NM23-H2, an estrogen receptor β-associated protein, shows diminished expression with progression of atherosclerosis

Katey Rayner,1 Yong-Xiang Chen,1 Benjamin Hibbert,1 Dawn White,1 Harvey Miller,1 Edith H. Postel,2 and Edward R. O’Brien1

1University of Ottawa Heart Institute, Ottawa, Ontario, Canada; and
2Robert Wood Johnson Medical School, New Brunswick, New Jersey

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Rayner K, Chen Y-X, Hibbert B, White D, Miller H, Postel EH, O’Brien ER. NM23-H2, an estrogen receptor β-associated protein, shows diminished expression with progression of atherosclerosis. Am J Physiol Regul Integr Comp Physiol 292: R743–R750, 2007. First published August 3, 2006; doi:10.1152/ajpregu.00373.2006.—While estrogen receptor (ER) profile plays an important role in response to estrogens, receptor coregulators act as critical determinants of signaling. Although the clinical effects of ovarian hormones on various normal and pathological processes are an active area of research, the exact signaling effects on, for example, the vessel wall, are incompletely understood. Hence, we sought to discover proteins that associate with ERB, the isoform that shows upregulated mRNA expression after arterial injury. Using a yeast two-hybrid screen we identified NM23-H2, a multifaceted metastasis suppressor candidate protein, as an ERB-associated protein. Although NM23-H2 was immunodetected in arteries from young subjects (27 ± 6 yr, 14 men and 6 women) with benign intimal hyperplasia, expression was diminished in fatty streaks/atheromas and altogether absent in advanced atherosclerotic lesions. Both nm23-H2 mRNA and protein were expressed by vascular cells in vitro. Treatment with 17β-estradiol and an ERB-selective agonist, diarylpropionitrile, increased protein expression of NM23-H2; an effect that was not seen with ERa-selective agonist, propylpyrazole-triol. Estrogen also prompted nuclear localization of NM23-H2 protein in human coronary smooth muscle cells (SMCs). An in vitro mimic of inflammation decreased the expression of NM23-H2 in SMCs, which was restored on addition of estrogen and dependent on the estrogen receptor. In summary, we report the novel association of NM23-H2 with ERB and show for the first time its expression in vascular cells and demonstrate regulation of its expression and localization by estrogen. In that the abundance of NM23-H2 diminishes with both the advancement of atherosclerosis and inflammation, this ERB-associated protein may play an important role in mediating the vasculoprotective effects of estrogens.

aortic sclerosis; receptors

ELEVATED CARDIOVASCULAR MORBIDITY and mortality rates among postmenopausal women remain a major health concern in Western societies. The most obvious explanation for the relative protection against cardiovascular events in premenopausal women is the presence of ovarian hormones. However, large randomized clinical trials fail to demonstrate the expected cardiovascular benefits of hormone replacement therapy (HRT) in postmenopausal women (9, 31, 32). While there are several important caveats to these clinical studies, there is a critical need to better understand how estrogens act at the level of the vessel wall (17, 30).

Estrogens act via two estrogen receptors (ERα and ERβ) that can exist as either homo- or heterodimers (4, 10, 21). While the two receptors share structural similarities in certain regions (the A/B domain has only 30% sequence identity between ERB and ERα), unique proteins that associate with ERs are important in determining the biological effects of the hormone ligand (6, 7, 10, 16, 21, 28). When activated, the receptors translocate to the nucleus and modulate transcriptional activity through interactions with estrogen response elements. In addition, these receptors participate in signaling cascades at the cell membrane and therefore have the potential to function entirely independently of gene regulation (13). ERs, as well as ER-associated proteins, may show differential tissue expression patterns and are instrumental in estrogen signaling. In male arteries, ERβ is the predominant receptor expressed in the intima, media, and adventitia, and data are emerging to suggest its expression is correlated with the degree of atherosclerosis (12). Moreover, the expression of ERβ mRNA is markedly upregulated after vascular injury in male arteries (11, 15). Recent evidence demonstrates that ERβ expression correlates with the degree of calcification of atherosclerotic coronary arteries, an effect that is not seen with ERα (2). Therefore, ERβ plays an important, yet unidentified, role in atherogenesis and the progression of atherosclerosis. To date, however, there is little information regarding ERβ-associated proteins. For example, Mendelsohn’s group discovered Mad2 to specifically interact with ERβ; however, the functional significance of this interaction remains to be seen (23).

