REVIEW

Sex differences in vascular function: implication of endothelium-derived hyperpolarizing factor

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Abstract

The vascular endothelium plays a crucial role in the regulation of vascular homeostasis by controlling vascular tone, coagulation, and inflammatory responses. These actions are exerted by endothelial factors including nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). The greater incidence of cardiovascular disease (CVD) in men and postmenopausal women compared with premenopausal women implies a vasoprotective phenotype of females, which may be influenced by sex hormones. These hormones, particularly estrogen, have modulatory effects on the endothelium and circulating cells that have been implicated in vascular inflammation and in the development of CVD. EDHF seems to be the predominant endothelial factor in the resistance vasculature of females and this mediator could afford the beneficial cardiovascular risk profile observed in premenopausal woman. In this review, we discuss sex differences in EDHF biology and how sex hormones can modulate EDHF responses. We also review the implication of sex hormone-dependent regulation of EDHF in inflammatory processes, platelet function, and repair after vascular damage, each of which have a critical role in several aspects of the pathogenesis of CVD.

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Introduction

A lower incidence of cardiovascular disease (CVD) in premenopausal women compared with age-matched male counterparts and postmenopausal women (Lerner & Kannel 1986, Barrett-Connor 1997) suggests that ovarian hormones underlie a protective effect on the cardiovascular system. Indeed, a wealth of evidence from observational and experimental studies, in both animals and humans, supports the concept of a protective effect of estrogens in the cardiovascular system. Thus, it was wholly unexpected when the Women's Health Initiative (WHI), a large-scale trial with menopausal hormone treatment (MHT), often also described as hormone replacement therapy (HRT) in postmenopausal women aged 50–79 was terminated early due to a surprising increase in the incidence of cardiovascular events (The Women's Health Initiative Steering Committee 2004). However, further secondary analysis of the WHI data sheds some light on the apparent paradoxical findings (Hsia *et al.* 2006); whilst overall there was an increase in events, separation of the cohorts by age demonstrated a significant protection of MHT in women with fewer menopausal years. This issue was later confirmed in a following ancillary study measuring coronary artery calcification in women aged 50–59 (Manson *et al.* 2007). It is clear that estrogens offer a number of protective beneficial effects; however, MHT is complicated by a number of detrimental actions that depend upon age, the type of hormone preparation, and mode of application, issues that all influence the target as well as the mechanism of action of estrogen. Understanding the molecular pathways that underlie the beneficial effects of estrogens may, therefore, identify novel strategies that could be taken to harness the therapeutic potential of MHT safely.

Numerous studies have been conducted in an attempt to more clearly understand the protective effects of estrogens in the cardiovascular system, and a number of targets/pathways/mediators have been proposed (Mendelsohn & Karas 1999, 2005, Orshal & Khalil 2004, Villar *et al.* 2006). Perhaps, of all of the effects that have been attributed to estrogens, there is one particular characteristic of these sex hormones, which has been demonstrated repeatedly to contribute to the beneficial effects in the cardiovascular system i.e. estrogen-induced or enhanced activation of the endothelium. It is now accepted that changes in endothelial function are instrumental

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in the vascular inflammation that is an early and crucial event in the pathogenesis of a number of cardiovascular disorders (e.g. atherosclerosis, ischemia/reperfusion (I/R) injury). Endothelium-derived factors not only alter the tone and growth of the underlying smooth muscle, but also regulate the reactivity of circulating white cells, erythrocytes, and platelets and govern vascular permeability. Indeed, the endothelium is critical in maintaining an anti-inflammatory, and thereby anti-atherogenic, influence on the blood vessel wall. In the early stages of several inflammatory CVDs, a switch in endothelial phenotype occurs from a protective anti-inflammatory to a pro-inflammatory phenotype, characterized by the production of pro-inflammatory mediators. Much evidence supports the thesis that estrogens upregulate the synthesis, release, and activity of protective endothelial factors and suppress the expression of pathogenic mediators by the endothelium and, thereby, preventing this apparent change in phenotype. The majority of these actions have been attributed to estrogens acting on the estrogen receptors (ER)α and ERβ, both of which have been identified in the vasculature (Mendelsohn & Karas 2005). More importantly, it is this protective action that is thought to underlie the protection of females from CVD (Villar *et al.* 2006).

Perhaps the most-described phenomenon relating to estrogen-induced changes in endothelial activity is the impact these hormones have on vascular tone mediated via stimulation of the release of endothelium-derived vasodilators. The most significant vasodilator factors that the endothelium releases are prostacyclin (PGI_2; Moncada *et al.* 1976), nitric oxide (NO; Ignarro *et al.* 1987, Palmer *et al.* 1987, Furchgott & Vanhoutte 1989, Moncada & Higgs 2006), and endothelium-derived hyperpolarizing factor (EDHF; Chen *et al.* 1988, Griffith 2004, Ahluwalia & Hobbs 2005). The biological activity and functions of endothelium-derived NO and PGI_2 in the cardiovascular system are well established and include not only vasodilatation but also inhibition of platelet aggregation, leukocyte recruitment, and smooth muscle growth (Moncada & Vane 1981, Moncada & Higgs 1991, 2006). Indeed, both of these factors have been identified as important targets for estrogen activity and there are many recent excellent reviews that the reader is referred to on this matter (Orshal & Khalil 2004, Mendelsohn & Karas 2005, Villar *et al.* 2006). However, the nature and role(s) of EDHF in the vasculature remain controversial and whether estrogens modulate its expression and function is also uncertain. Recently, we have proposed that the functional remit of EDHF may extend beyond its vasodilator effects and that, as its fellow endothelial vasodilators, it may also inhibit leukocyte recruitment and platelet reactivity and thereby provide a compensatory pathway in situations (such as CVD) where endothelial NO or PGI_2 synthesis/activity is suppressed (Ahluwalia & Hobbs 2005). Indeed, this is particularly relevant when one considers that basal NO synthesis is thought to inhibit EDHF release/activity and that EDHF responses are often exposed in an environment where NO synthesis has been repressed (Bauersachs *et al.* 1996). This review will describe and discuss the evidence investigating the possibility that alterations in EDHF may underlie the sex differences in CVD and whether estrogens are implicated.

