Invited Review

The Endothelium in Health and Disease —A Target for Therapeutic Intervention

Chris R. TRIGGLE[1], Morley HOLLENBERG[1], Todd J. ANDERSON[1], Hong DING[2], YanFen JIANG[1], Lisa CERONI[1], William B. WIEHLER[1], Ella S.M. NG[1], Anthie ELLIS[1], Karen ANDREWS[2], John J. MCGUIRE[1] and Malarvannan PANNIRSELVAM[1]

[1]*Smooth Muscle Research Group and Departments of Pharmacology & Therapeutics and Medicine, Faculty of Medicine, University of Calgary, Alberta, Canada, T2N 4N1*
[2]*Microvascular Biology Research Group, School of Medical Sciences, RMIT University, Bundoora, Victoria 3083, Australia*

Abstract

In this review we discuss the contribution of NO, prostacyclin and endothelium-derived relaxing factor — endothelium-derived hyperpolarizing factor, or EDHF, to vascular function. We also explore the hypotheses (1): that tissues can store NO as nitrosothiols (RSNOs) and (2) that such RSNO stores can be modulated by physiological and pathophysiological processes. Notably in the microcirculation, EDHF appears to play an important role in the regulation of vascular tone. Leading candidates for EDHF include extracellular potassium (K^+), an epoxygenase product, hydrogen peroxide and/or a contribution from myoendothelial gap junctions. Data from our laboratory indicate that in mouse vessels, different endothelium-dependent vasodilators, such as acetylcholine and protease-activated receptor (PAR) agonists, release different endothelium-derived relaxing factors. The combination of two K-channel toxins, apamin and charybdotoxin, inhibits EDHF activity in most protocols. Endothelial dysfunction is considered as the major risk factor and a very early indicator of cardiovascular disease including the cardiovascular complications of type I & types II diabetes. Impaired endothelium-dependent vasodilatation results primarily from a decreased synthesis of endothelium-derived nitric oxide (NO) and/or an increase in the production of reactive oxygen species such as superoxide. We have shown that the administration of tetrahydrobiopterin, an important co-factor for nitric oxide synthase (NOS) partially restores endothelial function (1) in leptin-deficient mice (db/db) with spontaneous type II diabetes, as well as (2) in human vascular tissue harvested for coronary artery bypass grafting (CABG). These data suggest that a deficiency in the availability of tetrahydrobiopterin plays an important role in vascular dysfunction associated with Type II diabetes. In addition, changes in the contribution of EDHF occur in vascular tissue from the db/db mice suggesting a compensatory increase in EDHF production; whether this alteration in EDHF production is physiological or pathophysiological remains controversial.

Key words: nitric oxide, EDHF, potassium channels, diabetes, endothelial dysfunction

Correspondence to: Dr. Chris R. Triggle, Smooth Muscle Research Group and Department of Pharmacology & Therapeutics, Faculty of Medicine, University of Calgary, Alberta, Canada, T2N 4N1
Phone: +1-403-220-8312 Fax: +1-403-270-2211 e-mail: triggle@ucalgary.ca

Introduction

The endothelium plays a key role in the short- and long-term regulation of the cardiovascular system and is the source of many factors that influence blood flow, blood coagulation as well as angiogenesis. A key factor produced by the endothelium is the gas, nitric oxide, NO. NO is an important vasodilator that also possesses platelet anti-aggregatory and pro- (and anti-) angiogenic activity. However, the synthesis and release of other vasoactive factors may also be altered in disease states and that may also contribute to endothelial dysfunction has yet to be explored in depth.

