

BRIEF REVIEW

FACTORS, FICTION AND ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR

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SUMMARY

1. The principal mediators of vascular tone are neural, endothelial and physical stimuli that result in the initiation of dilator and constrictor responses to facilitate the control of blood pressure. Two primary vasodilatory stimuli produced by the endothelium are nitric oxide (NO) and prostaglandins. An additional endothelium-dependent vasodilatory mechanism is characterized as the hyperpolarization-mediated relaxation that remains after the inhibition of the synthesis of NO and prostaglandins. This mechanism is due to the action of a so-called endothelium-derived hyperpolarizing factor (EDHF) and is dependent on either the release of diffusible factor(s) and/or to a direct contact-mediated mechanism.

2. Most evidence supports the concept that 'EDHF' activity is dependent on contact-mediated mechanisms. This involves the transfer of an endothelium-derived electrical current, as an endothelium-derived hyperpolarization (EDH), through direct heterocellular coupling of endothelial cells and smooth muscle cells via myoendothelial gap junctions (MEGJ). However, there is a lack of consensus with regard to the nature and mechanism of action of EDHF/EDH (EDH(F)), which has been shown to vary within and between vascular beds, as well as among species, strains, sex and during development, ageing and disease.

3. In addition to actual heterogeneity in EDH(F), further heterogeneity has resulted from the less-than-optimal design, analysis and interpretation of data in some key papers in the EDHF literature; with such views being perpetuated in the subsequent literature.

4. The focus of the present brief review is to examine what factors are proposed as EDH(F) and highlight the correlative structural and functional studies from our laboratory that

demonstrate an integral role for MEGJ in the conduction of EDH, which account for the heterogeneity in EDH(F), while incorporating the reported diffusible mechanisms in the regulation of this activity. Furthermore, in addition to the reported heterogeneity in the nature and mechanism of action of EDH(F), the contribution of experimental design and technique to this heterogeneity will be examined.

Key words: artery, connexin, endothelium, experimental design, gap junction, smooth muscle, vascular tone, vasoconstrictor, vasodilator.

WHAT IS EDH(F)?

The aim of the present brief review is to provide a critical overview of the endothelium-derived hyperpolarizing factor/endothelium-derived hyperpolarization (EDH(F)) field, with a focus on the role of gap junctions in the EDH(F) phenomenon. More extensive reviews on endothelium-derived hyperpolarizing factor (EDHF) are provided by McGuire *et al.*,¹ Campbell and Gauthier,² Ding and Triggle³ and Griffith.⁴

Briefly, the arterial endothelium produces three vasodilatory factors: nitric oxide (NO), prostaglandins and EDH(F). Classically, EDH(F) is the hyperpolarization and associated relaxation remaining after the inhibition of the synthesis of NO synthase (and, thus, NO) and prostaglandins. The two primary mechanisms that can account for EDH(F) activity rely on either diffusible- and/or contact-mediated mechanisms. Those that are dependent on the release of a diffusible substance, for which there is yet to be unequivocal evidence, are due to EDHF. Those that are dependent on the direct contact of endothelial cells (EC) and smooth muscle cells (SMC) via myoendothelial gap junctions (MEGJ) are due to the transfer of an electrical current, as an endothelium-derived hyperpolarization (EDH).^{4–9} In both cases, the net result is the hyperpolarization of the adjacent smooth muscle with subsequent vessel dilation. For clarity, the term EDH(F) will be used here to refer to both a diffusible- or contact-mediated mechanism.

Regardless of whether a diffusible- or contact-mediated mechanism is involved in EDH(F) activity, it is accepted that its action is dependent on the release of intracellular calcium and the activation of a specific pattern of potassium channels. The activation of receptors and/or application of physical stimuli, such as shear stress, results in a rise in intracellular EC calcium.^{1,4,10}

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Subsequently, this results in the activation of small (S) and intermediate (I) conductance calcium-activated potassium channels (K_{Ca}) located on EC and, in some cases, the activation of EC or SMC large (B) K_{Ca} .¹ This channel activation results in the generation of an EDH or the release of an EDHF, which is subsequently transmitted to the adjacent SMC layer either via MEGJ or diffusion.^{1,2} Indeed, it is agreed that EDH(F) activity is blocked by the application of K_{Ca} antagonists, such as apamin (SK_{Ca} antagonist) and charybdotoxin (non-selective IK_{Ca} and BK_{Ca} antagonist, with additional effects at voltage-dependent potassium channels³) in combination^{1,2} or apamin and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM)-34 (IK_{Ca} antagonist) in combination^{4,11,12} in the case of SK_{Ca} - and IK_{Ca} -dependent responses, or by iberiotoxin in the case of BK_{Ca} -dependent responses.²

