CHAPTER

6

Characteristics of the Endothelium in Both Sexes

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INTRODUCTION

Interest in studying sex as a biological and decisive variable of several diseases has grown in the last few years, mainly after the publication of *Exploring the Biological Contributions to Human Health: Does Sex Matter?* [1].

Several works published in scientific literature refer to sexual differences, inherent to a biological context, classifying them without distinguishing sex and gender. Currently, it is still possible to find overlapping between these terms in literature, without the correct distinction between them.

The term "sex" refers to the biological and physiological condition of the individual, human and animal, categorized as male and female. Indicators of this condition are sex chromosomes, gonads, internal reproductive organs, and external genitalia. The term "gender" refers to attributes that involve economic, social, and cultural aspects associated with the conception of being a man or a woman [1,2].

In sex chromosomes, genes are expressed differently according to the sex of the organism, since females have XX chromosomes while males have XY chromosomes. There are 1100 genes in chromosome X, and most of them are not expressed in chromosome Y. In addition, cellular mosaic created by the inactivation of chromosome X provides a biological advantage for females, that is, genes expressed in chromosome X are randomized between alleles proceeding from the father and the mother, while in males these genes are exclusive from the maternal X chromosome [3]. It is also important to consider the different meiotic processes and gene imprinting [1,4,5].

For decades, most of the research involving cardiovascular disease and the endothelium used males as experimental models, and extrapolated the results to females. In addition, many studies conducted in cell cultures do not specify the sex of the cell strain used. The gene imprint due partially to the presence of both "X" chromosomes in females and the "Y" chromosome in males may influence several biochemical and molecular pathways differently, which shall be decisive for cellular physiology [6,7].

Behavioral, anatomic, physiological, cellular, and molecular differences between males and females are common characteristics observed in several vertebrate species. Some of the differences are already evident on birth, mainly due to the inherent influence of sex chromosomes and, to a lesser extent, to fetal exposure to gonadal sex hormones. Currently, it is known that may differences observed may appear with sexual maturity, since the concentration of gonadal sex hormones are different since the intrauterine period and last the entire life [8–10].

Cardiovascular diseases are the largest cause of morbimortality in developed countries in both

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sexes [11]. Women of childbearing age have lower risk of developing cardiovascular diseases than men in the same age group; however, after menopause, in which estrogen plasmatic concentration reduces, the risk of cardiovascular diseases becomes similar in both sexes [11–14]. There is scientific consensus that men and women respond differently to risk factors, regarding the development and severity of cardiovascular diseases [15].

Most studies evaluating the differences associated with sex in the cardiovascular system focus especially on the endothelial function, since vascular endothelium is an important tissue to regulate cardiovascular homeostasis and may present differences between males and females [16,17]. Cardiovascular diseases such as arterial hypertension and coronary diseases are more severe in young men, as well as in several animal experiment models, than women/females of the same species and age group [11,18,19]. Sexual differences in the progression and severity of cardiovascular diseases may be attributed, largely, to differentiated regulation of the endothelial function, which may depend on part on the male and female gene imprints, and largely on the hormonal regulation of cardiovascular functional regulating molecules, such as some receptors, α-adrenergics and bradykinin B2, as well as endothelial nitric oxide synthase enzyme (eNOS) [20].

It was demonstrated that acetylcholine (ACh), an endothelium-dependent vasodilator, binds to the muscarinic receptors and promotes higher vasodilatation of aorta rings isolated from female than in males, in both normotensive rat strains [21] and SHR (spontaneously hypertensive rats) strains [22,23] (Fig. 6.1). These animals also displayed differences associated with sex in endothelial regulation of vasoconstrictor agents, such as angiotensin-II, endothelin-1, and noradrenaline [24]. The reduced plasmatic concentration of estrogen promoted by the surgical removal of the ovaries (ovariectomy) reduces ACh vasodilation in comparison to SHR females with ovaries and hormonal treatment with 17β-estradiol or conjugated equine estrogens was effective in restoring the reduced ACh vasodilation in ovariectomized SHR females [25,26] (Fig. 6.2).

In 1996, Taddei et al. studied sexual differences in endothelial function associated with aging. By measuring forearm blood flow change after administration of ACh (endothelium-dependent vasodilator) or sodium nitroprusside (Nitric Oxide donor) in normotensive and essential hypertension men and women, they observed constant and age-related maximum ACh vasodilation decline in normotensive and hypertensive men. In contrast, women (normotensive and hypertensive) showed only a slight reduction in ACh vasodilation per year, until middle age

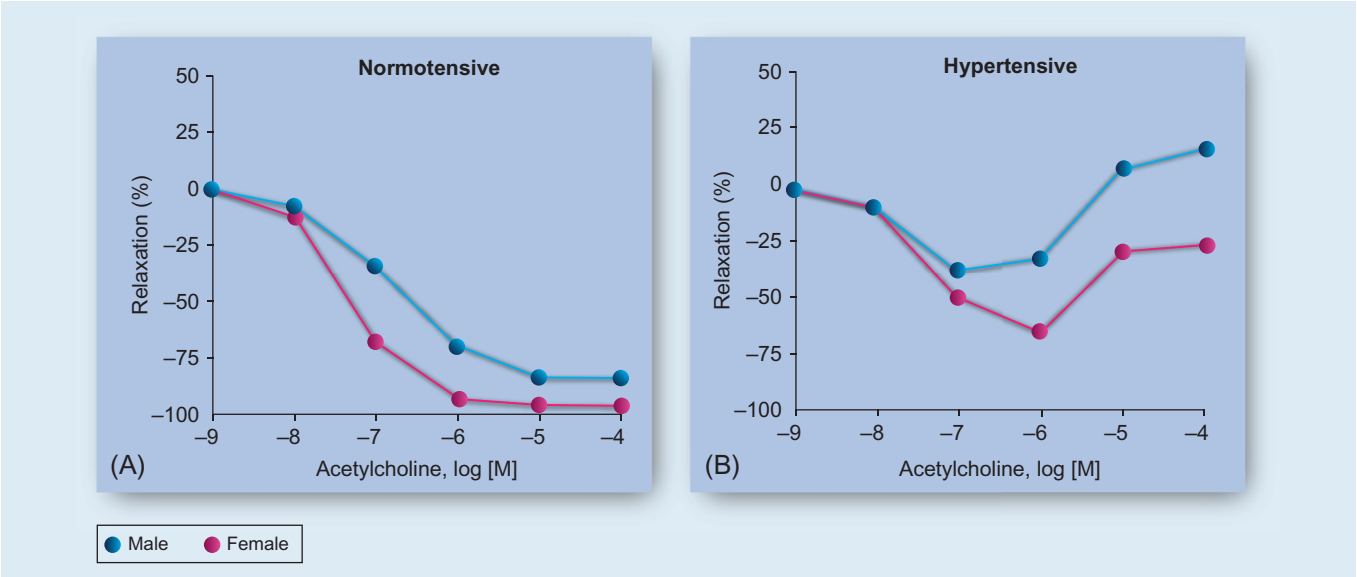

FIG. 6.1 Relaxation with acetylcholine, an endothelium-dependent vasodilator, is smaller in aorta rings, with endothelium, isolated from normotensive (Wistar) and hypertensive (SHR) male rats when compared to normotensive (Wistar) and hypertensive (SHR) females. Adapted from Kauser K, Rubanyi GM. Gender difference in endothelial dysfunction in the aorta of spontaneously hypertensive rats. Hypertension 1995;25:517–23.

