Biochemical Pharmacology, Vol. 54, pp. 1059–1070, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00039-7

COMMENTARY

Hyperpolarizing Factors

John Quilley, David Fulton and John C. McGiff*
DEPARTMENT OF PHARMACOLOGY, NEW YORK MEDICAL COLLEGE, VALHALLA, NY, 10595, U.S.A.

ABSTRACT. There is now overwhelming evidence for factors, other than nitric oxide (NO), that mediate endothelium-dependent vasodilation by hyperpolarizing the underlying smooth muscle via activation of Ca^{2+}-activated K^+ channels. Although the identity of endothelium-derived hyperpolarizing factor (EDHF) remains to be established, cytochrome P450 (CYP)-dependent metabolites of arachidonic acid (AA), namely, the epoxides, fulfill several of the criteria required for consideration as putative mediators of endothelium-dependent hyperpolarization. They are produced by the endothelium, released in response to vasoactive hormones, and elicit vasorelaxation via stimulation of Ca^{2+}-activated K^+ channels. Our studies in the rat indicate that, of the epoxides, 5,6-epoxyeicosatrienoic acid (5,6-EET) is the most likely mediator of NO-independent, but CYP-dependent coronary vasodilation in response to bradykinin. Studies in the rat kidney, however, support the existence of additional EDHFs as acetylcholine also exhibits NO-independent vasodilation that is unaffected by CYP inhibitors in concentrations that attenuate responses to bradykinin. In some blood vessels, NO may tonically suppress the expression of CYP-dependent EDHF. In the event of impaired NO synthesis, therefore, a CYP-dependent vasodilator mechanism may serve as a backup to a primary NO-dependent mechanism, although they may act in concert. In other vessels, particularly microvessels, an EDHF may constitute the major vasodilator mechanism for hormones and other physiological stimuli. EDHFs appear to be important regulators of vascular tone; alterations in this system can be demonstrated in hypertension and diabetes, conditions associated with altered endothelium-dependent vasodilator responsiveness. BIOCHEM PHARMACOL **54**;10:1059–1070, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. endothelium-derived hyperpolarizing factor; arachidonic acid; nitric oxide; endothelium; cytochrome P450-AA; bradykinin

The discovery by Furchgott and Zawadzki [1] of an absolute requirement for an EDRF† in the expression of vasodilation by acetylcholine and other vasodilator agonists ushered in a new era in our perception of vascular control. The endothelium is now recognized to elaborate a variety of vasoactive factors and to play a critical role in the regulation of vascular tone [2]. By 1987 it was established that EDRF was NO or a congener that released NO [3, 4]. The importance of NO to the regulation of vascular tone was underscored upon the introduction of inhibitors of NOS that prevented the formation of NO from L-arginine. Administration of these inhibitors *in vivo* resulted in vasoconstriction and elevation of blood pressure, indicating a continuous basal release of NO [5]. Studies of isolated organs and blood vessels revealed that NO is the primary mediator of endothelium-dependent responses to many vasodilator agents [6]. *However, the importance of NO-independent endothelium-derived relaxing factors in mediating or modulating responses to vasoactive hormones and, thereby, regulating blood flow and perfusion pressure, cannot be overlooked.* Indeed, depending on the species, tissue, agonist, and experimental conditions, NO cannot fully account for the vasodilator/vasorelaxant activity of many endogenous vasodilators, most notably, bradykinin and acetylcholine, which are the agonists of reference for probing endothelium-derived factors [7–9]. Moreover, in some vascular beds, endothelium-dependent vasodilator responses to bradykinin and acetylcholine were unaffected by inhibition of NO synthesis, prompting the conclusion that responses in these vasculatures were independent of NO and, therefore, mediated by another relaxant factor, designated EDHF [10]. However, this assumption may not be entirely correct, as inhibition of NO synthesis may be required to uncover the operation of a reserve system that is also capable of fully relaxing blood vessels and is expressed only when the NO system is compromised, i.e. that expression of hyperpolarizing factors may, under certain circumstances, require the elimination of NO [11, 12]. Alternatively, in some vascular beds such as the coronary and renal, EDHF may be an integral component of the dominant vasodilator mechanism invoked by a specific stimulus such as pulsatile pressure [13] in contradistinction to shear stress, the stimulus for NO release [14].

Based on the use of NOS inhibitors, it is apparent that in large conduit vessels, such as the thoracic aorta, NO is the principal mediator of endothelium-dependent relaxant re-

* Corresponding author. Tel. (914) 594-4075; FAX (914) 347-4956.

† *Abbreviations:* EDRF, endothelium-derived relaxing factor; EDHF, endothelium-derived hyperpolarizing factor; NO, nitric oxide; NOS, nitric oxide synthase; CYP, cytochrome P450; AA, arachidonic acid; PLC, phospholipase C; PLA_2, phospholipase A_2; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; K_{Ca}, calcium-activated potassium; K_{ATP}, ATP-sensitive potassium; and K_V, voltage-gated potassium.

sponses [15]. Smaller and more peripheral vessels exhibit greater dependency on NO-independent mechanisms [16]. In addition, vascular beds such as those of the kidney and heart exhibit pronounced NO-independent responses, although NO clearly is involved in the modulation of basal tone in these vascular beds [17, 18]. As endothelium-dependent vasodilation is demonstrable in the presence of mechanistically dissimilar inhibitors of NO-dependent responses such as oxyhemoglobin [19–23], $FeSO_4$ [24], and methylene blue [20, 25, 26], which bind, inactivate, or prevent the action of NO, NO-independent relaxation cannot be an artifact produced by the NOS inhibitor. These observations provide substantial evidence for the existence of factors, other than NO, that mediate endothelium-dependent relaxation. The capacity of the endothelium to form one or more hyperpolarizing factors that dilate blood vessels in response to vasoactive hormones and physiological stimuli is noteworthy and is the subject of this review. In particular, the origin of EDHFs from the metabolism of AA by CYP-dependent monooxygenases, the third pathway of AA metabolism—cyclooxygenase and lipoxygenase being the first and second pathways—will be examined.

MECHANISM OF VASODILATION

Agonist-induced hyperpolarization of smooth muscle [27, 28] was demonstrated well before the obligatory role of the endothelium in acetylcholine-induced relaxation of arterial smooth muscle. The contribution of the endothelium to smooth muscle hyperpolarization by the release of one or more factors, the EDHFs, was recognized within a decade of Furchgott's discovery [29]. Smooth muscle hyperpolarization in response to agonist stimulation, although endothelium dependent, is not inhibited by nitroarginine [8, 30, 31], methylene blue [20, 25, 26], or hemoglobin [19–23], distinguishing it from NO. Recently, NO has been shown to hyperpolarize vascular smooth muscle, both directly [32] and indirectly, via cyclic GMP [33, 34]. However, NO cannot account for the endothelium-dependent hyperpolarization that persists in the presence of NO antagonists, nor does exogenous NO hyperpolarize smooth muscle of all blood vessels [21, 35].

Activation of K^+ channels is the mechanism underlying endothelium-dependent hyperpolarization of vascular smooth muscle. This is supported by evidence demonstrating that variations in extracellular K^+, but not Cl^-, control the amplitude of endothelium-derived hyperpolarization [20, 36], that the reduction in membrane potential is associated with enhanced rubidium efflux [20], an index of K^+ movement, and that hyperpolarization is prevented by K^+ channel antagonists [30, 37]. Activation of the electrogenic Na^+/K^+-ATPase, by exchanging 3 Na^+ for 2 K^+, has been implicated as the source of hyperpolarization in canine coronary arteries based on the inhibitory effect of ouabain [38], although this may reflect non-specific antagonism of K^+ channels.

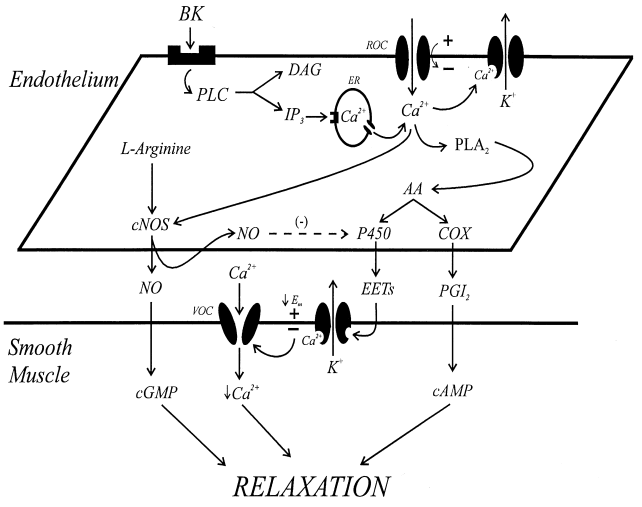

FIG. 1. Schematic representation of the formation, release, and potential interactions of endothelium-derived relaxant mediators.