A Healthy Endothelium is Anti-Atherogenic

Collectively, the endothelium is a major "organ" in the body. In an adult, the total endothelium mass is about 500 grams, located mainly in the pulmonary circulation. The endothelium is formed by a single layer of cells lining the inner wall of the vasculature, serving both as a semi-permeable barrier and as a regulator of vascular tone by releasing various vasoactive substances: vasodilators (nitric oxide, prostacyclin, endothelium-derived hyperpolarizing factor, bradykinin, adrenomedullin, C-natriuretic peptide), vasoconstrictors (endothelin-1, angiotensin-II, thromboxane A_2, prostaglandins, hydrogen peroxide (H_2O_2) and free radicals) (McGuire *et al.*, 2001). The L-arginine-nitric oxide (NO) pathway is thought to be the most important enzymatic vasodilator source. In addition to its function as a vasodilator, NO released from endothelial cells is also a potential inhibitor of the aggregation and adhesion of platelets to the vascular wall. NO also controls the expression of proteins involved in atheroma formation, decreasing the expression of the chemo-attractant protein MCP-1, and of surface adhesion molecules such as CD11/CD18, P-selectin, VCAM-1 and ICAM-1 (Li and Forstermann, 2000). Additionally, NO reduces vascular permeability and decreases the rate of oxidation of low-density lipoprotein (LDL), thereby preventing the pro-atherogenic effect of LDL, and inhibiting the proliferation of vascular smooth muscle cells. Endothelial function can be readily measured in both animal models and in humans. In humans, the status of the endothelium is thought to be an important surrogate marker of atherosclerotic activity (Anderson, 1999; Verma and Anderson, 2002). A reduction in the bio-availabilty of NO (defined as endothelial dysfunction resulting from a reduced vasodilatory response to acetylcholine) can result in cardiovascular dysfunction, with a concomitant increase in morbidity and mortality. Several recent studies have demonstrated that attenuation of endothelium-dependent vasodilation is associated with an increased risk of cardiovascular complications during long-term follow-up. These data point to the endothelium as an appropriate therapeutic target (Halcox *et al.*, 2002).

NO Stores in Tissues

As already stated, one of the most important substances that the endothelium produces is *nitric oxide*, or *NO*. NO is released from the endothelium either by "*chemical activation*" such as by the neurotransmitter, acetylcholine (ACh), or by "*mechanical activation*" as a result of the

shear stress created by blood flowing across the endothelium. NO is synthesized within the endothelial cells from L-arginine via the action of endothelial nitric oxide synthase (eNOS) and it is thus assumed that the cellular source of NO in the vasculature is derived from one of the three isoforms of NOS (*i.e.*, eNOS, neuronal NOS, termed nNOS, or the inducible NOS, iNOS). However, an increasing body of data suggests that there is a comparatively stable store of NO in vascular tissues and that the origin of this store cannot be linked causally to the activity of eNOS (Andrews *et al.*, 2002; Andrews *et al.*, 2003). These data suggest that there is more than one mechanism whereby NO can be synthesized in endothelial cells, that NO can be stored in endothelial and vascular smooth muscle cells, and that this "*pre-formed NO*" can be released from the cells by physiological stimuli. Most likely NO is stored in tissues as nitrosothiols (RSNOs). We have explored the contribution of such stores to the photorelaxation phenomenon that was first described by Furchgott *et al.* (1955) and Andrews *et al.* (2002, 2003). Figure 1 summarizes potential mechanisms whereby such stores can be modulated.