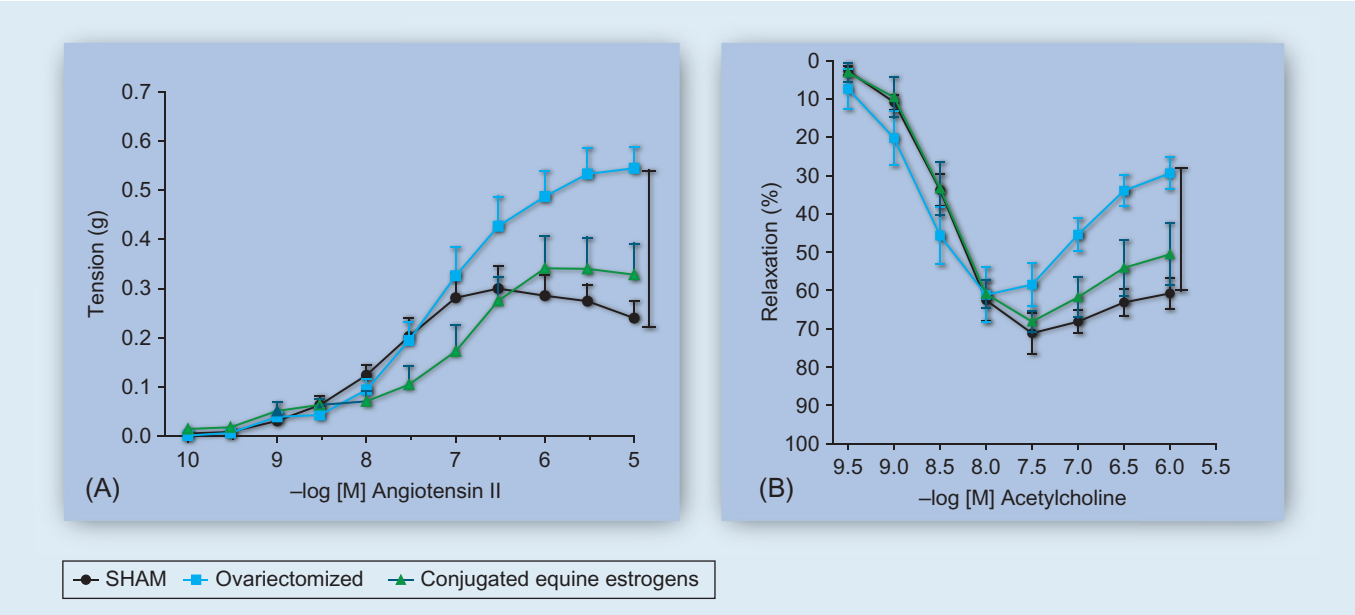

FIG. 6.2 (A) Angiostesin-II vasoconstriction in aortic rings with endothelium isolated from ovariectomized SHR females is increased when compared to aortic rings isolated from control SHR females (SHAM-operated), and those of ovariectomized females treated with conjugated equine estrogens. (B) Relaxation with acetylcholine, an endothelium-dependent vasodilator, is reduced in aortic rings isolated from ovariectomized SHR females when compared to those of control SHR females (SHAM-operated), and those of ovariectomized females treated with conjugated equine estrogens. Adapted from Costa TJ, Ceravolo GS, dos Santos RA, et al. Association of testosterone with estrogen abolishes the beneficial effects of estrogen treatment by increasing ROS generation in aorta endothelial cells. Am J Physiol Heart Circ Physiol 2015;308:H723–32.

(~50 years old). After that, the decline in endothelium-dependent vasodilator response accelerated and became more accentuated in comparison to men [27]. With that, they also evaluated the influence of postmenopause and, consequently, estrogen deficiency in endothelium-dependent vasodilation [27].

Arterial pressure values of women of childbearing age are lower than those of men of the same age group, and this difference has been attributed mainly to female gonadal sex hormones. Frequently, arterial pressure decreases during pregnancy, since in this period the concentration of circulating estrogen and progesterone are high [28–30] and in postmenopause, when the plasmatic concentration of estrogen is reduced, arterial pressure increases [31].

ESTROGENS, PROGESTERONE, AND TESTOSTERONE—MECHANISMS OF VASCULAR ACTION

The endothelial function of both sexes may suffer the influence of two important variables: sex chromosomes and sex hormones. In the prenatal period, the impact of sex chromosomes and genetic regulation contribute greatly to determining sexual differences. Female human umbilical vein endothelial cells (FHUVECs) have higher expression of eNOS messenger ribonucleic acid (mRNA) than male human umbilical vein endothelial cells (MHUVECs) [32], although in this cell type the protein expression of estrogen (ERα, ERβ e GPER) and androgen receptors (AR) do not differ between sexes [32].

After birth and during the entire life, sex-dependent characteristics are also determined by chronobiology of gonadal sex hormones. Therefore, influences of chromosomes and sex hormones may act together or in parallel to define the sex-dependent phenotype. During their lives, men and women are exposed to different concentrations of gonadal sex hormones. The difference includes variation between sexes and variations that are intrinsic to females for example, during the menstrual period and hormone decline after menopause.

The main sex hormones in men and women (estrogen, progesterone, and testosterone) act on

specific receptors in target cells to promote multiple actions on nonsexual tissues, including the cardiovascular system [33–38]. Steroid receptors were identified on the plasmatic membrane, cytosol, and nucleus of target cells [39]. Vascular endothelium expresses all subtypes of estrogen receptors (ER), as well as AR and progesterone receptors (PR) [22,40,41].

As gonadal sex hormones are liposoluble, they penetrate the cell and cross the plasmatic membrane by passive diffusion, and when they connect to specific receptors, forming the hormone-receptor complex, they promote genomic and nongenomic effects [34–38]. The nongenomic effects occur regardless of gene transcription and protein synthesis. These are considered quick effects, which occur in a matter of seconds or minutes, after the formation of the hormone-receptor complex [42] and involve the activation of kinase, ion channels present in the membrane, and production of nitric oxide (NO) [43,44].

On the other hand, genomic effects are delayed in response to the nuclear translocation of the hormone-receptor complex, positively or negatively regulating the gene and/or protein expression of some target genes [37] (Fig. 6.3).

The classic actions of the estrogens (17β-estradiol, estrone, and estriol) occur through three receptors, two of which are nuclear, called alpha (ERα/ERS1) [45] and beta (ERβ/ERS2) [46], and one membrane G-protein coupled receptor (GPR30) [47] (currently called GPER). The three types of receptors [48–50], as well as aromatase enzyme to metabolize androgens into estrogens [51], are expressed in both in smooth muscles and vascular endothelium of males and females.

The estrogen, coupled to its receptors associated with the membrane, ERα, or coupled to protein G, GPER, activates PI3K (phosphatidylinositol 3-kinase) and MAPK (mitogen activated kinase-like protein) signaling, contributing with NO generation in the vascular endothelium [52]. Although less studied, the ERβ located in the plasmatic membrane may activate MAPK, ERK and Src (extracellular signal-regulated kinase) pathways, and therefore modulate phosphorylation of several proteins involved in cell migration and proliferation processes [53].