Although hyperpolarization of vascular smooth muscle can be demonstrated to directly effect a reduction in vascular tone [39, 40], the mechanism by which this occurs is incompletely understood. The mechanism of vasodilation in response to reduced membrane potential is widely reported to be a consequence of the closure of smooth muscle voltage-sensitive Ca^{2+} channels, a reduction in Ca^{2+} influx and, subsequently, a loss of vascular tone (Fig. 1) [41]. A steep relationship exists between membrane potential and Ca^{2+} influx, viz. a depolarization or hyperpolarization of as little as 3 mV can increase or decrease Ca^{2+} influx, respectively, by up to 2-fold [42]. In the isolated perfused heart, nitroarginine-insensitive vasodilation to bradykinin and responses to cromakalim, a K^+ channel agonist, are inhibited effectively with the voltage-sensitive Ca^{2+} channel antagonist nifedipine, which does not affect responses to nitroprusside [43]. However, this relationship becomes tenuous in conduit vessels, such as the aorta and large diameter coronary arteries, where nitroarginine-insensitive relaxant responses to bradykinin [44] and cromakalim [45] are not antagonized effectively by nifedipine. This may relate to a greater dependency of smaller arteries on calcium entry through voltage-gated channels to maintain vascular tone as opposed to receptor-operated calcium channels in larger vessels. Indeed, voltage-gated calcium channel antagonists are more potent vasodilators in smaller caliber arteries [46, 47]. The mechanism by which hyperpolarization produces relaxation in larger arteries is not well understood, and may relate to inhibition of PLC and both reduced calcium release and increased sequestration of Ca^{2+} in the endoplasmic reticulum [48]. *These discrepancies highlight the differences in calcium handling in smooth muscle across the vascular tree and suggest that hyperpolarization produces vascular relaxation through more than one mechanism.*

The potassium channel mediating agonist-induced hyperpolarization in vascular smooth muscle or in the endo-

thelium has not been fully defined. Different potassium channels exist within both of these cell types, including K_{Ca}, K_V, and K_{ATP} channels [42]. Most evidence to date, based on the use of the K_{ATP} antagonist glibenclamide, excludes the contribution of K_{ATP} channels [12, 30, 49–53]. K_{Ca} channels have been implicated in the vast majority of reports that are based on a pharmacological approach [43, 52, 54, 55]. K_{Ca} channels can be subdivided broadly into three subclasses: large, intermediate, and small conductance. Most of the antagonists of K_{Ca} channels do not discriminate between these subtypes. However, within the past several years antagonists of specific classes of K_{Ca} channels have been derived from the venoms of scorpions and bees. These peptides have enabled investigators to identify the contribution of large and small conductance K_{Ca} channels to endothelium-derived relaxant responses. Not surprisingly, not all of these antagonists are entirely selective. For example, charybdotoxin, an inhibitor of K_{Ca} channels, has been shown to inhibit both large and intermediate conductance K_{Ca} channels [56, 57], as well as several classes of K_V channels [58]. Based on the use of charybdotoxin and selective inhibitors of large and small conductance K_{Ca} channels, we have implicated the possible contribution of intermediate conductance K_{Ca} channels to the NO-independent vasodilator responses of bradykinin [59]. To confirm the identity of the K^+ channel, pharmacological inhibition of K^+ channels should be complemented with electrophysiological techniques. However, because of practical limitations, difficulties arise when microvessels are involved or are perfused *in situ* within an organ. A further complication is the inability to distinguish between the actions of the K^+ channel antagonists on smooth muscle or on the endothelium.

The elevation of Ca^{2+} within endothelial cells, from internal [60, 61] and/or extracellular sources [62], and the participation of calmodulin [63, 64] are critical for the synthesis or release of hyperpolarizing factor (Fig. 1). Sustained elevation of intracellular Ca^{2+} is facilitated by endothelial hyperpolarization, which renders the cytosol more electronegative and enhances the gradient for Ca^{2+} influx [65]. In contrast to vascular smooth muscle cells, endothelial cells are devoid of voltage-sensitive Ca^{2+} channels [66, 67] and, as a consequence, Ca^{2+} entry is not retarded by hyperpolarization or Ca^{2+} channel antagonists. The primary K^+ channel responsible for the agonist-induced reduction in membrane potential of endothelial cells is a K_{Ca} channel [62, 65], such that the elevation of intracellular Ca^{2+} facilitates K^+ efflux, which, as part of a positive feed-forward loop, promotes further Ca^{2+} influx. Although hyperpolarization is clearly important in the influx of Ca^{2+} in endothelial cells, the synthesis of NO, which is also dependent on Ca^{2+} and calmodulin, may not depend entirely on this reduction in membrane potential. High K^+ solutions or K^+ channel antagonists, in concentrations that eliminate agonist-induced hyperpolarization of endothelial cells, do not prevent the synthesis of NO completely [11, 12, 65]. The initial transient increase in intracellular Ca^{2+}, arising primarily from emptying of intracellular stores, is apparently sufficient to switch on the synthesis of NO, which then outlives the elevation of intracellular Ca^{2+} [68]. Serotonin [69], oxotremorine [21], and ADP can all induce the Ca^{2+}-dependent release of NO from the endothelium without a corresponding reduction in membrane potential of either the endothelium or the smooth muscle. In contrast, all of the endothelium-dependent vasodilators that reduce the membrane potential of smooth muscle also hyperpolarize endothelial cells [70, 71], implying either that the synthesis of a hyperpolarizing factor requires the sustained phase of Ca^{2+} influx or that smooth muscle and endothelial cell hyperpolarization are closely linked. Because agonist-induced hyperpolarization of endothelial cell membranes is remarkably similar in amplitude and duration to that seen in vascular smooth muscle, it has been postulated that passive transmission of the electrical signal from the endothelium to smooth muscle may occur via myoendothelial bridges such as gap junctions [36].

GAP JUNCTIONS

Gap junctions are channels between cells that facilitate exchange of second messengers such as Ca^{2+} and inositol phosphates as well as the conduction of electrical signals. When injected into cells, dye tracers, such as lucifer yellow, diffuse to adjacent cells via gap junctions, enabling the identification of discrete junctions between cells. Endothelial cells as well as smooth muscle cells are electrically and dye interconnected, permitting efficient coordination of electrical signals within these cell types [72, 73]. However, the presence of gap junctions between endothelium and smooth muscle has not been demonstrated histologically, but several types of myoendothelial junctions do exist and may permit conduction of electrical signals from smooth muscle to endothelium [74]. This unidirectional propagation may be possible because of the electrical coupling of the smooth muscle, viewed as a functional unit with a large surface area, conducting to the comparatively small surface area of endothelial cells. However, in smaller blood vessels using non-sulfated dye tracers, the reverse has been shown, i.e. unidirectional dye transfer from endothelium to smooth muscle [75]. It is conceivable, therefore, that electrical signals can be transmitted passively from endothelium to smooth muscle without the contribution of a diffusible factor. Alternatively, the elevation of calcium within the endothelium may result in the efflux of calcium ions to the smooth muscle via gap junctions. Localized calcium elevation or "calcium sparks" have actually been shown to produce relaxation via activation of smooth muscle K_{Ca} channels [76]. In larger diameter vessels there is considerable evidence to exclude the contribution of gap junctions. Inhibition of gap junctions with halothane, heptanol, or octanol, at concentrations that equal or exceed those used to inhibit dye transfer, an index of gap junction permeability, has not suppressed effectively endothelium-dependent

hyperpolarization of smooth muscle or vascular smooth muscle relaxation attributed to hyperpolarization [52, 74, 77]. Moreover, direct application of electrical pulses to the endothelium does not evoke an electrical response in the smooth muscle [78]. Further, the ability to transfer hyperpolarization from one blood vessel to another is strong evidence for the release, from the endothelium, of a diffusible factor that evokes hyperpolarization of the smooth muscle [30, 79, 80]. On a cautionary note, many of the above findings have not been or cannot be tested in the microcirculation where unidirectional gap junctions from endothelium to smooth muscle have been demonstrated.

