Proteasome Activity as a Target of Hormone Replacement Therapy–Dependent Plaque Stabilization in Postmenopausal Women

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Abstract—The molecular mechanisms of the atheroprotective effect evoked by hormone replacement therapy in postmenopausal women is not well known. Recently, we have demonstrated enhanced activity of the ubiquitin-proteasome system in human atherosclerotic plaques and evidenced that it is associated with inflammatory-induced plaque rupture. Therefore, we hypothesized that hormone replacement therapy may exert the cardioprotective effects modulating the ubiquitin-proteasome activity. To investigate this possibility, this study examined the differences in inflammatory infiltration, as well as ubiquitin-proteasome activity, between asymptomatic carotid plaques of postmenopausal women with and without concomitant hormone replacement therapy. Plaques were obtained from 20 postmenopausal women treated with hormone replacement therapy (current users) and 32 nontreated women (never-users) enlisted to undergo carotid endarterectomy for extracranial high-grade ($>70\%$) internal carotid artery stenosis. Plaques were analyzed for macrophages, T lymphocytes, human leukocyte antigen-DR+ cells, ubiquitin-proteasome system, nuclear factor κB, inhibitor of nuclear factor κBβ, tumor necrosis factor-α, nitrotyrosine, matrix metalloproteinase-9, and collagen content (immunohistochemistry and ELISA). Compared with plaques from current users, plaques from never-users had more macrophages, T lymphocytes, and human leukocyte antigen-DR+ cells ($P<0.001$); more ubiquitin-proteasome activity, tumor necrosis factor-α, and nuclear factor κB ($P<0.001$); and more nitrotyrosine and matrix metalloproteinase-9 ($P<0.001$), along with a lesser collagen content and inhibitor of nuclear factor κBβ levels ($P<0.001$). This study supports the hypothesis that hormone replacement therapy inhibits plaque ubiquitin-proteasome activity by decreasing oxidative stress generation in postmenopausal women. This effect, in turn, might contribute to plaque stabilization by inhibiting the activation of nuclear factor κB–dependent inflammation, responsible for plaque rupture. (*Hypertension*. 2008;51:1135-1141.)

Key Words: hormone replacement therapy ■ atherosclerotic plaque ■ inflammation ■ ubiquitin-proteasome activity

Cardiovascular disease is the leading cause of death in postmenopausal women.[1,2] There is a strong link between menopause and an increased incidence of cardiovascular disease, and observational studies suggest that postmenopausal hormone therapy, including various estrogen preparations with or without a progestin (most commonly a synthetic progestin), reduces cardiovascular disease risk by about half.[2,3] Hormone replacement therapy (HRT) use was also associated with a lesser extent of angiographically assessed coronary atherosclerosis.[4,5] An atheroprotective effect of estrogens is plausible and is especially attributed to a direct effect on the arterial wall, an antioxidant action, and a favorable effect on inflammation.[6] In prospective studies, administration of ovarian hormones to postmenopausal women has been shown to decrease oxidative stress markers, such as superoxide anion and peroxynitrite, and proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin-1, that play a central role in the pathogenesis of atherosclerosis, including plaque instability.[7,8] Although these studies have demonstrated a clear vasoprotective effect of estrogen, the cellular/molecular mechanisms of ovarian hormones responsible for the antioxidant and anti-inflammatory actions remain unclear in humans. Because there is emerging evidence that the ubiquitin-proteasome system (UPS), the major pathway for nonlysosomal intracellular protein degradation in eukaryotic cells, induces inflam-

mation in both the initial stage and progression of atherosclerosis,[9] we hypothesized that HRT may exert the cardioprotective effects modulating the UPS activity. To investigate this possibility, this study examined the differences in inflammatory infiltration, as well as ubiquitin-proteasome activity, between asymptomatic carotid plaques of postmenopausal women with and without concomitant HRT. Moreover, because the upregulation of oxidative stress may activate the UPS,[10] the localization of nitrotyrosine, a modified amino acid produced by reactive superoxide anion (O^{2-}),[11] in atherosclerotic lesions was measured quantitatively.