EDHF

EDHF hyperpolarizes and relaxes vascular smooth muscle and, thereby, plays a fundamental role in the regulation of vascular tone (Garland *et al.* 1995, Busse *et al.* 2002). The physiological significance of EDHF is emphasized by the finding that as one descends the vascular tree, the role of NO diminishes whereas the influence of EDHF increases (Shimokawa *et al.* 1996) implicating EDHF in the control of local blood flow and, therefore, determination of peripheral resistance and systemic blood pressure. It is now accepted that EDHF release from the endothelial cell occurs following the opening of endothelial small conductance calcium-activated potassium channel (SK_{Ca}) and intermediate conductance calcium-activated potassium channel (IK_{Ca}; Busse *et al.* 2002). Its activity, on the vascular smooth muscle, involves activation of the Na^+/K^+-ATPase and the inwardly rectifying potassium channel (K_{IR}). However, the identity of this factor, or factors, remains uncertain. Since the first report of EDHF (Chen *et al.* 1988), several putative candidate factors have been proposed including hydrogen peroxide (H_2O_2; Shimokawa & Morikawa 2005), metabolites of arachidonic acid such as epoxyeicosatrienoic acids (EETs; Fleming & Busse 2006), potassium ions (Edwards & Weston 2004), and most recently C-type natriuretic peptide (CNP; Ahluwalia & Hobbs 2005); alternatively, EDHF may be an electrical coupling through myoendothelial junctions (Feletou & Vanhoutte 2006). Yet, none have been confirmed as a universal EDHF (Feletou & Vanhoutte 2006). Indeed, this 'EDHF heterogeneity' has led to the proposal that multiple EDHFs exist and that its identity varies between organs and species studied. Moreover, the release of more than one EDHF in a given vascular bed also seems to occur (Busse *et al.* 2002, Villar *et al.* 2007*b*). As a consequence of this intra- and inter-tissue and species heterogeneity, it is unlikely that a single unique EDHF exists (unlike the role of NO as EDRF). Furthermore, whether sex differences in EDHF activity might underlie the differences in susceptibility to CVD is unclear. In light of this, we have reviewed the evidence investigating this possibility focusing on the role that EDHF might play in mediating the influence of the endothelium on vascular reactivity in terms of vascular tone, leukocyte recruitment, platelet activation, and vessel repair.

Sex differences in vascular tone: EDHF

Endothelium-derived factors, particularly NO, play an important role in the paracrine regulation of vascular tone (Orshal & Khalil 2004, Villar *et al.* 2006). However, there is a growing body of evidence suggesting that EDHF is also

crucial in this modulation, especially given the fact that in the resistance vasculature the predominant mediators implicated in endothelium-dependent relaxation in males are NO and PGI_2, whereas in females it is EDHF (McCulloch & Randall 1998, Pak *et al.* 2002, Scotland *et al.* 2005*b*).

We developed an endothelial NO synthase/cyclooxygenase 1 (eNOS/COX-1) double knockout (dKO) mouse, or 'EDHF mouse', to explore the potential role of EDHF in vascular function. This model is unique because it circumvents the notorious lack of selective EDHF antagonists and the possible complications seen when using the classical inhibitors, L-nitro-arginine-methyl ester and indomethacin, to remove NO- and PGI_2-dependent relaxations (Chauhan *et al.* 2003*a*). In these animals, we have shown that males are hypertensive whereas females are normotensive. Moreover, the administration of the endothelium-dependent dilator bradykinin, to female dKOs, causes a dose-dependent decrease in arterial blood pressure, while this vasoactive peptide has no effect in the male dKO animals; this result was mirrored in mesenteric arteries *in vitro*, where endothelium-derived relaxation stimulated by acetylcholine was significantly greater in females. Since EDHF plays a more prominent role in endothelium-dependent relaxation of resistance versus conduit arteries (Shimokawa *et al.* 1996), data from the EDHF mouse suggests that sex hormones regulate EDHF activity and that this may play a critical role in the maintenance of physiological levels of blood pressure and endothelial function in females (Scotland *et al.* 2005*b*). Consequently, enhanced EDHF function in females compared with males (McCulloch & Randall 1998, Pak *et al.* 2002, Scotland *et al.* 2005*b*) might afford the cardioprotective phenotype of females.

It is thought that estrogen activity underlies this sex difference in the prevalence of EDHF responses. Indeed, an estrogen deficit in animals, achieved by ovariectomy, reduces EDHF responses induced by several different stimuli and this effect is reversed by treatment with 17β-estradiol (Huang *et al.* 2001, Liu *et al.* 2001, 2002, Chataigneau *et al.* 2004, Xu *et al.* 2005). Also, EDHF responses are reduced during periods of the estrus cycle when estrogen levels are lowest (Lucca *et al.* 2000). In contrast, recent studies have indicated that ovariectomy does not affect the magnitude of acetylcholine-mediated relaxation in the rat mesenteric artery (Chataigneau *et al.* 2004, Nawate *et al.* 2005). However, when the EDHF- and NO-mediated components of this endothelium-dependent relaxation were examined separately, it was observed that while the magnitude of the response remained relatively unaltered, ovariectomy reduced the magnitude of the EDHF-mediated component but enhanced the NO-mediated component of the response (Chataigneau & Schini-Kerth 2005), supporting a role for estrogen in regulation of EDHF-mediated vasorelaxation. However, these findings, especially with respect to the effect on NO-mediated responses, should be interpreted with caution since a substantial body of evidence clearly demonstrates that estrogens enhance NO production within blood vessels, indeed many of the beneficial actions of estrogens have been attributed to this very characteristic (for reviews, see Orshal & Khalil 2004, Mendelsohn & Karas 2005, Murphy & Steenbergen 2007). Moreover, in middle-aged rats, acetylcholine-mediated hyperpolarization is upregulated in females and this effect is reduced by ovariectomy and recovered by 17β-estradiol treatment (Sakuma *et al.* 2002). Interestingly, the EDHF-mediated component of acetylcholine-induced vasodilatation is enhanced after myocardial infarction only in females (Csanyi *et al.* 2006), a finding that perhaps hints at an endogenous EDHF-mediated damage-limiting mechanism. The exact nature of the mechanisms underlying the enhanced EDHF vasoactivity observed in females is by no means clear, but we have attempted to consider the main proposed pathways below in terms of putative EDHF candidates.

