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Novel aspects of endothelium-dependent regulation of vascular tone

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The vascular endothelium plays a crucial role in the regulation of vascular homeostasis and in preventing the initiation and progress of cardiovascular disease by controlling mechanical functions of the underlying vascular smooth muscle. Three vasodilators: nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor, produced by the endothelium, underlie this activity. These substances act in a co-ordinated interactive manner to maintain normal endothelial function and operate as support mechanisms when one pathway malfunctions. In this review, we discuss recent advances in our understanding of how gender influences the interaction of these factors resulting in the vascular protective effects seen in pre-menopausal women. We also discuss how endothelial NO synthase (NOS) can act in both a pro- and anti-inflammatory action and therefore is likely to be pivotal in the initiation and time course of an inflammatory response, particularly with respect to inflammatory cardiovascular disorders. Finally, we review recent evidence demonstrating that it is not solely NOS-derived NO that mediates many of the beneficial effects of the endothelium, in particular, nitrite acts as a store of NO released during pathological episodes associated with NOS inactivity (ischemia/hypoxia). Each of these more recent findings has emphasized new pathways involved in endothelial biology, and following further research and understanding of the significance and mechanisms of these systems, it is likely that new and improved treatments for cardiovascular disease will result.

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The endothelium is as a highly specialized, metabolically active organ lining the luminal side of all blood vessels that plays an integral role in the maintenance of vascular homeostasis, mediated by a number of endothelium-derived factors. The endothelium releases an array of vasoactive mediators that 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. Moreover, it appears that alterations in the capacity of the endothelium to release these mediators may be a major precipitating factor in many cardiovascular disease states.

ENDOTHELIUM-DERIVED VASODILATORS

Of the vasodilator factors that the endothelium can release, prostacyclin (PGI_2),[1] nitric oxide (NO),[2–4] and endothelium-derived hyperpolarizing factor (EDHF)[5–7] are the most significant.

PGI_2 is synthesized by cyclooxygenase (COX) isozymes, of which, essentially, two have been identified. COX-1 is a constitutive enzyme expressed in the vascular endothelium and thought to contribute to the maintenance of vascular homeostasis (a splice variant of COX-1, sometimes referred to as COX-3, has also been reported[8]). COX-2 is an inducible isozyme that is thought to be expressed in the cardiovascular system (and immune cells) only during pathogenic episodes, although there is some evidence to suggest that this isoform is constitutively present in human endothelial cells.[9] PGI_2 elicits smooth muscle relaxation by activating specific cell-surface receptors (IP) that are G-protein-coupled to adenylyl cyclase and thereby elevate cyclic adenosine monphosphate levels.[10]

NO is synthesized from L-arginine by NO synthases (NOS) and causes vasodilatation via activation of soluble guanylyl cyclase generating cyclic guanosine monophosphate.[11] Under physiological conditions, two 'constitutive' forms of NOS play a role in NO production, predominantly endothelial NOS (eNOS) and to a lesser extent neuronal NOS.[12] A third isoform, inducible NOS (iNOS), is expressed in a number of inflammatory cell types and has an essential role in vascular inflammation.[13] The constitutive enzymes produce low-level NO, important for maintaining vascular homeostasis, whereas iNOS activity results in 'high-output' NO

production and this is thought to underlie its activity in inflammation.

The identity of EDHF remains uncertain, although several candidates have been proposed including K^+ ions, cyclic adenosine monphosphate, cytochrome *P*450 2C products, H_2O_2, spread of electrotonic current and, most recently, C-type natriuretic peptide (CNP).[7] However, it is universally accepted that EDHF release/transmission from the endothelial cell occurs following opening of endothelial SK_{Ca} and IK_{Ca}.[14] Moreover, hyperpolarization of vascular smooth muscle associated with EDHF activity, in the main, involves activation of the Na^+/K^+-ATPase and Kir.

There is considerable evidence to support the concept that these substances not only act as vasodilators but they play a multi-faceted role in vascular homeostasis, including inhibition of mitogenesis, platelet aggregation, and the extravasation of leukocytes.[7,11,15–17]

PHYSIOLOGICAL INTERACTION OF ENDOTHELIUM-DERIVED MEDIATORS

In addition to their own distinct profile of activity, there is clear evidence that these three endothelial mediators work co-operatively in a complex but integrated manner to maintain the health of the vasculature. Most notably, it appears that each individual mediator possesses the capacity to interact with components of the synthesis/activation pathways for the other mediators and thereby manipulate their activity. These relationships have been best characterized in terms of maintenance of vascular tone and occur at two levels: first, in the physiological regulation of vessel diameter, and second, as a compensatory mechanism activated when the expression or activity of an alternate mediator is deficient. For instance, endothelial regulation of vascular tone is not uniformly consistent in terms of the contribution of the three main vasodilators, NO, PGI_2, and EDHF. Although NO is the predominant endothelium-derived vasodilator in conduit arteries, as one descends the vascular tree the role of NO diminishes, whereas the influence of EDHF increases.[18] In this way, the absolute vasodilator capacity of the arterial system is maintained. Studies investigating the relationship between NO and EDHF have indicated that basal NO tonically inhibits EDHF responses.[19,20] Indeed, in many blood vessels EDHF responses are only evident once NO production has been inhibited.[19] The exact mechanisms involved in this interaction between NO and EDHF remain unknown.