The nature and mechanism of EDH(F) apparently varies within and between vascular beds and among species, strains, sex and during development, ageing and disease,¹⁻³ as well as with variable experimental conditions and between laboratories.⁴ A proposal for unifying the role of EDH(F) and heterocellular coupling has recently been put forward by Griffith.⁴ This scheme incorporates many of the proposed EDH(F) and questions others, for which there is debatable evidence.

DIFFUSIBLE FACTORS

Contact-mediated mechanisms represent the simplest explanation of EDH(F) activity, as a purely electrical event. However, the release of diffusible factor/s from the endothelium, at a concentration sufficient to change that of the internal elastic lamina and the local environment surrounding the innermost layer of SMC, has also been proposed to account for EDH(F) activity. This substance then putatively effects the activation of SMC receptors and ion channels to initiate smooth muscle hyperpolarization and relaxation.¹⁻⁴

Diffusible factors proposed as EDHF include K^+ ions, epoxyeicosatrienoic acids (EET), H_2O_2 ,^{1,2} and C-type natriuretic peptide (CNP).¹³ N^G -Nitro-L-arginine methyl ester (L-NAME)-insensitive NO has also been suggested to account for EDHF activity.^{14,15} In addition, *S*-nitrosothiols have been suggested to contribute to EDHF activity,¹⁶ although the evidence for the endothelial dependence of this response requires further investigation.

Potassium ions

Several studies have supported the proposal that K^+ ions are an EDHF in some vessels.^{1,3,4,17} Indeed, since the original proposition that K^+ ions were an EDHF, this hypothesis has received much attention. Basically, this scheme involves the activation of EC K_{Ca} and the subsequent EC efflux of K^+ from these channels. The resultant potassium 'cloud'¹⁷ then reportedly diffuses across the internal elastic lamina to act as an EDHF by evoking smooth muscle hyperpolarization and relaxation via the activation of smooth muscle Na^+/K^+ -ATPase and inwardly rectifying potassium channels,¹⁷ key channels for the modulation of ionic mechanisms that are reportedly sensitive to the application of ouabain and barium, respectively. Antagonism of the EDHF response by these blockers is used as defining evidence for K^+ as an EDHF. In its

current form, this mechanism is referred to as the 'potassium cloud hypothesis'.¹⁷

A complication to this hypothesis is the efflux of K^+ from SMC that arises as a result of depolarization, which would contribute to the basal level of K^+ surrounding vascular cells and thus suppress the K^+ /EDHF effect. At a simplistic level, the term 'potassium cloud' is misleading, in that it implies the presence of a global cloud of potassium surrounding the vascular cells when, in fact, any physiologically relevant change in the K^+ concentration will be transient and localized. Indeed, a more plausible scenario is that the K^+ flux acts at restricted localized sites (microdomains), as has been described in SMC and other cell types.¹⁸

Interestingly, the most recent version of the 'potassium cloud hypothesis' includes a role for MEGJ in the action of K^+ as EDHF.¹⁷ However, once a role for MEGJ is included in this mechanism, a role for K^+ as a diffusible EDHF may be redundant, because the EDHF phenomenon can be explained simply through the action of EDH. As alluded to above, a potential scenario where the diffusion of K^+ may play a role in the EDHF activity could arise if there is a close spatial relationship between MEGJ and K_{Ca} distribution (as well as, perhaps, with sites of calcium extrusion), in the form of microdomains, where highly localized changes in K^+ concentrations could play a role in the coordination and modulation of heterocellular EDH(F) signalling (CJ Garland and SL Sandow, unpubl. obs., 2004). Although evidence for similar functional microdomains in SMC and other cell types is well documented,¹⁸ it is interesting to speculate that this scenario may be the case in EC of resistance vessels, such as the mesenteric bed of the rat, where functional studies have suggested this to occur.¹⁹ Further anatomical support for the existence of microdomains in EC is not currently available in resistance vessels and, thus, a role for K^+ in this scenario is speculative.

