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—— ORIGINAL ARTICLE ——

Correlation between estrogen receptor α expression, collagen content and stiffness in human uterine arteries

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Aim of the study. To investigate the relationship between estrogen receptor α expression, collagen content and distensibility of the human uterine artery.
Study design. Uterine arteries ($n = 13$) obtained from women undergoing hysterectomy were mounted for length–tension relations and thereafter frozen for later determination of collagen and estrogen receptor α contents.
Results. A negative correlation between estrogen receptor α content and collagen content ($r = -0.76$, $n = 13$) and a positive correlation between collagen content and passive tension at 1.4 L_o ($r = 0.72$, $n = 13$) in the uterine artery were detected. No difference was found between pre- and postmenopausal women concerning the estrogen receptor α content, the collagen content or maximal active force.
Conclusions. Our data show a functional correlate to the expression of estrogen receptors α, as a high content of estrogen receptor α correlates with a lower collagen concentration, indicating that estrogen through activation of estrogen receptor α protects against vascular collagen accumulation making the vessel more distensible.

Key words: collagen; estrogen receptors; vascular stiffness

Increased stiffness with age has been observed both in the human (1) and rat (2) thoracic aorta as well as in the rat carotid artery (3). Stiffness has also been determined noninvasively by ultrasound in the human abdominal aorta, and was shown to increase with age, with the increase occurring at a higher age among women than among men (4).

In the rat carotid artery, stiffness was shown to increase with age in parallel with the increase in collagen content (3). The concentration of collagen in the human thoracic aorta has also been shown to increase with age (5). Estrogen treat-

ment has been shown to prevent collagen and elastin accumulations in the rat aorta, leading to a higher proportion of elastic fibers (6), and also to decreased stiffness of the rat carotid artery (7).

Even though the increased incidence of atherosclerotic disease among women after menopause to some degree can be counteracted by estrogen treatment (8) some premenopausal women still suffer from ischemic heart disease. Further, secondary prophylaxis with estrogen treatment after ischemic heart disease has not been proven as effective (the HERS-study; 9). One reason for this could be a lower level of estrogen receptors (ERs) in the vessel walls of some women. An association between a lower expression of ERs and coronary atherosclerosis in premenopausal,

but not postmenopausal, women has been reported from autopsy material (10). Two types of estrogen receptors, ERα and ERβ, have been characterized to date. Both ERα and ERβ are expressed in the vascular wall (11). Estrogen receptor α has been reported to be the ER subtype mediating the cardiovascular protective effect of estrogen (12,13).

In animal studies an increased collagen content correlates with the development of diet-induced atherosclerosis (14). Likewise in humans, an increased collagen concentration has been reported in atherosclerotic blood vessels (15). In monkeys with diet-induced atherosclerosis, stiffness was reported to increase together with the total collagen content (16).

The degree of atherosclerosis is not uniform throughout the body (17), but a positive correlation between the severity of atherosclerosis in the uterine and coronary arteries has been reported (18). As the uterine artery could be obtained easily from women undergoing hysterectomy at various ages we found the uterine artery a suitable preparation for the present study. The question addressed here is whether a correlation between the collagen concentration and stiffness in the human uterine artery exists, and whether the expression of ERα correlates with the collagen content. In the same vessel the collagen concentration was measured and correlated to the amount of ERα and the distensibility of the vessel.

Materials and methods

Tissue sampling

Specimens of the ascending uterine artery were obtained from women undergoing abdominal or vaginal hysterectomy for various benign reasons [menometrorrhagia (7), uterovaginal prolapse (5) and pelvic pain (1)]. The study was approved by the local Ethics Committee at Lund University and each woman gave informed consent for the experiment. Menopause was considered to have occurred when 1 year had passed without bleeding. The mean age in the premenopausal group was 51 ± 1.4 years (n = 8), and in the postmenopausal group 62 ± 4.2 years (n = 5). Time since menopause in the postmenopausal group was 14 ± 3.3 years. In the postmenopausal group one woman was treated for hyperlipidemia and in the premenopausal group one woman had a diagnosed hypertension. The remainder of the women had no known risk factors for cardiovascular disease. One woman used estrogens in doses high enough for systemic effects and her

estrogen treatment was started 1 year before the hysterectomy, while she was still menstruating.