Gap junctions Gap junctions facilitate chemical and electrical communication between coupled cells (Sandow & Hill 2000). There is substantial support for the concept that EDHF, rather than being a 'factor' *per se*, might simply represent the transfer of hyperpolarizing current from the endothelial cell to the smooth muscle, a phenomenon facilitated by the myoendothelial gap junction (Griffith 2004). This thesis is supported by several studies using blockers of gap junctional communication that have demonstrated selective inhibition of vasodilator responses attributable to EDHF (Griffith 2004). These junctional structures are formed by intracellular channels that are composed of different members of the connexin (Cx) protein family (Griffith 2004). Endothelial cells express Cx37, Cx40, and Cx45, whereas smooth muscle cells express Cx43 and Cx45 (Gabriels & Paul 1998, Theis *et al.* 2001). Myoendothelial gap junctions occur in greater density in resistance compared with conduit arteries and, consequently, it has been suggested that the gap junctions may play a role in the hormonal modulation of vascular relaxation (as an EDHF candidate). Indeed, it has been proposed that a decrease in the vascular expression of Cx43 alone or concomitantly with Cx40 could afford the reduction in EDHF-mediated responses in rat mesenteric arteries of ovariectomized rats (Liu *et al.* 2002, Xu *et al.* 2002, Nawate *et al.* 2005); this was substantiated by the observation that when animals were treated with 17β-estradiol, all responses were normalized (Chataigneau & Schini-Kerth 2005, Nawate *et al.* 2005). In addition, it has been suggested that the increased EDHF-mediated relaxation, associated with pregnancy in rats and humans (Pascoal & Umans 1996, Gerber *et al.* 1998, Kenny *et al.* 2002), is in part due to enhanced gap junctional communication at least in response to bradykinin in subcutaneous small arteries of pregnant women (Luksha *et al.* 2004, Lang *et al.* 2007). However, in rat middle cerebral arteries myoendothelial gap junction frequency does not correlate with the reduced EDHF responses observed in females compared with males (Sokoya *et al.* 2007).

C-type natriuretic peptide CNP has been described as an EDHF in the mesenteric and coronary circulation (Barton *et al.* 1998, Chauhan *et al.* 2003*b*, Hobbs *et al.* 2004). This peptide belongs to a family of structurally similar vasoactive peptides that have vasodilator and diuretic actions and play an important role in maintenance of blood volume and blood pressure (Levin *et al.* 1998, Fowkes & McArdle 2000, Scotland *et al.* 2005*a*). This peptide is widely expressed throughout the vasculature, being found in particularly high concentrations in vascular endothelial cells (Stingo *et al.* 1992) where it plays a role in the local regulation of vascular tone. Its biological effects are mediated not only via natriuretic peptide receptor (NPR)-B (in a cGMP-dependent manner), but also via NPR-C, which is classically termed a 'clearance receptor' that removes natriuretic peptides from the circulation. However, this receptor also possesses an intracellular G-protein-binding domain and can couple to Gi/o G-proteins (Anand-Srivastava *et al.* 1996) and this is involved in part in a recently elucidated signal transduction pathway(s) underlying the vasorelaxant activity of CNP and EDHF (Villar *et al.* 2007*b*).

Interestingly, previous studies have identified sex differences in the vasorelaxant activity of CNP. In porcine coronary arteries, relaxations to CNP are greater in female than in males (Barber *et al.* 1998) and the highest CNP mRNA concentrations in mice are found in the uterus and ovaries, and this expression is increased further still during pregnancy, a period of high estrogen concentration (Stepan *et al.* 2000). Moreover, 17β-estradiol increases CNP gene expression in the uterus of ovariectomized mice (Acuff *et al.* 1997) and in rats, the estrous cycle regulates CNP content in both the ovary and uterus (Huang *et al.* 1996). Taken together, these studies suggest that modulation of CNP release/activity by sex hormones may contribute to the enhanced EDHF activity in females.

Hydrogen peroxide Several studies have suggested that H_2O_2 acts as an EDHF in animal and human blood vessels (Matoba *et al.* 2000, 2002, Miura *et al.* 2003). In endothelial cells, eNOS and some oxidases including cytochrome P450 (CYP) epoxygenases, cyclooxygenases, lipoxygenases, NAD(P)H, and xanthine oxidase can be sources of superoxide, which is dismutated spontaneously, or by superoxide dismutase (SOD), to H_2O_2 (Fleming *et al.* 2001, Shimokawa & Matoba 2004). This dismutation is prinicipally a mechanism utilized within the vasculature to inactivate superoxide and in this way sustain an antioxidant status. However, it is also clear that H_2O_2 *per se* has multiple functions within the vasculature, not only as a potentially damaging reactive oxygen species, but also possibly as a physiological regulator of vascular reactivity including the regulation of vascular tone (Miller *et al.* 2007*a*). Furthermore, there is some evidence to suggest that estrogens as well as enhancing antioxidant status by elevating SOD levels also simultaneously provide a vasodilator. It is thought that endothelial Cu/Zn SOD plays a principal role in the hyperpolarization responses elicited by H_2O_2 (Morikawa *et al.* 2003, Shimokawa & Matoba 2004) and several studies have identified sex differences in H_2O_2 responses. For example in Cu/Zn SOD KO mice, myogenic tone is increased in arteries of females compared with male controls (Veerareddy *et al.* 2004). This apparent difference in the H_2O_2-dependent regulation of tone may be a consequence of enhanced synthesis since physiological concentrations of estrogen stimulate the formation of reactive oxygen species, predominantly H_2O_2, in endothelial cells. The endothelial oxidases involved in this process appear to be within the mitochondrial respiratory chain and xanthine oxidase (Felty *et al.* 2005, Felty 2006). However, it is likely that alterations in direct synthesis are not the sole mechanism and that estrogens also effect changes in dismutation of free radicals and therefore H_2O_2 generation. In support of such a mechanism is the finding that ovariectomy reduces Cu/Zn SOD protein expression in mice; an effect reversed following *in vivo* estradiol supplementation (Muller-Delp *et al.* 2003). However, in contrast are studies in the cerebral circulation of the rat that show no difference in SOD expression between male and female animals (Miller *et al.* 2007*a*,*b*).

Epoxyeicosatrienoic acids EETs, generated following metabolism of arachidonic acid by CYP epoxygenases, have been considered as potential candidates for EDHF due to their capacity to hyperpolarize and relax vascular smooth muscle cells by activating K_{Ca} channels (Campbell *et al.* 1996, Campbell & Falck 2007). Accordingly, sex differences in the vasoactive responses to endogenously generated EETs have been reported. In particular, it has been proposed that EETs mediate flow-induced dilatation in gracilis arterioles of female rats and mice, while, in contrast, this response is largely mediated by NO in arterioles of male animals (Wu *et al.* 2001, Huang *et al.* 2005). Although, in male mice expressing depressed eNOS expression, flow-induced dilatation remains unchanged; an effect that the authors propose to be due to compensatory upregulation of EET synthesis (Sun *et al.* 2007). These responses in gracilis arterioles of female mice are dependent on estrogen since EET-mediated flow-induced dilatation is abolished by ovariectomy and restored by estrogen replacement (Huang *et al.* 2001). This effect of estrogen is likely due to an upregulation in endothelial EET synthesis, rather than alterations in the sensitivity of vascular smooth muscle to these eicosanoids, since the enhanced vasodilator response to shear stress is associated with upregulation of CYP activity (Huang *et al.* 2004). Furthermore, recent studies, in human coronary arterioles, suggest that exogenously applied EETs induce dilatation that is unaffected by sex (Larsen *et al.* 2006). Thus, it seems likely that at least a component of estrogen-mediated enhancement of EDHF activity relates to alterations in CYP epoxygenase activity.

