isoform of cyclooxygenase, is induced in macrophages, epithelial cells and fibroblasts by several pro-inflammatory stimuli leading to release of prostaglandins (16). 15-Keto-dihydro-$PGF_{2\alpha}$, a major metabolite of the primary $PGF_{2\alpha}$, is increased in inflammatory response and can be used as an index of lipid peroxidation through the cyclooxygenase pathway (17). We have recently developed highly specific radioimmunoassays by raising antibody in rabbits against 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ (17, 18). By applying these assays, we have shown that oxidative modification of arachidonic acid through nonenzymatic and enzymatic lipid peroxidation pathways are involved in endotoxin induced inflammation in sep-

Abbreviations used: COX, cyclooxygenase; PG, prostaglandin; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$; 15-keto-dihydro-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; CCl_4, carbon tetrachloride; BMD, bone mineral density; QUS, quantitative ultrasound.

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tic shock (11), hepatotoxin induced oxidative injury (19), cerebral oxidative injury after resuscitation from cardiac arrest (12), various rheumatic diseases (20) and experimental diabetic pregnancy (21). However, very little is known on the involvement of oxidative stress and bone mass. The main aim of this study was to investigate whether oxidative stress may have an impact on bone mass in man.

MATERIALS AND METHODS

Study subjects and bone density measurements. The study population and bone density measurements have previously been presented in detail (22). Briefly, 61 women and 61 men were randomly selected from a Swedish population register to represent ages between 22 and 85 years. Bone mineral density of the total body, lumbar spine, and proximal femur were measured by dual energy X-ray absorptiometry (DPX-L, Lunar), and BMD of the forearm with single energy X-ray absorptiometry (DTX-100 bone densitometer, Hologic). In addition, quantitative ultrasound (QUS; Achilles Lunar) measurements at the calcaneus were performed to assess the speed of sound (SOS) and broadband ultrasound attenuation (BUA). The precision error in our laboratory, is about 1% for the DPX-L and 0.2 and 2.6% for SOS and BUA, respectively. Using a questionnaire, subjects were asked about smoking habits, calcium intake, diseases, medical therapies, and physical activity. The women were asked about reproductive history and use of postmenopausal hormone replacement therapy (HRT). Fasting blood and urinary samples were collected and stored frozen at −70°C until analysis. Urinary samples were available from 53 men and 48 women, of whom 19 were premenopausal and 29 postmenopausal. Nine of the postmenopausal women had ever used HRT.

Radioimmunoassays of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$. Unextracted urinary samples were analyzed for 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$, by highly specific and validated radioimmunoassays at our laboratory as described elsewhere (17, 18). The cross-reactivity of the 8-iso-$PGF_{2\alpha}$ antibody with 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, TXB_2, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$ and 8-iso-$PGF_{3\alpha}$, respectively was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6%. The detection limits of the 8-iso-$PGF_{2\alpha}$ assay was about 23 pmol/L. The cross-reactivity of the 15-keto-13,14-dihydro-$PGF_{2\alpha}$ antibody with $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, PGE_2, 15-keto-13,14-dihydro-PGE_2, 8-iso-15-keto-13,14-dihydro-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$, TXB_2, 8-iso-$PGF_{3\alpha}$ was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001, 0.01%, respectively. The detection limit was 45 pmol/L. The urinary levels of 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ were adjusted for U-creatinine concentration.

Statistical analyses. We used standard linear regression models to examine the relationship between the urinary 8-iso- or 15-keto-dihydro-$PGF_{2\alpha}$/creatinine ratio and bone density measurements. Tobacco use and obesity could be regarded either as confounders or as mediators of the effect of oxidative stress on BMD. Therefore, two different models were used in the analyses; an age- and sex-adjusted model and a multivariate model also including body mass index, smoking and snuff status (never, former, and current use). We also tested to include other potential confounders such as hypertension, diabetes mellitus and other diseases, physical activity, age at menarche, HRT, and other medical therapies, but the inclusion of these factors had only marginal effects on the beta-coefficients and were therefore not included in the analyses presented in the tables. The continuous variables were kept in their original form.