We hypothesized that associated coregulators of ERβ may mediate important estrogenic effects in human vascular tissue. To identify candidate proteins, we performed a yeast two-hybrid screen using the aforementioned unique A/B region of ERβ. Recently, we published the first of these findings, demonstrating an interaction between ERβ and heat shock protein 27, and suggesting that this interaction might attenuate ERβ

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transcriptional activity (19). Herein, we report the discovery of NM23-H2 as another ERβ-associated protein that is expressed in the artery wall. NM23-H2 is a multifaceted metastasis suppressor candidate protein that acts as a nucleoside diphosphate kinase, transcriptional regulator, and DNA nuclease (24–26). As will be described, there is diminished expression of NM23-H2 with progression of atherosclerotic disease stage. Moreover, we note that NM23-H2 is capable of facilitating estrogen signaling in vitro. Hence, it is intriguing to consider that NM23-H2 plays an important role in maintaining vascular homeostasis by mediating the vasculoprotective effects of estrogens.

MATERIALS AND METHODS

Briefly, our studies involve two major components: 1) the association of NM23-H2 with ERβ and the regulation of NM23-H2 expression by estrogen, and 2) the demonstration of attenuated NM23-H2 expression in atherosclerotic human coronary arteries and smooth muscle cells (SMCs) subjected to an inflammatory stimulus.

Cell culture and treatment. Human aortic and coronary artery SMCs and endothelial cells (ECs) of female origin were obtained from Cambrex Bioscience (Walkersville, MD). HeLa and MCF-7 cells were obtained from American Type Culture Collection (Manas- sas, VA). Cells were maintained in phenol red-free DMEM (Invitrogen, Burlington, ON) supplemented with 10% FBS (Wisent, Saint-Jean-Baptiste de Rouville, QC, Canada), gentamycin (Sabex, Boucherville, QC, Canada), and Fungizone (Invitrogen). SMCs between passages 5 and 9 were used for all experiments. Before all treatments, SMCs were rendered quiescent by serum-starvation (DMEM with 0.5% FBS) for 24 h. The duration of all 17β-estradiol (E2; Sigma, St. Louis, MO) treatment was 24 h unless otherwise indicated.

RNA extraction and Northern blot analysis. Total RNA was extracted from smooth muscle, endothelial, HeLa, and MCF-7 cells using TRI Reagent (Sigma) as per the manufacturer’s instructions. The collected RNA was run on a agarose gel and blotted overnight to a nylon membrane. A randomly primed 32P-labeled probe (New England Biolabs, Beverly, MA) overnight at 20 V, 4°C. Membranes were then subjected to Western blot analysis using the following antibodies: a rabbit polyclonal anti-NM23-H2 (diluted 1:1,000; E. H. Postel), a rabbit polyclonal anti-ERβ (1 µg/ml; Affinity Bioreagents), a monoclonal anti-α (1:250; Sigma), and a polyclonal anti-DsRed (BD Biosciences).

Immunofluorescence. SMCs in culture were fixed by using freshly prepared 4% paraformaldehyde in PBS for 15 min on ice. Cell membranes were permeabilized using 0.1% Triton X-100 for 15 min, and nonspecific proteins were blocked using 2% BSA in PBS for 1 h. Primary antibodies (anti-NM23-H2, E. H. Postel; anti-ERβ, Novus Biologicals, Littleton, CO) were incubated overnight at 4°C (1:200 titer for nm23-H2, 1:100 titer for ERβ), washed with PBS, incubated with a secondary antibody (Texas Red tag for ERβ, fluorescein tag for NM23-H2; Vector Laboratories, Burlington, ON) at a 1:100 dilution for 30 min at room temperature, and subsequently washed with PBS. Cell nuclei were counterstained with Hoechst 33258 (1 µg/ml) for 15 min at room temperature, and cells were visualized on an Olympus BX60 fluorescence microscope.