Methods

Study Population

Patients were recruited from the outpatient department for internal medicine of the teaching hospital at the Second University of Naples, cardiovascular surgery units of V. Monaldi Hospital Naples, and cardiovascular surgery units of Sassari Hospital from January 2003 to June 2007. Among them we selected postmenopausal women with asymptomatic carotid stenosis (according to North American Symptomatic Carotid Endarterectomy Trial classification), enlisted to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis.[12] Asymptomatic patients underwent a baseline clinical examination, as well as medical history, and had never developed neurologic symptoms or cerebral lesions assessed by computed tomography. All of the patients received computed tomography or MRI. The sample size for the planed study was estimated in 52 patients (32 postmenopausal women without HRT and 20 postmenopausal women with concomitant HRT). HRT was defined as oral or transdermal use of estrogens combined with progestin. HRT use during the study was assessed by medicine inventory at baseline clinical examination. Furthermore, the postmenopausal women answered a specific questionnaire about medicines that were used for menopausal complaints. This questionnaire included information about medicines that were used before the beginning of the study, the date of the beginning and end of treatment, route of administration, and duration of use. Information from medicine inventory during the study and this specific questionnaire were used to classify the subjects. The women who never used HRT were classified as "never-users." The women who had already used HRT were classified as "current users." The current-user women used a continuous 17β-estradiol patch (50 μg/d) plus cyclic oral medroxyprogesterone acetate (10 mg/d for 12 days per month). Information on duration of treatment was available for 18 of the 20 current users who answered the specific questionnaire. A duration of ≥4 years was attributed to the 18 women who used HRT. All of the participants had not undergone surgically induced menopause and had not had a menstrual period in the preceding year. All of the subjects had no clinical or laboratory evidence of heart failure, previous stroke, valvular defects, malignant neoplasms, or secondary causes of hypertension. Carotid sonography was performed on a single ultrasound machine (Aloka 5500). During the observational period, dietary counseling was added to the previous therapy. The study was approved by the ethics committee, and informed written consent was obtained for each patient.

Laboratory Analysis

Plasma glucose and serum lipids were measured by enzymatic assays in the hospital's chemistry laboratory. Serum follicle-stimulating hormone and estradiol concentrations were measured by radioimmu-

noassay using commercially available kits (Boehringer Mannheim). All of the vascular inflammatory markers were measured at Mitsubishi Biomedical Laboratory Co.

Atherectomy Specimens

After surgery, the specimens were cut perpendicular to the long axis into 2 halves. The first half was frozen in liquid nitrogen for the following ELISA analysis. A portion of the other half of the specimen was immediately immersion fixed in 10% buffered formalin. Sections were serially cut at 5 μm, mounted on lysine-coated slides, and stained with hematoxylin-eosin and with the trichrome method. Carotid artery specimens were analyzed by light microscopy.

Immunohistochemistry

After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen. Similar regions of the plaque were analyzed. Serial sections were incubated with specific antibodies: anti-ubiquitin, anti-proteasome 20S, smooth muscle actin (vascular smooth muscle cell), anti–human leukocyte antigen-DR locus, anti-CD68, and anti-CD3 (Dako); anti-inhibitor of nuclear factor κBβ (NF-κB; IkB-β) and anti–matrix metalloproteinase (MMP)-9 (Santa Cruz); and anti–TNF-α (R&D). Specific antibodies that selectively recognize the activated form of NF-κB (p65 and p50 subunits, Santa Cruz) were used. Analysis of immunohistochemistry was performed with a personal computer–based quantitative 24-bit color image analysis system (IM500, Leica Microsystem AG).