In a similar manner, the contribution of PGI_2 (and other COX products) to endothelium-dependent relaxation is often evident only after inhibition of NOS,[21] and accordingly, this is thought to be owing to a tonic inhibitory effect of NO. There is evidence to suggest that NO can both enhance and inhibit COX activity and expression. These disparate effects of NO are likely to reflect the specific nitrogen oxide species involved and its concentration.[22,23]

There are several reports demonstrating upregulation of PGI_2 or EDHF in an environment where NO production has been suppressed. For example, in gracilis muscle arterioles of male eNOS knockout (KO) mice, endothelium-dependent dilatation is maintained despite the absence of NO; this compensation was shown to be PGI_2-dependent as the response was markedly attenuated by the COX inhibitor indomethacin.[24] Evidence supports the concept that this provision of PGI_2 originates from the upregulation of COX enzyme expression or activity.[25,26] However, evidence also supports the thesis that EDHF is upregulated in eNOS KO mice.[27,28] Our own studies have demonstrated that, whilst endothelial NO tonically antagonizes myogenic constriction in resistance arteries of wild-type (WT) mice, in eNOS KO animals rather than an exaggerated myogenic reactivity, these responses remain normal as EDHF is upregulated to replace the moderating influence of NO.[27] This upregulation of EDHF activity is thought to be linked to NO-mediated inhibition of EDHF.[19,20] Exactly how this is brought about is uncertain, but studies using NO donors in rabbit carotid and porcine coronary arteries demonstrate an NO-mediated suppression of EDHF-mediated dilatation associated with an interference with the synthesis and/or release of EDHF rather than its mechanism of action.[19,20] Indeed, patch-clamp recordings in vascular smooth muscle cells revealed that NO donors, which did not directly affect resting membrane potential or EDHF-induced hyperpolarization, markedly attenuated EDHF release from a donor segment.[20] Similarly, chronic inhibition of NO synthesis causes a change in the factors mediating endothelium-dependent relaxation; several studies have demonstrated a compensatory increase of PGI_2 production and upregulation of COX expression.[24,29,30] This compensatory upregulation of PGI_2 and/or EDHF following suppression of NO production is also observed in models of cardiovascular disease and in humans with cardiovascular disease.[21,26,29,31]

There have been a number of excellent recent reviews that have described in detail the roles of each of the three vasodilators and their interactions,[32–34] and the impact of cardiovascular disease on their expression and function,[35,36] and therefore these areas will not be dealt with in this review. Here, we focus on some areas of endothelial biology that have come to the fore recently, in particular, the impact of gender on endothelial responses, the role of the endothelium in determining inflammatory iNOS expression, and evidence demonstrating alternative endothelium-dependent sources of endogenous NO.

GENDER DIFFERENCES IN ENDOTHELIUM-DEPENDENT RESPONSES

Several lines of evidence support the existence of gender differences in endothelium-dependent vasodilator responses in animals[37–39] and humans.[40,41] In sum, the data support the concept that endothelium-dependent vasodilatation is enhanced in pre-menopausal females. Functionally, this difference in endothelial reactivity[42,43] is thought to contribute to the apparent protection of pre-menopausal women against

cardiovascular disease.[44] Many inflammatory cardiovascular diseases are associated with a prevalence of 'endothelial dysfunction', a phenomenon often attributed to the loss or suppression of NO biological activity.[35,45] Indeed, in many cases, endothelial dysfunction precedes the onset of disease and therefore this phenomenon may play an important role in pathogenesis.[46–48]

ESTROGENS AND VASCULAR FUNCTION

The mechanisms involved in enhanced endothelial function in females seem (intuitively) to be linked to female sex hormones. For example, flow-mediated dilatation (FMD) of the brachial artery is increased in females compared to males, a difference temporally associated with the increase in serum estradiol concentration that occurs during the menstrual cycle. Indeed, the difference in FMD disappeared during the M phase when serum levels of both estrogen and progesterone were similar to those in male subjects.[49] Similarly, vasodilator responses to bradykinin are increased mid-cycle in healthy women, when estrogen levels are at their highest.[50] Further, young women with premature ovarian failure (or premature menopause) have impaired endothelial function that has been directly linked to sex steroid deficiency, as treatment of these individuals for 6 months with estradiol restored endothelial function.[51] Ovariectomy also creates an estrogen deficient state and in such patients endothelium-dependent vasodilatation in the forearm is also attenuated.[52] Together, these findings provide strong support for the thesis that endogenous sex hormones, particularly estrogen, play an important role in maintaining endothelial function in healthy females.

There is an immense body of evidence demonstrating the beneficial effects of estrogen on endothelial and vascular function. Studies of post-menopausal women and ovariectomized (OVX) animals[52–58] have demonstrated that estrogen administration improves endothelium-dependent vasodilatation induced by vasodilators or flow.[49–61] Similarly, the endothelial dysfunction in women who suffer menopause in the earlier stages of life, owing to surgical intervention, is considerably improved following estrogen treatment;[62] this intimates that estrogen treatment in post-menopausal women does not simply reverse age-related endothelial dysfunction[63] but rather that it reverses dysfunction owing to a loss of female sex hormones. In the vasculature, estrogen has been demonstrated to act in both a genomic and non-genomic manner to influence endothelial function (for review see [42,64,65]). Relatively high micromolar concentrations of estrogen (i.e., 100–1000 greater than physiological levels) have direct acute vasodilator effects[66] and this action has been ascribed, in part, to endothelium-dependent vascular relaxation.[54,67] Alternatively, chronic exposure of vascular cells and tissues to physiological concentrations of estrogens alters the expression of a number of proteins involved in the synthesis and activity of NO, PGI_2, and EDHF.[65] Thus, the vasodilator effects of estrogens are believed to play a major role in exerting a hypotensive and anti-inflammatory phenotype in pre-menopausal women that suppresses the development of atherosclerosis.

HORMONE REPLACEMENT THERAPY AND VASCULAR FUNCTION

Despite the considerable body of evidence supporting a cardioprotective role of estrogens, some studies have reported that estrogen-induced improvement in FMD only occurs in younger post-menopausal women[68,69] and that estrogen treatment does not augment endothelial function.[70] As a result, if and how any effect of estrogens on vascular reactivity might translate to cardioprotection is a highly controversial issue. This debate was fuelled by the failure of hormone replacement therapy (HRT) in large-scale clinical trials to confer protection from cardiovascular diseases in post-menopausal women.[71,72] It is thought that the disappointing, and clearly unexpected, results of these trials may have been owing to the timing of HRT administration with respect to the onset of menopause;[43] treatment should be initiated at the start of menopause before the onset of significant changes in cardiovascular function. This is an issue that is being investigated by the recently set up Kronos early estrogen prevention study.