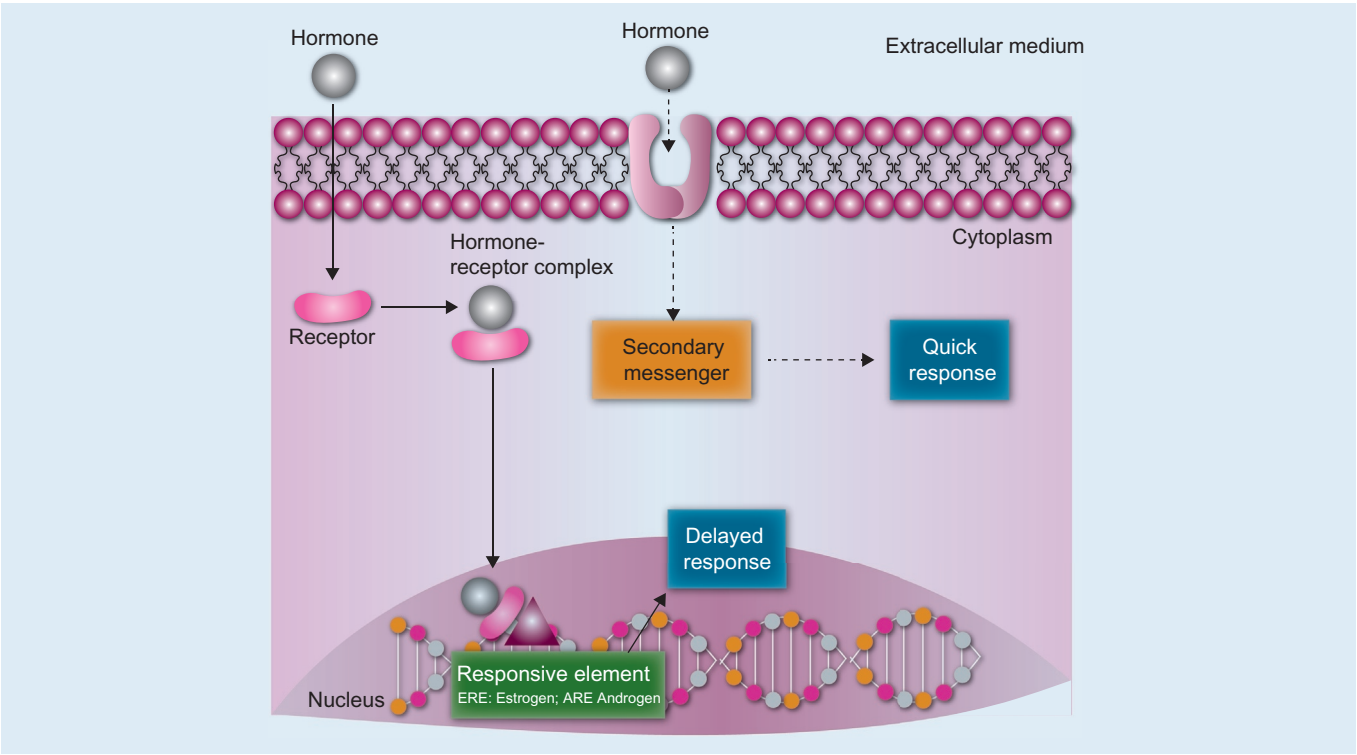

FIG. 6.3 Overview of the genomic and nongenomic action mechanisms of sex hormones. *ERE*, estrogen responsive element; *ARE*, androgen responsive element.

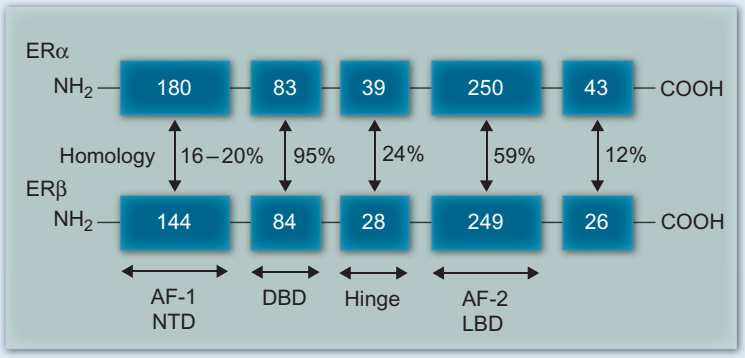

FIG. 6.4 Functional domains and homology of the estrogen receptor subtypes: ERα and ERβ. *NTD*, (NH_2)-terminal domain; *DBD*, DNA binding domain; *LBD*, ligand binding domain (see text for abbreviations).

The nuclear ERs consist of four functional domains:

(1) (NH_2)-terminal domain (NTD, N-terminal domain);
(2) DNA binding domain (DBD);
(3) Hinge domain; and
(4) Ligand binding domain (LBD), located in the carboxy terminal portion (COOH) of the receptor (Fig. 6.4).

The NTD contains an autonomous transcriptional activation region called AF-1 (activation function 1), which regulates transcription specifically in each gene and each cell. This regulation domain is considerably different in both ERs (only 16%–20% homology) and, in some cases, in ERβ, the AF1 may be significantly modified or absent [54]. The DBD is next to the NTD, highly conserved between ERα and ERβ (95% homology). The DBD is a domain structured as two zinc fingers able to recognize specific deoxyribonucleic acid (DNA) sequences in target genes (called estrogen responsive element, or ERE). The Hinge domain is essential for receptor dimerization, and is also a rotation point (therefore a "hinge"), which is fundamental for the receptor to acquire several conformations required to link to DNA. Regardless of the high-DBD homology in both receptor subtypes, suggesting that they bind to the DNA in similar fashion, the low homology between the NTD domains and Hinge indicates that both receptors may move differently and modulate different forms of gene transcription.

The LDB domain is in the carboxy terminal portion of the receptor, which contains the hormone biding site responsible for most of the functions activated by the agonist, such as dimerization of the receptor and translocation to the nucleus, besides attracting gene transcription co-regulator molecules, by means of autonomous activation region, called AF-2 (activation function 2) [55,56]. Differently from the great homology between the two DBD receptors, the LBD ERα and ERβ domains show lower homology (~59%), suggesting that the affinity between the agonists/antagonists for the receptor subtypes may differ significantly.

Estrogens influence vascular reactivity by direct effects on endothelial cells [57]. Vascular protection of estrogens in females is mediated partially via ERα [58,59], while in males this effect has more participation of ERβ [60,61]. In fact, mesenteric arterioles of ERβ knockout males have increased response to phenylephrine [62] and higher pressure levels than ERβ knockout females [62,63].

Due to the alternative splicing process, several forms of ERα have been described, although only a few has shown physiopathological relevance: ERαΔ3 which loses exon 3, which codifies part of the DBD and consequently its transcriptional capacity; and ERα36 and ERα46, variants with lower molecular weight (36 and 46 kDa, respectively) than the original ERα (with 66 kDa) [64]. For the ERβ, until the moment, at least four isoforms have been described: ERβ2, ERβ3, ERβ4, and ERβ5 [55]. In most cases, alternative splicing receptors lose their function or part of it and may act as negative domains that is able to inhibit the action of native ERs by forming a dimer with these receptors and inhibiting their action [64]. It is believed that the ratio between native receptors and their alternative splicing may change the response to estrogen and lead to tissue dysfunction.