Sirius Red Staining for Collagen Content

After dehydration, the sections were observed under polarized light after being placed on coverslips. The sections were photographed with identical exposure settings for each section.

Biochemical Plaque Assays

Plaques were lysed and centrifuged for 10 minutes at 10 000*g* at 4°C. After centrifugation, 20 μg of each sample was loaded, electrophoresed in polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. Each determination was repeated 3 times. Ubiquitin, IkB-β, MMP-9, TNF-α, and nitrotyrosine levels were quantified in plaques using a specific ELISA kits (Santa Cruz, R&D Systems, and Imgenex). Nuclear extracts from plaque specimens were obtained as described by Ohlsson et al.[13] We used a specific antibody that selectively recognizes the activated form of the NF-κB p65 and p50 subunits. NF-κB binding to B sites was assessed using the Trans-AM NF-κB p65 and p50 transcription factor assay kits (Active Motif Europe, Rixensart). For the quantitative measurement of the proteasome 20S activity, a specific SDS activation kit (Boston Biochem) was used. Nitrotyrosine was assayed into the plaque tissue with a kit supplied by Hycult Biotech.

Macrophages Extraction From Atherosclerotic Plaques

Macrophages were selectively extracted from plaques as described by de Vries et al.[14] Biochemical assays on cell homogenates for ubiquitin and proteasome 20S determinations were performed as illustrated earlier.

Measurement of O^{2-}

Production of O^{2-} was measured as the superoxide dismutase–inhibitable reduction of cytochrome C, as described previously.[15]

Statistical Analysis

Data are presented as means±SDs. Continuous variables were compared among the groups of patients with 1-way ANOVA for normally distributed data and the Kruskal-Wallis test for nonnor-

Table 1. Characteristics of Study Patients

Patients Characteristics	HRT Current-User Group (N=20)	HRT Never-User Group (N=32)
Age, y	61±6	62±8
Estradiol, pg/mL	94.7±26.8*	12.8±3.6
FSH, IU/L	26.7±12.6*	61.7±6.6
Clinical characteristics		
Family history of IHD, n (%)	12 (60)	17 (53)
Family history of diabetes, n (%)	9 (45)	14 (44)
Hypercholesterolemia, n (%)	11 (55)	17 (53)
Diabetes, n (%)	10 (50)	15 (47)
Cigarette smoking, n (%)	6 (30)	10 (31)
Hypertension, n (%)	13 (65)	20 (63)
Coronary artery disease, n (%)	7 (35)	11 (34)
BMI, kg/m^2	27.6±4	28.1±5
Clinic SBP, mm Hg	128±11	127±10
Clinic DBP, mm Hg	82±6	81±7
Heart rate, bpm	74±11	76±12
Serum creatinine, μmol/L	78.7±1.9	79.3±2.2
Blood glucose, mmol/L	6.04±0.6	6.05±1.1
Total cholesterol, mmol/L	5.69±0.12	5.71±0.10
HDL cholesterol, mmol/L	1.26±0.13	1.23±0.09
Triglycerides, mmol/L	1.95±0.41	1.97±0.39
Stenosis severity, %	77.1±5.1	76.7±4.4
Active therapy		
Aspirin, n (%)	18 (90)	29 (91)
Calcium channel blocker, n (%)	4 (20)	6 (19)
Statin, n (%)	19 (95)	30 (94)
ACE inhibitor, n (%)	12 (60)	19 (60)
Diuretic agent, n (%)	9 (45)	15 (47)
AT$_1$ antagonist, n (%)	6 (30)	11 (34)
Hypoglycemic agents, n (%)	9 (45)	14 (44)
Insulin, n (%)	3 (15)	4 (13)

Data are presented as means±SDs unless otherwise specified. FSH indicates follicle-stimulating hormone; IHD, ischemic heart disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACE, angiotensin-converting enzyme; HDL, high-density lipoprotein.