Epoxyeicosatrienoic acids

There is evidence of a role for EET in EDH(F) activity in some vascular beds.^{1,2} Epoxyeicosatrienoic acids are cytochrome P450 epoxygenase metabolites of phospholipase-dependent arachidonic acid production, which putatively activate smooth muscle BK_{Ca} to result in hyperpolarization and arterial relaxation in cerebral, coronary and renal arteries of several species.^{1,2} Indeed, although there is evidence that EET play an integral role in EDH(F) activity in some vascular beds, EET are not a universal EDH(F), in that in many vascular beds, EDH(F) activity is not sensitive to the application of iberiotoxin, a BK_{Ca} antagonist.⁴ Furthermore, it is not clear whether EET activity is related to the participation of EET in the facilitation of autocrine pathways that generate hyperpolarization via mechanisms that are indistinct from alternative agonist-induced pathways that result in an analogous activation of an EDH(F)-type response.⁴

Hydrogen peroxide

In human and mouse mesenteric and porcine coronary arteries, H_2O_2 has been proposed to act as an EDHF.²¹⁻²⁴ However, a primary problem with these studies is that the appropriate time and concentration controls for catalase, as an H_2O_2 antagonist, were not undertaken and, indeed, the proposal that H_2O_2 is an EDHF in these vascular beds is not consistent with several other studies under-

taken in the same vascular beds (see below). Beny and von der Weid,²⁵ for example, have shown that EDHF and H₂O₂ are distinct factors in porcine coronary arteries, where Pomposiello *et al.*²⁶ have demonstrated that catalase, an enzyme inhibitor of H₂O₂ activity, has no effect in porcine coronary vessels, although, at 300 U/mL, catalase did abolish the endothelium-independent relaxation to exogenously generated H₂O₂ after 45 min incubation. Catalase has been shown to have no effect on EDHF in the bovine ciliary, rat saphenous and mesenteric and human radial and subcutaneous arteries.^{9,27–30} In this light, several studies have shown that H₂O₂ can cause a vasoconstriction^{31,32} that can be attenuated by a 20 min incubation in 100 U/mL catalase.³³ Furthermore, in a membrane potential-independent manner, reactive oxygen species, such as H₂O₂, have been reported to variably activate SMC K_{Ca}, ATP-sensitive potassium channels and Na⁺/K⁺-ATPase and to modulate the sensitivity of the contractile apparatus to calcium,^{4,10} thus playing additional roles unrelated to EDHF, but complicating any speculative role for H₂O₂ in EDHF activity. Indeed, in contrast with the original proposition that H₂O₂ was an EDHF in mouse mesenteric vessels, Ellis *et al.*³⁴ provide evidence that H₂O₂ is not an EDHF in these vessels. Indeed, Ellis *et al.*³⁴ found that an inhibitory effect of catalase does not provide definitive evidence that H₂O₂ is critical to a given vascular response.¹⁰

In any event, the physiological relevance of H₂O₂ as an EDHF is simply questioned based on the observation that the concentration of H₂O₂ produced in response to endothelial stimulation (10–60 nmol/L;³⁵ see also Griffith⁴) is substantially less than the 3–100 μmol/L of H₂O₂ required to elicit a 30–90% relaxation in human mesenteric vessels²² or the 0.1 and 1 mmol/L of H₂O₂ required to elicit a 60 and 100% relaxation, respectively, in porcine coronary arteries.²⁵ In addition, concentration-dependent effects of H₂O₂ are critical to the question of whether physiological or pathophysiological effects are observed, because H₂O₂ can mediate vascular cell proliferation, apoptosis, hyperplasia, cell adhesion and migration, as well as having effects on arterial tone.¹⁰ Indeed, predominant evidence supports the proposition that H₂O₂ is not involved in the hyperpolarization-dependent EDHF response and that it is not an EDHF.^{10,36}