Although this theory has been confirmed in several types of gynecological cancer, participation of

the ER variants in the vascular endothelium still needs to be clarified. On the other hand, studies have described that isoform ERα46 may facilitate its connection to the plasmatic membrane, and therefore improve endothelial function by quick activation of eNOS [65]. However, increased ERα46 expression over ERα66 (native) in cytosol significantly changes the genomic effects induced by estrogen on the vascular wall [66–68]. Up to now, ERs have been the sex hormone receptors with the best structural and functional characterization, although other nuclear receptors are also expressed in the vascular wall and have endothelial function modulation effects.

Progesterone is a natural steroid hormone, produced by gonads, by adrenal cortex, and by placenta. There are several progesterone derivatives, such as: medroxyprogesterone, norgesterone, and acetate, which have similar activities. The PR are called A (PR-A) and B (PR-B). Although they are codified by a single gene, the gene that codifies the PRs uses separate promoters and different translation starting points to produce two isoforms, which are practically identical, except for an additional group of amino acids in the N-terminal portion of PR-B [69]. Although PR-A and PR-B share several structural domains, the transcriptional activity is distinct and measures their own genes with physiological response and effect with little overlap [70]. Both PRs were identified in smooth muscle and vascular endothelium of humans, mice, rats, rabbits, and primates [71]. PR-B is expressed equally in the aorta of men and women, while PR-A has higher expression in females [72]. The role of progesterone the endothelium is relevant, but is not as well characterized as the effects of estrogens. These actions have been generally associated with regulation of angiogenesis process in tumors [73], although the isolated effects of progesterone also have been associated with decreased arterial pressure and antiinflammatory potential [40,74–76]. It was demonstrated that acute administration of progesterone induced quick vasodilation (nongenomic pathway) in the coronary artery of ovariectomized female rhesus macaque (*Macaca Mulatta*) [77]. In endothelial cell cultures, the administration of progesterone increases, by means of genomic pathway, eNOS activity, and production of NO [78]. For nongenomic signaling, the membrane progesterone receptors (mPR) are strong candidates. The mPRs are receptors with seven transmembrane domains coupled to protein G, and have five subtypes: mPRα, mPRβ, mPRγ, mPRδ and mPRε. The mPRs are expressed both in human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells. Treatment with both progesterone and the specific mPRα agonist increases NO production quickly and reduces the concentration of cAMP, suggesting that the receptor is coupled to an inhibitory protein G. On the other hand, treatment with specific PR agonist does not cause the same effect on NO production [30]. The influence of these mPRs on the vascular wall of both sexes still requires clarification.

Despite direct vascular effects, it is believed that progesterone may antagonize estrogen's effects. Administration of progesterone in an ovariectomized mouse treated with 17β-estradiol reduced the antioxidant effects of estrogen, leading to increased nicotinamine adenine dinucleotide phosphate (NADPH) oxidase activity and reduced level of mRNA antioxidant enzymes as manganese-dependent superoxide dismutase (MnSOD) and extracellular superoxide dismutase (SOD) [79]. In vascular endothelium of females, progesterone inhibits arginine transportation through cationic amino acid transporter 1, impairing eNOS activity [80].

Testosterone is the main natural androgen produced in men and women, being responsible for male sexual characteristics, libido, and increase of bone and muscle mass in both sexes [81]. Testosterone exercises its actions by interacting with the target receptor, one of them being the cytosolic receptor belonging to the family of steroid hormone nuclear receptors, and the other located in the plasmatic membrane [82]. In the cardiovascular system, these receptors are expressed in smooth muscle cells and in vascular endothelium [41,82,83].

Although they are associated with male characteristics, the gene that codifies the testosterone receptor is located in the X chromosome, is codified by eight exons, and one of its products is a protein with molecular weight of ~110 kDa [84]. Similar to the ERs, the AR have a LBD and the AF-1 and AF-2 domains that recognize androgen response elements (AREs) in DNA [84–86]. When activated by its agonist, the AR is translocated to the nucleus and binds, in its dimerized form, to the target gene AREs, activating or repressing the expression of such genes. Changes in AR sequence consist mainly of highly polymorphic trinucleotidic repeats (CAG) in exon 1 and the number of repeats is inversely correlated to the transcriptional activity of the androgen target genes [87]. In men, the number of CAG repeats is not correlated to total or free testosterone serum concentration, but few CAG repeats entail low levels of high-density lipoproteins (HDL) and reduced vasodilation

mediated by brachial arterial flows, therefore increasing the risk of developing cardiovascular diseases [88]. Also similarly to the ERs, testosterone may exercise nongenomic actions by means of activation of an AR located in the plasmatic membrane [82].

The role of androgens on the cardiovascular system is still controversial. Studies have shown both beneficial and harmful effects of these hormones [89–91]. For example, in men, low concentration of testosterone is associated with higher body mass index, higher waist circumference, diabetes, hypertension, low HDL, and risk of developing coronary arterial diseases [90–92], while in women in postmenopause, high concentration of testosterone is associated within sulin resistance, metabolic syndrome, and cardiovascular diseases [93].

Androgens exercise specific effects on each sex regarding functions regulated by endothelial cells, including angiogenesis and interaction between monocytes and the endothelium via AR. MHUVECs exposed to dihydrotestosterone (DHT) increased the gene expression of vascular cell adhesion molecule-1 (VCAM-1), effect abolished when the AR receptor antagonist, hidroxyflutamide, is used. However, when the HUVECs are from female donors, the phenomenon was not observed [94]. It has been shown in studies developed in vivo and in vitro that endogenous androgens are required for angiogenesis in males, but not in females [95].

Testosterone may exercise part of the effects on the vascular endothelium by means of metabolization of estrogen by the aromatase enzyme present in the endothelial cell. In fact, administration of testosterone in HUVECs decreased VCAM-1 gene and protein expression due to the conversion in estrogen [96]. However, testosterone may have a direct effect on the vascular endothelium, since in rat aorta endothelial cells, the hormone increases the production of NO, which was abolished in the presence of the androgen receptor antagonist (flutamine), but not with the aromatase inhibitor (anastrol) [97].

ACTION OF SEX HORMONES ON ENDOTHELIUM-DERIVED RELAXATION FACTORS AND ENDOTHELIUM-DERIVED CONTRACTING FACTORS

Nitric Oxide (NO)

The NO molecule is able to promote vascular relaxation, induce angiogenesis, and inhibit vascular smooth muscle cell proliferation, leukocyte adhesion, platelet aggregation, and thrombosis, among other functions. It is formed from the transformation of L-arginine into L-citrulline by a family of enzymes called nitric oxide synthases (NOS), present in several tissues. Mammals have three NOS isoforms, of which two are constitutive isoforms, the endothelial NOS (eNOS/NOS3) and the neuronal NOS (nNOS/NOS1), and one inducible isoform, inducible NOS (iNOS/NOS2), produced in response to inflammatory stimuli [98]. The increase in NO production or bioavailability via gonadal sex hormones, mainly estrogen, may involve several mechanisms, such as increased protein expression of eNOS [99], reduced generation of reactive oxygen species (ROS), such as superoxide anion [100,101], increased intracellular calcium ($[Ca^{2+}]$) in endothelial cells [102], activation of the PI3K pathway [103], decreased asymmetric dimethylarginine (ADMA) an eNOS endogenous inhibitor, and increased concentration of L-arginine (Fig. 6.5) [104].