Studies exploring the direct effects of HRT on the vascular endothelium have also contributed to the controversy. Several investigations show an improvement in endothelial function in post-menopausal women on HRT.[73–75] However, HRT is commonly composed of both estrogens and progestins (the latter to reduce risk of endometrial cancer induced by long-term estrogen use[76,77]) and there is evidence that progestins also alter endothelial function. For instance, progesterone attenuates the favorable effects of estradiol on endothelium-dependent dilatation in coronary arteries of OVX dogs[78] and in healthy post-menopausal women.[79–81] In contrast, progesterone has also been shown to cause vasodilatation in coronary and mesenteric resistance arteries.[82–85]

TESTOSTERONE AND VASCULAR FUNCTION

An alternative perspective to gender-dependent protection is that an increase in male sex hormones, principally testosterone, is detrimental to vascular function. For example, young women with polycystic ovary syndrome have associated high levels of androgens and impaired brachial artery FMD responses.[86] In addition, hypogonadal men display exaggerated FMD responses that are reduced in matched controls following treatment with testosterone.[87] In contrast, femoral arteries from male testicular feminized mice, which have reduced levels of circulating testosterone, exhibited reduced endothelial-dependent vasodilatation to acetylcholine (ACh),[88] and testosterone implant therapy improves, rather than depresses, brachial artery FMD in post-menopausal women using HRT.[89] Moreover, trials of testosterone in heart failure demonstrate an improvement of functional capacity in men.[90] The beneficial effects of testosterone in the latter studies may in part be owing to local aromatization of testosterone to estradiol within the vascular wall. This

possibility is supported by the observation by Lew *et al.*[91] that suppression of endogenous estrogen production with an aromatase inhibitor impairs FMD in young healthy men, intimating that endogenous estrogens may play a direct regulatory role in endothelial function in males. In an analogous manner to estrogen and progesterone, testosterone can induce vasodilatation through endothelium-dependent mechanisms in arteries, including male and female canine coronary conductance and resistance arteries,[92] rat aorta, and mesenteric arteries.[93–95]

GENDER-RELATED DIFFERENCES IN ENDOTHELIAL MEDIATORS: NO

Hormonal modulation of endothelial and vascular function may be via interaction with the NO pathway. Many studies have shown that NO plays a major role in the beneficial effect of sex hormones on endothelium-dependent vasodilatation (for reviews see [65,66]). Several animal studies have also demonstrated clear increases in basal NO levels in the presence of estrogens.[39,96,97] Moreover, ovariectomy reduces basal NO to levels equivalent in males,[98–101] an effect that is reversed by treatment of OVX animals with estradiol (for review see [66]). Additionally, 17-beta-estradiol (17β-estradiol) enhances the sensitivity to endothelium-dependent vasodilators in various blood vessels in both males and OVX females, an effect that often is attributed to elevated endothelial NO production.[102,103] However, some studies report no change, or even a decrease, in NO bioactivity following ovariectomy and chronic treatment with 17β-estradiol.[99,100,104–106] The explanation for these conflicting results is difficult to pinpoint but may reflect heterogeneity with respect to the role of endogenous NO in endothelium-dependent relaxation throughout the vascular tree. Interestingly, perusal of the literature demonstrates that basal NO production does appear to be elevated in both conduit and resistance arteries in response to estrogens; in general, however, instances in which augmented NO-mediated responses are thought to underlie enhanced endothelium-dependent dilatation occur in larger conduit arteries,[103,107–109] whereas in resistance arteries, changes in NO bioactivity do not appear to play a role in the altered vascular responsiveness.[104,110,111]

Clinical investigations are in agreement with the many animal studies and demonstrate enhanced circulating levels of NO in pre-menopausal females compared to males,[112,113] and in post-menopausal women on estrogen replacement therapy.[114] Additionally, treatment of human endothelial cells in culture with 17β-estradiol increases both basal and stimulated NO release.[115] Taken together these studies suggest that estrogens elevate endothelial NO production and that this may underlie the elevated levels of circulating NO prevalent in females.

Both genomic and non-genomic mechanisms have been proposed to account for estrogen-induced augmentation of NO production. In terms of the genomic effects, activation of specific estrogen receptors (membrane bound and/or cytosolic) causes nuclear translocation (of the hormone-receptor complex) and activation of estrogen response elements in the eNOS promoter, increasing enzyme expression and NO synthesis. This phenomenon has been demonstrated in cultured endothelial cells and arterial preparations.[43,44,66,116,117] The effects of estrogen on the NO pathway may also be related to its antioxidant effects.[118,119] In human umbilical vein endothelial cells, 17β-estradiol inhibits nicotinamide adenine dinucleotide phosphate hydrogenase oxidase expression and the generation of reactive oxygen species and peroxynitrite.[120] Furthermore, estrogen decreases the generation of superoxide anions from cultured bovine aortic endothelial cells and thereby enhance NO bioactivity.[121]

The non-genomic effects of estrogens are mediated via an upregulation of eNOS activity rather than expression. In this case, interaction of estrogen with estrogen receptors α and β (both found in human endothelial cells; see for review[65,122,123]), but primarily the former, results in 'fast' eNOS activation.[124–127] This is thought to occur via several pathways.[128] The principal mechanism involves phosphorylation of eNOS by protein kinase B/Akt, analogous to shear-stress-induced increases in eNOS activity.[129] However, there is also evidence suggesting that alterations in the expression of accessory proteins such as calmodulin, heat shock protein-90, and caveolin-1 also mediate the estrogenic effects on eNOS activity.[71,103,130] However, this issue is controversial as some studies report opposing effects of estrogens on accessory protein expression directly contradicting the latter hypothesis.[131–134]

The detrimental effect of progesterone on endothelium-dependent relaxation appear to be related to decreased eNOS levels, and increased consumption of NO by superoxide anions.[135] The endothelium-dependent effects of testosterone are likely to be mediated at least, in part, through NO production. Studies in rat aorta and mesenteric arterial bed and coronary arteries from rabbit and dogs have demonstrated that testosterone causes acute endothelium-dependent vasorelaxation that is, in part, mediated by the activation of eNOS.[92,93,95,136] However, Li and Duckles[137] showed that an NOS inhibitor had no effects on vasorelaxation to testosterone in rabbit coronary artery and aorta.