TABLE 1

	Women (n = 48)		Men (n = 53)	
	Mean	SD	Mean	SD
Age (years)	55.9	17.4	55.7	17.7
Weight (kg)	68.4	11.8	75.9	10.9
Height (cm)	165.2	7.0	175.4	7.9
Body mass index (kg/m^2)	25.1	4.4	24.6	2.9
8-Iso-$PGF_{2\alpha}$ (nmol/l)	3.8	2.6	5.6	3.4
Creatinine (mmol/l)	10.8	5.1	15.0	7.8
8-Iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.35	0.14	0.38	0.14
15-Keto-dihydro-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.48	0.16	0.50	0.22
BMD lumbar spine (g/cm^2)	1.11	0.16	1.17	0.16
BMD femoral neck (g/cm^2)	0.91	0.16	0.95	0.17
BMD total body (g/cm^2)	1.11	0.10	1.19	0.10
BMD distal forearm (g/cm^2)	0.34	0.07	0.44	0.08
BUA (dB/mHz)	113.9	4.9	117.9	14.0
SOS calcaneus (m/s)	1528	41	1535	41
	n	Percent	n	Percent
Hypertension	7	15	3	6
Diabetes mellitus	1	2	1	2
Never smoker	32	66	22	41
Former smoker	8	17	19	36
Current smoker	8	17	12	23
Never snuffer	47	98	39	73
Former snuffer	0	0	3	6
Current snuffer	1	2	11	21

RESULTS

The characteristics of the participants are outlined in Table 1. Nine out of 48 women (19%) whereas 23 out of 53 men (44%) were current smokers or snuffers.

Bone Density Measurements and 8-Iso-$PGF_{2\alpha}$ Levels

As seen in Table 2, higher levels of 8-iso-$PGF_{2\alpha}$ were significantly related to lower BMD at the lumbar spine (P = 0.04) and total body (P = 0.001) as well as to lower ultrasound measurements at the heel (BUA P = 0.01 and SOS, P = 0.03) in the age- and sex-adjusted model. When BMI, smoking, and snuffing were also added to the model, the association remained at total body (P = 0.001), distal forearm (P = 0.04), and heel (BUA, P = 0.04 and SOS, P = 0.03).

Bone Measurements and 15-Keto-dihydro-$PGF_{2\alpha}$ Levels

We then examined the association between 15-keto-dihydro-$PGF_{2\alpha}$, a $PGF_{2\alpha}$-metabolite and an indicator of inflammation, and bone density measurements (Table 3). There was a negative association only at the femoral neck in the age- and sex-adjusted model, which did not persist after multivariate adjustment.

TABLE 2

Association between Adjusted Bone Density and 8-Iso-$PGF_{2\alpha}$

	Age- and sex-adjusted model			Multivariate model[a]		
Variable	β-coefficient	SD	*P* value	β-coefficient	SD	*P* value
BMD lumbar spine (g/cm^2)	−0.22	0.11	0.04	−0.19	0.11	0.11
BMD femoral neck (g/cm^2)	−0.17	0.10	0.09	−0.16	0.10	0.12
BMD total body (g/cm^2)	−0.21	0.06	0.001	−0.21	0.06	0.001
BMD distal forearm (g/cm^2)	−0.08	0.04	0.06	−0.10	0.05	0.04
BUA (dB/mHz)	−23	9.1	0.01	−20	9.4	0.04
SOS calcaneus (m/s)	−54	24	0.03	−55	26	0.03

[a] Model including age (continuous), sex (female/male), body mass index (continuous), smoking status (never, former, current), and snuff status (never, former, current).