In several studies, the NO released by the vascular endothelium of females was higher than in males, probably due to the higher expression/activity of eNOS observed in females [105,106]. In fact, aortic rings isolated from SHR females showed higher ACh vasodilation and higher phenylephrine vasoconstriction after incubation with NOS inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester) when compared to SHR males [107].

Progesterone and testosterone also may increase NO production, positively modulating expression and eNOS activity in Wistar female aortas [78,108]. In Wistar female aortas, progesterone increases NO production, positively regulating eNOS activity [97,98]. In Wistar normotensive female endothelial cell culture, acute treatment with testosterone increases NO production via RA activation [97].

The role of estrogen as potent NO stimulator becomes evident when reduced endogenous levels of estrogen, due to ovariectomy, decreased the expression of eNOS in female aortas of normotensive Sprague-Dawley [109] and hypertensive SHR [25]. Hormonal treatment with conjugated equine estrogens in ovariectomized SHR females restored the mRNA expression of eNOS, and consequently improved endothelial function [25].

Although the effects of estrogen on NO production are well characterized in females, biological effects are less known and more controversial in males. In males, it has already been shown that acute and chronic administration of estrogen improves

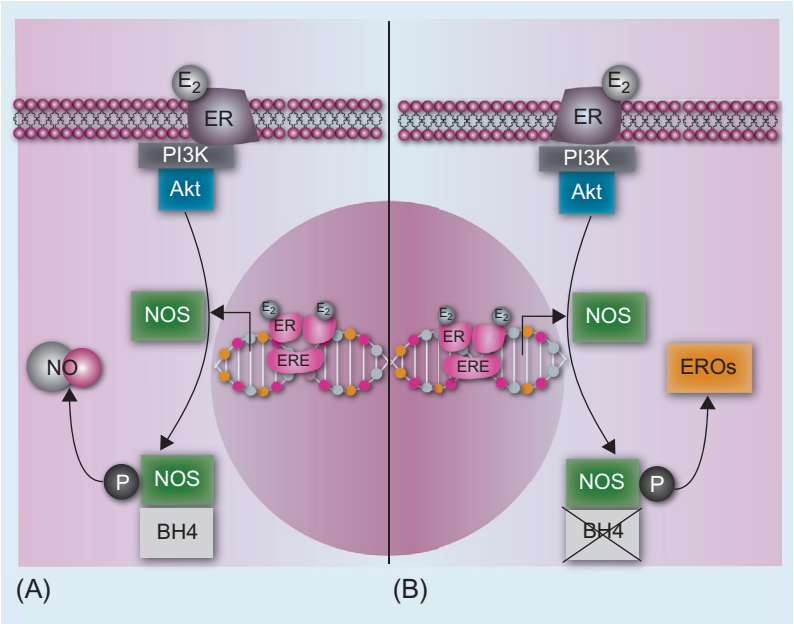

FIG. 6.5 Representation of estrogen genomic and nongenomic signaling pathway in NO production regulation in young (A) and old (B) females. *ER*, estrogen receptor; *E_2*, estrogen; *NOS*, nitric oxide synthase; *BH4*, tetrahydrobiopterin; *ROS*, reactive oxygen species; *PI3K*, phosphatidylinositol-3-Kinase; *ERE*, estrogen responsive element. Adapted from Murphy E. Estrogen signaling and cardiovascular disease. Circ Res 2011;109:687–96.

endothelial function and increase release of NO in carotids and aorta of Sprague-Dawley and SHR, respectively [110,111]. On the other hand, the estrogen may increase vascular damage in males by activating iNOS [112]. The opposite and controversial effects of estrogen in males and females may result from the differential expression of ER subtypes in both sexes [52, 113].

Prostaglandin (PG)

In addition to NO, endothelial cells produce and release prostacyclin (PGI_2), a vasodilator prostaglandin produced by conversion of arachidonic acid by cyclooxygenase (COX). Although PGI_2 is the main prostanoid produced in endothelial cells, the balance between production of vasodilator prostanoid and vasoconstrictor prostanoid, such as thromboxane A_2 (TXA_2), is extremely important to regulate vascular tonus [114]. Therefore, sexual differences in endothelium-dependent relaxation of macro and microvessels of rats and mouse may be partially explained by the imbalance in the production of prostanoids derived from COX-1 or COX-2 [115–117].

Deletion of the PGI2 receptor in C57B16 females reduced cardiovascular protection of ovariectomized females treated with estrogen [46], suggesting that PGI2 is one of the important mediators in vascular protection of females. Other studies have demonstrated that estrogen was able to modulate vascular function by decreasing the production of vasoconstricting prostanoids [26].

Effects of estrogen on gene and protein expression of COX-1 and COX-2 on the endothelium are still controversial. On the one hand, estrogen increases mRNA expression and COX-2 protein expression on women uterine circulation endothelium; [118] on the other hand, it reduced its expression on female mice dermis microcirculation and vena cava [119], showing that regulation of COX expression by estrogen may be specific for each vascular bed.

In Sprague-Dawley males, surgical removal of testicles (orchiectomy) increased COX-2 protein expression in the aorta, and induced unbalanced production of vasodilator and vasoconstrictor prostanoids, with predominant production of vasoconstrictor prostanoids [120]. Despite that fact, chronic administration of testosterone in males orchiectomized Fisher-344 strain increased vascular tonus of cerebral arteries, by increasing TXA_2 [121], showing that the physiological levels of sex gonadal hormones are important for the control of the vascular tone, since they may influence the production of various vasoactive agents.

ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTORS (EDHFS)

The EDHFs are vascular relaxation mediators with an important role in tonus control. Studies have proposed that the contribution of EDHFs for vascular relaxation increases in vascular beds and physiopathological conditions in which there is reduced participation of NO [122]. The release of EDHFs may be modulated by binding the agonist to specific receptors and by the shear stress on the blood vessel walls. The EDHFs may act on resistance or conductance arteries, but the vasodilator effect is more pronounced in resistance arteries of humans and experimental models [123,124].

Estrogen can also regulate vascular relaxation mediated by EDHFs, being one of the possible mechanisms by means of which hormonal treatment exercises a protective effect on the cardiovascular system [125]. Studies have shown reduced hyperpolarization mediated by EDHF in mesenteric bed arterioles of Wistar females submitted to ovariectomy, which was reversed after treatment with 17β-estradiol [125].

The differences associated with sex in the contribution of EDHFs in resistance artery relaxation have been described in several vascular beds, although opposite effects have been observed [126,127]. In resistance arteries of the mesenteric bed and other peripheral beds, the release of EDHFs is higher in females than in males [126]. On the other hand, in cerebral circulation, the contribution of EDHFs for vascular relaxation is smaller in females than in males [126]. Despite the lower contribution of EDHF in females, the infarcted area after induced ischemia in Wistar and stroke-prone SHR (animals prone to cerebrovascular accident) is greater in males than in females. These results suggest greater postischemia cerebral protection in females, as it is independent from EDHF release [128–130].

Although the nature of EDHFs is unknown, the sexual difference in vasodilation mediated by hyperpolarization may be correlated to the soluble epoxide hydrolase (EHs) enzyme, responsible for metabolizing epoxyeicosatrienoic acids, important vasodilator and candidate to one of the EDHFs [126].