Immunohistochemistry. Cross sections of normal coronary arteries from 20 individuals who died as a result of noncardiovascular causes were obtained from the coroner’s service at the Vancouver Hospital and Health Sciences Center (Vancouver, Canada) (20). Proximal segments of the left anterior descending, the left circumflex, and the right coronary arteries were harvested from 14 men (with a total of 34 artery segments) and 6 women (with a total of 17 artery segments) (mean age: 27 ± 6.4 yr). All tissue specimens were immersed fixed with 10% neutral buffered formalin and embedded in paraffin. These postmortem arteries were harvested within 6 h of death, and previously we demonstrated the preserved protein and mRNA content of these arteries (3, 8). To identify histopathological features of these arteries, sections were stained with hematoxylin and eosin, Masson’s trichrome, and Movat’s pentachrome stain. Histopathological classification of lesions was done according to the methods of Stary (29). Immunolabeling was performed using the following antibodies: a rabbit polyclonal anti-human NM23-H2 (E. H. Postel; dilution 1:200), an EC marker [von Willebrand factor 8 (anti-vWF8) 1:1,000; Dako, Carpenteria, CA], an SMC-specific marker [α-smooth muscle actin (anti-αSMA) 1:100, Dako], a macrophage-specific marker (anti-CD68, 1:100, Dako), and a T-lymphocyte-specific marker (anti-CD45R, 1:100, Dako). Biotinylated anti-rabbit or anti-mouse IgG (Vector Laboratories) was used as a secondary antibody. Immunohistochemistry was performed as described previously (1). Briefly, tissue sections were deparaffinized and incubated with 10% horse or goat serum (Vector Laboratories) for 20 min to minimize the nonspecific binding of the primary antibody before incubation overnight with one of the primary antibodies in a 4°C moisture chamber. Tissue sections were then incubated with the appropriate secondary antibody for 30 min at room temperature. To inhibit endogenous peroxidase activity, tissue sections were incubated with 3% H2O2 for 30 min before incubation with peroxidase-labeled streptavidin (Vector Laboratories) for 30 min. Visualization of positive immunolabeling was made possible by the addition of the standard peroxidase enzyme substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma), which resulted in a brown color reaction product. Hematoxylin and eosin was used as the nuclear counterstain. PBS washes were used between each of the aforementioned steps. The number of NM23-H2-positive cells in the vascular wall was counted for each section, and then a numerical grade was assigned as follows according to the number of NM23-H2-positive and ERβ-positive cells: score of 0 (−), no positively-labeled cells; score of 1 (+), 1 to 30; score of 2 (+), 31 to 60; and score of 3 (+), ≥61 in each section of coronary artery.

For the immunohistochemical-positive controls, the tissue blocks retrieved from human breast carcinoma tissue were used for
NM23-H2 and ERβ immunostaining. For negative control, nonimmune mouse and rabbit IgGs were used instead of the respective primary antibodies.

ELISA. The human coronary artery SMCs (HCASMCs) of female origin were grown to confluence in 24-well dishes containing 10% FBS DMEM. The NM23-H2 expression was quantified by using an ELISA as previously described (1). Briefly, the cells were treated by LPS (5, 10 µg/ml; Sigma) with or without E2 (100 nM), E2 (20 nM, 100 nM) with or without ICI 182,780 (10 µg/ml; Tocris, Ellisville, MO), a potent ERβ-selective agonist diarylpropionitrile (DPN) (10 nM; Tocris) with or without ICI (10 µg/ml), and an ERα-selective agonist propylpyrazole-triol (PPT) (10 nM; Tocris) with or without ICI 182,780 (10 µg/ml) for 24 h, respectively, and then fixed with 4% paraformaldehyde. Following tripticates washes in PBS and blocking with 3% skim milk powder for 1 h, the cells were incubated overnight at 4°C with an rabbit anti-NM23-H2 (diluted 1:1,000 in 0.1% BSA/PBS) primary antibodies, washed three times with PBS and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 in 0.1% BSA/PBS; Dako) at room temperature for 30 min, respectively. Three washes with PBS were again performed before binding of the second antibody was detected by adding the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma). The reaction was stopped by adding 25 µl of sulfuric acid and transferring the media into 96-well plates to read the optical density (OD) at 450 nm by using a microplate reader (Bio-Rad). After the media were transferred, the cells were washed with PBS and the cell nuclei were stained with hematoxylin and eosin as the nuclear counterstain. (Sigma) for 20 min.