Sections of the uterus containing the main branch of the uterine artery were removed and placed in ice-cold Krebs solution for transportation to the laboratory. The Krebs solution contained in mM: NaCl 122, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, and glucose 11.5. pH was adjusted to 7.4 at $37\,°C$. The uterine artery running on the lateral side of the uterus was dissected free under a microscope and the adventitia removed, leaving the media and intima. The vessel was cut into 3–5-mm long rings. For length–tension relation experiments, the endothelium was removed by pulling a needle several times through the lumen. Separate experiments showed that after this treatment no relaxation occurred in response to acetylcholine.

The arterial rings were mounted isometrically on parallel pins of stainless steel with a diameter of 1.0 mm, one fixed and the other connected to a force transducer. The arteries were allowed to accommodate for approximately 1 h in Krebs solution with 2.5 mM Ca^{2+} at a preload of approximately 1 mN/mm. After two conditioning contractures elicited by high-K^+-solution (140 mN) the muscle was shortened and a length–tension relation was run according to the following protocol: (1) accommodation at each muscle length in nominally Ca^{2+}-free solution for 15 min; (2), high K^+-solution including 10 μM of norepinephrine and 2.5 mM Ca^{2+} for 5 min; (3) nominally Ca^{2+}-free solution for 10 min; and (4) change to a new length and then start again from step 1. Experiments were carried out at $37\,°C$.

Using a microscope the distance between the pins at each step during the length–tension experiment was measured and from this the circumference of the muscle calculated. As the diameter of the uterine arteries differed, all values of passive and active tension were calculated as mN/mm^2 muscle area using the wet weight and the circumference of the muscle and assuming a density of 1.06 mg/mm^3.

The circumference of the muscle at which maximal active force was produced is called L_o and the corresponding passive force F_o. A length–tension relation was constructed by plotting each active force, i.e. force above passive force at each circumference, and passive force, i.e. force above baseline, against muscle circumference expressed as fractions of L_o.

After the mechanical experiment was completed the muscle was weighed and frozen for later measurements of collagen content, i.e. the collagen

content was always measured in the same tissue as that used to make the length–tension relation.

Collagen measurements

The muscles were stored at $-80\,°C$. After freezing the muscles were dried for 4 h at $90\,°C$. The duration of the drying period was tested in preliminary experiments to be sufficient. Thereafter the muscles were defattified in acetone for 3 days, dried again and weighed, and subsequently hydrolyzed in 6 M HCl for approximately 16 h at $106\,°C$ and evaporated. The content of hydroxyproline was analyzed as described by Stegemann and Stalder (19). Briefly this is a colorimetric method including oxidation of hydroxyproline with chloramine-T and coupling of the chromogen formed with Ehrlich's aldehyde in a strong perchloric acid, and read in a photometer at 550 nm. Collagen content in percentage of dry defattified weight was then calculated using a conversion factor of 7.46, corresponding to the commonly accepted percentage of hydroxyproline in collagen of 13.4%.

Determination of estrogen receptors α

For quantitative determination of ERα, samples (at least 20 mg) of the uterine artery immediately adjacent to the segment used for the length–tension experiments were immediately frozen at $-80\,°C$ until analysis. The tissues were pulverized with a microdismembrator, and then dissolved in buffer (10 mM Tris, 1.5 mM EDTA, 5.0 mM Na_2MoO_4, 1.0 mM monothioglycerol, pH 7.4). The homogenate was centrifuged at 100 000 g for 60 min at $0\,°C$, whereafter the content of ERα in the supernatant was measured with an enzyme immunoassay according to the kit instructions (Abbott Laboratories, Diagnostic Division, Chicago, IL). The Abbott antibody is a rat monoclonal antibody (17) that recognizes ERα but not ERβ. The sensitivity of the Abbott ER enzyme immunoassay monoclonal system is approximately 1.5 fmol ER/ml, and values below this were considered to be below the detection limit. ERα content was expressed as fmol ERα/protein. Protein was determined as described by Lowry et al. (21), using bovine serum albumin as standard.

Statistics

Values are presented as means $\pm$ SEM. Statistical significance was evaluated by using Student's t-test for unpaired comparisons. Correlation was tested using Pearson's r constant. $p < 0.05$ was regarded as statistically significant.