GENDER-RELATED DIFFERENCES IN ENDOTHELIAL MEDIATORS: PGI_2

The contribution of PGI_2 to endothelium-dependent relaxation is also thought to be modulated by hormonal status. A number of publications have documented enhanced PGI_2 production following estrogen treatment of endothelial cells and blood vessels of several species, including humans.[138–140] Often this elevation in PGI_2 is associated with an elevation in COX expression,[141,142] but more specifically it appears this enhancement is the result of an upregulation of dilator prostaglandins and downregulation of constrictor prostaglandins.[139,143,144] For instance, estrogen treatment enhances prostanoid-mediated vasodilatation in middle cerebral arteries isolated from OVX female rats, whereas in the absence

of estrogen arachidonic acid was actively converted to a COX-1-dependent constrictor.[139] In ovine arteries, it has been suggested that this switch is related to an estrogen-specific elevation of PGI_2 synthase expression.[142] Estrogen also suppresses production of a COX-sensitive vasoconstrictor[143] and together these effects would shift the balance in favor of vasodilator prostaglandin production. In contrast, some studies show no role for COX and prostaglandins on the estrogen-induced enhancement of endothelium-dependent relaxation.[145–147]

Upregulation of prostaglandin synthesis in response to an absence of NO bioactivity has been well-documented.[27,30] This cross-talk between the NOS and COX pathways is also thought to be modulated by estrogens. In mouse cerebral arteries, from animals treated chronically with an NOS inhibitor or with transgenic disruption of the eNOS gene, estrogen treatment *in vivo* increases levels of vascular COX-1 expression and PGI_2 production.[148] Moreover, in OVX animals loss of estrogen is associated with a decrease in the NO component of endothelium-dependent vasodilatation and an upregulation of the COX component,[148] whereas estrogen treatment has been shown to produce an apparent upregulation of the NO component of the endothelium-dependent relaxation and a concomitant depression of the COX-product component.[149]

The influence of other sex hormones on PGI_2 bioactivity remains unclear. For example, progesterone exerts a direct relaxant effect on rat aorta that involves COX activation and increased PGI_2 production,[150] and both progesterone and medroxyprogesterone acetate increase cultured human umbilical vein endothelial cell PGI_2 production in a receptor-dependent manner by enhancing COX-1 and COX-2 expression and activities.[151] Yet, the concomitant administration of progesterone with estrogen prevents the stimulatory effects of estrogen on PGI_2 production in cultured human umbilical vein endothelial cells.[152] Akin to progesterone, testosterone also causes acute vasorelaxation that is dependent on endothelium-derived prostanoids.[94] However, testosterone treatment decreases PGI_2 production in cultured aortic vascular smooth muscle cells[153] and in the aorta of female rats.[154]

GENDER-RELATED DIFFERENCES IN ENDOTHELIAL MEDIATORS: EDHF

A growing body of evidence supports the thesis that EDHF is upregulated in females compared to males.[155] However, as the chemical identity of EDHF still remains elusive (see [7]), selective antagonists of EDHF are lacking and therefore the true (patho)physiological role(s) of EDHF remains equivocal. As a consequence, EDHF activity is regularly defined as the response that persists in the presence of combined inhibition of NO and PGI_2 synthesis (i.e., by blocking NOS and COX, respectively). This definition, however, has proved problematic as inhibitors of NOS and COX are not isoform-selective, do not result in complete inhibition, and often have complicating inherent hemodynamic effects *in vivo*. Moreover, NO and PGI_2 can also elicit dilatation via hyperpolarization of vascular smooth muscle resulting in difficulties in delineating the actions of these endothelium-derived vasodilators.[156,157] To circumvent these difficulties, we developed an eNOS/COX-1 double-knockout (dKO) mouse or 'EDHF mouse'.[104] In these animals, we observed that whilst males were markedly hypertensive, the female animals were not. Indeed, this gender effect was duplicated in mice deficient in either eNOS or COX-1, indicating that irrespective of genotype females are protected against the hypertensive consequences of deletion of these genes. Moreover, we reported that administration of bradykinin in female dKOs caused potent dose-dependent depressor responses whilst this endothelium-dependent vasodilator had no effect in the male dKO animals (Figure 1). These findings provided convincing evidence that the cardioprotective phenotype of females (at least in terms of blood pressure) are mediated via upregulation of EDHF rather than NO and/or PGI_2 bioactivity, as has been proposed previously. Indeed, examination of resistance arteries from these animals clearly demonstrated that whilst NO and PGI_2 are the predominant endothelium-dependent vasodilators in isolated arteries from males, in female blood vessels this response is due principally to EDHF.[104]

Augmentation of the EDHF component of the dilator response in females to endothelium-dependent stimuli, including shear stress, ACh, and bradykinin, has also been