*$P<0.05$ vs never-user group.

mally distributed data. The Kolmogorov-Smirnov test was used to assess whether continuous variables were normally distributed or not. We compared data using a Kruskal-Wallis test for triglycerides and insulin. When differences were found among the groups, Bonferroni correction was used to make pairwise comparisons. The Bonferroni correction was used in the comparison of ubiquitin, proteasome, p50, p65, IkB-β, MMP-9, and O^{2-} production values. A $P<0.05$ value was considered statistically significant. All of the calculations were performed using the computer program SPSS 12 (SPPS Inc).

Results

Preoperative Characteristics of the Patients

Demographic data for the study population are presented in Table 1. The percentage of carotid diameter reduction, risk factors, and concomitant therapy did not differ among the groups (Table 1). Anthropometric characteristics, routine blood chemical analyses, and BP were similar in all of the patients (Table 1). In the group of current users of HRT, the estradiol concentration was significantly higher ($P<0.01$) and the follicle-stimulating hormone concentration was significantly lower ($P<0.01$) compared with the group of never-users of HRT (Table 1).

Plaque Composition of the Study Patients: Is HRT Use Associated With a Stable Plaque Phenotype?

Compared with HRT current-user women, never-users of HRT had a significantly greater portion of plaque area occupied by macrophages ($P<0.01$), T lymphocytes ($P<0.01$), and greater expression of human leukocyte antigen-DR antigen ($P<0.01$), as well as smaller content of vascular smooth muscle cell ($P<0.01$) and interstitial collagen (Figure 1 and Table 2). MMP-9 levels were more abundant in never-users that in current-user lesions ($P<0.001$; Figure 1 and Table 2). Notably, estradiol levels were positively correlated with plaque interstitial collagen content ($r=0.36$; $P<0.01$) and vascular smooth muscle cell content ($r=0.38$; $P<0.01$) and were negatively correlated with plaque expression of macrophages ($r=-0.40$; $P<0.01$) and plaque expression of human leukocyte antigen-DR antigen ($r=-0.33$; $P<0.01$).

Oxidative Stress in the Plaque: Is HRT Use Associated With the Downregulation of Nitrotyrosine Levels?

Higher levels, as well as higher staining, of nitrotyrosine were found in plaques of never-users of HRT as compared with plaques of current users ($P<0.001$; Figure 2). A similar pattern was found for O^{2-} production (never-users: 8.13±1.8 pmol/L; current users: 1.96±0.77 pmol/L; $P<0.01$). Notably, oxidative stresses were strongly dependent from estradiol levels, as also reflected by the statistically significant negative correlation between plasma estradiol levels and plaque nitrotyrosine concentrations ($r=-0.48$; $P<0.01$).

Ubiquitin-Proteasome Activity in the Plaque: Does HRT Use Modulate Ubiquitin-Proteasome Activity in the Plaque Reducing Oxidative Stress?

Immunohistochemistry revealed higher staining of ubiquitin and proteasome 20S in inflammatory cells of HRT never-users as compared with inflammatory cells of the current users ($P<0.01$). A similar pattern of response was seen for ubiquitin plaque levels ($P<0.001$) and proteasome 20S plaque activity ($P<0.001$; Figure 3). Notably, both ubiquitin levels and proteasome 20S activity were positively correlated with plaque nitrotyrosine ($r=0.51$ and $r=0.41$, respectively; $P<0.01$) and negatively correlated with estradiol plasma levels ($r=-0.33$ and $r=-0.39$, respectively; $P<0.01$).