Female C57Bl6 mice, subjected to ovariectomies, showed increased protein expression of EHs, which was reverted with estrogen treatment. Studies in vivo have shown that female mice knock-out to EHs showed infarcted areas similar to males of the same species [131]. It is known that even a temporary reduction of estrogen can affect the vascular reactivity of mesenteric circulation microvessels, mediated by EDHF, because female C57B16 mice in the diestro phase in which the plasmatic concentration of estrogen is reduced, showed diminished response mediated by EDHF [125]. In Sprague-Dawley males, treatment with 17β-estradiol increased endothelium-dependent relaxation, via EDHFs, in isolated aorta rings [132]. Therefore, estrogen may promote vascular relaxation in different vascular beds of males and females via nonidentified EDHFs.

REACTIVE OXYGEN SPECIES (ROS)

ROS play an important role in endothelial function, either directly as vasodilator agents (H_2O_2) and vasoconstrictor agents ($ONOO^-$), or indirectly by reducing NO bioavailability. In the latter case, superoxide anion (O_2^-) quickly reacts with the NO, promoting its inactivation and decreasing its beneficial effects on the vascular wall [133–135]. ROS production and the bioavailability of NO are important factors to determine endothelial dysfunction [133–135].

It has been demonstrated in experiments developed in SHR mesenteric arterioles studied by intravital microscopy [136] and aortic rings isolates from SHR [137] that the ROS production was lower in females than that of males and, therefore, blood vessels of females tend to respond less strongly to vasoconstriction agonists. The prooxidant environment also is less accentuated in women, as shown by the lower plasmatic concentration of malondialdehyde and thiobarbituric acid reactive substances (TBARS), human oxidative stress markers [138,139]. Also, reduced plasmatic concentration of estrogen, induced by ovariectomy, in SHR [136,137] and Sprague-Dawley [140] females increased the concentration of superoxide anion in mesenteric arterioles [136] and aortas [137,140]. Hormonal treatment with 17β-estradiol [136,140] or conjugated equine estrogens [137] reduced the concentration of ROS in the aorta and mesenteric arterioles in the female experimental models described above [136].

The effect of the estrogens (estradiol, estrone, and estriol) in reducing ROS may be related to the phenolic structure of these hormones, since, regardless of the interaction with its receptors, they may remove

ROS. However, this effect was only observed in concentrations 1000 times higher than the physiological concentrations [141,142], while the estrogen antioxidant effects are observed in females that showed physiological estrogen levels [101].

Actions of sex hormones regarding ROS regulation have been associated with modulation of NADPH oxidase expression/activity, which requires recruitment of cytosolic subunits ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$), and association with membrane subunits ($gp91^{phox}$ and $p22^{phox}$). Changes in expression or phosphorylation of these subunits induce higher or lower enzyme activity, and consequently ROS production. Seven subunits equivalent to $gp91^{phox}$ have been described, called Nox, and four of them were described in the vasculature (Nox1, Nox2, Nox4, and Nox5) [143].

In 2004, Dantas et al. [136] showed within in vivo studies on SHR animals that there was lower ROS production in mesenteric arterioles of hypertensive females than males. The sexual difference in ROS generation in SHR vasculature was followed by higher protein expression in subnuits $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ of NADPH oxidase in males. In addition, the aging process, a physiological condition associated with ROS increase, showed higher increase and more anticipated generation of ROS in the male mouse aorta when compared to the female mouse. At 7 months of age (middle age in mice), males presented higher ROS production than young males (3 months). In females, this difference becomes evident only at 12 months of age (aging in mice). In these females, treatment with apocynin, NADPH oxidase inhibitor, reduced the ROS generation [144].

Male mouse knockout for Nox2 presented reduced angiostesin-II vasoconstriction in the middle cerebral artery in comparison to native mouse. Reduced vasoconstriction described above was higher in knockout males than in females [145]. In addition, ovariectomized SHR females showed increased production of ROS, associated with positive regulation of mRNA of $gp91^{phox}$, $p22^{phox}$ and protein of $p47^{phox}$, subunits of NADPH oxidase. Treatment with conjugated equine estrogens [25,137] or 17β-estradiol reduced ROS generation and mRNA expression of subunits $gp91^{phox}$, $p22^{phox}$ of NADPH oxidase [25], and protein of $p47^{phox}$ [137].

The effects of testosterone on ROS generation seem oppose the effects of estrogen. Ovariectomized SHR females treated with testosterone alone [19] or with testosterone associates with equine conjugated estreogens [25] presented an increase in the generation of vascular ROS, mainly due to the increase in the active (phosphorylation) of the $p47^{phox}$ subunit of NADPH oxidase [25].

ROS generation may also be related to mitochondria and its modulation by estrogens [146]. Reduction in ROS generation in situation such as reoxygenation, followed by anoxia, occurred in a more pronounced way in cardiomyocyte mitochondria in isolated culture from females than males [146]. Generation of mitochondrial ROS can be modulated by estrogens, since the ERα and ERβ receptors are present in the mitochondrial membrane [147,148]. The cells of the aerobic organisms have developed a complex system of antioxidant enzymes that maintain the control of the production of ROS, avoiding the cellular damage. By the action of SOD, O_2^- is transformed into hydrogen peroxide (H_2O_2). The H_2O_2 by catalase is convert to water (H_2O) and oxygen (O_2). Thus, the regulation of the expression or activity of antioxidant enzymes, influenced directly by gonadal sex hormones, may contribute to the circulating redox state [133–135].

It has been demonstrated that the aorta of ovariectomized SHR females showed reduced protein expression of SOD and catalase, which was corrected by treatment with conjugated estrogens [137]. Bellanti et al. [149] analyzed redox balance in mononuclear cells of the peripheral blood of premenopause women with bilateral ovariectomy (surgical menopause). This was evaluated 30 days after the surgery, without treatment with estrogen, and 30 days after treatment with estrogen. After surgery, increased oxidative stress was observed due to reduced expression of mRNA for SOD and glutathione peroxidase, and recovered after estrogen treatment. The expression of mRNA of catalase and glutathione transferase was not modified in any of these conditions. The authors concluded that menopause is associated with significant changes in antioxidant enzyme gene expression that, in turn, change the circulating redox state.

Females and males knockout for MnSOD were infused with nonhypertensive doses of Ang II, and the authors observed endothelial dysfunction associated to increased ROS in basilar artery rings more pronounced in males than in females [46]. In fact, they observed more SOD activity in vascular smooth muscle cell culture of females than in males [150].

It is important to emphasize that most of the works described above clearly showed the participation of antioxidant enzymes in the endothelial function of experimental animals and women in postmenopause; however, few results compare sexual differences. The sexual difference was found in vascular smooth muscle cells, in the brain, and in the liver, since in the latter tissues catalase and glutathione peroxidase are more expressed in females than in male rats [151,152].

RENIN-ANGIOTENSIN SYSTEM (RAS)

The RAS is an important hormone complex that regulates arterial pressure, salts, and bodily fluids. Angiotensin II (Ang II), main RAS vasoconstrictor peptide, acting on AT1 (AT1R) receptors, contributes to increase vasoconstriction and ROS generation [153].

Aortic rings isolated from SHR males were more responsive to Ang II when compared to SHR females in physiological estrus, although AT1R antagonism reduces vasoconstriction in both sexes [23]. Similar results were demonstrated in animals in aging process. Aorta rings of male CD-1 mice with 12 months of age were more responsive to Ang II than females of the same age [154].