C-Type natriuretic peptide

C-Type natriuretic peptide (CNP) has been proposed to act as an EDHF¹³ and, indeed, the data presented by Chauhan *et al.*¹³ are consistent with the activation of the CNP receptor C subtype playing a role in the EDH(F) phenomenon. However, in the same mesenteric vessels as examined in Sprague-Dawley rats by Chauhan *et al.*¹³ but in the mature Wistar rat, Sandow *et al.*³⁷ demonstrate that heterocellular coupling of EC and SMC accounts for EDH activity in this bed. Although the difference between the two studies could be related to strain variation, such a fundamental difference is unlikely and the specific reason for the discrepancy is unknown. Interestingly, in this light, the use of the non-selective gap junction antagonist glycyrrhetic acid (GA) and its derivatives have implicated a primary role for gap junctional coupling in EDH(F) activity in this vascular bed.^{38–41} Indeed, Chauhan *et al.*¹³ implicate a role for MEGJ in the proposal that CNP is EDH(F) via the use of α-GA, although at present this role is currently unknown but is being investigated (A Ahluwalia, pers. comm.). In any case,

a role for MEGJ in the activity of CNP as EDH(F) is based on the assumption that GA is a specific antagonist for MEGJ and because no control studies for the effects of GA were undertaken in the study of Chauhan *et al.*,¹³ this claim is open to question. Indeed, GA and its derivatives have been shown to block homocellular and MEGJ in this vessel,^{39,41} as well as having direct effects on the EC hyperpolarization to acetylcholine (ACh) via effects on phospholipase activity and EC SK_{Ca}, IK_{Ca} and Na⁺/K⁺-ATPase, irrespective of their putative effects at gap junctions.^{6,42} A limitation of future studies examining a potential role for CNP as an EDH(F) is the lack of availability of selective antagonists for the CNP receptor C subtype that is reported to mediate this response. Furthermore, specific limitations of the study of Chauhan *et al.*¹³ include the lack of a demonstration that the CNP-mediated relaxation can occur independently of the endothelium (which would thus demonstrate CNP action at the smooth muscle) and a lack of explanation of the observations that CNP evokes approximately 60–70% relaxation, whereas EDHF evokes approximately 100% relaxation. In addition, there is also a lack of explanation as to why the (non-specific) blockade of gap junctions with GA suppresses CNP activity or what effects barium alone has on the CNP- and EDHF-mediated relaxations, or the inclusion of appropriate control data to determine whether there was a basal release of CNP in these mesenteric vessels. Thus, a definitive role for CNP in EDH(F) activity remains to be elucidated.

L-NAME-insensitive NO

Endogenous or basal NO activity, which is insensitive to the application of NO synthase antagonists used in the routine study of EDH(F), has been suggested to account for EDH(F) activity.^{14,15} Current evidence suggests that, in some vascular beds under specific experimental conditions, this L-NAME-insensitive NO may account for a minor degree of EDH(F) activity and one not consistently observed in studies of the same vascular bed. For example, in the study of Chauhan *et al.*¹⁵ purporting to show that L-NAME-insensitive NO accounts for a significant portion of EDH(F) activity, 63% of hyperpolarization and 70% of relaxation to ACh remain after the addition of the NO scavenger oxyhaemoglobin (in the presence of L-NAME and indomethacin). Furthermore, in the caudal and saphenous arteries of the rat and mesenteric artery of the mouse, the NO scavengers hydroxocobalamin and carboxy-2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide (PTIO) have no effect on EDH(F),^{9,24,43} thus demonstrating a lack of an L-NAME-insensitive NO component in these vascular beds. Therefore, the contribution of endogenous NO to EDH(F) activity appears variable and, in many cases, non-existent. Further studies are required to determine the physiological relevance of this phenomenon.

CONTACT-MEDIATED MECHANISMS

Evidence supporting the critical role of MEGJ in EDH(F) activity comes primarily from structural and functional studies from our laboratory in Canberra and Tudor Griffith's^{4,36,44,45} laboratory in Cardiff. These studies, which illustrate the simplest explanation of EDH(F) activity, use electron microscopic identification of MEGJ, electrophysiological recordings from dye-identified EC and SMC

and myography with pharmacological interventions, as well as immunohistochemical methods for identifying the connexins and ion channels involved in the EDH(F) phenomenon. These studies are consistent with the hypothesis that EDH(F) is an electrical phenomenon involving the gap junctional transfer of an

EDH from the EC to the innermost layer of intimal SMC in the arterial wall for the subsequent generation of an arterial relaxation.

