A Therapeutic Target for Microvascular Complications in Diabetes: Endothelium-Derived Hyperpolarizing Factor

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Abstract: Vascular alterations in diabetes cause or contribute to the etiology of microvascular complications such as nephropathy, neuropathy, and retinopathy. The endothelium controls the vascular smooth muscle tone through the production of vasodilator mediators such as nitric oxide, prostacyclin, and a still-elusive endothelium-derived hyperpolarizing factor (EDHF). Although EDHF is a prominent vasodilator, particularly in smaller arteries, little attention has been paid to the potential role of EDHF responses in diabetes. EDHF function may involve the participation of mediators, including several diffusible factors and non-diffusible factors, (e.g., conduction of hyperpolarization via myoendothelial gap junctions). Indeed, in several vessels, cyclic adenosine 3',5'-monophosphate (cAMP) facilitates EDHF responses by enhancing electrotonic conduction via gap junctions. It has been demonstrated that the alterations in EDHF relaxation seen in mesenteric arteries from diabetic rats may be attributable to an increase in phosphodiesterase3 (PDE3) activity, leading to a reduction in the action of cAMP, and moreover the activity of protein kinase A (PKA) is decreased in such arteries. Although an improvement in EDHF responses has not been, as yet, the subject of any direct pharmaceutical effort, increasing cAMP/PKA signaling (e.g., by inhibiting PDE3 activity) has potential as an interesting therapeutic target in diabetic microvascular disease.

Key Words: EDHF, cAMP, diabetes, phosphodiesterase3, cilostazol, gap junction.

INTRODUCTION

Vascular complications are the main causes of morbidity and mortality in patients with diabetes, and vascular alterations cause or contribute to the etiology of diabetic complications such as nephropathy, neuropathy, and retinopathy [1-6]. Several lines of evidence suggest that endothelial dysfunction could play a key role in the development of both macro- and microangiopathy in patients with diabetes and in animal models of the disease [6-8]. Endothelial cells relax the tone of the underlying vascular smooth muscle cells by releasing a number of vasodilator substances, including nitric oxide (NO), prostacyclin (PGI2), and an as-yet-elusive endothelium-derived hyperpolarizing factor (EDHF). A consistent finding in diabetic animal models is the presence of vascular endothelial dysfunction. This has been linked to elevated oxidative stress and is characterized by impaired NO-mediated endothelium-dependent relaxation, as noted for numerous individual vessels and vascular beds, including aorta, basilar and cerebral arteries, corpus cavernosum, and renal and mesenteric vascular beds [7, 9-17]. Studies in diabetic patients have revealed comparable deficits [6, 7]. Although NO has generally been considered to be the principal mediator of endothelium-dependent relaxations, it has become increasingly clear that NO-independent endothelium-derived relaxing factors may have also important roles to play in local vasomotor control. The contribution made by EDHF to relaxation is dependent on vessel size, its effects being more prominent in the smaller, physiologically more important arteries than in larger ones [18, 19].

As yet, little attention has been paid to the potential role of an impaired release or impaired action of EDHF in diabetes. Since small-vessel dysfunction (such as occurs in retinopathy, nephropathy, and neuropathy) is one of the major complications seen in diabetes, an impairment of EDHF-mediated responses could make an important contribution to the mechanisms by which diabetes leads to vascular dysfunction. Thus, an improvement in EDHF signaling could be an interesting therapeutic target in cases involving diabetic vasculopathy.

In the present review, we will discuss (a) the possible mediators of EDHF activity, as well as their regulation and alterations in cellular signaling during diabetes, (b) the possible contribution of EDHF to the long-term vascular changes observed during diabetes, and (c) potential therapeutic modulation(s) of EDHF responses in diabetic states.

EDHF: NATURE AND PHYSIOLOGICAL IMPORTANCE

EDHF is defined as the non-NO/PGI2, endothelium-derived relaxing factor that mediates vascular smooth muscle hyperpolarization via a direct or indirect opening of vascular potassium (K+) channels. It is important to recognize this distinction from NO and PGI2, each of which can also hyperpolarize smooth muscle in a highly tissue-dependent manner. Moreover, NO may be linked to the presence of active tone, as well as to the cellular mechanisms associated with the activation of vascular tone. Thus, a complete elimination of the synthesis and contribution of NO and PGI2 must be achieved before it can be concluded that EDHF is involved in any given endothelium-dependent relaxation [20].