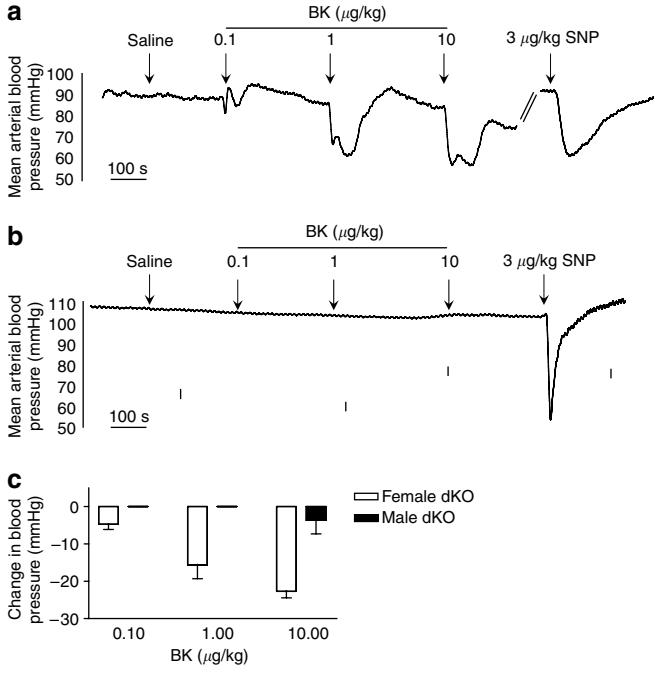

Figure 1 | Bradykinin (BK) reduces blood pressure in female but not male eNOS/COX-1 dKO mice *in vivo*. (**a**) Original trace depicting typical responses to bolus injections of BK and sodium nitroprusside (SNP) in (**a**) female and (**b**) male dKO mice. (**c**) Mean dose-dependent decrease in blood pressure to BK in female ($n = 4$) and male dKO mice ($n = 3$). Male significantly different ($P < 0.01$) from female (reproduced with permission by Scotland *et al.*[104])

demonstrated in a range of different arteries from various species.[40,104,158,159] This gender effect is thought to be owing to the activity of estrogen. Indeed, EDHF responses are reduced during diestrus, a period of comparatively low estrogen levels,[99] and elevated during pregnancy when estrogen levels are very high.[160] In addition, EDHF responses evoked by ACh,[71,99] shear stress,[161] ADP,[162] and Ca^{2+} ionophore[163] are all reduced by ovariectomy, an effect that is reversed by treatment with 17β-estradiol.[99,100,162,163] As EDHF plays a greater role in endothelium-dependent relaxation of resistance arteries than conduit arteries,[19] our findings in the dKO mice intimate that estrogen-induced regulation of EDHF may be critical in determining peripheral resistance in females and hence blood pressure.

Some studies have reported an inhibitory effect of estrogen on EDHF, although this appears to occur mainly within cerebral arteries. The EDHF response in isolated rat middle cerebral arteries is reduced in female rats and estrogen-treated OVX females as compared with male rats and vehicle-treated OVX females.[164] *In vivo* studies of pial arterioles in female rats, OVX rats, and OVX rats with oestrogen replacement came to the same conclusion that estrogen downregulates EDHF.[165]

The mechanism underlying estrogen-mediated upregulation of EDHF bioactivity merits further attention. Recent findings suggest that treatment of OVX rats with 17β-estradiol increases the expression of specific connexin proteins (connexins 40 and 43) that constitute myo-endothelial gap junctions[71,163] and have been suggested to play a key role in EDHF-mediated smooth muscle hyperpolarization.[166] In addition, estrogen enhances transcription of CNP in mouse uterus[167] and relaxations to CNP in female porcine coronary artery are greater than in male arteries.[168] As our work suggests that CNP acts as an EDHF in the mesenteric and coronary circulation,[169,170] it is possible that increased CNP release/activity may account for enhanced EDHF activity in females. Furthermore, EDHF responses are associated with opening of K_{Ca} channels and treatment of dogs with estrogen improves coronary vasodilatation mediated by activation of such channels.[171]

INTERACTION BETWEEN ENDOTHELIAL AND INDUCIBLE NOS IN THE REGULATION OF VASCULAR REACTIVITY

NO production from iNOS is the principal mediator of the microbicidal and tumoricidal actions of macrophages.[172] In fact, iNOS is expressed by many cell types in response to inflammatory cytokines (e.g., IL1-β, interleukin-2, interferon gamma, and tumor necrosis factor-α) and bacterial metabolites (e.g., lipopolysaccharide (LPS))[173] and the NO produced is cytotoxic and cytostatic to a number of pathogens and tumor cells.[174] However, inappropriate production of NO is also thought to lead to host damage; for instance, 'high-output' NO production from iNOS contributes to the pathophysiological changes observed in sepsis.[175] Thus, a tight regulation of the expression and activity of iNOS is vital. Recently, it has become apparent that eNOS-derived NO plays a key role in the regulation of iNOS expression, thereby conferring a novel, additional role for the endothelium in an inflammatory response and extending our understanding of the role of NO in particular.

FEEDBACK REGULATION OF NOS BY NO

Since the early 1990s it has been widely accepted that NO exerts an autoregulatory feedback on its synthetic enzymes. Both authentic NO and NO donors inhibit the activity of all three NOS isozymes.[176,177] Moreover, in endothelial cells, prior exposure to supramaximal concentrations of NO strongly suppressed NO biosynthesis in response to subsequent physiological stimuli.[177] As a consequence of these findings, it was hypothesized that high levels of NO, produced endogenously by iNOS, would inhibit eNOS activity. Such a mechanism might underlie the endothelial dysfunction associated with inflammatory cardiovascular disorders.