To identify whether the higher ubiquitin-proteasome levels observed in plaques were produced by macrophages, we repeated quantitative analyses on macrophages selectively extracted from 10 plaques randomly selected from each
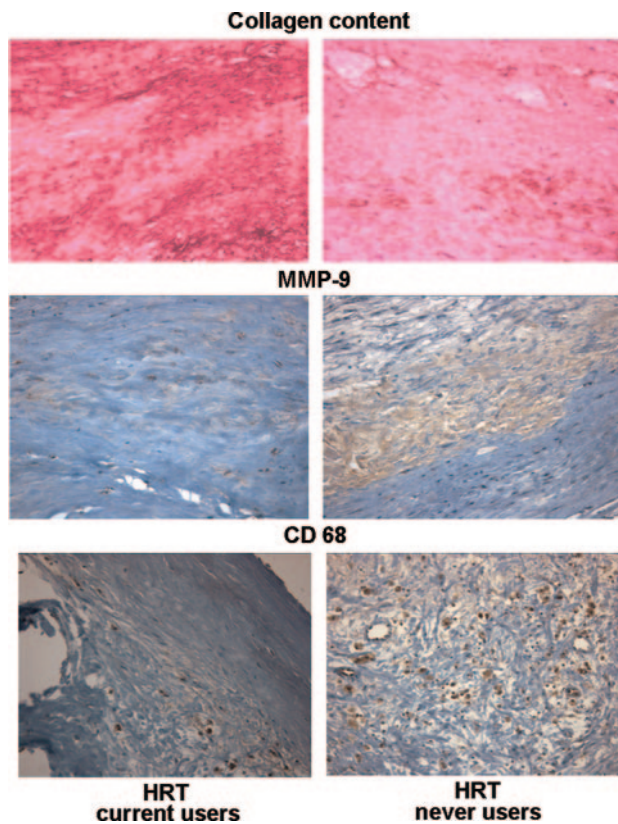
Collagen content

MMP-9

CD 68

| HRT current users | HRT never users |

Figure 1. Representative sections show immunochemistry for collagen content and sirius red staining (×400), for MMP (MMP-9; ×400) and macrophages (CD 68; ×400) in plaques of HRT current-user women and HRT never-user women. Similar regions of plaque are shown. Results are typical of HRT and untreated HRT women plaques.

groups. We observed that the never-user group had the highest of both ubiquitin levels (529.5 ± 121 ng/mg) and proteasome 20S activity (79.3 ± 27 pmol/mg) and the current-user group had the lowest (ubiquitin: 197 ± 58 ng/mg; proteasome 20S: 21.3 ± 10 pmol/mg; ubiquitin: $P < 0.05$; proteasome 20S: $P < 0.05$).

Inflammation in the Plaque: Does HRT Use Downregulate NF-κB Activity in the Plaque Reducing Ubiquitin-Proteasome Activity?

NF-κB activation, as reflected by the selective analysis of the activated form of both p50 and p65, was significantly higher in plaques of HRT never-users ($P < 0.01$) as compared with plaques of HRT current users. Immunohistochemistry and quantitative analyses revealed lower staining and levels for IkB-β in plaques of never-users as compared with plaques of current users ($P < 0.001$; Figure 4). Both immunohistochemistry and ELISA revealed markedly higher staining and levels of TNF-α in never-user lesions compared with the current-user lesions ($P < 0.001$; Figure 2). Notably, inflammation was negatively correlated with estradiol levels, as also reflected by the statistically significant negative correlation between estradiol plasma levels and TNF-α concentrations ($r = -0.44$; $P < 0.01$). Moreover, both ubiquitin levels and proteasome 20S activity were positively correlated with plaque TNF-α concentration ($r = 0.40$ and $r = 0.34$, respectively; $P < 0.01$).

Table 2.   Characteristics of Plaques Examined

Plaque characteristics	HRT Current-User Group (N=20)	HRT Never-User Group (N=32)
Macrophage-rich areas, %	$6 \pm 4*$	28 ± 3
T-cell–rich areas, %	$14 \pm 5*$	41 ± 15
HLA-DR–rich areas, %	$10 \pm 3*$	28 ± 9
VSMC-rich areas, %	$23 \pm 6*$	12 ± 4
Collagen content, %	$24.6 \pm 5.2*$	9.2 ± 3.1
MMP-9, μg/mg	$5.2 \pm 3.1*$	13.6 ± 7.3

Data are presented as means±SDs. VSMC indicates vascular smooth muscle cell.