EDHF-mediated responses-- to any agonists, such as acetylcholine (ACh) or bradykinin (BK), that stimulate G-
protein-coupled receptors— are associated with an increase in [Ca^{2+}]_{i} in the endothelial cell, and may be generated by substances that increase endothelial [Ca^{2+}]_{i} in a receptor-independent manner (e.g. Ca^{2+} ionophores and the sarcoplasmic- reticulum Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid). This implies that for EDHF-mediated responses, as for many other endothelial functions, an increase in endothelial [Ca^{2+}]_{i} is a crucial step [18, 21] (Fig. 1).

An increase in [Ca^{2+}]_{i} in endothelial cells leads to activation of Ca^{2+}-activated K^{+} channels and to membrane hyperpolarization (Fig. 1). In most instances, an EDHF-mediated vascular response (a) can be completely, or almost completely, inhibited by a combination of apamin, a small-conductance Ca^{2+}-activated K^{+} channel (SK_{Ca}) inhibitor, and charybdoxin, an intermediate-conductance Ca^{2+}-activated K^{+} channel (IK_{Ca}) inhibitor, and (b) lacks sensitivity to iberiotoxin, a large-conductance Ca^{2+}-activated K^{+} channel (BK_{Ca}) inhibitor [22]. Most likely, these effective channel toxins (apamin and charybdoxin) act on sites located on endothelial cells, and their inhibitory effect is probably related to a decrease in the driving force for Ca^{2+} entry, and hence to a reduction in the synthesis/release of EDHF [20].

Despite numerous studies aimed at identifying a specific factor responsible for endothelium-dependent hyperpolarization, the putative mediator of the EDHF response may actually be one, or a combination, of a number of candidates. Indeed, several different factors have been implicated as mediators, including diffusible factors [such as K^{+} ions, epoxyeicosatrienoic acids (EETs), hydrogen peroxide (H_{2}O_{2}), and C-type natriuretic peptide (CNP)] and non-diffusible, contact-mediated mechanisms [such as conduction of hyperpolarization via myoendothelial gap junctions (MEGJs)]. More extensive reviews on EDHFs have been published elsewhere [18, 20, 23-28].

Pharmacological evidence indicates that EDHF is a significant component not only of agonist-induced vasodilation, but also of shear stress-induced vasodilation [20, 29, 30]. The contribution of NO is greater in the larger diameter arteries than in the resistance arteries, but EDHF has been found in both sizes of blood vessels [31]. Further in the coronary and renal vascular beds EDHF even plays a major role in conduit arteries [18]. Some years ago, Shimokawa et al. [19] demonstrated an inverse relationship between eNOS expression and vessel size in the rat (aorta versus proximal and distal mesenteric arteries), and they also reported a more negative resting membrane potential in the distal mesenteric resistance vessels, suggesting that a release of EDHF may influence their basal tone. Several studies have produced evidence of clear alterations in EDHF-mediated responses in various pathological conditions [8, 26, 27], and also evidence of an important role for EDHF in anti atherogenic mecha-