DISCUSSION

In the present study, we used the urinary excretion of the F_2-isoprostane, 8-iso-$PGF_{2\alpha}$ as a marker of *in vivo* oxidative stress. The measurement of this chemically stable compound has several distinct advantages over other markers of oxidant stress: (1) It reflects a nonenzymatic process of lipid peroxidation of an ubiquitous, endogenous substrate (i.e., arachidonic acid) that is catalyzed by oxygen radicals (2, 10). Once released in free form from cell membranes or LDL, 8-iso-$PGF_{2\alpha}$ circulates in peripheral venous blood and excretes into the urine. This compound has been used previously to demonstrate enhanced lipid peroxidation in association with cigarette smoking (23), different rheumatic diseases (20) as well as after coronary artery reperfusion (24), etc.

We found that the formation and urinary excretion of 8-iso-$PGF_{2\alpha}$ was negatively related to BMD in individuals who were carefully characterized for other variables that could influence *in vivo* lipid peroxidation. In contrast, no association was seen in multivariate analysis between bone density measurements and 15-keto-dihydro-$PGF_{2\alpha}$, which is increased in inflammatory response, such as in endotoxemia (11), CCl_4-induced hepatotoxicity (19) and different rheumatic diseases (20). The distinct differences obtained with these two closely related substances demonstrate the high specificity of the assays.

Compared to former and never users of tobacco, current users had on average 33% higher ($P = 0.01$) 8-iso-$PGF_{2\alpha}$ levels consistent with previous studies (9). The higher 8-iso-$PGF_{2\alpha}$ levels in current smokers compared with former and never smokers could be regarded either as a confounder or as a mediator of the effect on bone mass. Obesity has also been reported to increase oxidative stress (25). We therefore presented results from analyses using two different models, a basic age- and sex-adjusted model and a multivariate model including age, gender, BMI and tobacco use. We also tested to analyze the data in a multivariate model including age, gender, and BMI, but excluding tobacco use, but only small changes in the beta-coefficients were found. Thus, the results were similar in the age- and sex-adjusted model and in a model also including tobacco use, BMI and other potential confounders, indicating that other factors have an impact on the levels of 8-iso-$PGF_{2\alpha}$. The diet appears to contain several such factors, including vitamin C (26), vitamin E (14), selenium, (27), and isoflavone phytestrogens (28). We find it intriguing that there are studies that suggest that

TABLE 3

Association between Adjusted Bone Density and 15-Keto-13,14-dihydro-$PGF_{2\alpha}$

	Age- and sex-adjusted model			Multivariate model[a]		
Variable	β-coefficient	SD	*P* value	β-coefficient	SD	*P* value
BMD lumbar spine (g/cm^2)	−0.09	0.09	0.30	−0.03	0.09	0.72
BMD femoral neck (g/cm^2)	−0.16	0.08	0.04	−0.11	0.08	0.19
BMD total body (g/cm^2)	−0.07	0.05	0.12	−0.05	0.05	0.36
BMD distal forearm (g/cm^2)	−0.01	0.03	0.69	−0.009	0.04	0.80
BUA (dB/mHz)	0.06	7.3	0.99	4.3	7.9	0.59
SOS calcaneus (m/s)	−5.1	18.9	0.79	−2.0	20.6	0.93

[a] Model including age (continuous), sex (female/male), body mass index (continuous), smoking status (never, former, current), and snuff status (never, former, current).

these dietary factors also have important effects on bone (29).

Age-adjusted rates of hip fracture incidence vary more than seven-fold in Europe; the highest rates are found in Northern Europe, particularly Norway and Sweden. Known risk factors cannot explain these observations; this indicates that hitherto unknown environmental factors, such as diet, contribute to the development of osteoporosis. When dietary patterns in Europe were compared the dietary intake of vitamin C was found to be lower in Northern Europe (30). Although studies on vitamin C intake and the relation to BMD have been somewhat conflicting, possibly because of the well-known limitations of dietary nutrient assessment questionnaires, a positive association has generally been found (31). Recently, vitamin C supplement use was shown to have a beneficial effect on BMD in postmenopausal women (29).

In conclusion, our results provide an important biochemical link between increased lipid peroxidation and reduced bone mineral density. Further studies are needed to elucidate the roles of pro- and antioxidants in osteoporosis.

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