OD readings were not simply due to differences in cell number that might have occurred as a result of treating HCASMCs with either LPS with or without E2, either E2 with or without ICI 182,780, DPN, and PPT. The OD reading measurement was therefore divided by the number of HCASMCs per millimeter squared to account for this possibility.

Statistics. Values are means ± SE. Comparisons of groups were performed by using a one-way ANOVA. Statistical significance was defined as P < 0.05 and is denoted by an asterisk.

RESULTS

NM23-H2 associates with ERβ. Using a yeast two-hybrid screen, we identified previously unreported protein interactions with the unique A/B domain of ERβ (19). From the initial library of 5.3 × 10⁶ clones, only three clones were found to represent true positive interacting proteins. One of the clones was identified as nm23-H2, as it shared 98.7% sequence identity with the reported NM23-H2 protein sequence in the open reading frame (Swiss-Prot accession no. P22392).

Vascular expression and interaction of NM23-H2 with ERβ. Because we are interested in the potential of NM23-H2 to modulate vascular effects of estrogen, we began by determining the expression profile of this protein in vascular cells in culture. NM23-H2 expression was examined in ECs and SMCs. Both HeLa and breast tumor-derived MCF-7 cells are known to express high levels of NM23-H2 and thus served as positive controls (14, 22). Northern blot analysis demonstrated the presence of a 650-bp mRNA in all of the cell types investigated (Fig. 1A). Similarly, protein was isolated from the

Fig. 1. Vascular expression of NM23-H2. Endothelial (ECs), smooth muscle (SMCs; both quiescent and serum-stimulated), HeLa, and MCF-7 cells were examined for NM23-H2 expression. A: Northern blot showing expression of the 650-bp mRNA nm23-H2 transcript and 28S rRNA. B: Western blot using antibodies specific for the 17.5-kDa NM23-H2 protein. C: coimmunoprecipitation of ERβ using an NM23-H2-specific antibody for the pull down and an ERβ-specific antibody for the detection. Omission of NM23-H2 antibody constituted the negative control (–ve control). Whole cell lysate from both HeLa and SMCs were probed using antibodies to ERβ. IP, immunoprecipitated. D: Western blot showing HeLa and MCF-7 cells, quiescent SMC (qSMC) and serum-stimulated SMCs (10% serum), and SMCs treated with serum supplemented with 100 nmol/l 17β-estradiol (E2). Antibodies specific for the 17.5-kDa NM23-H2 protein (top) and α-actin (bottom, loading control) were used for immunodetection. SMC expression of NM23-H2 is upregulated in response to E2.

NM23-H2 and ERβ immunostaining.
immunohistochemical analysis of human coronary arteries: Movat pentachrome stain (Fig. 1B). A higher molecular mass species of ~21 kDa was also observed in MCF-7 and HeLa cells, most likely representing NM23-H1 that may be detected with this polyclonal antibody (E. H. Postel, unpublished observations).