Experimentally, most of the evidence linking iNOS-derived NO production with eNOS dysfunction has been conducted using animal models of sepsis, induced primarily by the administration of the gram-negative bacterial cell wall component LPS or endotoxin.[175] In several species exposed to endotoxin, endothelial dysfunction occurs that is characterized by loss of NO-dependent responses[178,179]. Moreover, this phenomenon is associated with vascular iNOS expression.[180,181] The link between iNOS-derived NO and endothelial dysfunction was confirmed by studies in our laboratory using iNOS KO animals.[182] In WT animals, we demonstrated that LPS caused vascular iNOS expression associated with a 10-fold increase in circulating NO levels. This was accompanied by reduced eNOS expression and inhibition of endothelial function (demonstrated by suppression in the endothelium-dependent relaxation to ACh; see Figure 2). These effects of LPS were absent in iNOS KO animals proving unequivocally a role for iNOS in mediating endotoxemia-induced endothelial dysfunction.[183] In these studies, we concluded that iNOS-derived NO inhibits eNOS activity not only by biochemical means (e.g., negative feedback regulation at the heme active site as described above) but also genetic effects, as eNOS expression was suppressed in WT, but not iNOS KO, animals exposed to LPS. This work is supported by similar findings using iNOS inhibitors where LPS-induced suppression of endothelial responses evoked by eNOS activation is blocked.[183–185]

ENDOTHELIAL NOS-DERIVED NO AND REGULATION OF iNOS

In addition to the autoinhibitory actions of NO, recent evidence suggests that NO plays a dual role in the regulation of iNOS expression such that under certain conditions NO can act in a proinflammatory manner. This biphasic effect of NO has been shown to be mediated by changes in the transcription factor nuclear factor-kappa B (NFκB).[186] Inflammatory stimuli, including infectious organisms and cytokines, result in an elevation of free cytoplasmic NF-κB, which then translocates to the nucleus where it binds to the

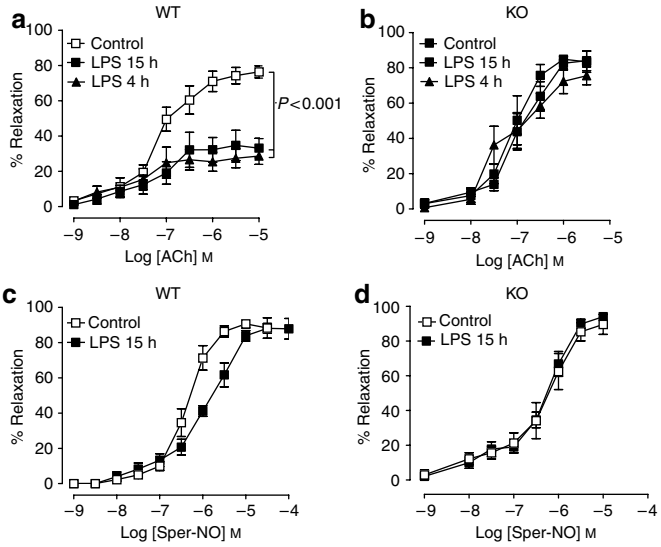

Figure 2 | iNOS underlies sepsis-induced endothelial dysfunction. Effect of LPS (12.5 mg/kg intravenously, 4 or 15 h pre-treatment) on relaxation responses of mesenteric resistance arteries. Concentration response curves to ACh in (**a**) WT and (**b**) iNOS KO ($n = 5$–14) and SPER-NO in (**c**) WT and (**d**) KO ($n = 7$–11) mesenteric resistance arteries. Values shown are mean ± SEM. Statistical analysis using two-way analysis of variance.

promoter regions of various inflammatory genes including iNOS.[187,188] We have shown that increasing concentrations of exogenous NO exert differential effects on LPS-induced iNOS expression in macrophages and that this is associated with changes in NF-κB activity. More specifically, at low concentrations of NO, NF-κB levels are raised and conversely, and at high NO concentrations, NF-κB levels and DNA-binding activity are suppressed.[186,189] Subsequently, we have demonstrated that the stimulatory effect of 'low' NO levels on macrophage iNOS expression is not only brought about by exogenously provided NO but also by endogenous eNOS-derived NO in a cyclic guanosine monophosphate-dependent process.[190,191] For example, stimulation of macrophages derived from the bone marrow of eNOS KO mice with LPS produces a suppressed (~50%) and delayed iNOS expression in comparison to cells from WT animals.[190] Moreover, our studies have shown that this effect of eNOS-derived NO to promote iNOS expression also extends to the vasculature *in vitro* and *in vivo* such that maximal expression and activity of LPS-induced iNOS is dependent on eNOS-derived NO. Consequently, we hypothesized that LPS causes an acute increase in eNOS activity (via an effect on heat shock protein-90 and protein kinase B/Akt) to generate levels of NO commensurate with its augmentation of NF-κB activity that then stimulates iNOS protein synthesis.[192,193]

Taken together, these data highlight a key, biphasic regulatory role for NO that governs iNOS expression. In the initial stages of an inflammatory response, eNOS-derived NO augments NF-κB activity and facilitates iNOS expression to expedite host defence. Following iNOS expression and 'high-output' NO production (e.g., to combat infection), the elevated levels of NO exert a negative effect on NF-κB activity and turn off subsequent iNOS transcription and inhibit eNOS expression and activity. Thus, it appears that although eNOS acts predominantly as an anti-inflammatory protein, producing an array of effects including endothelium-dependent vasodilatation, and inhibition of leukocyte and platelet activation, under the appropriate circumstances it fulfils an integral, proinflammatory role in facilitating iNOS expression (and potentially other proinflammatory proteins with NF-κB binding sites in their promoter region including COX-2, interleukin-6).

ALTERNATE SOURCES OF ENDOGENOUS NO

In contrast to endothelium-derived NO, which has well-established and important effects in the regulation of vascular tone, its oxidative metabolite nitrite was until recently considered to be physiologically inert. The only interest in the physiological generation of nitrite was as a stable metabolite and index of NO production (in combination with nitrate). However, evidence is now emerging that nitrite serves as a storage form of NO, which is released preferentially under acidic and/or hypoxic conditions and may thereby provide a vital reservoir of NO that supplements NOS activity during pathological episodes.