**Fig. (1).** Interactions between the endothelium and smooth muscle that may participate in the endothelium-derived hyperpolarizing factor (EDHF) phenomenon. Following stimulation of the endothelial cell (EC) with an agonist such as acetylcholine or bradykinin, there is a transient increase in [Ca^{2+}]_{i}, which leads to activation of Ca^{2+}-dependent K^{+} channels and membrane hyperpolarization. The hyperpolarization is conducted to the subjacent smooth muscle cell (SMC) via myoendothelial gap junctions, and may subsequently spread through the vessel wall via the homocellular gap junctions that couple smooth muscle cells. The elevations in cytosolic Ca^{2+} may also increase cAMP levels, thereby enhancing the electrical conductance of myoendothelial gap junctions. Epoxyeicosatrienoic acids (EETs), metabolites of arachidonic acid (AA), may contribute to endothelial hyperpolarization by opening store-operated channels and elevating [Ca^{2+}]_{i}, as well as by having possible direct effects on K_{Ca} channels. EETs may also stimulate the synthesis of cAMP by adenyl cyclase (AC). PKA, protein kinase A; e{, electrical charge; PL{, phospholipase A{, G, G protein.
domains of the first and second extracellular loops of Cx
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and rat mesenteric arteries can be inhibited
(a) by synthetic peptides homologous to the Gap 26 or 27
domains of the first and second extracellular loops of Cx
proteins, which interrupt intercellular communication in a
Cx-specific fashion, and (b) by 18α-glycyrrhetinic acid (18α-
GA), a lipophilic aglycone that disrupts gap-junction plaques
[24, 34-39]. A few years ago, an electrophysiological and
electromicroscopic study revealed that endothelial and
smooth muscle cells are electrically coupled via MEGJs in the
the rat mesenteric artery [40]. Moreover, in the mature rat
femoral artery, which lacks MEGJs, shows no EDHF-mediated
smooth muscle hyperpolarization of relaxation, despite the
generation of a charybdotoxin + apamin-sensitive hyper-
polarization in the endothelial cells [40].
An accumulating body of evidence indicates that the
second messenger cAMP regulates EDHF signaling in sev-
eral vessels [24]. Observations that the EDHF-type relaxa-
tions evoked by ACh in the rabbit iliac artery is dependent
on an elevation in the smooth muscle cAMP level and on
phosphorylation events mediated by protein kinase A (PKA)
suggest that even responses to agonists may not simply be
mediated by passive electrotonic mechanisms [41]. Endoge-

dous formation of cAMP may play an important role in the
EDHF phenomenon because agonists such as ACh are capa-
ble of promoting endothelial synthesis of this nucleotide
through a mechanism that is independent of the formation of
prostanoids [41, 42]. Indeed, in rabbit ilio-femoral arteries
the adenylyl cyclase (AC) inhibitor 2′,5′-dideoxyadenosine
(2′,5′-dDA) markedly attenuates the subintimal smooth mus-

cle hyperpolarization evoked by ACh, suggesting that cAMP
generated within the endothelium plays an important role in
facilitating electrical coupling via MEGJs during agon-

ist-induced responses [43,44]. This permissive role of cAMP
is likely to explain the ability of 2′,5′-dDA and PKA inhibi-
tion to attenuate EDHF-type relaxations in rabbit and rat
arteries [37, 41, 43, 45]. Interestingly, cAMP accumulation
can be suppressed by interrupting gap junctional communi-
cation using Cx-mimetic peptides or 18α-GA in the rabbit
iliac artery [41]. Hence, it is possible that chemical signaling
and gap junctions each contribute to the response to ACh
because, in addition to conferring electrical continuity, gap
junct ions allow direct transfer of signaling molecules <1 kDa
in size between coupled cells [24]. Furthermore, elevations
in smooth muscle cAMP levels have been shown to facilitate
electrotonic signaling within the vascular media, and thereby
to amplify and prolong the transmission of ACh-induced
hyperpolarizations to smooth muscle cells remote from the
endothelium [43]. This mechanism is likely to contribute to
the marked potentiation of EDHF-type relaxations reported
in the presence of (a) the phosphodiesterase (PDE) inhibitor
isobutylmethylxanthine (IBMX) or 8-bromo-cAMP in rabbit
iliac arteries [37, 41, 43], (b) IBMX in rat mesenteric arteries
[39], and (c) a cell-permeant cAMP analogue in rat renal
arteries [46]. Thus, cAMP would seem to play an important
role in the regulation of EDHF responses.