Results

Estrogen receptor α content

The amount of ERα in uterine arteries from the pre- and postmenopausal women was similar (2.5 ± 0.71 and 1.8 ± 0.82 fmol/mg protein, $n = 8$ and $n = 5$, respectively). No correlation between age and ERα content was detected.

Collagen content

A negative correlation existed between ERα content and collagen concentration, i.e. the collagen concentration was lower in arteries with a larger amount of ERα (Fig. 1; $r = -0.76$). When the postmenopausal arteries were excluded an even stronger negative correlation was found in the
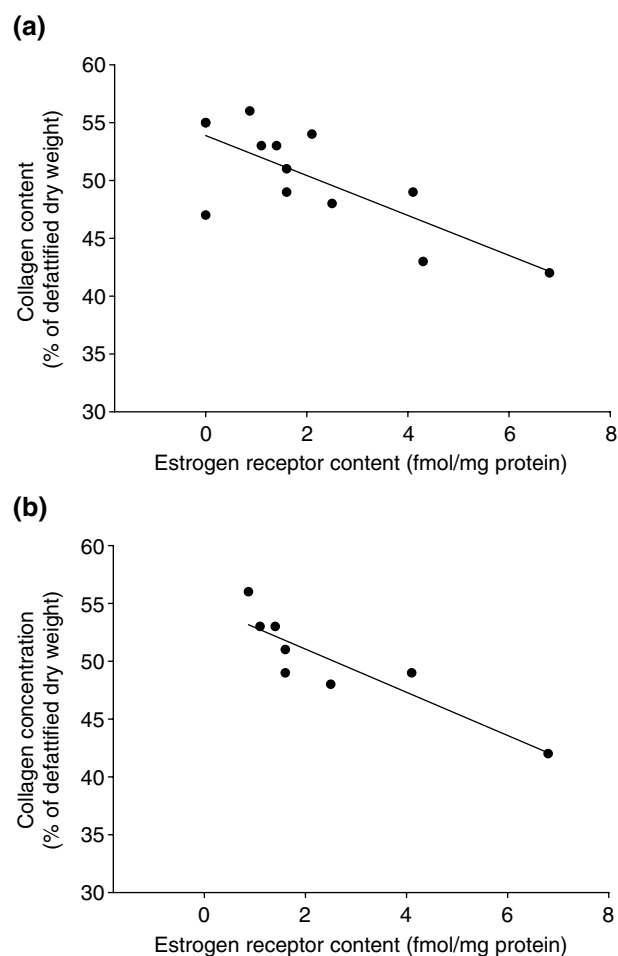

(a)

(b)

Fig. 1. Relation between the estrogen receptor content (fmol/mg protein) on the horizontal axis and collagen content (percentage of defattified dry weight) on the vertical axis in human uterine arteries from (A) both pre- and postmenopausal women ($n = 13$) (note: two individual preparations with 55% collagen and 0 ER) and (B) premenopausal women only; $n = 8$. A negative correlation between estrogen receptor content and collagen concentration in both groups was detected [$r = 0.76$ in (A) and $r = 0.89$ in (B); $p < 0.01$].

premenopausal group ($r = -0.89$). No correlation between age and collagen content was detected and the collagen content in arteries from the premenopausal and postmenopausal women was similar ($50 \pm 1.5\%$ and $51 \pm 2.5\%$; $n = 8$ and $n = 5$). No correlation was seen between the circumference at L_o and the collagen content.

Contractility

From each vessel a length–tension relation was constructed. The length at which maximal active force was evoked is called L_o and the corresponding passive force F_o. Summarized data of all the length–tension relations irrespective of collagen concentration in the muscles are shown in Fig. 2. From each individual length–tension relation, passive tension at 1.4 L_o, expressed both as its absolute value and as fractions of F_o, was extrapolated. These values of passive tension were then plotted against the collagen concentration of each muscle. Data from 13 muscles are summarized in Fig. 3. A statistically significant positive correlation between collagen concentration and passive tension at 1.4 L_o was disclosed, i.e. the higher the collagen concentration the larger the passive tension at 1.4 L_o. This correlation existed both when tension was expressed as its absolute value (mN/mm^2) ($r = 0.70$, $p < 0.01$) and as fractions of F_o ($r = 0.59$, $p < 0.05$). No correlation existed between absolute passive tension at L_o and the collagen concentration. The average maximal active force in uterine arteries from the premenopausal and postmenopausal women did not differ (12 ± 2.4 and 11 ± 6.5 mN/mm^2, $n = 8$ and $n = 5$, respectively; $p = 1.0$).