Sex differences in vascular inflammation

There are several studies supporting the thesis that sex hormones modulate the initiation and progression of inflammatory responses in females. Indeed, female sex and

estrogens appear to be protective against a range of inflammatory diseases including multiple sclerosis and Alzheimer's to atherosclerosis (Nilsson 2007). Similarly, estrogens appear to suppress the inflammatory responses in experimental models of inflammation in both *in vivo* and *in vitro* systems (Nathan *et al.* 1999, Mukherjee *et al.* 2002, Card *et al.* 2006). A significant proportion of this anti-inflammatory activity has been attributed to alterations in NO synthesis and activity (Thompson & Khalil 2003, Orshal & Khalil 2004); however, EDHF has also been proposed to play a role. For example, EDHF controls blood flow and, therefore, levels of shear stress at the blood vessel wall. These hemodynamic forces regulate endothelial activation (Malek *et al.* 1999) and consequently moderate leukocyte recruitment, platelet activation, and vessel repair mechanisms. Therefore, at atheroma-prone sites, where low levels of shear stress have been implicated in pathogenesis, shear stress-regulated EDHF-dependent responses may be important in limiting, or indeed preventing, atherosclerotic events (Selemidis & Cocks 2002). In the next section, we have examined the possibility that, just as endothelium-derived NO, EDHF may be more than simply a vasodilator (Ahluwalia & Hobbs 2005). In particular, we have discussed the possibility that EDHF might play an essential role in maintaining the anti-inflammatory phenotype of the endothelial cell and thereby limiting vascular inflammation and progression of CVD. We have specifically focused on the role of EDHF in leukocyte recruitment, platelet activation, and vessel repair; all important mechanisms involved particularly in the progression of atherosclerosis (Libby 2002).

Sex differences in leukocyte recruitment

Leukocyte recruitment is an early and pivotal event in vascular inflammatory responses that is prompted by the expression of, and consequent interaction between, leukocyte and endothelial cell adhesion molecules. The sequential steps of rolling, adhesion, and transmigration mediate this process via the activity of specific families of adhesion molecules that characterize each step (Butcher 1991, Springer 1994, Rao *et al.* 2007, Zarbock & Ley 2008). There is some evidence, from diverse animal models of inflammation, to suggest the existence of sex hormone-dependent male/female differences in leukocyte recruitment (Squadrito *et al.* 1997, Simoncini *et al.* 2000*b*, Eckhoff *et al.* 2002) with evidence supporting the thesis that estrogens have the capacity to alter all stages of the recruitment process.

Recently, we have shown that female mice exhibit reduced basal and interleukin (IL)-1β-stimulated leukocyte rolling compared with males (Villar *et al.* 2007*b*), although whether ovarian hormones underlie this effect is currently unknown. However, estrogen inhibits the leukocyte rolling response to diverse inflammatory stimuli including I/R injury, where, for example, estrogen treatment prevents leukocyte rolling in the mouse cremaster (Prorock *et al.* 2003).

Similarly, there is supporting evidence for an inhibitory effect of estrogen on the firm adherence of leukocytes. In hypercholesterolemic rabbits, monocyte adhesion to endothelial cells and transendothelial migration is retarded in females compared with male animals; an effect likely due to female sex hormone activity since ovariectomy enhances cell adhesion while supplementation of these animals with 17β-estradiol restores protection (Nathan *et al.* 1999). This protective effect of sex is reproduced in other inflammatory models, again including ischemia–reperfusion injury, where, for instance, female sex hormones decrease leukocyte adhesion in response to a transient forebrain ischemic insult (Santizo *et al.* 2000). In addition, 17β-estradiol reduces the granulocyte and monocyte/macrophage populations of injured vessels and limits leukocyte entry from adventitial/periadventitial tissues into injured vessels, thereby reducing the neointimal response to vascular injury (Xing *et al.* 2004).

Mechanistically, there is much evidence supporting the theory that estrogen-induced inhibition of leukocyte recruitment is due to an effect of female sex hormones on endothelial adhesion molecule expression and activity. 17β-estradiol suppresses monocyte endothelial adhesion stimulated by diverse stimuli including cytokines (Mikkola & St Clair 2002, Mori *et al.* 2004), LPS (Gao *et al.* 2006), and oxidized LDL (Suzuki *et al.* 1997), and various endothelial adhesion molecules have been implicated including intercellular and vascular adhesion molecule-1 (ICAM-1 and VCAM-1), P- and E-selectin in human (Caulin-Glaser *et al.* 1996, Simoncini *et al.* 2000*a*), and rabbit endothelial cells (Nathan *et al.* 1999). However, conversely 17β-estradiol and progesterone increase adhesion of leukocytes to human umbilical vein endothelial cells (HUVEC), stimulated by tumor necrosis factor-α (TNF-α) by increasing expression of E-selectin, ICAM-1, and VCAM-1 (Cid *et al.* 1994). In addition, there is some evidence suggesting that male sex hormones exert inhibitory effects on leukocyte recruitment. Indeed, testosterone inhibits VCAM-1 mRNA and protein expression in HUVECs, although this action is dependent upon conversion to 17β-estradiol via aromatase activity, an enzyme present in endothelial cells (Mukherjee *et al.* 2002). There is also evidence to suggest that rather than directly affecting adhesion molecule expression, estrogens suppress the expression of specific chemokines (chemoattractant cytokines) that are produced by endothelial cells among other cell types. In a model of balloon-induced vascular injury in rats MCP-1 and CINC-1, chemoattractants for monocytes and neutrophils respectively (Miller *et al.* 2004) were suppressed. Furthermore, in animal models of atherosclerosis, MCP-1 levels, which have been implicated in disease progression (Libby 2002), are reduced in females in an estrogen-dependent manner, an effect correlated with decreased atheroma formation (Pervin *et al.* 1998). Exactly what role EDHF might play, with respect to each of the proposed candidates in mediating these sex-dependent inhibitory effects is discussed below.