Role of Thiols

NO may be stored as labile NO-releasing intermediate compounds containing a thiol-NO linkage and the sulfhydryl moiety to which NO attaches is found in the side chain of cysteine, homocysteine, glutathione or the cysteine sulfhydryls present in proteins such as the abundant albumin. S-nitrosothiol compounds, or RSNOs, (Stamler *et al.*, 1992) may be critical determinants of the activity, transport and metabolic fate of NO. Adduct compounds such as S-nitrosocysteine (CysNO), S-nitrosoglutathione (GSNO) and S-nitroso-acetyl penicillamine (SNAP) can either decompose to liberate NO, and, as such, possess potent smooth muscle relaxant activity; or, such compounds may be involved in transnitrosation and S-thiolation reactions where they can affect signal transduction and enzyme activity. RSNOs have also been shown to inhibit platelet aggregation, and have longer half-lives than NO itself (Feelisch *et al.*, 1994). RSNOs have also been implicated to play a critical role in stress responses such as hypoxia (Lipton *et al.*, 2001). The reaction between NO and thiols is dependent on the presence of oxygen and the one-electron oxidation product of NO, NO^+, which is more likely to react with thiols than $NO^\bullet$ to form nitrosothiols (Stamler *et al.*, 1992). Some RSNOs are polar or too large to readily cross cellular membranes. Therefore, the entry of NO from RSNOs into cells is thought to involve a process called trans-nitrosation, where NO^+ is transferred from one thiol-containing protein to another to ultimately elicit its signal (Liu *et al.*, 1998). RSNOs may also mediate their cellular effects through stereo-specific receptor activation, particularly in the CNS (Davisson *et al.*, 1997). Similar functions for NO stores in the vasculature are also likely. We have recently begun to explore this hypothesis and have been able to measure the nitrosothiol, S-nitrosoglutathione (GSNO), in vascular tissue and demonstrate that such stores can be modulated by light:

Using analytical techniques recently developed in our laboratory, we have been able to quantify the nitrosothiol, S-nitrosoglutathione (GSNO), and its major NO decomposition product, nitrite, in vascular tissues. High Performance Liquid Chromatography (HPLC) was employed for the measurement of GSNO in mouse aortic tissues. In brief, tissues were homogenized in ice-