IDENTIFICATION OF HYPERPOLARIZING FACTORS

As a result of the work conducted by several groups, the term "hyperpolarizing factor" was coined by Taylor and Weston in 1988 [81] to account for endothelium-dependent, but NO-independent, vasodilator responses to acetylcholine and bradykinin, which were attributed to EDHF. There are major problems in characterizing this factor by means of K^+ channel inhibitors, as hyperpolarization of the endothelium may facilitate release of a relaxing factor. Blockade of a K^+ channel in the endothelium, by reducing calcium influx [65], could not only prevent the release of a vasorelaxant mediator but could also prevent its effect at the level of the smooth muscle. To circumvent such problems and to differentiate between release and action, a superfusion bioassay system is the simplest and most direct approach, analogous to that used by Moncada's group in characterizing the release and action of NO [82]. The use of "sandwich" preparations of arterial strips demonstrated hyperpolarization to be transferable from strips of guinea pig carotid artery with endothelium to coronary strips denuded of endothelium [30]. Similar approaches, with regard to proximity of donor and recipient vessels, have demonstrated transferable hyperpolarization in pressurized rat femoral arteries [80] and canine coronary arteries [79]. The requirement of close proximity of donor and recipient tissues suggests a labile mediator and, thus far, has precluded the use of interventions directed at either the endothelium or smooth muscle to distinguish effects on endothelial release of EDHF versus activity of EDHF on vascular smooth muscle. More recently, however, Mombouli *et al.* [83] and Harder *et al.* [84] have demonstrated the release of a diffusible hyperpolarizing factor using a more conventional superfusion bioassay. The separation of donor and detector tissue in these experiments has demonstrated conclusively that a diffusible factor, derived from the endothelium, activates smooth muscle K^+ channels. It remains to be defined whether endothelial and smooth muscle K_{Ca} channels mediating hyperpolarization at each site are the same or distinct.

Another major obstacle in identifying EDHF also becomes apparent when reviewing the literature, that is, the possibility, or even the probability, that more than one unidentified factor exists depending on the species, tissue, and agonist studied. Indeed, even within the same species it appears that different vessels may utilize different hyperpolarizing factors. The ability of NO [32], prostaglandin I_2 [85], and H_2O_2 [86] to hyperpolarize smooth muscle demonstrates the capacity of the endothelium to generate more than one hyperpolarizing factor (Fig. 1). Studies with acetylcholine and bradykinin show differences in the susceptibility of the NO-independent responses to agents that inhibit the vasodilator effects of these agonists. For example, our studies of the rat kidney show that the vasodilator response to bradykinin can be reduced by CYP inhibitors that do not affect the renal vasodilator effect of acetylcholine at the same concentrations [17]. However, the responses to both vasodilators can be prevented by K^+ channel inhibitors including non-specific agents such as tetraethylammonium and procaine and the specific K_{Ca} channel inhibitor, charybdotoxin [43, 59]. In contrast, iberiotoxin, which is considered to be more specific than charybdotoxin as an inhibitor of large conductance K_{Ca} channels, was without effect on either bradykinin or acetylcholine-induced renal vasodilation. Inhibitors of small conductance K_{Ca} channels, such as apamin, antagonize NO-independent vasodilator responses to bradykinin in porcine coronary arteries [52], but do not affect those in the rat renal [59] or coronary circulations (unpublished observation), raising the possibility that either distinct EDHFs activate different K^+ channels or that an EDHF can activate more than one type of K^+ channel. These observations raise some key issues, viz, that the endothelium elaborates more than one hyperpolarizing factor, and, more importantly, that the effect of the K^+ channel inhibitors may occur at the level of the endothelium to prevent hyperpolarization in response to receptor stimulation, an effect that diminishes the release of a vasodilator mediator.

A CYP-AA METABOLITE AS A HYPERPOLARIZING FACTOR

The established action of bradykinin to stimulate phospholipases and the ability of CYP and PLA_2 inhibitors to attenuate vasodilator responses support the proposal first suggested by Vanhoutte in 1987 that EDHF may be a labile CYP-derived arachidonate metabolite [87]. Indeed, several earlier studies, addressing the identity of EDRF, had shown that CYP inhibitors attenuated endothelium-dependent vasodilation [88], although these effects could be attributed to the ability of CYP inhibitors to inhibit NOS, which is a CYP-like enzyme [89]. In view of recent studies, the effects of these agents probably result from activity against enzyme systems that generate EDHF as well as NO. CYP-dependent metabolism of AA results in the formation of epoxides (EETs) by the epoxygenase pathway and HETEs by the $(\omega)(\omega - 1)$ hydroxylase pathway. Of these products, an EET is the most likely candidate as a putative EDHF, a concept that has received considerable support from several recent investigations [18, 51, 54]. Thus, the highest levels

of CYP within the vasculature are localized to the intima [90], and EETs are produced by the endothelium [91, 92]. Conduit vessels such as the aorta do not normally synthesize EETs [93]. Moreover, EETs are vasodilators, especially of smaller, more peripheral vessels such as intestinal microvessels [94], caudal [95], cerebral [96], renal [97], and coronary arteries [52, 98, 99], as well as resistance arterioles of the kidney [100, 101]. EETs hyperpolarize vascular smooth muscle, an essential property of an EDHF candidate, by increasing the open probability of K_{Ca} channels [54, 96, 97, 102].

Our studies also support the identity of EDHF as a CYP-AA metabolite, at least as the mediator of the coronary and renal vasodilator actions of bradykinin (Fig. 1). The vasodilator effect of bradykinin in the rat perfused heart, treated with nitroarginine and indomethacin to exclude any contribution of NO and prostaglandins, is abolished by inhibitors of both PLC and PLA_2 [103]. Moreover, 7-ethoxyresorufin, clotrimazole, and 17-octadecynoic acid (17-ODYA), an inhibitor of CYP-dependent metabolism of long-chain fatty acids, reduce the coronary vasodilator effect of bradykinin [18], which is dependent on the activity of K_{Ca} channels as it is blocked by charybdotoxin [43]. As three structurally distinct inhibitors of CYP reduce the vasodilator response to bradykinin, this provides strong evidence for a role of this system. The possibility that these agents attenuate endothelium-dependent responses by preventing the CYP-dependent synthesis of NO from hydroxy-arginine can be excluded as endothelium-dependent vasodilation and/or hyperpolarization can be demonstrated when NO is bound by hemoglobin [19–23] and when NO stimulation of guanylate cyclase is prevented by methylene blue [20, 25, 104]. Further, NOS inhibitors such as N^{ω}-nitro-L-arginine methyl ester abolish the stimulated increases in cyclic GMP associated with endothelium-dependent vasodilation [7]. As the inhibitors of CYP differ in their capacity to prevent the formation of EETs versus HETEs, their effects provide insights as to the identity of the putative mediator. Thus, clotrimazole, which was the most effective inhibitor of the bradykinin vasodilator response [18], is more specific for the epoxygenase pathway of CYP metabolism of AA, whereas 17-ODYA and 7-ethoxyresorufin, which produced less attenuation of the response, do not exhibit differential inhibitory activity towards epoxygenase versus ω-hydroxylase [105]. These results, therefore, are consistent with an EET mediator of CYP-dependent coronary vasodilator responses to bradykinin. Moreover, results of GC–MS analysis of coronary perfusates indicate that the ω-hydroxylase pathway of AA metabolism is negligible in the rat heart, whereas EET release can be demonstrated (≈1 ng/min). The concept of an EET as an EDHF has received support from several laboratories. Campbell *et al.* [54] in a comprehensive study showed, in bovine coronary arteries, that muscarinic receptor stimulation elicited endothelium-dependent release of EETs, which were vasorelaxant by virtue of their ability to increase the open probability of charybdotoxin-sensitive K_{Ca} channels.

Pharmacological experiments using inhibitors of CYP and K^+ channels have their own limitations: SKF-525A antagonizes acetylcholine receptors, ion channels, and the effects of EDRF [106–108]; 7-ethoxyresorufin inhibits NOS [109]; the imidazole CYP inhibitors such as clotrimazole have been reported to affect K^+ channels [110, 111], NOS [112], and intracellular Ca^{2+}/calmodulin [113], and, therefore, effects could result from dual actions in preventing the synthesis/release of a vasodilator mediator as well as preventing its effect on smooth muscle. To minimize the possible contribution of non-specific inhibitory actions of CYP inhibitors, we have used inhibitors differing chemically and mechanistically. We also obtained responses to reference vasodilators that do not utilize CYP-dependent mechanisms and that should be unaffected by the inhibitor. In addition, we have shown that clotrimazole, which greatly attenuated the coronary vasodilator effect of bradykinin, was without effect on vasodilator responses to either the K_{ATP} channel agonist, cromakalim, or SCA40, an alleged opener of K_{Ca} channels, indicating that the primary effect of clotrimazole was to inhibit CYP [43]. These data, coupled with those demonstrating an absolute requirement for PLA_2 in the coronary vasodilator action of bradykinin, strongly suggest a role for a CYP-derived metabolite of AA in the coronary and renal circulations of the rat. However, in contrast to the results reported by Hecker *et al.* [52], the porcine coronary artery response to bradykinin was unaffected by inhibitors of all three pathways of AA, prompting Weintraub *et al.* [114] to postulate the existence of a fourth pathway of AA metabolism. An alternative explanation is provided by the findings that EETs, like HETEs, may be stored in tissue phospholipids and released by activation of phospholipases [115, 116]. Depending on the capacity of a given tissue or cell type to generate and store epoxides, inhibitors of CYP may be expected to have variable effects. Thus, Weintraub *et al.* [114] reported that clotrimazole was without effect on vasorelaxant responses of porcine coronary arteries to bradykinin but attenuated those to AA, presumably by preventing CYP-dependent conversion. As this group have shown that vasorelaxant responses to bradykinin are dependent on PLA_2 activity, the results are consistent with a stored form of the mediator. It should be recognized, however, that the porcine coronary artery also differs from most tissues studied in that the relaxant response to bradykinin is unaffected by charybdotoxin [52].