Gap junctions Besides the implication of gap junction in controlling vascular tone, these cellular connections also play a role in leukocyte recruitment. It has been reported that neutrophils, monocytes, and lymphocytes can form functional gap junction channels and this coupling, primarily mediated by the ubiquitously expressed Cx43, is inhibited by pharmacological blockers of gap junctions and stimulated by inflammatory mediators including LPS (Oviedo-Orta & Howard Evans 2004). It is thought that the formation of these junctions facilitates intercellular communication not only via transfer of current between cells, but also by permitting the transfer of small molecules including ions, second messengers, and small peptides (see Neijssen *et al.* 2007) Functionally, leukocyte–endothelial interaction and transendothelial migration of leukocytes also appear to involve activity of gap junctions (Neijssen *et al.* 2007). In particular, Cx43 expression of leukocytes is associated with the adherence and recruitment of leukocytes stimulated by LPS or ischemia–reperfusion injury (Jara *et al.* 1995).

The exact functional role of these gap junctions with respect to cellular recruitment appears to vary dependent on the cell type. For example, inhibition of gap junctions increases neutrophil but decreases monocyte migration (Wong *et al.* 2004). The inhibitory effect of gap junction expression on PMN migration appears to involve heterotypic (i.e. more than one type of connexin) cell communication and, in particular, has been attributed to gap junctions comprised Cx40 and Cx37 (Zahler *et al.* 2003) and Cx43 (Parthasarathi *et al.* 2006). More recently, the functional consequences of this inhibitory activity have been highlighted in APOE KO mice (a model of atherosclerosis) also deleted for Cx37, where lesion formation was substantially enhanced (Wong *et al.* 2006). Several studies clearly demonstrate that the expression of Cx37, Cx40, and Cx43 are all regulated by estrogen (Di *et al.* 2001, Punyadeera *et al.* 2005), and it is likely that this regulation, to some extent, underlies the effects of female sex hormones on leukocyte recruitment, such that the enhancement of expression of the gap junctions may in part mediate the decreased inflammatory cell recruitment evident in female animal models of inflammation.

C-type natriuretic peptide Some evidence implicates CNP in inflammatory events since plasma levels of this peptide are elevated in patients with septic shock (Hama *et al.* 1994) and anti-inflammatory effects of CNP have been described in acute myocarditis (Obata *et al.* 2007). Our *in vivo* experiments have demonstrated that CNP inhibits leukocyte rolling in mice with elevated basal leukocyte activation induced by IL-1β and histamine; this effect appears to be mediated via attenuation of P-selectin expression (Scotland *et al.* 2005*c*). In addition, adenovirus mediated-expression of human CNP and suppresses ICAM-1 and VCAM-1 expression and macrophage infiltration in balloon-injured rabbit carotid arteries (Qian *et al.* 2002). Moreover, this endothelium-derived mediator suppresses infarct size and myocardial dysfunction in isolated mouse hearts (Hobbs *et al.* 2004). Whether the differences in inflammation prevalent in females are due to an enhancement of CNP synthesis or activity is for the present unknown.

Hydrogen peroxide Several studies have shown that H_2O_2 possesses pro-inflammatory properties. For example, experimental data suggest that endogenous H_2O_2 production induced in I/R and following inflammatory stimuli contributes to the consequent associated injury (Granger 1988). H_2O_2 also promotes neutrophil adhesion to endothelial cells and this effect appears to be mediated by P-selectin (Lewis *et al.* 1988, Patel *et al.* 1991). In addition, it has been suggested that increased H_2O_2 production contributes to endothelial NF-κB activation in aged rat arteries (Ungvari *et al.* 2007) and induces TNF-α mRNA in HUVECs (Valen *et al.* 1999). Moreover, H_2O_2 enhances the expression of both CD11b and CD18 on eosinophils (Nagata *et al.* 2000). It has also been reported that H_2O_2 plays an important role in cardioprotection against coronary I/R injury *in vivo* (Yada *et al.* 2006). While this latter evidence supports an anti-inflammatory role for H_2O_2, a greater body of evidence indicates a pro-inflammatory function for this molecule. These findings suggest that H_2O_2 is unlikely to underlie the beneficial effects on inflammation/leukocyte recruitment associated with EDHF.

Epoxyeicosatrienoic acids EETs have been described as powerful anti-inflammatory mediators since they inhibit NF-κB and IκB kinase and, consequently, prevent leukocyte adhesion to the vascular wall (Node *et al.* 1999). Moreover, at physiological concentrations, these agents inhibit cytokine-induced VCAM-1, ICAM, and E-selectin expression and the activity of pro-inflammatory enzymes including inducible NOS and COX-2 in human endothelial cells (Node *et al.* 1999, Campbell 2000). Overexpression of the EET-synthesizing CYP 2J2 also has similar effects and can protect against I/R injury *in vitro* and *in vivo* (Spiecker & Liao 2006). EETs also decrease pro-inflammatory prostaglandin levels in porcine aortic smooth muscle cells by competitive inhibition of COX enzymes (Fang *et al.* 1998) and attenuate cigarette smoke-induced lung inflammation and the accumulation of neutrophils, alveolar macrophages, and lymphocytes in bronchoalveolar fluid (Smith *et al.* 2005). Recently, it has been reported that the anti-inflammatory effects of EETs on endothelial cells may be mediated by α/γ peroxisome proliferator-activated receptor (PPAR; Liu *et al.* 2005, Wray & Bishop-Bailey 2007). Sex hormones might operate via such a pathway since it has been demonstrated that estrogen regulates PPAR expression (Campbell *et al.* 2003, Faddy *et al.* 2006). Although, more recent evidence in isolated arteries suggest that while PPARγ might enhance endothelial vasodilator release in conduit vessels, in resistance arteries activation of this receptor is inhibitory for EDHF release (O'Sullivan *et al.* 2006). Thus, EETs clearly mediate a number of anti-inflammatory effects, although, whether estrogens operate via regulation of their synthesis is as of yet uncertain.

Sex difference in platelet function

Platelets not only participate in vascular homeostasis but also contribute to the development of thrombotic events and play a critical role in the pathogenesis of atherosclerosis and CVDs. In this capacity, platelets provide an active surface for pro-coagulant reactions, expressing membrane receptors that affect platelet–platelet and platelet–vessel wall interactions, and releasing vasoactive substances and mitogenic cytokines (von Hundelshausen *et al.* 2007, Ruggeri & Mendolicchio 2007). There is evidence that sex hormones, especially estrogen, regulate platelet function. The presence of the ERs, ERα and ERβ, in platelets and their precursors, megakaryocytes (Jayachandran & Miller 2003, Bord *et al.* 2004), is thought to underlie the capacity of estrogen to regulate platelet function in a genomic and non-genomic manner, although ERβ appears to be the main receptor involved (Khetawat *et al.* 2000, Bord *et al.* 2004).