Discussion

Increased collagen content with age has been reported in the human aorta (5) and in the rat carotid artery (3). The absence of correlation between age and collagen content in our study could be explained by the narrow range of ages of the women. More interesting is the correlation between the ERα content and the collagen concentration. Even though no systematic morphometric analysis of the uterine arteries was performed in this study the correlation to collagen content could be argued as a link to atherosclerosis, as a higher total collagen concentration with increasing degrees of atherosclerosis in the human aorta has been found (15). In some preliminary experiments the morphometry of the vessel wall was examined, and intimal thickening as a sign of atherosclerosis was verified in some vessels. None of the women in the study group suffered from any severe cardiovascular
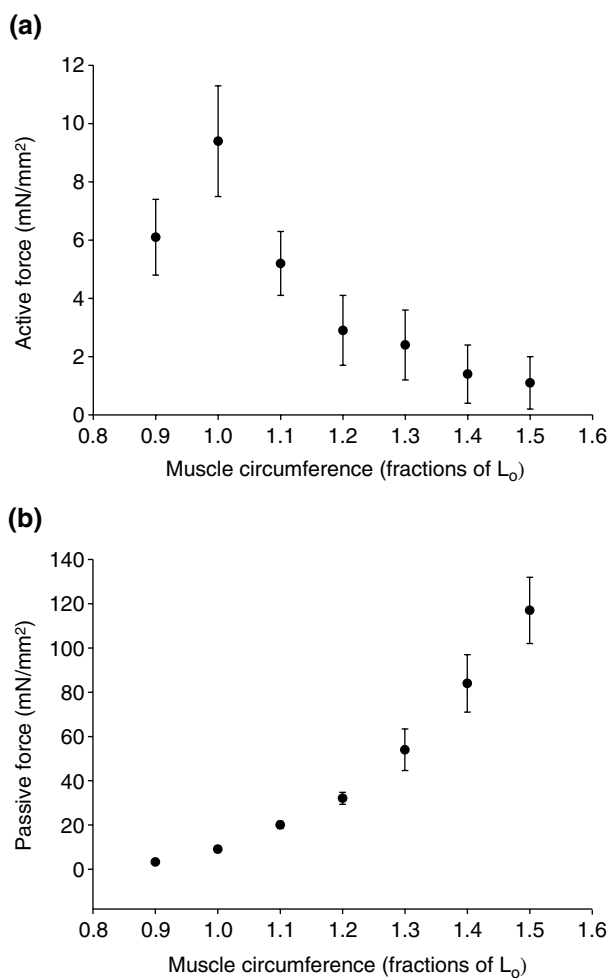

(a)

(b)

Fig. 2. Summarized data on all length–tension relationships obtained in the study irrespective of the collagen content in the individual artery. Active force (A) and passive tension (B) are expressed as mN/mm^2 and L as fractions of L_o.
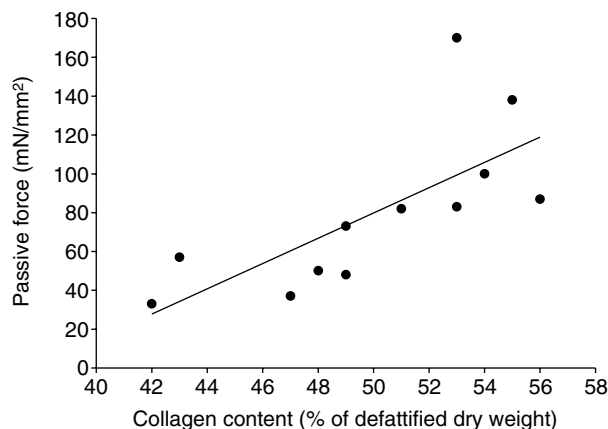

Fig. 3. Positive correlation between collagen concentration (percentage of defattified dry weight) on the horizontal axis and passive force at 1.4 L_o (mN/mm^2) on the vertical axis. $n = 13$, $r = 0.70$, $p < 0.01$.

disease, a fact that could explain why no larger signs of atherosclerotic disease were found in the uterine arteries.