Studies from our laboratory, which are the focus of this section of the present review, have examined the role of MEGJ in EDH activity. We have found that the distribution and activity of MEGJ is correlated with the presence of EDH within and between vascular beds, during development and in disease. In the proximal and distal mesenteric arteries of the rat, for example, gap junctions play a critical role in EDH activity^{39,41} where MEGJ are prevalent.⁴⁶ In this vascular bed, in collaborative studies with Marianne Tare in Helena Parkington's laboratory in Melbourne, we showed that the presence of EDH is correlated with the presence of MEGJ, whereas in the femoral artery a lack of MEGJ is correlated with the absence of EDH.³⁷ A similar situation is present in the lateral saphenous artery of the juvenile rat, where MEGJ are prevalent and EDH-mediated relaxation present.⁹ This is in contrast with the saphenous artery of the adult, where MEGJ were rare and EDH absent.⁹ The relationship between EDH and MEGJ is somewhat more complicated in disease states, such as in hypertension. In a comparative study of the caudal artery of the spontaneously hypertensive and normotensive Wistar-Kyoto rats, EDH activity was maintained in spite of an increase in the number of SMC layers in vessels from the hypertensive rat. This maintenance was found to be correlated with a concomitant increase in the incidence of MEGJ in the caudal artery of the hypertensive rat.⁴³

The above studies demonstrate that there is a direct relationship between the degree of EDH and the incidence of MEGJ. Indeed, EDH increases with an increase in the number of MEGJ per EC, whereas, conversely, it generally decreases with an increase in the number of SMC layers and vessel diameter (Fig. 1). Interestingly, although EDH is the predominant vasodilator in smaller vessels, it is present in some larger vessels (Fig. 1), such as the rabbit iliac, rat caudal and superior mesenteric arteries.^{41,43,45} In the rabbit iliac artery, cAMP has been proposed to enhance the spread of EDH via modulating gap junctional coupling within the multiple SMC layers, as well as at MEGJ.⁴⁷ Although conclusive biophysical evidence for this mechanism being relevant in larger vessels is lacking,⁴⁸ this mechanism may be of some importance for EDH activity.

These studies demonstrate that there is a consistent positive correlation between MEGJ and EDH activity within and between vascular beds and during development and disease. Although this correlation is not definitive evidence that contact-mediated mechanisms account for EDH(F) activity, to date these data provide the most conclusive and plausible explanation for this activity.