To test whether or not ERβ and NM23-H2 interact endogenously (i.e., in unmodified primary cells), NM23-H2 was immunoprecipitated from nontransfected SMCs and HeLa cells before being analyzed by Western blot analysis using an ERβ antibody. The NM23-H2-specific antibody immunoprecipitated the 55-kDa ERβ, with a band that was more prominent in SMCs compared with HeLa cells (Fig. 1C). A higher molecular mass band was also noted, and corresponded to a nonspecific interaction with the beads (e.g., also seen in negative control lane). Analysis of the residual flow-through lysate showed little ERβ remaining unbound following immunoprecipitation (data not shown). Analysis of whole cell lysates from both SMC and HeLa cells consistently demonstrated expression of ERβ, thereby acting as a positive control for these experiments.

To determine the ability of estrogen to modulate the expression of NM23-H2, SMCs from quiescent, serum-stimulated, and estrogen-stimulated cells were harvested and subjected to Western blot analysis using an antibody to NM23-H2. Estrogen treatment resulted in an increase in protein expression compared with both quiescent and serum-stimulated cells (Fig. 1D). These results indicate the ability of estrogen to regulate the expression of NM23-H2 in SMCs, prompting us to further examine its role in ERβ and estrogen signaling in vascular cells. To examine the functional consequences of the ERβ/NM23-H2 interaction, we performed colocalization studies using fluorochrome-labeled ERβ and NM23-H2 antibodies in serum-stimulated and estradiol-treated SMCs. Both ERβ and NM23-H2 were expressed and colocalized in the nucleus and cytoplasm of SMCs (Fig. 2A).

**NM23-H2 expression in human coronary arteries.** To gain insight into the potential in vivo role of NM23-H2 in vascular cells, we determined the pattern of expression of NM23-H2 in both normal and diseased human coronary arteries by using specific anti-NM23-H2 antibodies. Arteries with complex atherosclerotic lesions were devoid of NM23-H2 expression (data not shown); therefore we studied coronary arteries from subjects less than age 40 yr with either no histological evidence of atherosclerosis or minimal lesions that are the precursors of advanced disease. Of the 51 coronary artery cross sections examined, 26 had diffuse intimal thickening (a normal developmental finding that does not obstruct blood flow), 26 had nonobstructive fatty streaks (type I and type II lesions), and seven sections were considered to have type III or type IV atherosclerotic lesions. NM23-H2 expression was detected in both the endothelium and SMC layers of arteries with diffuse intimal thickening, but only the endothelium was immunopositive for NM23-H2 in those arteries with fatty streaks and/or more advanced atheromas (Fig. 2B). Intimal and medial expression of NM23-H2 was assessed in a semiquantitative manner and found to diminish with the progression of atherosclerotic disease stage (Fig. 2C). The immunolabeling pattern for NM23-H2 was not individual specific but rather dependent on the histopathology of the coronary artery. Interestingly, the attenuation of NM23-H2 expression in the intima (but not media) of these coronary arteries coincided with a decrease in ERβ expression (Fig. 2C). Given our interest in atherosogenesis and the potential disparity between the sexes, we analyzed the differences in NM23-H2 expression in male vs. female proximal left anterior descending coronary arteries from young patients. As demonstrated in Fig. 2D, there is a trend toward an increase in the number of NM23-H2-positive sections in women vs. men in all three layers of the vessel wall (media 83% vs. 43%; intima 83% vs. 50%, and luminal ECs 100% vs. 79%). We examined the ERβ expression in these arteries and compared male vs. female subjects. There was no difference between the sexes in ERβ expression in the media and luminal ECs but a trend toward an increase in ERβ expression in the intima (21% men vs. 50% women), paralleling NM23-H2 expression. However, conclusions from this analysis cannot be drawn due to the low sample number of young subjects in this study.

**Estrogen effects on NM23-H2 in human coronary artery SMCs.** Because atherosclerosis is an inflammatory disorder, we sought to determine the influence of an in vitro mimic of inflammation, LPS, on NM23-H2 expression. Increasing doses of LPS reduced SMC NM23-H2 protein expression, an effect that was partially reversed with the addition of E2 (Fig. 3A). Increasing doses of estrogen progressively augmented NM23-H2 expression, an effect that was blocked by the ER antagonist ICI 182,780 (Fig. 3A). Furthermore, the increasing expression of NM23-H2 in HCASMCs was only observed by using a selective ERβ agonist DPN but not an ERα-selective agonist PPT. Finally, this effect of ERβ-mediated induction of NM23-H2 expression was blocked by the ER antagonist ICI 182,170 (Fig. 3A).