Losordo et al. (10) reported a lower expression of ER in atherosclerotic coronary arteries than in normal coronary arteries from premenopausal women. In their study the expression of ER was measured using immunohistochemistry and the arteries were considered positive or negative in this aspect. In our study the ERα content in each artery was quantified using enzyme-immuno assay to make possible a correlation to the collagen content. The relation in the human uterine artery between degrees of atherosclerosis and ERα content has to be further investigated.

Aortic smooth muscle cells cultured in the presence of 17β-estradiol in the concentration $10^{-12}-10^{-6}$ M shows a decreased collagen synthesis compared with vehicle (22). Even though the premenopausal women in our study were all regularly menstruating, the exact level of estrogen in the tissue of the uterine artery is unknown. The estradiol level in premenopausal women varies between 0.2 and 0.5 nM during the menstrual cycle (23), which is in the same range that Beldekas et al. (22) found an effect of 17β-estradiol on collagen synthesis in cultured aortic smooth muscle cells. The action of steroids is generally believed to depend on the expression of steroid receptors in the target organ, and thus a stronger effect of estrogen on the uterine artery would be expected in women with higher amounts of ERα.

In the present study no significant correlation between the ERα content of the uterine artery and the menstrual status or age of the women was found. However, three uterine arteries showed no measurable ERα and these arteries were all from the postmenopausal women. The mean age in the premenopausal group was relatively high (51 ± 1.4 years), and it could not be excluded that the ERα content in arteries from younger women is higher. A lack of difference in ERα content between pre- and postmenopausal women using enzyme-immuno assay was also reported by Bergqvist et al. (27). A decrease in the concentration of progesterone receptors, but not of estrogen receptors, with age has been reported by Lantta et al. (28). In their study the receptors were identified and quantified by means of radioactive ligand exchange and the Scatchard plot assay.

To our knowledge no data on the collagen content of the human uterine artery have been reported and data from other human vessels are sparse. Cattell et al. (5) reported a collagen content of 18–31% in the human aorta. Data from animals are more abundant. The collagen content in rat aorta has been reported to be approximately 30% (6). Fischer and Llaurado (24) measured the collagen concentration in functionally different vascular segments, and found it to vary between 20% and 51%. In their study, the collagen content in the ascending aorta was 20% and in the abdominal aorta 46%. This is reflected by a significantly higher distensibility of the thoracic aorta than of the abdominal aorta *in vivo* (25). One of the highest collagen concentrations in their study, 48%, was found in the coronary artery, maybe with a function to limit the distensibility in an artery working in a contracting myocardium. The function of the uterine artery is to deliver blood to the uterus in varying amounts depending on the hormonal cycle. During pregnancy the flow through the uterine artery is dramatically increased. A high collagen concentration could in this situation facilitate a pathway with an unaltered pressure gradient to the fetus.

The only study known to the authors comparing stiffness and collagen content in the same vessel is that by Bruel and Oxlund (2) using rat thoracic aorta. They reported increased stiffness with age but no corresponding increase in collagen concentration. Instead they found accumulation of fluorescent material in collagen and elastin, indicating increased levels of advanced glycation endproducts. In the rat carotid artery stiffness measured *in vivo* was shown to increase with age associated with an increased collagen content (3).

Our findings of a correlation between absolute passive tension at 1.4 L_o and collagen content and absence of such correlation at L_o is in accordance with the interpretation by Roach and Burton (26) that the elastin fibers contribute to the elastic properties mainly at low pressures, while the collagen fibers would be stretched only at normal and higher pressures.

To summarize, the ERα content of the uterine artery was reflected in functional characteristics of the blood vessel in that a higher ERα concentration, and thus probably a higher sensitivity to estrogen, caused a lower collagen content and a more distensible artery. In the light of the HERS-study (9), where absence of secondary prophylaxis against ischemic heart disease by estrogen was reported, it is interesting to speculate if the lack of prevention of estrogen against ischemic heart disease could be because of a low expression of ERα in the atherosclerotic artery.

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