EDHF ACTIVITY IN DIABETIC STATES

Although the exact nature of the alterations in EDHF activity
that in occur disease states remains unclear, there is
emerging evidence to suggest that such alterations do occur
both in disease states, such as hypertension and diabetes, and
as a result of aging [7, 8, 26, 27, 33, 47]. Closer examination
of the literature reveals a species segregation of the findings
as follows: impaired EDHF activity in diabetic rats [15, 39,
48-51] and diabetic humans [52], but enhanced EDHF activity
in diabetic mice [53]. For example, Fukao et al. [48]
showed that both the amplitude and duration of the ACh-
induced hyperpolarization were significantly decreased in
mesenteric arteries from diabetic rats. Inhibition of NO syn-

thase did not affect the hyperpolarization response to ACh in
mesenteric arteries from either control or diabetic rats, im-
plying that the functional EDHF response in the rat mesen-

teric artery decreases as a result of diabetes. Furthermore,
Wigg et al. [50] demonstrated a selective impairment of
EDHF activity in smaller mesenteric resistance arteries, but
not in femoral arteries, from STZ-induced type 1 diabetic
rats, whereas NO-dependent responses studied in vitro were
actually preserved. This strongly suggests that the impair-
ment of endothelium-dependent relaxation seen in the diab-
etic mesenteric artery is attributable to the presence of a
reduced EDHF-dependent response. These results are con-
istent with the finding of a more pronounced influence of
EDHF in smaller vessels than in larger ones under normal
conditions (see above). In diabetes, selective damage to the
smaller resistance vessels, mediated via reduced EDHF ac-
tivity, may provide a possible mechanism for the microvas-
cular complications commonly observed in that disease.
Further, the agonist-induced relaxation attributed to EDHF in
human penile resistance arteries is greatly impaired in pa-
tients with type 1 and type 2 diabetes [52]. The factors medi-
ating the impairment of EDHF responses seen in diabetes are
currently under investigation (see below), but the fact that
this impairment occurs in a range of vascular beds (including
the epi neural arterioles of the sciatic nerve, the mesenteric
and carotid arteries, and renal arterioles) suggests that there
may be a common mechanism [27].

As mentioned above, there is emerging evidence to sug-
gest that gap junctions may play a role in EDHF activity.
Under high-glucose conditions, Cx43 expression and gap-
junction activity have been found to be greatly reduced in microvascular endothelial cells [54] and in aortic endothelial and/or smooth muscle cells [55]. Furthermore, a disruption of gap-junction pathways has been observed in the retinal microvasculature of STZ-induced diabetic rats [56]. An upregulation of protein kinase C, which is known to be involved in diabetic complications [57, 58], an alteration in Cx expression, and/or phosphorylation have been invoked to explain the changes in gap-junction function [55, 59, 60].

A recent in vitro study in our laboratory [39] confirmed that in the mesenteric artery of STZ-induced diabetic rats, ACh-induced EDHF-type relaxation was impaired. Moreover, the EDHF response was sensitive to 18α-GA, a gap-junction inhibitor, and further investigation revealed increased PDE activity in the diabetic arteries, specifically PDE3, and reduced cAMP levels [39]. In that study, PDE inhibitors enhanced the EDHF-type relaxations in mesenteric arteries from both control and diabetic rats, but the augmentation was greater in the diabetics, such that maximal relaxation amplitude was then similar to control. Under normal conditions, cAMP appears to facilitate the spread of current through gap junctions, thus enabling the potentiation and transmission of EDHF-mediated hyperpolarization to regions electrically distant from the endothelium (see above section). Hence, it is possible that a reduction in cAMP activity via an increase in PDE could contribute to the impairment of EDHF-type relaxation that is observed in the resistance vessels of diabetic rats (Fig. 2).

The intracellular level of cAMP is tightly regulated, both by control of its rate of synthesis (by ACs [61-63], in response to extracellular signals) and by control of its rate of hydrolysis (by PDE) [64-66]. Following an intracellular elevation of cAMP, reversible phosphorylation of several protein substrates (including the gap-junction component Cx by PKA modulates many physiological processes [67-69]. It is known that PKA is activated by cAMP, and that it comprises two regulatory (R) and two catalytic (C) subunits, and several pieces of evidence indicate that PKA activity is determined by the expression-balance between the C and R subunits [67, 70]. In recent studies, we have demonstrated that the abnormal vascular relaxation responsiveness seen in STZ-diabetic rats may be attributable not only to increased PDE activity [39], but also to decreases in PKA activity [71] and AC activity [72]. Possibly, the decreased PKA activity may result from an imbalance between PKA C and R subunit expressions [71], and the decreased AC activity may result from reductions in AC5/6 expressions [72]. Taken together, the above results suggest that the cAMP/PKA signaling pathway could be intimately involved in the specific alteration of the EDHF-mediated response in the mesenteric artery that is seen in diabetic rats (Fig. 2). Indeed, our recent study demonstrated that a selective PDE3 inhibitor could improve the EDHF response in the diabetic mesenteric artery (see below).