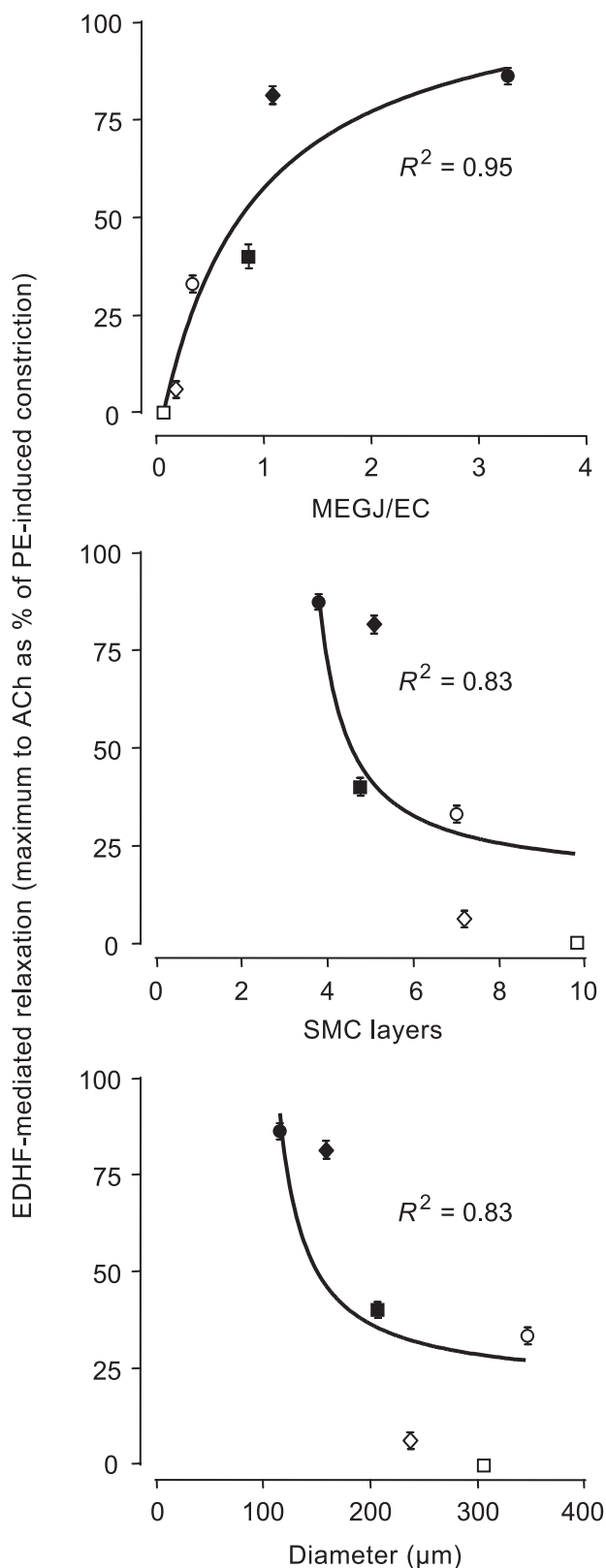


Fig. 1 Summary data demonstrating the relationship between acetylcholine (ACh)-induced endothelium-derived hyperpolarizing factor/endothelium-derived hyperpolarization (EDH(F)) activity and arterial morphology as the number of myoendothelial gap junctions (MEGJ) per endothelial cell (EC), per number of medial smooth muscle cell (SMC) layers and per vessel diameter. Individual data points are presented as the mean \pm SEM, with data being derived from earlier studies.^{9,37,43,46} Data were fitted with a one-phase exponential curve using Graphpad Prism (Graphpad Software, San Diego, CA, USA). PE, phenylephrine. (●), tertiary mesenteric (juvenile); (◆), primary mesenteric (juvenile); (■), primary/secondary mesenteric (juvenile); (○), caudal (adult); (◇), saphenous (adult); (□), femoral (adult).

ROLE OF DIFFUSIBLE FACTORS IN CONTACT-MEDIATED MECHANISMS

Direct electrical coupling is the most plausible mechanism to fully account for EDH activity. Indeed, there is increasing evidence that the diffusible factors that act as credible EDHF may, in fact, be associated with the modulation of gap junction activity and, specifically, of MEGJ⁴ for the transfer of EDH as the most plausible mechanism of their activity. These mechanisms are outlined below.

Potassium ions

The original hypothesis regarding the mechanism of action of K⁺ as EDHF has been modified to include a role for MEGJ.¹⁷ However, although K⁺ is involved in mediating EDH(F) activity, once a role for such MEGJ is included, no direct role for K⁺ as a diffusible EDHF is necessary for the transfer of an EDH. Indeed, in a series of experiments that repeated those in the original proposal that K⁺ was EDHF, the data in the original study could not be repeated.⁴⁹ In addition, several studies have questioned the nature of K⁺ as EDHF because barium and ouabain, which are used to define the role of K⁺ as an EDHF, do not universally block EDHF-mediated responses.^{1,3,4,17,50} Indeed, the efficacy of ouabain as a selective Na⁺/K⁺-ATPase antagonist has been questioned,^{4,51,52} whereby it has inhibitory effects on cell coupling via modulating gap junction function.⁵³ Indeed, ouabain may directly attenuate the transfer of EDH by its action at gap junctions.^{4,51,52} This action includes direct effects on gap junctional coupling, such as reducing connexin (Cx) expression through reduced Cx trafficking to the cell membrane, as well as modulating gap junction conductance.⁵² The implication of these observations is that the attenuation of an EDH(F) response by ouabain, as with high concentrations of potassium, does not necessarily provide evidence of the EDH(F) nature of the response.^{4,6,52} The demonstration that ouabain has direct effects on gap junctions and, thus, on EDH(F) are essentially control studies for the earlier work that relied on the use of ouabain to show that K⁺ was EDHF. Thus, based on these 'control' data,^{4,51,52} K⁺ ions are not an EDH(F) but, rather, may simply be involved in the modulation of the signal transduction pathways associated with gap junction function^{54,55} and, thus, with EDH activity.⁴ Further investigation is required to elucidate any potentially specific effects of ouabain on vasomotor responses and those at gap junctions. Indeed, this point is critical for the accurate interpretation of future EDH(F) data.