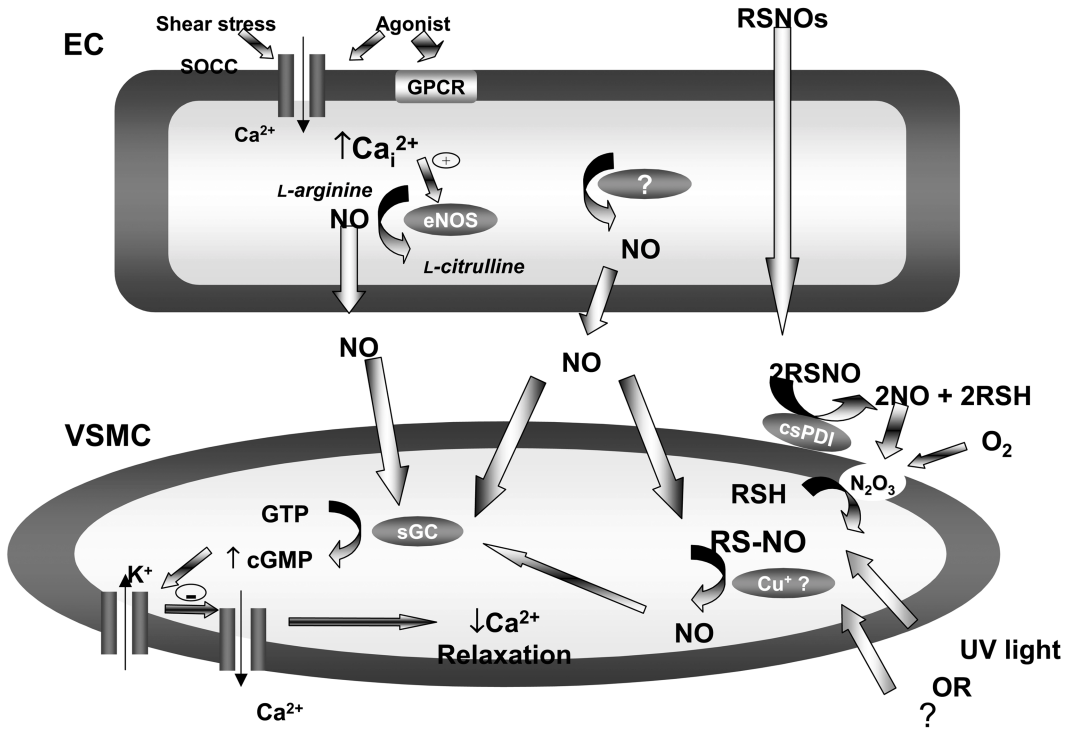

Fig. 1. Possible mechanisms involved in the synthesis, storage and release of nitric oxide (NO) and/or S-nitrosothiols (RSNOs) in endothelial cell (EC) and vascular smooth muscle cells (VSMC). Typically activation of G-protein coupled receptors (GPCR) by agonists or activation of stretch operated Ca^{2+} channels (SOCC) from the shear stress of blood flow in the membrane of ECs increases intracellular calcium (Ca^{2+}_i) levels, which in turn activates endothelial nitric oxide synthase (eNOS). The activation of eNOS results in the conversion of L-arginine to L-citrulline and the liberation of NO, which diffuses across to the VSMC and activates soluble guanylate cyclase (sGC) causing an increase in cGMP. By a number of possible mechanisms, K^+ channels open, Ca^{2+} channels close and a decrease in Ca^{2+}_i results, which is followed by vasodilation. Alternately, NO may be derived from an additional enzyme (ie xanthine oxidase) or a non-enzymatic pathway within the EC or NO liberated from RSNOs outside the cell (possibly from the circulating blood) by the cell surface protein disulfiole isomerase (csPDI) may provide an additional mechanism for the formation of RSNOs inside VSMCs. The liberated NO reacts with O_2 in the membrane to form N_2O_3, which reacts with intracellular thiols (GSH) at the cell cytosol interface to produce RSNOs. NO can also be liberated from RSNOs by irradiation with UV light which homolytically cleaves the S-N bond of RSNOs to release NO. Alternatively, a Cu^+ containing intracellular enzyme may catalyse the release of NO from RSNOs.

cold phosphate buffer (50 mM, pH 7.4) containing N-ethylmaleimide (5 mM) using a glass homogenizer, and the resulting homogenate was immediately analyzed after trichloroacetic acid (TCA: 2%) precipitation of proteins. Extracts were injected into an Agilent 1100 Series HPLC system equipped with a Hypersil BDS C_{18} column (125 × 4 mm, 5 μm) (Agilent Technologies, US) running at a flow rate of 0.55 mL/min using 99% phosphate buffer (50 mM, pH 4.5) and 1% acetonitrile. This method allows direct detection and quantification of GSNO in vascular tissues. Indeed, our preliminary data show that there was a small GSNO store (6 ± 0.7 nM; n=4) (Fig. 2) in mouse aorta, suggesting that NO can be stored in the form of RSNOs. Moreover, we have examined whether ultraviolet (UV) light could induce the release of NO from the GSNO store.

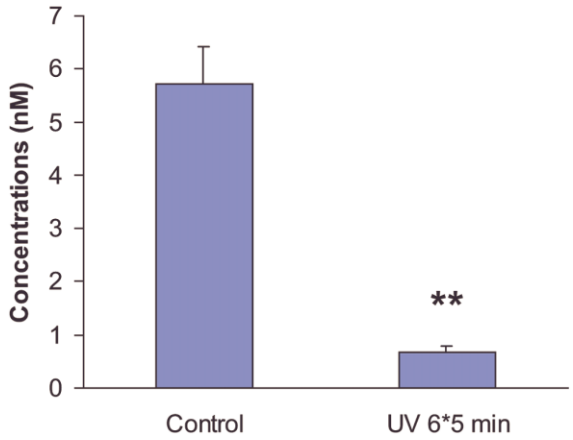

Fig. 2. Levels of GSNO in mouse aorta before (control) and after UV light exposure (UV 6 × 5 min). Aortic tissue was exposed to 30 min of UV light (6 times, 5 minute exposure). Data are presented as mean ± SEM. $^{**}P<0.01$ relative to control.