As noted, an EET is the most likely candidate to mediate vasodilator responses to bradykinin in the coronary circulation of the rat. In addition, Campbell *et al.* [54] have demonstrated increased EET formation by the bovine coronary artery in response to the cholinergic vasodilator, methacholine. However, in contrast to the rat coronary vasculature, which dilates to the greatest degree to the labile 5,6-EET (Fig. 2), bovine coronary arteries are equally relaxed by all four EET regioisomers that produce relaxation by opening K^+ channels [54]. These differences may

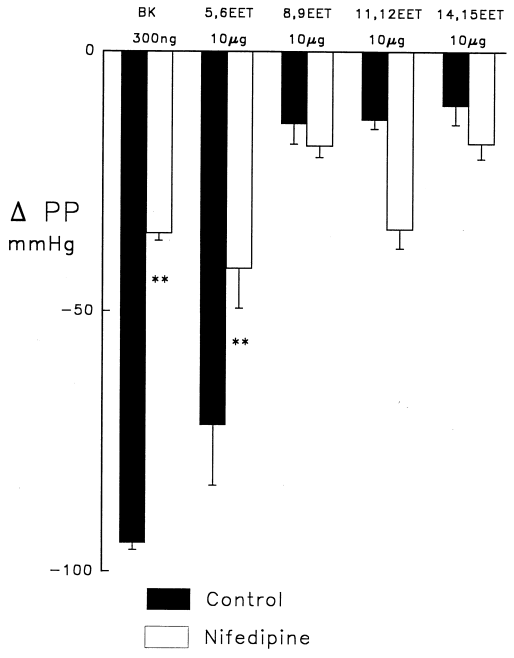

FIG. 2. Effect of nifedipine (5 nM) on vasodilator responses to bradykinin (BK; 300 ng) and epoxide regioisomers (10 μg) in the rat isolated, perfused heart constricted with nitroarginine (50 μM) and treated with indomethacin (2.8 μM). Values are means ± SEM, N = 4. Key: (**) $P < 0.01$.

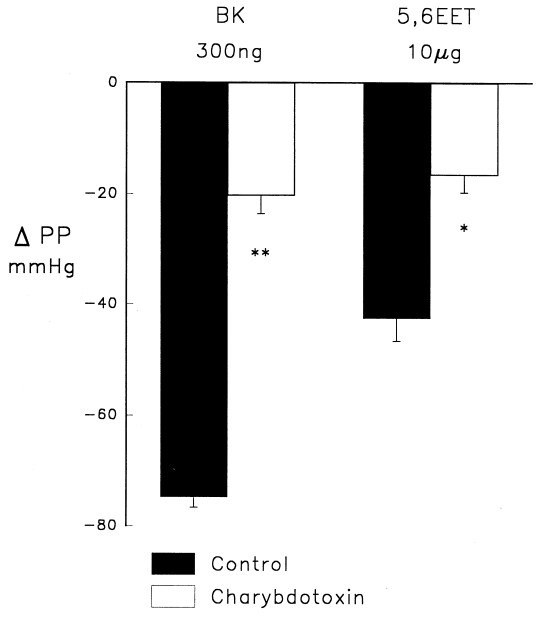

FIG. 3. Effect of charybdotoxin (15 nM) on vasodilator responses to bradykinin (BK; 300 ng) and 5,6-EET (10 μg) in the rat isolated, perfused heart constricted with nitroarginine (50 μM) and treated with indomethacin (2.8 μM). Values are means ± SEM, N = 4. Key: (*) $P < 0.05$, and (**) $P < 0.01$.

partially derive from the greater sensitivity of small vessels to the vasodilator/vasorelaxant effects of EETs. Further, our studies in the rat, using nifedipine, tend to exclude all of the EETs except 5,6-EET. Nifedipine, used as a probe for vasodilator mechanisms dependent on closure of voltage-sensitive Ca^{2+} channels following smooth muscle cell hyperpolarization, and which blocks the vasodilator effects of bradykinin and K^+ channel openers, attenuated the coronary vasodilator action of 5,6-EET, but not that of the other EET regioisomers (Fig. 2). Moreover, the coronary vasodilator effect of 5,6-EET, like bradykinin, was inhibited by charybdotoxin (Fig. 3). The 5,6-EET, although reported to produce cyclooxygenase-dependent renal vasoconstriction *in vivo* in the rat [117], is a direct vasodilator in Krebs perfused kidneys [101] and hearts (Fig. 2). The renal vasoconstrictor ability of 5,6-EET in the rat may arise from incorporation and metabolism in blood-borne elements such as platelets to form novel vasoconstrictor prostanoids. In addition, the ability of 5,6-EET to dilate the coronary circulation is not affected after the removal of the endothelium (unpublished observation), and, therefore, excludes the possibility that 5,6-EET directly elevates endothelial cell calcium to release the same mediator as bradykinin [118].

Although EETs are produced by the endothelium, are vasodilator in most vascular beds, and increase the activity of K^+ channels, they lack potency as vasodilators in isolated organs and large vessels where microgram quantities are usually required to elicit vascular effects [98, 100, 101]. For example, in the rat perfused heart, 10 μg 5,6-EET reduced perfusion pressure, an index of vasodilation, by approximately 70 mm Hg, whereas equivalent doses of the other EETs reduced perfusion pressure by 20 mm Hg or less. In contrast, nanogram doses of bradykinin elicit maximal vasodilation. However, EETs are reportedly more potent in vessels of smaller caliber [97]. The lack of potency of EETs has been attributed to their rapid uptake and acylation, their susceptibility to spontaneous degradation or metabolism by epoxide hydrolase to the inactive diol, as well as the requirement to traverse the endothelium in sufficient concentrations to induce a response. With respect to acylation of administered EETs, it is of interest to note that Mombouli *et al.* [83] were only able to demonstrate the release of a bioassayable factor in the presence of thimerosal to inhibit reacylation. However, in the rat heart we found that removal of the endothelium did not increase the sensitivity to EETs, which tends to exclude a major effect of reacylation by the endothelium in limiting the vasodilator response and which argues against the endothelium as a barrier (unpublished observation). Although competition with endogenous products could contribute to the reduced potency of CYP-derived eicosanoids and increased potency should be expected by treatment with a CYP inhibitor, this seems unlikely, as removal of the endothelium, the likely source of vascular EETs, did not influence the activity of administered EETs. Most studies with EETs have used racemic mixtures, whereas endogenous enzymatic synthesis results in the predominance of a specific enantiomer. In this regard, Roman's group has reported that 11*R*,12*S*-EET, but not 11*S*,12*R*-EET, is a potent dilator (nanomolar concentrations) of rat renal microvessels [97]. Indeed, the use of racemic mixtures may be misleading because of the possi-

bility of competition between the inactive and active isomers.

There are findings that raise potential objections to the proposed channel that EETs activate, i.e. whether they activate large or intermediate conductance K_{Ca} channels or both. Zou *et al.* [97] reported that 11,12-EET is a potent dilator of rat renal microvessels, an effect blocked by the selective inhibitor of large conductance K_{Ca} channels, iberiotoxin, which did not affect the vasodilator action of bradykinin [59] in kidneys treated with nitroarginine and indomethacin to isolate the CYP-dependent component of the response, and also was ineffective against the vasodilator response to 5,6-EET in the coronary circulation (unpublished observation). Although iberiotoxin, which is considered to be a more potent antagonist of large conductance K_{Ca} channels than charybdotoxin, was used at concentrations of up to 40 nM as compared with the 100 nM used by Zou *et al.* [97], it was completely ineffective. In contrast, charybdotoxin, which inhibits both large and intermediate conductance K_{Ca} channels, effectively antagonized coronary vasodilator responses to bradykinin and 5,6-EET (Fig. 3). Campbell *et al.* [54] have reported that EETs activate a large conductance, charybdotoxin-sensitive K_{Ca} channel of 240 pS in bovine coronary artery although the effects of iberiotoxin were not tested.