Ex vivo ADP-mediated platelet aggregation and ATP secretion *in vitro* are higher in platelets from postmenopausal women compared with premenopausal women and 17β-estradiol, medroxyprogesterone, or MHT suppresses this increase (Nakano *et al.* 1998). In addition, ADP-induced aggregation is greater in platelets isolated from male compared with female rats, and castration reduces aggregation in males but increases aggregation in females (Johnson *et al.* 1977). Also, while aggregation decreases with maturity in female pigs, the opposite is true in male pigs (Jayachandran & Miller 2002). These results are in concordance with human studies supporting the importance of timing of the initiation of MHT. It has been reported that the benefit of treatment only occurs in younger postmenopausal women and this benefit disappears with age (Manson *et al.* 2006, 2007); an effect that may relate to the action of estrogens on platelets. The Kronos Early Estrogen Prevention Study (KEEPS), currently in progress, was embarked upon to directly address the timing issue, and will assess the anti-atherosclerotic effects of conjugated equine estrogens alone and in combination with progesterone transdermally administrated in recently postmenopausal women (Harman *et al.* 2005). These studies should explain, at least in part, the controversy generated by some trials showing that MHT does not confer protection from CVD in postmenopausal women (Manson *et al.* 2003, Anderson *et al.* 2004).

Depletion of female sex hormones has been described to induce numerous changes in platelet reactivity; effects that are reversed following restoration of estrogen levels including increases in platelet aggregation and dense body ATP secretion (Jayachandran *et al.* 2003, 2005*a*), increased expression of CD40 and its ligand (Jayachandran *et al.* 2005*b*), increased platelet turnover, increase in ER-associated heat shock proteins (HSP70 and HSP90), matrix metalloproteinase-2 (which activates platelet aggregation and adhesion (Sawicki *et al.* 1997)), and platelet-derived growth factor-BB (Jayachandran & Miller 2002, 2003, Jayachandran *et al.* 2003). Many of these actions could contribute to the increased proliferative arterial response to injury that has been identified in ovariectomized animals (Bracamonte *et al.* 2002).

Many of the anti-aggregatory actions of estrogens on platelets can be attributed to an indirect action of these hormones on the endothelium, in particular via release of the endothelial factors NO and PGI_2, known to reduce platelet aggregation and adhesion. Whether EDHF might also play a role in this endothelium-dependent regulation is currently unknown. *In vitro*, 17β-estradiol-induced decreases in platelet aggregation have been associated with increased NO activity, as evidenced by elevated cGMP level expression in human platelets (Nakano *et al.* 1998), but also to increases in plasma levels of the stable PGI_2 metabolite 6-keto-PGF1α (Nakano *et al.* 2002).

Yet, a beneficial effect of female sex steroids on platelet reactivity remains controversial since estrogen can, under certain circumstances, induce a pro-aggregatory phenotype. Several studies show higher *ex vivo* platelet aggregation of platelets of female over male animals (Elam *et al.* 1980, Durand & Blache 1996) and women than men (Kelton *et al.* 1982, Haque *et al.* 2001). In addition, 17β-estradiol treatment and MHT increase the number of circulating activated platelets in postmenopausal women (Thijs *et al.* 2002); an effect that may relate to estrogen-induced increases in bone marrow megakaryocyte and pro-platelet formation, and platelet release in humans (Bord *et al.* 2000, Nagata *et al.* 2003). A pro-aggregatory action of female sex hormones is also supported by the finding that 17β-estradiol increases tissue factor (TF), a primary initiator of the coagulation pathway, mRNA and protein levels in porcine platelets (Jayachandran *et al.* 2005*b*). This effect becomes more significant when one considers that the TF pathway inhibitor (an inhibitor of TF-dependent secretion) is reduced by estrogen treatment in endothelial cells and in postmenopausal women using oral contraceptives (Luyer *et al.* 2001). Interestingly, unlike the potential pathways that might be implicated in the anti-aggregatory effects of estrogen, thrombin-induced platelet aggregation and activation of integrin α(IIb)β3 by 17β-estradiol is mediated through ERβ (Moro *et al.* 2005).

Just as endothelial factors have been implicated in the beneficial effects of estrogens on platelet reactivity, some studies also support a role for these very same factors in the detrimental effects on platelet function. Surprisingly, the release of PGI_2 is greater in platelets from ovariectomized pigs compared with male and female pigs (Miller *et al.* 1999). While, in contrast, endothelial cell PGI_2 production is decreased by physiological doses of female sex hormones (17β-estradiol, progesterone or combined treatment; Berge *et al.* 1990). In addition, certain progestins reduce the anti-aggregatory effect of endothelial cells by decreasing the expression of eNOS and the production of NO (Zerr-Fouineau *et al.* 2007). All these negative effects of sex hormones on platelet function likely contribute to the increased risk of thrombosis shown in postmenopausal women using MHT. Thus, while it is clear that estrogens can exert anti-aggregatory actions and that the endothelium is likely to play an important role in this response, the evidence is equivocal in terms of which endothelial mediator might be important in mediating this effect. In addition, another point of interest is that

it is not only the female sex hormones that alter platelet activity, but also androgens have been shown to increase the production and reactivity of platelets (Shapiro *et al.* 1999, Weidemann & Hanke 2002). What role EDHF might have to play in mediating the effects of sex hormones on platelet reactivity has not been extensively studied, but below we have reviewed the evidence that EDHF might play a role.

C-type natriuretic peptide CNP inhibits platelet–leukocyte interactions by downregulating P-selectin expression and preventing thrombin-induced platelet aggregation of human blood (Scotland *et al.* 2005*c*). This study taken together with the anti-inflammatory effects described above, suggests that CNP likely represents an important anti-atherogenic endothelial mediator. However, further studies are needed to confirm whether the effects of estrogens on platelets are due to CNP activity.

Hydrogen peroxide Some studies have demonstrated that H_2O_2 has a pro-thrombotic influence on the vascular wall. H_2O_2 induces platelet aggregation (Li *et al.* 2007), potentiates thrombin-induced platelet aggregation (Naseem & Bruckdorfer 1999), and is involved in the enhancement of Ca^{2+} mobilization observed in platelets from diabetic patients which results in platelet hyperactivity (Redondo *et al.* 2005). These findings suggest a detrimental effect, if anything, of H_2O_2 on platelet function, and in combination with the pro-inflammatory effects of H_2O_2 described above, suggest that it acts in a pro-atherogenic manner rather than in an anti-atherogenic manner (unlike EDHF).