To investigate the potential role of NM23-H2 to modulate estrogen signaling via ERβ, we performed the following in vitro experiments. Because endothelial cell NM23-H2 expression in human coronary arteries persisted throughout the progression of atherosclerosis, we surmised that SMCs may be the target of estrogen regulation by NM23-H2 in the vessel wall. Treatment of SMCs with E2 upregulated NM23-H2 protein expression and showed increased nuclear localization of

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Fig. 2. NM23-H2 and estrogen signaling and expression in human coronary arteries. A: human SMCs in culture were plated, and 24 h later were stimulated with 10% serum SMC (left) or 10% serum plus 100 nM E2 SMC (right) for 30 min. Cells were immunolabeled with fluorescent antibodies to ERβ (red), NM23-H2 (green), and a Hoechst stain for the nuclei (blue). Merged photos show both green and red output signals. Original magnifications were ×400. B: immunohistochemical analysis of human coronary arteries: Movat pentachrome stain (first row), smooth muscle α-actin (SMα; second row), von Willebrand Factor 8 (vWF8; third row), and NM23-H2 (fourth row). Tissue sections were from arteries without atherosclerosis but showing benign diffuse intimal thickening (DIT; left), and atherosclerotic arteries with fatty streaks (type-I lesion, middle), and atheromas (type-III lesion, right). NM23-H2 immunopositive endothelium is denoted by arrow heads; NM23-H2 immunopositive smooth muscle cells (SMCs) are denoted by arrows. Original magnification: lower power (×20), high power (×400). M, media; I, intima; and L, lumen. C: semiquantitative analysis of the %NM23-H2 immunopositive (top) and %ERβ immunopositive (bottom) sections in the medial (left) and intimal layers (right) of tissue sections described above (%P ≤ 0.05 and ***P ≤ 0.001 compared with DIT). D: semiquantitative analysis of the NM23-H2 expression (left) and ERβ expression (right) in left anterior descending coronary artery sections in men vs. women. The number of sections determined to be NM23-H2 positive (NM23-H2+) or ERβ positive (ERβ+) in all sections were counted and expressed relative to the total number of sections.
NM23-H2 compared with cells treated with serum alone. Therefore, cells were treated with either serum alone or serum supplemented with E2, and the number of NM23-H2-positive nuclei were counted and expressed relative to total number of nuclei per high power field (Fig. 3, B and C). As anticipated from our initial observations, SMCs treated with E2 for 30 min showed 93% more NM23-H2 immunopositive nuclei compared with cells treated with serum alone ($P < 0.001$). This effect was diminished after 24 h with only 21% more NM23-H2 immunopositive nuclei in E2-treated cells compared with control ($P < 0.05$). Therefore, treatment of SMCs with E2 promptly increased the nuclear translocation of NM23-H2 compared with cells treated with serum alone.

DISCUSSION

Physiological and pathological control of the response to steroid hormones is critically linked to the relative abundance of specific regulatory proteins that associate with their respective receptors (5). Because we are interested in the potential role of estrogen in vascular disease, we sought to identify and characterize proteins that might associate with ERβ, the ER isoform that is known to be correlated with atherosclerosis in male arteries and whose mRNA upregulated in response to inflammatory stimuli and estrogens. NM23-H2 expression was analyzed using an ELISA in cultured HCASMCs. Cells were treated with or without LPS and triol (PPT) with or without ICI 182,780, respectively. Expression was measured as a function of optical density at 450 nm and normalized to cell number.

Normal diffuse intimal thickening (DIT) sections or atherosclerotic sections (lesion types I–V) were counted and expressed relative to the total number of sections: men, 14; women, 6.