The relative lack of potency of EETs as renal and coronary vasodilators, in our hands, as well as the evidence linking CYP activity with AA release in the expression of vasodilator responses to bradykinin, prompted us to evaluate other potential mediators. For example, it is recognized that CYP-dependent metabolism results in the generation of free radicals such as superoxide [119], which, in turn, can lead to the formation of hydrogen peroxide, a vasodilator that activates K^+ channels [120] and hyperpolarizes smooth muscle [86]. Alternatively, we hypothesized that a CYP-dependent eicosanoid such as an EET may react with free radicals to yield a more active vasodilator. We demonstrated that CYP-dependent conversion of AA resulted in the generation of superoxide, but were unable to show that bradykinin stimulated superoxide formation in the perfused heart or that free radical scavengers affected the vasodilator action of bradykinin [121].

INTERACTIONS OF NO AND P450

The evidence pointing to a CYP-AA product acting as an EDHF, at least that released in response to bradykinin in the rat heart and kidney, invites some interesting possibilities regarding interactions or associations with NO. Thus, endotoxin shock, which is associated with induction of NOS and release of large amounts of NO, is known to result in inhibition of hepatic CYP-dependent metabolism. The mechanism underlying this long-known defect was elucidated by Khatsenko *et al.* [122], who provided evidence for an inhibitory effect of NO on CYP, presumably by binding to the heme prosthetic group. We used rat renal cortical microsomes as a source of CYP to confirm that NO or a donor of NO inhibits the NADPH-dependent conversion of [^{14}C]AA (unpublished observation). In keeping with a negative effect of NO on CYP-AA metabolism, we found that inhibition of NOS in the rat kidney increases the release of 20-HETE (Fig. 4). Thus, in vascular tissues where NO and CYP are expressed, CYP-dependent activity may be tonically inhibited by the production of NO. Consequently, inhibition of NOS and removal of the tonic inhibitor may enhance CYP activity, resulting in enhanced formation of a CYP-dependent EDHF, which then mediates the vasodilator effect of bradykinin when the NO-dependent system is compromised. Thus, the contribution of EDHF to vasodilation may vary according to the levels of NO, serving as a backup to a primary NO-dependent system in vessels where NO is highly expressed or dominating in those tissues where the levels of NO are low. EDHF expression is greater in microvessels than in large blood vessels and may be a major determinant of vascular tone in small resistance vessels. It is at the level of the microcirculation that NO–CYP interactions may be most important. This concept provides an explanation for some of our results in the rat heart, where inhibition of NOS does not affect the vasodilator action of bradykinin [10]. However, it should be pointed out that in the porcine coronary artery, inhibition of NO production with nitroarginine does not uncover or enhance hyperpolarization produced by bradykinin [8].

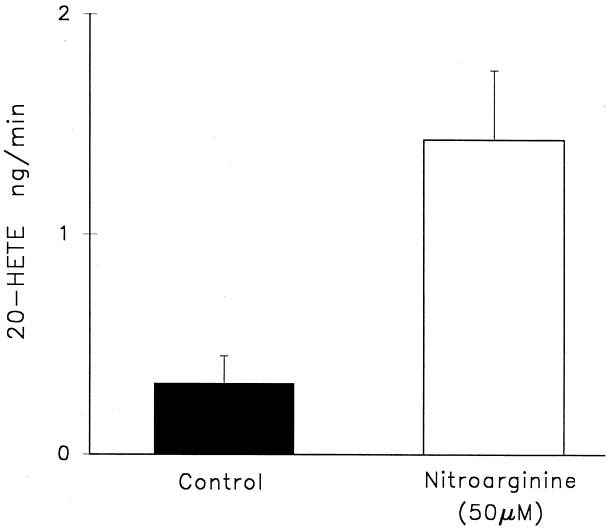

FIG. 4. Effect of inhibition of NO synthesis with nitroarginine on the release of 20-HETE, measured by GC–MS, into the perfusate of the rat isolated kidney constricted with phenylephrine to elevate perfusion pressure to approximately 180 mm Hg. Values are means ± SEM, N = 4.

REGULATION OF VASCULAR TONE BY EDHF

Although the evidence for an EDHF is considerable, definition of its contribution to the regulation of vascular tone is limited by the absence of a definitive assay and by the lack of selective inhibitors of K_{Ca} channels that can be

used *in vivo*. This limitation may not be easily overcome, as K^+ channels are ubiquitous, and inhibition of cardiac K^+ channels can have pronounced effects on cardiac rhythm. Most of the selective inhibitors of K_{Ca} channels are isolated from various toxins and, as such, their application *in vivo* may be limited by toxic effects related to channel inhibition. A further limitation is the lack of availability of agonists for specific types of K_{Ca} channels that are required to ensure that interventions aimed at reducing synthesis/release of EDHFs do not also affect the target K^+ channel. If an EDHF is an important regulator of vascular function, then specific inhibition of its formation or action should result in increased vascular tone and, possibly, elevation of blood pressure. However, inhibition of CYP produces little effect on blood pressure, except under conditions of salt-loading in Dahl-R rats in which inhibition of epoxygenase rendered the animals salt-sensitive, an effect that may relate to diminished capacity of the kidney to excrete salt rather than a vascular effect [123]. Nonetheless, in perfused organ systems, we have found that K^+ channel inhibitors that affect intermediate conductance K_{Ca} elevate perfusion pressure even in the presence of NOS inhibition, suggesting a role for these channels in the regulation of vascular tone [43]. Because of the nature of these channels, i.e. activation by calcium, the possibility arises that these channels are activated as a result of increased vascular tone in response to NOS inhibition rather than tonic release of an EDHF [124]. This may explain why there is no increase in blood pressure following CYP inhibition, viz. tonic release may not be required as channels are already active. Indeed, removal of the endothelium does not depolarize vascular smooth muscle as would be expected under conditions of continuous basal release of EDHF [125]. It has been proposed that pulsatile pressure is the stimulus for basal EDHF [13], whereas shear stress stimulates NO formation. Further developments in this area await identification of EDHF and the availability of specific probes, e.g. specific inhibitors of the CYP isozyme responsible for the generation of the putative mediator, which, in turn, awaits a method for reliably detecting EDHF. Unlike studies of NO, cultured endothelial cells cannot be used, as CYP activity is rapidly lost upon passage [126] and the use of fresh endothelial cells is mandated, which poses practical problems regarding harvesting of sufficient amounts of tissue.

EDHF AND ALTERED VASCULAR RESPONSIVENESS

Hypertension and diabetes mellitus are conditions associated with impairments of endothelium-dependent vasodilation [127], which are generally attributed to reductions in NO, possibly resulting from its increased inactivation [128]. However, the available evidence also suggests that vasodilator mechanisms that utilize K^+ channels are also modified in these conditions [7, 129]. In hypertension, enhanced activity may constitute a protective mechanism, whereas in diabetes, diminished activity may contribute to some vascular complications.

There is evidence that hypertension results in a compensatory increase in the activity of K^+ channels and, possibly, increased synthesis/release of the putative hyperpolarizing factors, the EETs. Thus, the vasorelaxant effects of K^+ channel openers are amplified in vessels from hypertensive rats, a phenomenon that could reflect increased activity of voltage-gated Ca^{2+} channels [130] such that hyperpolarization, which promotes closure of these channels and reduces intracellular calcium, elicits greater responses. Further, vasodilator responses to agonists, namely bradykinin, that utilize EDHF are increased in kidneys of spontaneously hypertensive rats (SHR) [7]. Greater expression of a hyperpolarizing mechanism could result from increased synthesis/release of an EDHF, increased responsiveness to the EDHF, decreased degradation, or a combination of these. Our preliminary findings support the last as renal vasodilator responses to bradykinin are enhanced, associated with increased release of EETs into the perfusate of kidneys from SHR. Renal vasodilator responses to cromakalim are also increased in SHR.

Diabetes, on the other hand, is associated with diminished responsiveness to vasodilators that activate K^+ channels [131]. Similarly, CYP-dependent vasodilator responses to bradykinin in the rat heart and kidney are reduced by diabetes [132]. Analysis of renal perfusates from diabetic animals reveals dramatically reduced levels of 20-HETE, an index of CYP activity. These changes persist even following administration of AA, excluding possible deficiencies in substrate or PLA_2. When renal cortical microsomes from diabetic animals are incubated with excess NADPH, the deficiency in AA metabolism is no longer apparent [133]. A possible mechanism for this has been proposed by Cameron and Cotter [134] to account for reduced synthesis of NO in diabetes—that increased activity of aldose reductase, an NADPH-dependent enzyme, reduces the availability of cofactors such as NADPH that are required for NOS activity. The activity of CYP, which is also an NADPH-dependent enzyme, could be reduced in a similar way consequent to increased activity of aldose reductase to produce sorbitol. Preliminary data showing that inhibition of aldose reductase partially restores vasodilator responses to bradykinin in diabetic rat hearts are consistent with this hypothesis [132].

This work was supported by NIH Grants HL492750 and RO1 HL 25394 and by AHA Grant 940-318.