Epoxyeicosatrienoic acids EETs inhibit platelet aggregation (Fitzpatrick *et al.* 1986, Jiang *et al.* 2004) and platelet adhesion to endothelial cells. This is thought to be mediated by EET-induced platelet hyperpolarization and inhibition of P-selectin expression (Krotz *et al.* 2004). However, as with CNP, there have been no studies to date directly assessing whether EET synthesis might underlie the beneficial effects of estrogens on platelet function.

Sex differences in vessel repair

Endothelial damage is a crucial event in the development of vascular diseases such as atherosclerosis, vein-graft atherosclerosis and angioplasty-induced restenosis (Ip *et al.* 1990). Many studies suggest that estrogen replacement therapy reduces the number of coronary events after coronary angioplasty in postmenopausal women (O'Brien *et al.* 1996, O'Keefe *et al.* 1997, Khan *et al.* 2000). However, others investigating directly the relationship between MHT with 17β-estradiol alone or in combination with progesterone have shown no benefit on the progression of atherosclerosis (bu-Halawa *et al.* 1998, Herrington *et al.* 2000, Hodis *et al.* 2003). However, these negative studies have been conducted, invariably, in older postmenopausal women with pre-existing stenoses and, as mentioned previously, there is a growing understanding that timing of treatment post menopause onset determines whether hormonal treatment is largely beneficial or not (Manson *et al.* 2006, 2007). However, in postmenopausal women with no evidence of pre-existing vascular disease, 17β-estradiol treatment is associated with reduced progression of carotid artery atherosclerosis and, more recently, this has been further substantiated by the demonstration that those women with the highest free estradiol levels were those with the least progression of atherosclerosis (Karim *et al.* 2008).

Mechanistically, there is support for the concept that estrogens might reduce the atherosclerotic burden by inhibiting hyperplasia. In a porcine model of restenosis, local delivery of 17β-estradiol decreases neointimal hyperplasia after coronary angioplasty without stent implantation (Chandrasekar & Tanguay 2000). Also, administration of this hormone enhances re-endothelialization and endothelial function in rats and pigs and reduces intimal hyperplasia after percutaneous transluminal coronary angioplasty (Krasinski *et al.* 1997, Concina *et al.* 2000, Chandrasekar *et al.* 2001, Kyriakides *et al.* 2006). Furthermore, estrogen-coated stents are associated with reduced neointimal formation in porcine models (New *et al.* 2002) and in humans (Abizaid *et al.* 2004). In addition, neointimal formation in response to balloon injury of the rat carotid artery is attenuated in females compared with males (Chen *et al.* 1996). Moreover, ovariectomy is associated with a greater increase of neointima formation, and 17β-estradiol treatment inhibits myointimal proliferation after injury (Chen *et al.* 1996, Oparil *et al.* 1999, Bakir *et al.* 2000). Taken together, the combined effects of enhancing endothelial formation but repressing smooth muscle proliferation have clear beneficial effects in limiting the atherosclerotic burden in the damaged blood vessel.

Migration and proliferation of vascular smooth muscle cells in vascular tissue are two important events for development of myointimal thickening after vascular injury (Libby *et al.* 1992, Newby & Zaltsman 2000). In cell culture, 17β-estradiol inhibits the migration and proliferation of vascular smooth muscle cells of rodents (Kolodgie *et al.* 1996, Akishita *et al.* 1997) and humans (Dai-Do *et al.* 1996). It is generally accepted that proliferation of smooth muscle cells from the media is involved in intimal hyperplasia; however, it has been suggested that the bone marrow could also supply circulating vascular progenitor smooth muscle and endothelial cells. Indeed, 17β-estradiol enhances activation and recruitment of endothelial progenitor cells and contributes to improved neovascularization preserving cardiac function after myocardial infarction (Strehlow *et al.* 2003, Iwakura *et al.* 2006). It is thought that this favorable effect is exerted predominantly through ERα and to a lesser extent through ERβ (Hamada *et al.* 2006), perhaps with the involvement of eNOS (Iwakura *et al.* 2006). The possibility that estrogen-induced modulation of EDHF activity might underlie these effects is reviewed below.

Gap junctions Gap junctions may play a role in the response to vessel injury since Cx43 expression is enhanced in smooth muscle cells of atherosclerotic human, murine, and rodent