Epoxyeicosatrienoic acids

In studies of cultured EC, EET have been shown to modulate homocellular gap junctions,⁵⁶ thus providing a potential mechanism for a modulatory role for EET in EDH action.⁴ Griffith⁴ suggests that EET activity may be related to a complex interaction of calcium and potassium homeostasis, cAMP and arachidonic acid activity and electrotonic signalling (see Fig. 3 in Griffith⁴ and also Dhein⁵⁰). Indeed, EET have been suggested to modulate EC K_{Ca} activity,⁵⁷ thus providing a further mechanism for their potential role in modulating EDH, independent of acting directly as an EDHF. Further studies of the role of EET in EDH activity in intact vessels are required to clarify these proposals.

Hydrogen peroxide

There is some evidence that H₂O₂ can affect gap junction activity and calcium homeostasis, two factors that are integral for EDH activity. Depending on the experimental conditions, studies have shown that H₂O₂ can both increase⁵⁸ and decrease⁵⁹ gap junctional coupling and effect changes in intracellular calcium homeostasis, both in cultured cells and in intact arteries.⁵⁹⁻⁶¹ Although no specific evidence is currently available to support this proposal, these observations provide potential support for a mechanism to link the putative role of H₂O₂ as an EDH(F) with the MEGJ dependence of the EDH phenomenon.

C-Type natriuretic peptide

The putative action of CNP as an EDH(F)¹³ may be via acting as yet another factor that facilitates electrical coupling through gap junctions, although any putative mechanism for this is unknown. Indeed, any putative action for CNP as EDH(F) cannot be associated directly with the gap junctional transfer of CNP from EC to SMC because gap junctions are limited to passing substances of ≤ 1 kDa and CNP has a molecular weight of approximately 2.2 kDa (A Ahluwalia, pers. comm.). Interestingly, in the study of Chauhan *et al.*¹³ proposing that CNP is an EDH(F), the response is sensitive to the combination of barium and ouabain, an observation that this is not a universal characteristic of EDH(F) in this, the rat mesenteric vascular bed.⁴⁹ Indeed, because ouabain is recognized as a non-specific gap junction antagonist, this result may, in fact, reflect an MEGJ dependence of EDH(F) in the mesenteric bed of the rat, as demonstrated by Sandow *et al.*³⁷

Myoendothelial gap junctions, EDH and gap junction inhibitors

The demonstration of the dependence of EDH activity on gap junctions relies, in part, on the specific pharmacological inhibition of gap junctions. Unfortunately, there are a number of limitations regarding this methodology. The primary one of these relates to the dependence on the use of gap junction inhibitors that have not been characterized adequately in terms of their specificity and mechanism of action. Currently, there is no unequivocal evidence that the available gap junction inhibitors are specific,⁶² let alone selective, for gap junctions, be they heterocellular or homocellular. Indeed, unfortunately, to date few studies have examined this problem in detail and few have performed the defining experiment of examining the effect of these agents on cell input resistance, whereby an increase in input resistance would provide key data on the gap junction antagonist effects of these agents. Of the studies that have undertaken such technically demanding experiments, the data are not consistent and are incomplete, although this may reflect, in part, the heterogeneity in the Cx composition of vascular gap junctions.⁶³

Much of the current evidence for the gap junction and, specifically, MEGJ dependence of EDH relies on the use of the licorice derivatives (the GA and carbenoxolone; see above for an outline of non-specific actions), the Cx-mimetic peptides (Gap26,⁴³ Gap27⁴⁰ and Gap27,^{37,43} which, based on putative selectivity, are the current gap junction inhibitors of choice^{4,9,44}) and, decreasingly, with the long-chain alcohols, such as heptanol.^{64,65} However, there is little

equivocal evidence that these agents are gap junction specific and that they do not induce other non-gap junctional effects. Although there is well-documented (and often ignored) evidence for the non-specific effects of the licorice derivatives (see above) and heptanol,^{64,65} the Cx-mimetic peptides have not yet been equivocally tested for specificity and nor is their mechanism of action known. In this regard, a primary issue with the use of the Cx-mimetic peptides relates to the apparent requirement to use very high concentrations and long incubation times to attenuate gap junction activity.⁴ Interestingly, others report significant effects with lesser concentrations of the peptide/s and reduced incubation times.^{62,66,67} Clearly, there is a pressing need for these issues to be addressed.

WHY IS THERE SUCH A DISPARITY OF VIEWS AS TO THE NATURE AND MECHANISM OF ACTION OF EDH(F)?