References

1. Furchgott RF and Zawadzki JV, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288:** 373–376, 1980.
2. Vane JR, Anggard EE and Botting RM, Regulatory functions of the vascular endothelium. *N Engl J Med* **323:** 27–36, 1990.

3. Palmer RM, Ferrige AG and Moncada S, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327:** 524–526, 1987.
4. Ignarro LJ, Buga GM, Wood KS, Byrns RE and Chaudhuri G, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* **84:** 9265–9269, 1987.
5. Rees DD, Palmer RM and Moncada S, Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* **86:** 3375–3378, 1989.
6. Rees DD, Palmer RM, Hodson HF and Moncada S, A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br J Pharmacol* **96:** 418–424, 1989.
7. Cachofeiro V and Nasjletti A, Increased vascular responsiveness to bradykinin in kidneys of spontaneously hypertensive rats. Effect of N^{ω}-nitro-L-arginine. *Hypertension* **18:** 683–688, 1991.
8. Pacicca C, von der Weid PY and Beny JL, Effect of nitro-L-arginine on endothelium-dependent hyperpolarizations and relaxations of pig coronary arteries. *J Physiol (Lond)* **457:** 247–256, 1992.
9. Nagao T, Illiano S and Vanhoutte PM, Heterogeneous distribution of endothelium-dependent relaxations resistant to NG-nitro-L-arginine in rats. *Am J Physiol* **263:** H1090–H1094, 1992.
10. Baydoun AR and Woodward B, Effects of bradykinin in the rat isolated perfused heart: Role of kinin receptors and endothelium-derived relaxing factor. *Br J Pharmacol* **103:** 1829–1833, 1991.
11. Cowan CL and Cohen RA, Two mechanisms mediate relaxation by bradykinin of pig coronary artery: NO-dependent and -independent responses. *Am J Physiol* **261:** H830–H835, 1991.
12. Adeagbo AS and Triggle CR, Varying extracellular $[K^+]$: A functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J Cardiovasc Pharmacol* **21:** 423–429, 1993.
13. Popp R, Bauersachs J, Fleming I and Busse R, Pulsatile stretch ensures the continuous release of EDHF in porcine coronary arteries. *Pflugers Arch* **433**(suppl)**:** R29, 1997.
14. Buga GM, Gold ME, Fukuto JM and Ignarro LJ, Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension* **17:** 187–193, 1991.
15. Palmer RM, Rees DD, Ashton DS and Moncada S, L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem Biophys Res Commun* **153:** 1251–1256, 1988.
16. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M and Takeshita A, The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* **28:** 703–711, 1996.
17. Fulton D, McGiff JC and Quilley J, Contribution of NO and cytochrome P450 to the vasodilator effect of bradykinin in the rat kidney. *Br J Pharmacol* **107:** 722–725, 1992.
18. Fulton D, Mahboubi K, McGiff JC and Quilley J, Cytochrome P450-dependent effects of bradykinin in the rat heart. *Br J Pharmacol* **114:** 99–102, 1995.
19. Beny JL, Brunet PC and van der Bent V, Hemoglobin causes both endothelium-dependent and endothelium-independent contraction of the pig coronary arteries, independently of an inhibition of EDRF effects. *Experientia* **45:** 132–134, 1989.
20. Chen G, Suzuki H and Weston AH, Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br J Pharmacol* **95:** 1165–1174, 1988.
21. Komori K, Lorenz RR and Vanhoutte PM, Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. *Am J Physiol* **255:** H207–H212, 1988.
22. Huang AH, Busse R and Bassenge E, Endothelium-dependent hyperpolarization of smooth muscle cells in rabbit femoral arteries is not mediated by EDRF (nitric oxide). *Naunyn Schmiedebergs Arch Pharmacol* **338:** 438–442, 1988.
23. Nishiye E, Nakao K, Itoh T and Kuriyama H, Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin. *Br J Pharmacol* **96:** 645–655, 1989.
24. Cocks TM and Angus JA, Evidence that contractions of isolated arteries by L-NMMA and NOLA are not due to inhibition of basal EDRF release. *J Cardiovasc Pharmacol* **17:** S159–S164, 1991.
25. Komori K and Vanhoutte PM, Endothelium-derived hyperpolarizing factor. *Blood Vessels* **27:** 238–245, 1990.
26. Brayden JE, Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am J Physiol* **259:** H668–H673, 1990.
27. Funaki S and Bohr DV, Electrical and mechanical activity of isolated vascular smooth muscle of the rat. *Nature* **203:** 192–194, 1964.
28. Kuriyama H and Suzuki H, The effects of acetylcholine on the membrane and contractile properties of smooth muscle cells of the rabbit superior mesenteric artery. *Br J Pharmacol* **64:** 493–501, 1978.
29. Bolton TB, Lang RJ and Takewaki T, Mechanism of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. *J Physiol (Lond)* **351:** 549–572, 1984.
30. Chen G, Yamamoto Y, Miwa K and Suzuki H, Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *Am J Physiol* **260:** H1888–H1892, 1991.
31. Nagao T and Vanhoutte PM, Hyperpolarization contributes to endothelium-dependent relaxations to acetylcholine in femoral veins of rats. *Am J Physiol* **261:** H1034–H1037, 1991.
32. Bolotina VM, Najibi S, Palacino JJ, Pagano PJ and Cohen RA, Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368:** 850–853, 1994.
33. Robertson BE, Schubert R, Hescheler J and Nelson MT, cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am J Physiol* **265:** C299–C303, 1993.
34. Miyoshi H and Nakaya Y, Endotoxin-induced nonendothelial nitric oxide activates the Ca^{2+}-activated K^+ channel in cultured vascular smooth muscle cells. *J Mol Cell Cardiol* **26:** 1487–1495, 1994.
35. Beny JL and Brunet PC, Neither nitric oxide nor nitroglycerin accounts for all the characteristics of endothelially mediated vasodilatation of pig coronary arteries. *Blood Vessels* **25:** 308–311, 1988.
36. Brunet PC and Beny JL, Substance P and bradykinin hyperpolarize pig coronary artery endothelial cells in primary culture. *Blood Vessels* **26:** 228–234, 1989.
37. Nagao T and Vanhoutte PM, Hyperpolarization as a mechanism for endothelium-dependent relaxations in the porcine coronary artery. *J Physiol (Lond)* **445:** 355–367, 1992.
38. Cohen RA and Vanhoutte PM, Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. *Circulation* **92:** 3337–3349, 1995.
39. Mekata F, The role of hyperpolarization in the relaxation of smooth muscle of monkey coronary artery. *J Physiol (Lond)* **371:** 257–265, 1986.