The ovariectomy procedure in SHR females increased vasoconstriction to Ang II, equating with SHR males. This response was corrected after treatment of the females with 17β-estradiol [136], suggesting that estrogen may reduce the response to Ang II. The sexual difference regarding response to Ang II is partially related to the capacity of estrogen in reducing AT1R mRNA expression [155] and increase AT2R after vascular injury [156]. In fact, it was demonstrated that AT1R expression in aortas was higher in males that in SHR females. However, AT2R expression was higher in females than in SHR males [23]. Activation of AT2R in blood vessels is associated with vasodilation by increased production of NO, by means of eNOS, FHDE, and the B2 receptor of bradykinin [157].

Okomura et al. [156] demonstrated that, after vascular occlusion and induction of inflammatory process, young female mouse presented higher AT2R expression in femoral artery than males, but this difference was smaller in old females with reduced estrogen levels. The sexual difference in AT2R expression in the cardiovascular system is due to the X chromosome, as in the iliac arter of male knockout mouse for the Y chromosome, the vasoconstricting response to Ang II became the same as that of females [157].

Estrogen also may act on the angiotensin converting enzyme (ACE), responsible for converting angiotensin I into Ang II. In large vessels, such as the aorta, estrogen reduced the expression of mRNA of ACE and the plasmatic content of this enzyme, reducing local and systemic production of Ang II and the deleterious effects of this peptide on the endothelium [158]. The lower plasmatic content of ACE contributes to increasedbioavailability of Ang 1-7 [1,8,32,60,74,128,159] and bradykinin, which has vascular actions that are opposite from Ang II, therefore maximizing the beneficial effects of estrogen [160]. In fact, Sullivan et al. [161] demonstrated that the production of renal Ang 1-7 [1,8,32,60,74,128,159] was higher in females that in SHR males.

ENDOTHELINS (ETS)

In 1985, Hickey et al. [162] described a vasoconstrictor polypeptide derived from the endothelium that regulates vascular muscle contractility. Later, this potent vasoconstrictor peptide of 21 amino acids was isolated and called endothelin-1 (ET-1) [163]. Currently, three different endogenous isoforms of the 21 amino acids peptide (ET-1, ET-2, and ET-3) and three of 31 amino acids (ET-$1^{1\text{-}31}$, ET-$2^{1\text{-}31}$ and ET-$3^{1\text{-}31}$) were identified [164–166]. There are two main subtypes of endothelin receptors: ETA and ETB, that belongs to the super family of receptors coupled to protein G and expressed in the endothelium, smooth vascular muscle, and mesangial cells [166].

ET-1 is responsible for promoting potent vasoconstriction, cellular growth, and inflammation, other than stimulating ROS generation, deposition of collagen in tissues, and expression of adhesion molecules in endothelial cells [165]. ET-1 binds mainly with the ETA receptor, but the existence of this subtype of endothelin receptor in the endothelium is still controversial. However, in human aortic endothelial cells, it was demonstrated that the presence of ETA e ETB receptors and peptide ET-1 is predominantly nuclear (including its envelope) [167]. ETB is expressed in vascular endothelial cells and its activation by ET-1 releases NO and prostaglandin [168].

The endothelin system, i.e., ET-1 and endothelin receptors, contribute to sexual differences present in cardiovascular diseases and arterial hypertension [169]. Women have a lower quantity of endothelin receptors in the saphenous vein, in the ration ET_A to ET_B when compared to men [170]. The expression of vascular mRNA of ET_B is increased in rats with DOCA-salt hypertension (uninefrectomized rats treated chronically with deoxycorticosterone acetate and sodium chloride) when compared to females [171]. The vasoconstriction induced by ET-1 is two times higher in samples of saphenous vein of men submitted to bypass surgery then in women submitted to the same procedure [170].

The sex hormones have a modulatory action on endothelin plasmatic concentration. In both hypertensive and nonhypertensive patients, the plasmatic concentration of ET-1 is higher in men than in women [165,169,172]. The concentration of ET-1 in plasma changes according to the stage of the menstrual cycle, suggesting a modulatory action of estrogens on endothelins. During the menstrual period, in which the concentration of circulating estrogen is lower, the concentration of plasmatic ET-1 is higher than in the follicular and luteal phases [108]. During the gestational period, concentration of ET-1 decreases. In female transgenders, treatment with estradiol and progestational substance cyproterone acetate decreased the plasmatic concentration of ET-1, while in male transgenders treated with testosterone the concentration of ET-1 increased [108].

ESTROGEN HORMONE TREATMENT—CLINICAL STUDIES

Observational studies suggested that estrogen treatment reduced the risk of mortality due to cardiovascular complications in postmenopause women in 30%–50% [173–175]. The study conducted with the nurses of Framingham Hospital (Nurse Health Study) was considered one of the largest observational studies regarding cardiovascular system of hormonal therapy. In this study, more than 48,000 women were followed for a period of 10 years. After adjustments by age and cardiovascular risk factors, the authors concluded that women who used estrogen therapy presented lower risk of developing acute coronary diseases or death due to cardiovascular diseases [173,176].

These results stimulated smaller clinical studies—randomized and double-blind—to evaluate the effects of treatment with estradiol in endothelium-dependent vasodilation mediated by brachial artery flow in postmenopause women. One of these studies showed that the hormone has endothelium-dependent vasodilator effects [176]. Not only with chronic treatment with 17β-estradiol, but acute treatment as well, have an effect on vascular tissue of women. Besides that Gilligan et al. demonstrated that intra-arterial infusion of physiological doses of estradiol in postmenopause women, with or without diagnosed coronary atherosclerosis, promoted endothelium-dependent vasodilation. The same was observed in normotensive women that were submitted to ovariectomy procedures (surgical menopause) and treated with transdermal estradiol [177,178]. However, large randomized, double-blind, and placebo-controlled clinical tests raised doubts regarding the beneficial effects of hormone therapy with conjugated equine estrogens on the cardiovascular system.

The purpose of the HERS (Heart and Estrogen/Progestin Replacement Study) was to evaluate the role of estrogen in secondary prevention of cardiovascular diseases. Started in the 1990s, the study followed a group of 2763 women with average age of 66.7 years, who received conjugated equine estrogen (0.625 mg) associated with medroxyprogesterone acetate (2.5 mg) for a period of 4.1 years. In this study it was observed that hormone therapy increased coronary and venous thromboembolism events in the first year of follow-up after acute myocardial infarction [179]. In the second segment of HERS, HERS II, which was expanded by 2.7 years and started to evaluate the role of estrogen in primary prevention of cardiovascular diseases, no difference was observed in respect to acute nonfatal myocardial infarction, death by coronary diseases, or other cardiovascular events, except for nonfatal ventricular arrhythmia, which was higher in the group treated with estrogen [180].

Afterwards, the Women's Health Initiative (WHI), the largest study regarding the effects of hormone therapy on women's health (more than 15,000 women in postmenopause), evaluated the effects of therapy with conjugated equine estrogen associated with medroxyprogesterone in primary prevention of cardiovascular diseases. The study demonstrated that hormone therapy may result in increased risk of cardiovascular events such as myocardial infarction and cerebrovascular accident [181,182].

Several questions emerged from these randomized studies (HERS and WHI). One of them is that administration of conjugated equine estrogens, concomitantly with progestogens, may influence the impact of the hormone on the vascular endothelium [182].