It had previously been suggested that NO may be transported in the blood in the form of a more stable S-nitrosothiol, by interacting with free cysteine residues in, for example, albumin[194,195] or hemoglobin.[196–200] Although early studies, supporting this thesis, suggested high circulating levels (μM),[194,201] subsequent reports have measured considerably lower levels. Indeed, using an electron paramagnetic resonance spectrometry assay for S-nitrosothiols that we recently developed, we found that plasma S-nitrosothiols were below the limit of detection of 25 nM in 9 of 12 healthy volunteers.[202] Such low levels of S-nitrosothiols question their potential physiological relevance.[203–206] In contrast, the levels of plasma nitrite we measured were much higher ~420 nM,[202] in line with other studies that indicate a range of 0.1–0.5 μM[207–211] (the majority (>60%) is carried by erythrocytes; Dejam *et al.*[212]), although tissue concentrations of nitrite are even greater than plasma, with levels in the rat heart of 5–40 μM and aorta in the low μM range.[213,214]

The main source of nitrite has been considered to be from the oxidation of NO derived from NOS, with one study showing that 70% or more of plasma nitrite was derived from eNOS.[210] However, there is also a non-endothelium-dependent source of nitrite derived from the diet, either directly from dietary nitrite (used to preserve meat and protect against botulism) or indirectly from the enterosalivary circulation of dietary nitrate (principally from green leafy vegetables). In the latter, up to 25% of ingested nitrate is concentrated in the salivary glands, secreted in the saliva and then reduced by bacterial nitrate reductases on the surface of the tongue producing nitrite, which is then swallowed.[207,215–217]

Although high concentrations of nitrite have long been known to cause vasodilatation, even until quite recently, it was thought that nitrite at near-physiological concentrations lacked vasodilatory activity.[211] Evidence for a vasodilatory action of nitrite *per se* dates back to 1953 when Furchgott relaxed rabbit aortic strips with high concentrations of acidified nitrite;[218] however, the mechanism of dilatation was unknown. More recently, Modin *et al.*,[219] demonstrated that physiological concentrations of nitrite relaxed rat aortic rings at acidic pH 6.6, a pH reminiscent of ischemia, via the generation of NO (through intermediates including nitrous acid (HNO_2)) and the consequent activation of soluble guanylyl cyclase. However, other studies have demonstrated vasodilatation under non-ischemic conditions, where acidosis is unlikely to account for NO production. For example, the ingestion of large amounts of oral nitrite reduces blood pressure *in vivo* in spontaneously hypertensive rats.[220–222] The mechanism likely to account for the majority of the vasodilator effects of nitrite under normal physiological conditions is via the reduction of nitrite to NO by deoxyhemoglobin. The oxidation of human deoxyhemoglobin by nitrites producing methemoglobin and NO was first described by Doyle *et al.*[223] in 1981. However it was not until 2003 that the physiological relevance was demonstrated by Gladwins group who demonstrated vasodilator activity of near-physiological concentrations of intra-arterial nitrite into the human forearm[224] associated with elevations of iron-nitrosylated hemoglobin, the product of the reaction between NO and deoxyhemoglobin. This study followed Gladwin's previous work demonstrating circulating arterial–venous plasma nitrite gradients in the human forearm, which increased during hand-grip exercise, suggesting that nitrite was providing a source of NO.[204] Tsuchiya *et al.*,[225,226] subsequently, showed that the ingestion of nitrite also produced nitrosylhemoglobin, and moreover the co-administration of nitrite with the NOS inhibitor, N^G-nitro-L-arginine methyl ester, over 3 weeks attenuated the development of hypertension.

An additional 'nitrite reductase' that may contribute to nitrite-induced vasodilatation is xanthine oxidoreductase (XOR). In addition to the hypoxemia and increased deoxyhemoglobin that develops during ischemia, the acidosis that develops creates a reducing environment that may favor the chemical reduction of nitrite to NO, as described above. Indeed, this was demonstrated by Zweier *et al.*,[214] in 1995 in the isolated perfused Langendorff heart where NO is produced from nitrite once the pH drops below 6.0 following 10 min global ischemia. Such a source of NO may be particularly important, as the normal production of NO from NOS and L-arginine is dependent on oxygen, which is rapidly depleted in ischemia. At the time, Zweier considered it likely that the degree of acidosis *per se* was sufficient to account for the nitrite reduction, without the need for enzymatic activity. We have more recently demonstrated that this production of NO from nitrite is dependent on endothelial XOR in both rat and human myocardium (and this in the absence of any exogenously applied superoxide dismutase).[227] XOR, a complex molybdoflavoprotein, was a likely candidate as it possesses structural similarity to bacterial and plant nitrate/nitrite reductases and is an endothelial enzyme that had previously been shown in its isolated purified form to reduce nitrite to NO, a process that is enhanced in acidic conditions and under low oxygen tensions.[228,229] Moreover, the activity of XOR is upregulated in atherosclerosis (which predisposes to myocardial ischemia/infarction): in patients with coronary artery disease, endothelium-bound XOR activity was found to be increased by $>200\%$.[230] We found that the application of both low and high concentrations of nitrite (10 and 100 μM), far from being inactive or damaging, had marked functional effects in the Langendorff model of ischemia–reperfusion: nitrite was highly protective in terms of improving the recovery of left ventricular function, reducing coronary tone on reperfusion and reducing infarct size.[227] These effects were blocked by the NO scavenger carboxy-PTIO, suggesting that the protective effect of nitrite was through its conversion to NO. This therefore suggests the involvement of XOR in a protective process. XOR is usually associated with causing damage through its production of oxygen-free radicals, including superoxide anion.[231] However, as our data suggest, if XOR is presented with nitrite as an alternative substrate, the resultant effects of its activity may be protective, via its production of NO rather than damaging. Also nitrite will compete with oxygen for electrons across XOR, which may inhibit the reduction of oxygen to potentially damaging superoxide. The cytoprotective effects of similar, and even lower, concentrations of nitrite have subsequently been demonstrated in ischemia–reperfusion experiments in the mouse heart and liver *in vivo*.[232] Nitrite reduction in these studies was associated with the formation of iron-nitrosylated hemoglobin, suggesting the same mechanism involving deoxyhemoglobin previously demonstrated for vasodilatation. These studies therefore support a role for nitrite as an important store of protective NO during ischemia–reperfusion. The cytoprotective effects of nitrite do not appear to be limited to the heart and the liver: nebulized sodium nitrite was shown by Hunter *et al.*,[233] to produce a marked reduction in hypoxia-induced pulmonary hypertension in newborn lambs by causing a selective pulmonary vasodilatation. Also, the intravenous infusion of sodium nitrite over 2 weeks caused complete inhibition of cerebral vasospasm in a model of a ruptured intracranial aneurysm in primates.[234]