Given the important role of inflammation in atherogenesis, we showed that the inflammatory mediator, LPS, reduced NM23-H2 expression, thereby mirroring the attenuated expression in atherosclerotic arteries. Interestingly, E2 supplementation partially restored SMC expression of NM23-H2, an effect that was dependent on the ER. NM23-H2 expression was only increased in response to a highly selective ERβ agonist, DPN, but not with an ERα-selective agonist. Moreover, E2 upregulated SMC expression of NM23-H2 and prompted its nuclear translocation. Hence, we surmised that estrogens may modulate the regulatory role of NM23-H2 in estrogen signaling. Experiments that specifically address this hypothesis are ongoing.

Taken together, these data suggest that NM23-H2 may be an important regulator of vessel wall homeostasis and estrogen responsiveness. While we acknowledge the limitations of these studies to a causal relationship between NM23-H2 and atherosclerosis, as an ERβ coactivator and potentiatior of the antimigratory effects of E2, it follows that early attenuation of NM23-H2 expression with advancement of atherosclerosis would result in a reduction in the salutary effects of estrogens on atherogenesis. Although E2 may augment NM23-H2 levels, this effect may be lost with the reduction in estrogen levels that occurs with the onset of menopause. Theoretically, HRT should be capable of restoring NM23-H2 expression levels; however, there may be a window of opportunity for the initiation beyond which the beneficial effects on NM23-H2 expression are less pronounced or lost. Certainly, in clinical practice, HRT is often initiated in women who are already several years postmenopause (17, 18). Hence, understanding the role of NM23-H2 and ERβ as a woman approaches and enters menopause may be very important in appreciating the therapeutic profile of HRT. For example, if the NM23-H2 pathway has ceased to function and cannot be reactivated, HRT may actually produce unwanted side-effects (e.g., hypercoagulable state) (27). Finally, while these data highlight the specific vascular interplay between NM23-H2 and ERβ, we cannot exclude the possibility that other coregulatory proteins, as well as alternate ERs forms (e.g., ERα/β heterodimers or ERα homodimers), also contribute to vessel wall homeostasis and require further study.

Table 1. Analysis of lesion types for the cross sections used for NM23-H2 and estrogen receptor-β analysis

<table>
<thead>
<tr>
<th>Sex</th>
<th>DIT</th>
<th>Lesion Type I–II</th>
<th>Lesion Type III–V</th>
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<tbody>
<tr>
<td>Men</td>
<td>14% (2/14)</td>
<td>50% (7/14)</td>
<td>36% (5/14)</td>
</tr>
<tr>
<td>Women</td>
<td>50% (3/6)</td>
<td>35% (2/6)</td>
<td>17% (1/6)</td>
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Fig. 3. NM23-H2 expression and localization in human coronary artery smooth muscle cells (HCASMCs) in vitro. A: NM23-H2 expression in response to inflammatory stimuli and estrogens. NM23-H2 expression was analyzed using an ELISA in cultured HCASMCs. Cells were treated with or without LPS and E2 (left), E2 with or without ICI 182,780 (middle), and a potent ERβ-selective agonist diarylpropionitrile (DPN) and an ERα-selective agonist proplyprazoletril (PPT) with or without ICI 182,780 (right). Respectively. Expression was measured as a function of optical density at 450 nm and normalized to cell number per millimeter squared. NS, not significant. B: immunofluorescence of NM23-H2 in HCASMCs treated with or without E2 HCASMCs in culture labeled with anti-NM23-H2 antibodies (green; left) and Hoechst 33258 nuclear stain (blue, right) after 30 min of serum stimulation alone (control; top), or with the addition of E2 (100 nmol/l; bottom). Shown are NM23-H2-positive and -negative nuclei. Original magnifications were ×400. C: E2 promoted NM23-H2-immunopositive nuclear localization. NM23-H2-positive nuclei were counted and expressed as %total number of nuclei per high-power field (magnification ×200). Cells were counted after 30 min or 24 h of estradiol treatment and compared with cells treated with serum alone. Multiple fields with multiple cells were used for analysis.
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