Progesterone is commonly administered with estrogen to reduce the risk of developing endometrial cancer; however, little is known regarding its effects on the cardiovascular system. Sorensen et al. [182a] demonstrated, in a randomized study, that administration of estrogen together with norethisterone—a progestogen, in women in postmenopause—did not improve dilation mediated by brachial artery flow, which is reduced in menopause. On the other hand, McCrohon et al. [183] demonstrated that medroxyprogesterone did not interfere in vasodilation promoted by estrogen in the brachial artery of women in postmenopause [183]. The therapeutic branch of the WHI study, which analyzed hormone therapy with estrogen alone, did not show differences regarding cardiovascular results; furthermore, it was interrupted in advance due to increased risk of breast cancer [184].

Another important point to be considered is the age in which estrogen therapy begins, since in the studies described the groups of women were in postmenopause, on average, for 10 years. This could lead to erroneous interpretations, because little is currently known about the relationship between the vascular effects of estrogens and the changes resulting from the vascular aging process [182]. In this regard, emerged the timing hypothesis or therapeutic opportunity window, a hypothesis created by WHI researchers proposing that the potential benefits mediated by estrogen to prevent cardiovascular diseases only appear when the hormone therapy is initiated before the deleterious effects of aging or before vascular dysfunction subclinical is present in the vascular wall [185,186].

In fact, the ELITE (Early versus Late Intervention Trial with Estradiol) clinical study, which evaluated 673 women with less than 6 and more than 10 years in postmenopause, observed that treatment with 17β-estradiol reduced the progression of atherosclerosis, measured in the carotid by ultrasound, in women who were in postmenopause for less than 6 years [187].

In men, plasmatic concentration of testosterone also has favorable direct and indirect effects on the cardiovascular system. The replacement of testosterone in elderly men with heart failure is associated with improved physical activity capacity, muscular strength, and glucose metabolism [188]. In men with angina pectoris, acute administration of testosterone promotes vasodilation in coronaries, by means of the potassium channel [189]. In contrast, transsexual women who received chronic administration of testosterone showed reduced vasodilation response induced by nitrates, increased concentration of triglicerides (TG), total cholesterol, low density lipoproteins (LDL), and apolipoprotein-B, as well as reduced levels of HDL [190].

Given these reports, the conclusion is that several aspects regarding the action of gonadal sex hormones on the cardiovascular system are controversial, and still are not completely clarified. It is important to emphasize that the endothelial effects induced by the action of gonadal sex hormones and sexual differences depend on the vascular bed, the animal model used, the plasmatic concentration of hormones, and the association between the different hormones.

NEW EXPERIMENTAL APPROACHES

Sex differences in several cardiovascular diseases is a well-established fact and regardless of the significant importance of gonadal sex hormones on regulating the mechanisms involved in these differences, it is still unknown if the expression of sexually dimorphous genes or if the sexual differences, that are intrinsic to the cells itself, may influence sexual dimorphism in cardiovascular physiopathology [191].

Currently, in this context, besides being widely used to study endothelial physiology and pathology regarding the cardiovascular system, HUVECs have also been used to study sexual differences present in cardiovascular diseases [32].

Curiously, when primary cultures of HUVECs obtained from male (MHUVECs) and female (FHUVECs) donors were studied independently, several sexual differences were noted; for example, MHUVECs synthesize higher concentrations of prostacyclins and prostaglandins E2 than FHUVECs when stimulated with thrombin, a molecule with important role in platelet aggregation [192]. In HUVECs stimulated with DHT, it was shown that in MHUVECs this androgen had proinflammatory and proatherogenic action, increased migration of endothelial cells, proliferation, tubulogenesis, and

production of endothelial vascular growth factor, while there were no similar changes in FHUVECs, suggesting that androgens can regulate vascular changes differently in both sexes [94–96,193].

Other studies showed that FHUVECs presented higher cell migration and proliferation rates, higher expression of genes associated with metabolism, stress, and immune response, as well as higher expression of eNOS when compared to MHUVECs. In addition, after these cells were submitted to shear stress, the cells proceeding from FHUVECs showed higher expression of eNOS, SOD-1, and HO-1 (Heme Oxygenase 1) genes, as well as lower expression of the ET-1 gene when compared to MHUVECs. On the other hand, MHUVEC cells showed higher production of hydrogen peroxide, higher expression of beclin-1, and LC3-II/LC3-I ratio (microtubule-associated protein 1 light chain 3), indicating higher autophagic activity in these cells when compared to FHUVECs [32,191].

It is important to emphasize that no differences were found regarding the expression of estrogen and AR (ER-α, ERβ, GPER, and AR) and aromatase 5α-redutase 1 and 5α-redutase 2 enzymes (convert testosterone in DHT) in HUVECs proceeding from both sexes, as well as no differences in Akt (protein kinase B) and mTOR (mammalian target of rapamycin) expression, a pathway involved in several cellular processes, including proliferation, growth, and survival [32,159]. Furthermore, images obtained using an inverted microscope also did not show differences in size, shape, and morphology of cells proceeding from MHUVECs and FHUVECs. On the other hand, images obtained using a transmission electronic microscope revealed that MHUVECs and FHUVECs showed different ultrastructural patterns. In this context, cells proceeding from MHUVECs showed, for example, pinocytic vesicles distributed uniformly in the cell membrane, several autophagic vacuoles, and absence of lipid vacuoles, while cells proceeding from FHUVECs showed, for example, pinocytic vesicles distributed eccentrically in the cell membrane, several lysosomes, and lipid vacuoles, which could justify the sexual dimorphism found in several cell responses [32].

Sexual differences observed in endothelial cells can contribute to improve the comprehension of endothelial functions on cardiovascular diseases. Together, the findings described herein emphasize the importance of the use of male and female cells in cellular culture experiments, which should be applied not only to endothelial cells, but also to other types of cells, organs, and tissues, as well as using both sexes in studies to understand the mechanisms involved in the development and progression of cardiovascular diseases.

CLINICAL PERSPECTIVE

Regardless of evidence on sexual differences in cardiovascular system regulation, effective treatment of cardiovascular diseases in women in pre or postmenopause is a difficult issue in medicine, mainly due to the lack of understanding of the mechanisms involved in the initial stage of cardiovascular diseases, symptoms, and menopause process. Cardiovascular diseases are the main cause of death of women in postmenopause, and therefore should receive high priority among women health issues. However, there is still an alarming gap regarding the knowledge and understanding of the effects of estrogen on the cardiovascular system and general awareness of the medical and scientific societies regarding how to treat cardiovascular diseases in women. Medical training is still dominated by studies conducted in men, and therefore the clinical trends and instructions on drug and procedure recommendations are focused on this data. The lack of crucial information and the differences in the data available regarding women cardiovascular system regulation many times lead to inappropriate diagnosis and treatment. Therefore, women are still treated in the same way as men, regardless of the notable sexual differences in cardiovascular function. Increased awareness regarding risk factors and specific treatment for cardiovascular diseases in women is required to allow early diagnosis and more effective treatment.

CONCLUSIONS

Considering the importance of sex for the differences in cardiovascular morbimortality, it is important to have different diagnosis and therapeutic strategies for men and women. In basic research, the use of both sexes, male and female, in experimental and cellular models, is decisive to characterize the vascular effects of gonadal sex hormones.

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