Other enzymes besides XOR may act as nitrite reductases: these include the mitochondrial enzymes cytochrome *c* oxidase[235] and the bc_1 complex,[236] with the latter demonstrating significant nitrite-reductase activity. Also, it would follow that if deoxyhemoglobin has nitrite-reductase activity, deoxymyoglobin might be expected to possess similar activity, and indeed myoglobin has been shown to have nitrite-reductase activity in its deoxy form.[235,237]

Although most of the evidence to date for the physiological effects of nitrite appears to relate to it acting as a store

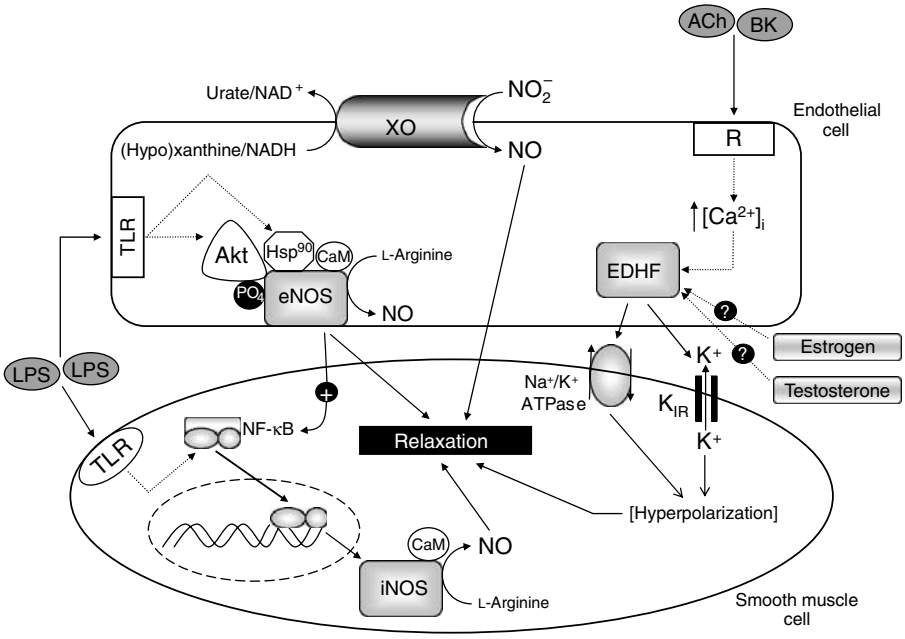

Figure 3 | Endothelial regulation of vascular smooth muscle tone. Schematic depicting novel mechanisms of endothelial regulation highlighted in this review, that is, endothelial cell pathways invoked by LPS, nitrite (NO_2^-), and sex hormones resulting in vasodilatation. LPS binds to toll-like receptors (TLR) resulting in eNOS activation that consequently stimulates smooth muscle iNOS expression and function, generating high levels of vasodilator NO. Nitrite is reduced by xanthine oxidase (XO), utilizing either xanthine or nicotinamide adenine dinucleotide hydreogenase as substrate, to generate NO that results in vasodilatation. Sex hormones interefere with the EDHF pathway resulting in an upregulation of vasodilatation in females in comparison to males.

of NO, there is very recent evidence that nitrite also has direct effects. Bryan *et al.*[213] found that nitrite increases cyclic guanosine monophosphate levels and heat shock protein-70 expression, and decreases cytochrome *P*450 activity and heme oxygenase-1 expression in a variety of tissues, without conversion to NO, suggesting that it is a signalling molecule in its own right, such a role remains to be substantiated.

In sum, nitrite appears to represent the most important storage form of NO, with conversion by nitrite reductases such as hemoglobin, myoglobin, and XOR resulting in vasodilatation and cytoprotection. These findings highlight the importance of the normal physiological actions of nitrite, but also provide scope for the development of novel pharmacological and therapeutic uses of nitrite in disease.

CONCLUSION

The endothelium plays a pivotal role in maintaining cardiovascular homeostasis preventing the initiation and progress of vascular disease. Endothelium-derived vasodilators including NO, PGI_2, and EDHF play a key role in these processes. Recent reports have further highlighted the importance of these mediators and mechanisms, which coordinate their bioactivity to optimize the cytoprotective effects of the endothelium. This work has also provided further insight into the gender difference in cardiovascular disease, with EDHF appearing to play a prominent role in conferring a cytoprotective phenotype in females. Moreover, it is becoming clear that eNOS can act in both a pro- and anti-inflammatory action and therefore is likely to be pivotal in the initiation and time course of an inflammatory response, particularly with respect to inflammatory cardiovascular disorders. However, it is not solely NOS-derived NO that mediates many of the beneficial effects of the endothelium; in recent years, the physiological significance of nitrite as a store of NO has come to the fore and this appears to be heightened in pathological episodes associated with NOS inactivity (e.g., ischemia/hypoxia). Each of these more recent findings has emphasized new pathways involved in endothelial biology (Figure 3), and following further research and understanding of the significance and mechanisms of these systems, it is likely that new and improved treatments for cardiovascular disease will result.

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