40. Parkington HC, Tonta MA, Coleman HA and Tare M, Role of membrane potential in endothelium-dependent relaxation of guinea-pig coronary arterial smooth muscle. *J Physiol (Lond)* **484:** 469–480, 1995.
41. Gurney AM, Mechanisms of drug-induced vasodilation. *J Pharm Pharmacol* **46:** 242–251, 1994.
42. Nelson MT and Quayle JM, Physiological role and properties of potassium channels in arterial smooth muscle. *Am J Physiol* **268:** C799–C822, 1995.
43. Fulton D, McGiff JC and Quilley J, Role of K^+ channels in the vasodilator response to bradykinin in the rat heart. *Br J Pharmacol* **113:** 954–958, 1994.
44. Kilpatrick EV and Cocks TM, Evidence for differential roles of nitric oxide (NO) and hyperpolarization in endothelium-dependent relaxation of pig isolated coronary artery. *Br J Pharmacol* **112:** 557–565, 1994.
45. Kuhberger E, Kukovetz WR and Groschner K, Cromakalim inhibits multiple mechanisms of smooth muscle activation with similar stereoselectivity. *J Cardiovasc Pharmacol* **21:** 947–954, 1993.
46. Mustafa SJ and Askar AO, Effect of calcium entry blockers and adenosine on the relaxation of large and small coronary arteries. *Life Sci* **38:** 877–885, 1986.
47. Hintze TH and Vatner SF, Comparison of effects of nifedipine and nitroglycerin on large and small coronary arteries and cardiac function in conscious dogs. *Circ Res* **52:** I139–I146, 1983.
48. Quast U, Do the K^+ channel openers relax smooth muscle by opening K^+ channels? *Trends Pharmacol Sci* **14:** 332–337, 1993.
49. Nakashima M, Mombouli JV, Taylor AA and Vanhoutte PM, Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. *J Clin Invest* **92:** 2867–2871, 1993.
50. Kitagawa S, Yamaguchi Y, Kunitomo M, Sameshima E and Fujiwara M, NG-nitro-L-arginine-resistant endothelium-dependent relaxation induced by acetylcholine in the rabbit renal artery. *Life Sci* **55:** 491–498, 1994.
51. Zygmunt PM, Waldeck K and Hogestatt ED, The endothelium mediates a nitric oxide-independent hyperpolarization and relaxation in the rat hepatic artery. *Acta Physiol Scand* **152:** 375–384, 1994.
52. Hecker M, Bara AT, Bauersachs J and Busse R, Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J Physiol (Lond)* **481:** 407–414, 1994.
53. Cowan CL and Cohen RA, Different mechanisms of relaxation of pig coronary artery to bradykinin and cromakalim are distinguished by potassium channel blockers. *J Pharmacol Exp Ther* **260:** 248–253, 1992.
54. Campbell WB, Gebremedhin D, Pratt PF and Harder DR, Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* **78:** 415–423, 1996.
55. Bauersachs J, Hecker M and Busse R, Display of the characteristics of endothelium-derived hyperpolarizing factor by a cytochrome P450-derived arachidonic acid metabolite in the coronary microcirculation. *Br J Pharmacol* **113:** 1548–1553, 1994.
56. Garcia ML, Knaus HG, Munujos P, Slaughter RS and Kaczorowski GJ, Charybdotoxin and its effects on potassium channels. *Am J Physiol* **269:** C1–C10, 1995.
57. Brugnara C, Armsby CC, DeFranceschi L, Crest M, Martin Euclaire MF and Alper SL, Ca^{2+}-activated K^+ channels of human and rabbit erythrocytes display distinctive patterns of inhibition by venom peptide toxins. *J Membr Biol* **147:** 71–82, 1995.
58. Garcia-Calvo M, Leonard RJ, Novick J, Stevens SP, Schmalhofer W, Kaczorowski GJ and Garcia ML, Purification, characterization, and biosynthesis of margatoxin, a component of *Centruroides margaritatus* venom that selectively inhibits voltage-dependent potassium channels. *J Biol Chem* **268:** 18866–18874, 1993.
59. Rapacon M, Mieyal P, McGiff JC, Fulton D and Quilley J, NO-independent renal vasodilation to bradykinin: Role of K^+ channels. *Br J Pharmacol* **118:** 1504–1508, 1996.
60. Weintraub NL, Stephenson AH, Sprague RS, McMurdo L and Lonigro AJ, Relationship of arachidonic acid release to porcine coronary artery relaxation. *Hypertension* **26:** 684–690, 1995.
61. Marchenko SM and Sage SO, Mechanism of acetylcholine action on membrane potential of endothelium of intact rat aorta. *Am J Physiol* **266:** H2388–H2395, 1994.
62. Cheung DW and Chen G, Calcium activation of hyperpolarization response to acetylcholine in coronary endothelial cells. *J Cardiovasc Pharmacol* **20**(Suppl 12): S120–S123, 1992.
63. Illiano S, Nagao T and Vanhoutte PM, Calmidazolium, a calmodulin inhibitor, inhibits endothelium-dependent relaxations resistant to nitro-L-arginine in the canine coronary artery. *Br J Pharmacol* **107:** 387–392, 1992.
64. Nagao T, Illiano S and Vanhoutte PM, Calmodulin antagonists inhibit endothelium-dependent hyperpolarization in the canine coronary artery. *Br J Pharmacol* **107:** 382–386, 1992.
65. Kukovetz WR, Graier WF and Groschner K, Contribution of agonist-induced hyperpolarization to Ca^{2+} influx and formation of EDRF in vascular endothelial cells. *Jpn J Pharmacol* **58**(Suppl 2): 213P–219P, 1992.
66. Takeda K and Klepper M, Voltage-dependent and agonist-activated ionic currents in vascular endothelial cells: A review. *Blood Vessels* **27:** 169–183, 1990.
67. Adams DJ, Barakeh J, Laskey R and van Breemen C, Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J* **3:** 2389–2400, 1989.
68. Blatter LA, Taha Z, Mesaros S, Shacklock PS, Wier WG and Malinski T, Simultaneous measurements of Ca^{2+} and nitric oxide in bradykinin-stimulated vascular endothelial cells. *Circ Res* **76:** 922–924, 1995.
69. Frieden M and Beny JL, Effect of 5-hydroxytryptamine on the membrane potential of endothelial and smooth muscle cells in the pig coronary artery. *Br J Pharmacol* **115:** 95–100, 1995.
70. von der Weid PY and Beny JL, Effect of Ca^{2+} ionophores on membrane potential of pig coronary artery endothelial cells. *Am J Physiol* **262:** H1823–H1831, 1992.
71. Chen G and Cheung DW, Characterization of acetylcholine-induced membrane hyperpolarization in endothelial cells. *Circ Res* **70:** 257–263, 1992.
72. Beny JL and Gribi F, Dye and electrical coupling of endothelial cells *in situ*. *Tissue Cell* **21:** 797–802, 1989.
73. Beny JL and Connat JL, An electron-microscopic study of smooth muscle cell dye coupling in the pig coronary arteries. Role of gap junctions. *Circ Res* **70:** 49–55, 1992.
74. Beny JL and Pacicca C, Bidirectional electrical communication between smooth muscle and endothelial cells in the pig coronary artery. *Am J Physiol* **266:** H1465–H1472, 1994.
75. Little TL, Xia J and Duling BR, Dye tracers define differential endothelial and smooth muscle coupling patterns within the arteriolar wall. *Circ Res* **76:** 498–504, 1995.
76. Nelson MT, Cheng H, Rubart M, Santana LF, Bonev D, Knot HJ and Lederer WJ, Relaxation of arterial smooth muscle by calcium sparks. *Science* **270:** 633–637, 1995.
77. Kuhberger E, Groschner K, Kukovetz WR and Brunner F, The role of myoendothelial cell contact in non-nitric oxide-, non-prostanoid-mediated endothelium-dependent relax-