*$P < 0.05$ vs with HRT never-user group.

Discussion

We reported previously that oxidative stress-dependent UPS overactivity contributes to the clinical instability of atherosclerotic plaques in hypertensive,[15] diabetic,[16] and symptomatic patients[17] by promoting plaque rupture induced by NF-κB–dependent inflammation. Now, in the present report, we provide evidence for the critical involvement of UPS in the process of plaque stabilization realized by HRT in postmenopausal women. In particular, we evidence an inhibitory effect of HRT on UPS activity in human atherosclerotic lesions, show in humans the possibility of UPS activity regulation in an oxidative stress-dependent fashion, and finally we associate the inhibition of UPS with the reduction of inflammation during HRT. Lower expression and activity of UPS was found in specimens obtained from carotid lesions of postmenopausal women treated with HRT compared with specimens obtained from women never treated with HRT. Notably, the hypothesis that UPS activity suppression by HRT may have a protective impact on plaque phenotype is also supported in this study by its negative correlation with the MMP-9 expression and by a positive correlation with plaque collagen content in HRT-treated women. Previous studies[18–20] reported the ability of HRT to reduce atherosclerotic plaque inflammatory burden and to slow lesion evolution toward rupture. Moreover, estrogen treatment greatly reduced neutrophil and monocyte/macrophage numbers into arteries of ovariectomized rats.[21] In prospective studies, administration of ovarian hormones to postmenopausal women has been shown to negatively modulate most inflammatory markers, with significant decreases in E-selectin, soluble vascular cell adhesion molecule-1, soluble intercellular adhesion molecule-1, and TNF-α.[7] However, these studies did not delineate the cellular/molecular effects of ovarian hormones on vascular inflammation and on the pathogenesis of vascular disease and did not provide any evidence regarding the involvement of UPS in pathophysiology of HRT-dependent plaque stabilization. In our study, macrophages, T lymphocytes, and HDLA-DR+ inflammatory cells were more abundant in HRT never-user plaques and represented the major source of ubiquitin-proteasome activity, suggesting the presence of an active inflammatory reaction in never-user plaques. Moreover, concomitantly higher expression of ubiquitin and proteasome was found in plaque macrophages
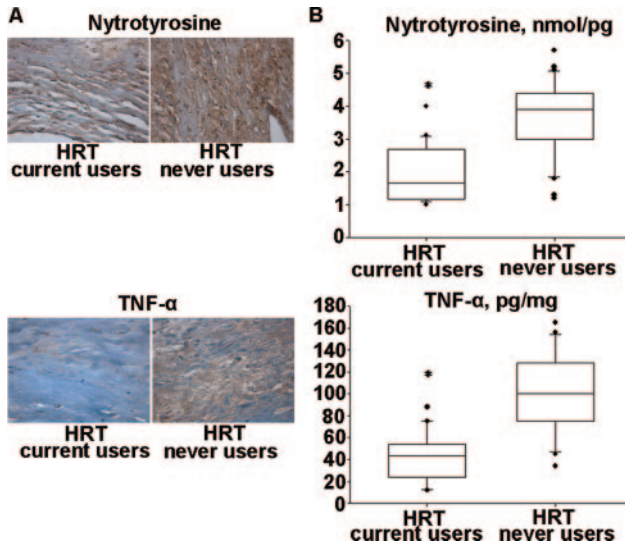
Figure 2. A, Representative sections show immunochemistry for nitrotyrosine ($\times$400) and TNF-α ($\times$400) in plaques of HRT current-user women and HRT never-user women. Similar regions of plaque are shown. Results are typical of HRT and untreated HRT women plaques. B, ELISA for nitrotyrosine levels and TNF-α in plaques of HRT current-user women and HRT never-user women (boxplot, a plot type that displays the median; 10th, 20th, 25th, and 75th percentiles; range; and extreme values). *$P<0.05$ vs HRT never-users.