**EDHF: A THERAPEUTIC TARGET**

Several studies have suggested that impairments of EDHF-mediated responses may be present in disease states, indicating the potential for therapeutic interventions [26]. For instance, chronic treatment with an angiotensin-converting-enzyme inhibitor, with an angiotensin-receptor antagonist, or with a diuretic normalizes EDHF-mediated responses in spontaneously hypertensive rats [26, 73]. Moreover, treatment with folate, with an aldose-reductase inhibitor, with calcium dobesilate (an angioprotective agent), with evening primrose oil (an ω-6 γ-linoleic acid-containing oil), or with trientine (a metal chelator) restores impaired EDHF-mediated responses in diabetic states [26, 52, 74-76]. In addition, dietary supplements and exercise have beneficial

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**Fig. (2).** EDHF and potential new therapeutic targets in the diabetic state. cAMP facilitates EDHF-type relaxation by enhancing electrotonic conduction via gap junctions. The reduced EDHF-type relaxation present in the diabetic state may be attributable to an increase in PDE3 activity, leading to a reduction in the action of cAMP, and the reduced PKA activity may be due to an imbalance between the expressions of PKA catalytic and regulatory subunits. Cilostazol (PDE3 inhibitor) improves EDHF-type relaxations in diabetic rats via an increase in cAMP/PKA signaling. AC, adenylyl cyclase; PKA, protein kinase A; ϵ+, electrical charge; SKCa/IKCa, small-/intermediate-conductance Ca2+-activated K+ channel; R, receptor; G, G protein.
effects on EDHF responses [26]. However, the mechanisms underlying these drug-induced and adjunct-induced improvements in EDHF responses remain poorly understood. EDHF-mediated responses are clearly affected in a variety of pathological conditions, and the fact that the above therapeutic or adjunct interventions can restore these responses suggests that an improvement in EDHF-mediated responses might contribute to the observed beneficial effects of such interventions. Especially interesting is the possibility that an enhancement of EDHF-mediated responses might contribute to improvements in diabetic microvascular complications such as retinopathy, nephropathy, and neuropathy (since EDHF plays important roles in the microvasculature). In a recent study [45], we noted that in STZ-induced diabetic mesenteric arteries: (a) treatment with cilostazol, a selective PDE3 inhibitor, improved ACh-, A23187-, and cyclopiazonic acid induced EDHF-type relaxations, (b) the ACh-induced cAMP accumulation in mesenteric arteries was more sustained in cilostazol-treated than in cilostazol-untreated STZ rats, (c) the EDHF-type relaxation was significantly decreased by a PKA inhibitor in the cilostazol-treated group, but not in the cilostazol-untreated group, (d) cilostazol treatment improved both the relaxations induced by cAMP analogs and the PKA activity level, and (e) PKA C subunit (Cat α) protein was significantly decreased, but the R subunit RI β was increased (and the latter effect was significantly diminished by cilostazol treatment). These results strongly suggest that cilostazol improves EDHF-type relaxations in STZ rats via an increase in cAMP/PKA signaling (Fig. 2). Although cilostazol is used clinically in several diseases, such as peripheral artery disease [77], the above study [45] (a) provided the first evidence of its potential as a therapeutic drug for the improvement of EDHF-mediated responses in diabetic states, and (b) strongly suggested that the reduced EDHF-mediated responses seen in diabetes may be improved by manipulation of the cAMP/PKA pathway (Fig. 2). These findings should stimulate further interest in cilostazol as a potential therapeutic drug for use against diabetes-associated vascular disease, especially microvascular disease.

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