ation of porcine coronary artery. *Br J Pharmacol* **113:** 1289–1294, 1994.
78. Beny JL, Endothelial and smooth muscle cells hyperpolarized by bradykinin are not dye coupled. *Am J Physiol* **258:** H836–H841, 1990.
79. Feletou M and Vanhoutte PM, Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol* **93:** 515–524, 1988.
80. Kauser K, Stekiel WJ, Rubanyi G and Harder DR, Mechanism of action of EDRF on pressurized arteries: Effect on K^+ conductance. *Circ Res* **65:** 199–204, 1989.
81. Taylor SG and Weston AH, Endothelium-derived hyperpolarizing factor: A new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol Sci* **9:** 272–274, 1988.
82. Moncada S, Palmer RM and Gryglewski RJ, Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc Natl Acad Sci USA* **83:** 9164–9168, 1986.
83. Mombouli JV, Bissiriou I, Agboton VD and Vanhoutte PM, Bioassay of endothelium-derived hyperpolarizing factor. *Biochem Biophys Res Commun* **221:** 484–488, 1996.
84. Harder DR, Campbell WB, Pratt PF, Jacobs ER and Gebremedhin D, Bioassay of endothelium derived cytochrome P450-dependent hyperpolarizing factor from bovine coronary arteries. *FASEB J* **10:** A301, 1996.
85. Parkington HC, Tare M, Tonta MA and Coleman HA, Stretch revealed three components in the hyperpolarization of guinea-pig coronary artery in response to acetylcholine. *J Physiol (Lond)* **465:** 459–476, 1993.
86. Beny JL and von der Weid PY, Hydrogen peroxide: An endogenous smooth muscle cell hyperpolarizing factor. *Biochem Biophys Res Commun* **176:** 378–384, 1991.
87. Vanhoutte PM, Vascular physiology: The end of the quest? *Nature* **327:** 459–460, 1987.
88. Singer HA, Saye JA and Peach MJ, Effects of cytochrome P-450 inhibitors on endothelium-dependent relaxation in rabbit aorta. *Blood Vessels* **21:** 223–230, 1984.
89. Marletta MA, Nitric oxide synthase: Aspects concerning structure and catalysis. *Cell* **78:** 927–930, 1994.
90. Abraham NG, Pinto A, Mullane KM, Levere RD and Spokas E, Presence of cytochrome P-450-dependent monooxygenase in intimal cells of the hog aorta. *Hypertension* **7:** 899–904, 1985.
91. Rosolowsky M, Falck JR and Campbell WB, Synthesis and biological activity of epoxyeicosatrienoic acids (EETs) by cultured bovine coronary artery endothelial cells. *Adv Prostaglandin Thromboxane Leukot Res* **21A:** 213–216, 1991.
92. Rosolowsky M and Campbell WB, Synthesis of hydroxyeicosatetraenoic (HETEs) and epoxyeicosatrienoic acid (EETs) by cultured bovine coronary artery endothelial cells. *Biochim Biophys Acta* **1299:** 267–277, 1996.
93. Pfister SL, Falck JR and Campbell WB, Enhanced synthesis of epoxyeicosatrienoic acids by cholesterol-fed rabbit aorta. *Am J Physiol* **261:** H843–H852, 1991.
94. Proctor KG, Falck JR and Capdevila J, Intestinal vasodilation by epoxyeicosatrienoic acids: Arachidonic acid metabolites produced by a cytochrome P450 monooxygenase. *Circ Res* **60:** 50–59, 1987.
95. Carroll MA, Garcia MP, Falck JR and McGiff JC, 5,6-Epoxyeicosatrienoic acid, a novel arachidonate metabolite. Mechanism of vasoactivity in the rat. *Circ Res* **67:** 1082–1088, 1990.
96. Gebremedhin D, Ma YH, Falck JR, Roman RJ, VanRollins M and Harder DR, Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am J Physiol* **263:** H519–H525, 1992.
97. Zou AP, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR and Roman RJ, Regional and stereospecific effects of 11,12-epoxyeicosatrienoic acids on vascular tone and K^+ channel activity in renal arterioles. *Hypertension* **24:** 382, 1994.
98. Rosolowsky M and Campbell WB, Role of PGI_2 and epoxyeicosatrienoic acids in relaxation of bovine coronary arteries to arachidonic acid. *Am J Physiol* **264:** H327–H335, 1993.
99. Rosolowsky M, Falck JR, Willerson JT and Campbell WB, Synthesis of lipoxygenase and epoxygenase products of arachidonic acid by normal and stenosed canine coronary arteries. *Circ Res* **66:** 608–621, 1990.
100. Carroll MA, Balazy M, Margiotta P, Falck JR and McGiff JC, Renal vasodilator activity of 5,6-epoxyeicosatrienoic acid depends upon conversion by cyclooxygenase and release of prostaglandins. *J Biol Chem* **268:** 12260–12266, 1993.
101. Fulton D, Balazy M, McGiff JC and Quilley J, Possible contribution of platelet cyclooxygenase to the renal vascular action of 5,6-epoxyeicosatrienoic acid. *J Pharmacol Exp Ther* **277:** 1195–1199, 1996.
102. Hu S and Kim HS, Activation of K^+ channel in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acid. *Eur J Pharmacol* **230:** 215–221, 1993.
103. Fulton D, McGiff JC and Quilley J, Role of phospholipase C and phospholipase A_2 in the nitric oxide-independent vasodilator effect of bradykinin in the rat perfused heart. *J Pharmacol Exp Ther* **278:** 518–526, 1996.
104. Menon NK, Pataricza J, Zehetgruber M and Bing RJ, A model to study physiological activation of phospholipase A_2 and vasorelaxation by lysophosphatidylcholine. *Life Sci* **47:** 1941–1948, 1990.
105. Harder DR, Campbell WB and Roman RJ, Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone. *J Vasc Res* **32:** 79–92, 1995.
106. Choo LK, Malta E and Mitchelson F, Investigation of the antimuscarinic and other actions of proadifen *in-vitro*. *J Pharm Pharmacol* **38:** 898–901, 1986.
107. Pinto A, Abraham NG and Mullane KM, Arachidonic acid-induced endothelial-dependent relaxations of canine coronary arteries: Contribution of a cytochrome P-450-dependent pathway. *J Pharmacol Exp Ther* **240:** 856–863, 1987.
108. Xie JM, Wang Y and Greenberg SS, SKF-525A does not inhibit release of endothelium-derived relaxing factor from rat thoracic aorta and dog mesenteric and femoral artery. *Blood Vessels* **28:** 475–489, 1991.
109. Bennett BM, McDonald BJ, Nigam R, Long PG and Simon WC, Inhibition of nitrovasodilator- and acetylcholine-induced relaxation and cyclic GMP accumulation by the cytochrome P-450 substrate, 7-ethoxyresorufin. *Can J Physiol Pharmacol* **70:** 1297–1303, 1992.
110. Brugnara C, de Franceschi L and Alper SL, Inhibition of Ca^{2+}-dependent K^+ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. *J Clin Invest* **92:** 520–526, 1993.
111. Brugnara C, Armsby CC, Sakamoto M, Rifai N, Alper SL and Platt O, Oral administration of clotrimazole and blockade of human erythrocyte Ca^{++}-activated K^+ channel: The imidazole ring is not required for inhibitory activity. *J Pharmacol Exp Ther* **273:** 266–272, 1995.
112. Dudek RR, Conforto A, Pinto V, Wildhirt S and Suzuki H, Inhibition of endothelial nitric oxide synthase by cytochrome P-450 reductase inhibitors. *Proc Soc Exp Biol Med* **209:** 60–64, 1995.
113. Wolff DJ, Datto GA, Samatovicz RA and Tempsick RA, Calmodulin-dependent nitric-oxide synthase. Mechanism of inhibition by imidazole and phenylimidazoles. *J Biol Chem* **268:** 9425–9429, 1993.
114. Weintraub NL, Joshi SN, Branch CA, Stephenson AH, Sprague RS and Lonigro AJ, Relaxation of porcine coronary

artery to bradykinin. Role of arachidonic acid. *Hypertension* **23:** 976–981, 1994.
115. Capdevila JH, Kishore V, Dishman E, Blair IA and Falck JR, A novel pool of rat liver inositol and ethanolamine phospholipids contains epoxyeicosatrienoic acids (EETs). *Biochem Biophys Res Commun* **146:** 638–644, 1987.
116. Zhu Y, Schieber EB, McGiff JC and Balazy M, Identification of arachidonate P-450 metabolites in human platelet phospholipids. *Hypertension* **25:** 854–859, 1995.
117. Takahashi K, Capdevila J, Karara A, Falck JR, Jacobson HR and Badr KF, Cytochrome P-450 arachidonate metabolites in rat kidney: Characterization and hemodynamic responses. *Am J Physiol* **258:** F781–F789, 1990.
118. Graier WF, Simecek S and Sturek M, Cytochrome P450 mono-oxygenase-regulated signalling of Ca^{2+} entry in human and bovine endothelial cells. *J Physiol (Lond)* **482:** 259–274, 1995.
119. Bondy SC and Naderi S, Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem Pharmacol* **48:** 155–159, 1994.
120. Vega Saenz de Miera E and Rudy B, Modulation of K^+ channels by hydrogen peroxide. *Biochem Biophys Res Commun* **186:** 1681–1687, 1992.
121. Fulton D, McGiff JC, Kaminski P, Wolin MS and Quilley J, Evidence against a P450-derived free radical as the mediator of the NO-independent vasodilator effect of bradykinin in the rat perfused heart. *J Pharmacol Exp Ther* **280:** 702–709, 1997.
122. Khatsenko OG, Gross SS, Rifkind AB and Vane JR, Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. *Proc Natl Acad Sci USA* **90:** 11147–11151, 1993.
123. Makita K, Takahashi K, Karara A, Jacobson HR, Falck JR and Capdevila JH, Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. *J Clin Invest* **94:** 2414–2420, 1994.
124. Brayden JE and Nelson MT, Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* **256:** 532–535, 1992.
125. Nagao T and Vanhoutte PM, Endothelium-derived hyperpolarizing factor and endothelium-dependent relaxations. *Am J Respir Cell Mol Biol* **8:** 1–6, 1993.
126. Guzelian PS, Bissell DM and Meyer UA, Drug metabolism in adult rat hepatocytes in primary monolayer culture. *Gastroenterology* **72:** 1232–1239, 1977.
127. Quilley J, Sarubbi D and McGiff JC, Influence of diabetes mellitus on renal vascular responses to bradykinin. *Agents Actions* **38:** 31–35, 1992.
128. Tesfamariam B, Selective impairment of endothelium-dependent relaxations by prostaglandin endoperoxide. *J Hypertens* **12:** 41–47, 1994.
129. Asano M, Masuzawa-Ito K and Matsuda T, Charybdotoxin-sensitive K^+ channels regulate the myogenic tone in the resting state of arteries from spontaneously hypertensive rats. *Br J Pharmacol* **108:** 214–222, 1993.
130. Watts SW, Finta KM, Lloyd MC, Storm DS and Webb RC, Enhanced vascular responsiveness to Bay K 8644 in mineralocorticoid- and *N*-nitro arginine-induced hypertension. *Blood Press* **3:** 340–348, 1994.
131. Mayhan WG, Effect of diabetes mellitus on response of the basilar artery to activation of ATP-sensitive potassium channels. *Brain Res* **636:** 35–39, 1994.
132. Quilley J, McGiff JC, Mieyal P, Rapacon M and Fulton D, NO-independent coronary vasodilation to bradykinin in diabetes. *Hypertension* **28:** 552, 1996.
133. Fulton D, McGiff JC and Quilley J, Cytochrome P450 arachidonate metabolites (P450-AA): Deficit in diabetes mellitus. *FASEB J* **9:** A113, 1996.
134. Cameron NE and Cotter MA, Impaired contraction and relaxation in aorta from streptozotocin-diabetic rats: Role of polyol pathway. *Diabetologia* **35:** 1011–1019, 1992.