obtained from the carotid lesions of postmenopausal female HRT never-users of compared with specimens obtained from female current users of HRT. In agreement with the difference in ubiquitin-proteasome staining pattern, the histological milieu of the lesions appears different with regard to cellularity, but not in the degree of vessel stenosis, suggesting that lesions from HRT never-users and HRT current users are only different with regard to inflammatory burden. Hence, the
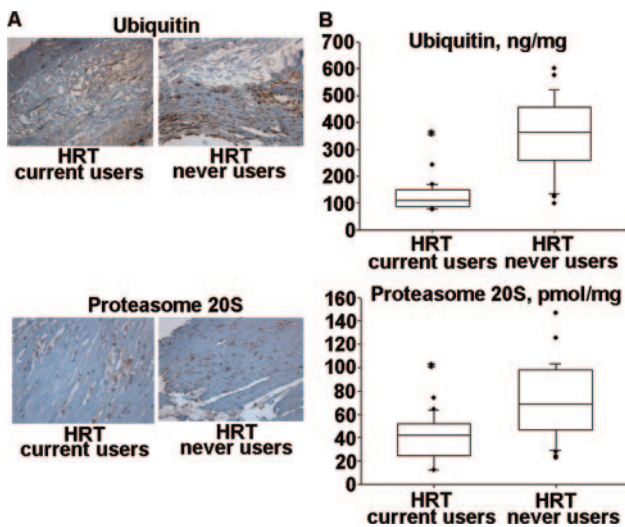

Figure 3. A, Representative sections show immunochemistry for ubiquitin ($\times$400) and proteasome ($\times$400) in plaques of HRT current-user women and HRT never-user women. Similar regions of plaque are shown. Results are typical of HRT and untreated HRT women. B, ELISA for ubiquitin levels and specific SDS activation kit for proteasome 20S in plaques of HRT current-user women and HRT never-user women. *$P<0.05$ vs HRT never-users.
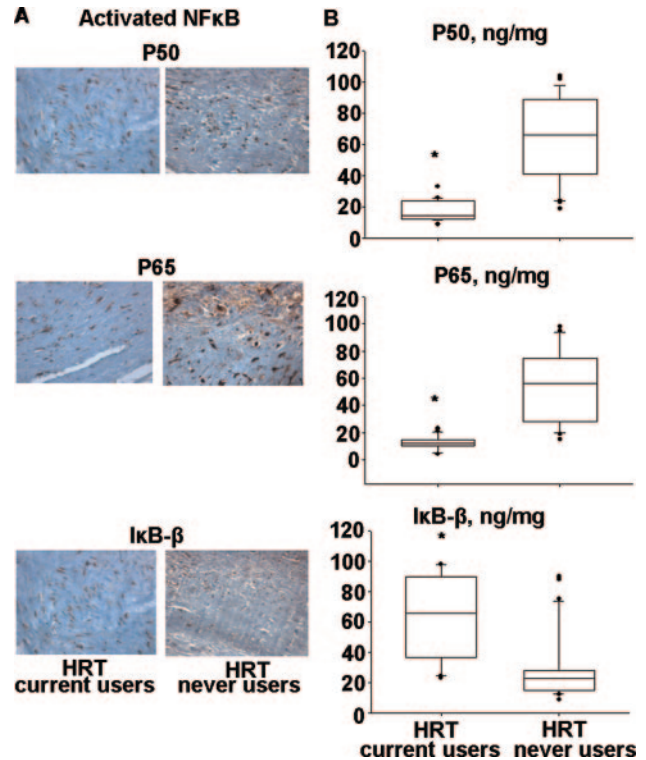