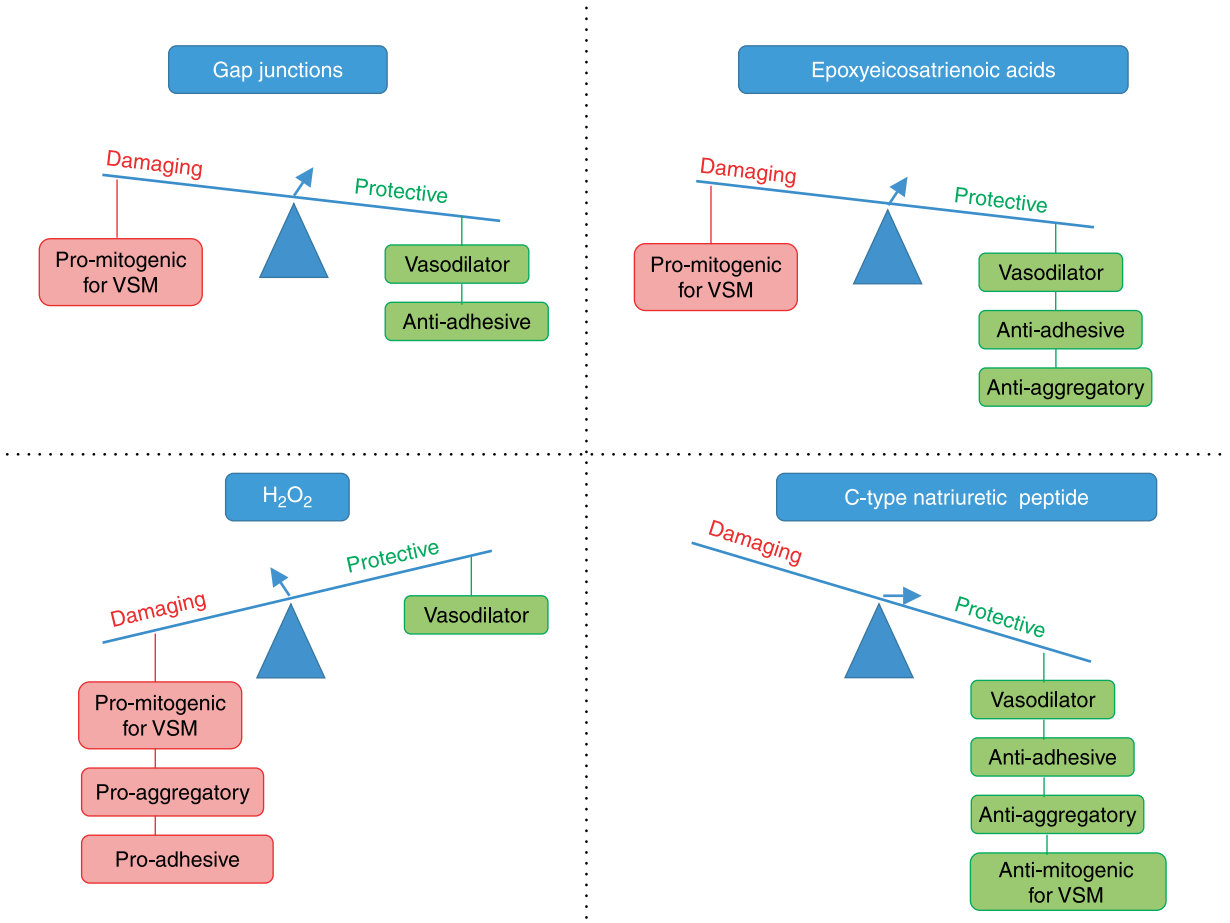

Figure 1 Impact of estrogen-induced changes in gap junctions, epoxyeicosatrienoic acids, H_2O_2, and C-type natriuretic peptide on vascular homeostasis.

arteries (Blackburn *et al.* 1995, Yeh *et al.* 1997, Kwak *et al.* 2002). Moreover, a critical role for Cx43 has been reported in human saphenous vein during the development of intimal hyperplasia (Deglise *et al.* 2005) and restenosis (Plenz *et al.* 2004) and Cx43 decreases smooth muscle cell proliferation and limits neointima formation after vascular injury (Chadjichristos *et al.* 2006). Conversely, regeneration of the endothelium following carotid artery injury in rats, a process essential for wound healing, is associated with increasing expression of Cx37, Cx40, and Cx43 (Yeh *et al.* 2000). More detailed investigations of the significance of altered connexin expression is required before any clear consensus on the role

Table 1 The effect of estrogens on the vascular expression/synthesis of some of the main candidates for endothelium-derived hyperpolarizing factor (EDHF)

	Effect of estrogen on expression/synthesis	**References**
EDHF candidate		
Myoendothelial gap junctions	↑	Liu *et al.* (2002), Xu *et al.* (2002) and Nawate *et al.* (2005)
	NC	Sokoya *et al.* (2007)
CNP	↑	Huang *et al.* (1996), Acuff *et al.* (1997) and Stepan *et al.* (2000)
H_2O_2	↑	Felty *et al.* (2005) and Felty (2006)
	NC	Miller *et al.* (2007*b*)
EETs	↑	Huang *et al.* (2004) and Sun *et al.* (2007)

NC, no change.

of gap junction expression in mediating the beneficial effect of estrogens in vessel repair can be made.

C-type natriuretic peptide CNP overexpression by adenoviral gene transfer in rabbit vein grafts accelerates re-endothelialization and dramatically reduces the intima formation that develops when it is grafted to the carotid artery (Ohno *et al.* 2002). Also, CNP inhibits vascular smooth muscle migration and proliferation (Cahill & Hassid 1991, Furuya *et al.* 1991). Taken together, these studies suggest that endothelium-derived CNP facilitates repair after vascular injury although again further investigations are required to determine whether CNP might underlie the effects of estrogens.

Hydrogen peroxide In general, reactive oxygen species participate in processes such as growth and migration of vascular smooth muscle cells, which contribute to the development of atherosclerosis. In particular, H_2O_2 has been described as a proliferative mediator and an inducer of apoptosis (Konishi *et al.* 2004, Wang & Huang 2005) and it has been used as a tool to induce vascular injury. As such it is unlikely that this mediator underlies the beneficial effects of estrogens in vessel injury.

Epoxyeicosatrienoic acids Exogenous ETTs inhibit vascular smooth muscle migration in response to growth factors and overexpression of CYP2J2 in these cells attenuates the migratory effect (Sun *et al.* 2002). In human vascular endothelial cells, overexpression of CYP2C9, or administration of EETs, increases cell proliferation (Michaelis *et al.* 2003). Together this data suggests a beneficial profile for EETs as enhances of endothelial but suppressors of smooth muscle cell proliferation. However, EETs have also been shown to induce cell proliferation in smooth muscle cells (Graber *et al.* 1997, Potente *et al.* 2002) an effect thought to contribute to the pro-angiogenic effects of EETs confirmed by several studies (Fleming 2007) and thus it is uncertain at present whether alterations in EET expression might be beneficial or pathogenic in vessel injury.

Summary

The existence of sex differences in CVD has suggested that sex hormones may proffer cardiovascular protection to females. The endothelium plays a crucial role in vascular homeostasis mediating the effects of endothelial-derived factors that are regulated by sex hormones (in particular estrogen). These effects thought to play an important role in cardioprotection observed in younger (premenopausal) women. Besides the regulatory role of sex hormones on vascular tone, estrogen also modulates the recruitment of circulating cells, platelet function, and several processes in vascular repair after injury. While NO and PGI_2 are well characterized in this regard, the significance of EDHF is increasing (despite its identity still being controversial) with respect to its vasodilator activity and its role as a vasoprotective mediator, particularly in females. In this review, we have highlighted that EDHF is not only a potent vasodilator but is also implicated in several aspects of cardiovascular inflammation, platelet function, and vascular repair, and therefore this factor may represent an endogenous protective mechanism against atherosclerosis that is more active in females than males. Not all putative EDHFs described in this review possess beneficial effects in terms of the atherogenic processes, but it is important to bear in mind that the vast majority of the studies were carried out in male animals and subjects, in which NO and PGI_2 are the predominant endothelial factors and therefore the positive effects of EDHF might have been overlooked. Nonetheless, in terms of anti-atherogenic potential of the proposed EDHF candidates, it appears as if CNP possesses the most appropriate bioactive profile (Fig. 1, Table 1) since it not only reduces vascular smooth muscle tone and proliferation, but also leukocyte and platelet reactivity and promotes endothelial growth. In sum, we suggest that sex hormones regulate several atherogenic events in which EDHF appears to play an important role and that this factor might contribute to the cardioprotective phenotype that women enjoy before menopause.

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