The conventional reason given for the disparity of views as to the nature and mechanism of action of EDH(F) is that there is heterogeneity within and between arteries, species, sex, strain and disease states.^{1-4,10,17} However, a further cause of the heterogeneity relates to the less-than-optimal design, analysis and interpretation of data present in some key papers in the EDH(F) literature. Although some earlier studies can be seen as flawed with hindsight, this is not necessarily the case, because they may, in fact, represent significant contributions to the EDHF literature through their role in advancing the evolution of the field. Unfortunately, this is not always the case and the perpetuation of now potentially misleading data is problematic. In any case, it is recognized that there is variation in the nature and mechanism of EDH(F) between laboratories,⁴ thus questioning the relevance of the data and conclusions of some studies.

The problems of experimental technique, with regard to the design, analysis and interpretation of data that contribute to the reported heterogeneity in the nature and mechanism of action of EDH(F) in the literature include the following:

1. The use of selected agonists, antagonists and/or modulators of the investigators' choice and interest, but not those that may indicate an alternative nature or mechanism of EDH(F).^{1,3,4,17} That is, for example, an investigator may be interested in EET or gap junctions to account for EDH(F) activity, but may limit the investigation to the use of antagonists of the mechanism of their interest, rather than of alternative pathways. This results in a potential for a bias in favour of a particular putative EDH(F).^{1,3,4,17}

2. The lack of control data for the effects of agonists, antagonists and other modulators. For instance, in the studies of Matoba *et al.*²¹⁻²³ examining the role of H₂O₂ as an EDH(F), justification should be provided as to the incubation time with catalase (2 h or longer), as well as the high (and variable) concentration of catalase that was used.

3. The clear need for greater transparency with regard to variability in cell, vessel and species specific responses, as a result of a specific receptor and channel population and associated signal transduction pathways.⁶³

4. Making inappropriate comparative analyses between studies, including a lack of consideration of strain,^{68,69} age,^{9,70-72} sex,⁷³⁻⁷⁶ the use of intact versus isolated tissue and tension versus pressurized myography,⁶³ as well as variation in the classification of arterial branching patterns.^{39,41,46} Indeed, such characteristics are

often not stated in the methods section of papers and, thus, result in an inability to make comparative analyses between studies.

5. Lack of clarity and relevance as to the experimental protocol. For instance, under conditions of little or no vascular tone, the use of buffers (such as HEPES)⁷⁷ that have non-physiological effects, the use of preconstrictor agents that adversely effect channel activity⁴ (such as the effect of U46619 on SK_{Ca}⁷⁸ and the effect of the GA and related compounds on a variety of cell processes), as outlined above.

6. Extrapolation of data to other vascular beds. For example, Chauhan *et al.*^{13,15} examined EDH(F) in mesenteric vessels of the mature male Sprague-Dawley rat, but extrapolate the data to be applicable to the vasculature as a whole. Although several studies have made such claims,^{1,3,4} this contention merely confuses the field because there is no evidence to justify this point of view.

CONCLUSIONS

The nature and mechanism of action of EDH(F) can apparently differ along and between vascular beds, between species, strains, sex and during development, ageing and disease. This heterogeneity can be explained through the action of heterocellular coupling. Indeed, contact-mediated mechanisms represent the simplest explanation of EDH(F) activity and involve the transfer of an endothelium-derived electrical signal to the smooth muscle via MEGJ, as EDH. Of the putative diffusion-mediated mechanisms, K⁺ ions have received much attention in the literature and although they may not be an EDHF, they are involved in the signal transduction pathways associated with the generation of the EDH and they may be involved in the modulation of gap junction activity. In a similar manner, there is good evidence for a role of EET in EDH(F) activity in some vascular beds, although this role may be confined to a modulatory role of homo- and heterocellular coupling, as well as modulating the K_{Ca} component of the EDH mechanism. The role of CNP as an EDH(F) is yet to be clarified, but may also be related to the modulation of EDH activity. Predominant evidence supports the proposition that H₂O₂ is not an EDH(F), although, again, its activity may be related to the modulation of gap junction function and, thus, of EDH. N^G-Nitro-L-arginine methyl ester-insensitive NO may account for a degree of EDH(F) activity in some vascular beds, but the extent of this is limited to only a minor part of such activity. Although the nature and mechanism of action of EDH(F) is due, in part, to actual heterogeneity, it is also unfortunately due to a lack of consistent and sound scientific methodology.

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