Figure 4. A, Representative sections show immunohistochemistry for activated NF-κB: p50 ($\times$400), p65 ($\times$400), and IkB-β ($\times$630) in plaques of HRT current-user women and HRT never-user women. Similar regions of plaque are shown. Results are typical of HRT and untreated HRT women plaques. B, Levels of activated NF-κB (specific Trans-AM p50 and p65 subunit assay kit) and IkB-β (ELISA) in plaques of HRT current-user women and HRT never-user women. *$P<0.05$ vs HRT never-users.

differences in plaque behavior likely stem from differences in the presence of stimuli (ie, oxidative stress, as evidenced by high nitrotyrosine levels, and high O^{2-} production in never-user women) for selective expression of ubiquitin proteasome, capable of disrupting plaque stability via NF-κB induction.

NF-κB is normally bound to IkB in the cytosol; this binding prevents its movement into the nucleus.[22] Various cellular stimuli, such as oxidative stress, induce ubiquitination of phosphorylated IkBs and subsequent degradation by the proteasome.[23] Degradation of IkBs results in unmasking of the nuclear localization signal of NF-κB dimers, which subsequently translocates to the nucleus, where it induces the transcription of proinflammatory cytokines like TNF-α that play a central role in plaque instability progression.[24] In line with this construct, our findings also suggest that HRT may reduce phosphorylation and degradation of IkBs via the suppression of ubiquitin-proteasome activity, thus reducing NF-κB activation. According to the response-to-injury theory,[25] plaque progression may be linked to an inflammatory-proliferative response to an injurious stimulus, constituted by oxidative stress. Previous reports evidenced the involvement of the ubiquitin-proteasome system in NF-κB activation, particularly under conditions of aggravated oxidative stress. Of note, it has been shown that oxidative stress reduction can suppress the ubiquitin system in endothelial cells by reducing

the expression of components of its enzymatic machinery, such as ubiquitin-binding proteins.[26] Moreover, the hypothesis that UPS activity reduction by HRT is largely dependent on the reduction of oxidative stress is also supported by the observation that UPS activity reduction in plaques is associated with comparable reduction in both O^{2-} and nitrotyrosine levels. According to this hypothesis, many data evidenced the antioxidative properties of HRT.[27,28] The antioxidative properties of estrogens probably result from 2 mechanisms. The first arises because estrogens are important free-radical scavengers because of the hydroxyphenolic structure of their molecules.[29] The second possible mechanism is connected with their influence on the endogenous antioxidative enzyme system.[30] Capel et al[31] were the first who suggested that sex hormones may have antioxidative properties by increasing cellular antioxidative enzyme activity. The stimulatory effect of sex steroids on cellular antioxidative enzyme activity was also confirmed by others who observed significant, cycle phase-related changes in glutathione peroxidase. In this context, our data may suggest a novel mechanism by which estrogen replacement therapy reducing oxidative stress and ubiquitin-proteasome activity may mediate inflammatory activity in atherosclerotic plaques of HRT-treated postmenopausal women.

Perspectives

This study proposes an interesting hypothesis for addressing the missing link between HRT and plaque stabilization in postmenopausal women by demonstrating the inhibition of the functional UPS/oxidative stress axis in human atherosclerotic lesions and by providing evidence that it is associated with plaque stabilization possibly by suppression of the NF-κB–induced inflammation promoting plaque rupture. However, it is worth noticing that, for statins and antagonists of the rennin-angiotensin system, 2 of the most successful drugs in cardiovascular diseases, an antioxidative and a proteasome inhibitory effect have been described.[32,33] Whether this is an effect that relates to their clinical benefit, however, awaits further investigation. These findings are also potentially important from a practical standpoint, because they raise the interesting possibility that modification of the UPS activity by HRT might provide a novel form of therapy for plaque stabilization of elderly women with atherosclerotic disease and prevention of acute ischemic syndromes.

Disclosures

None.

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