Aortae were repeatedly (six times) exposed to 5 minutes of UV light every 5 minutes for a total of 30 minutes. Interestingly, the minute amount of GSNO was depleted following illumination with light (Fig. 2). To support our contention that NO was liberated from the NO stores, we measured the concentrations of nitrite in aortic tissues before and after UV light exposure. Using the DAN fluorometric technique with excitation and emission wavelengths of 380 nm and 450 nm respectively (Misko *et al.*, 1993), an increase in nitrite was observed after mouse aorta was irradiated with light (data not shown). Our preliminary results demonstrate that vascular GSNO stores can be modulated by light. These data are summarized in Fig. 2.

Non-NOS Sources of NO in the Vasculature

If NOS is not the sole source of NO, then what is the origin of this NO store? Recent studies have suggested enzyme-independent chemical reactions and/or non-NOS enzymatic sources may be able to generate NO independently of NOS. Indeed, chemiluminescence studies have shown that reactions between L or D-arginine and H_2O_2 can produce NO (Nagase *et al.*, 1997). It has been reported that NO can be produced by the reduction of nitrite in the ischaemic heart and it has also been proposed that the acidic-metabolic vasodilatation seen in rat aorta under hypoxic conditions might arise following the conversion of nitrite into NO (Modin *et al.*, 2001). The production of NO from acidified nitrite is a commonly used approach to generate NO under experimental conditions (Cocks and Angus, 1990). As such, it is interesting that a by-product of cellular metabolism and thus a non-enzymatic source of NO could have a physiological (or indeed pathological) role in regulating vascular tone. A potential non-NO synthase enzymatic source is, not surprisingly, cytochrome P_{450} (CYP), which in addition to being structurally similar to NOS, has been reported to be able to generate NO in a NADPH and oxygen-dependent manner (Karasu, 2000). In addition, xanthine oxidase is thought to catalyze the reduction of nitrite to generate NO in the presence of NADH (Zhang *et al.*, 1998).

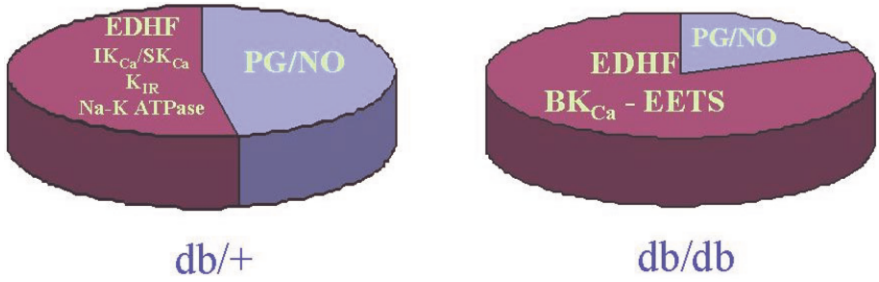

Fig. 3. Relative contribution of bradykinin-induced, EDHF-mediated relaxation in db/+ and db/db mice. Bradykinin-induced EDHF- (non-NO, non prostaglandin) mediated relaxation is sensitive to a combination of blockers of IK_{Ca} (charybdotoxin) and SK_{Ca} (apamin) as well as to a combination of inhibitor/blocker of Na/K ATPase (ouabain) and KIR channel (barium), but not to blocker of BK_{Ca} (iberiotoxin) whereas bradykinin-induced EDHF-mediated relaxation is sensitive to blocker of BK_{Ca} (iberiotoxin) and probably mediate through 17-ODYA sensitive pathway namely EETs.

The storage (and release) of NO in cells as nitrosothiols provides a provocative argument that NO is not simply an autocrine, or paracrine signal, but that it may also function as a true blood/tissue borne hormone. We hypothesize that NO is released from a store in one cell and, because of its unique ability to cross cell membranes readily, acts on distant cells either to "signal" a cell response and/or to be stored as a nitrosothiol. Thus, the hypothesis originally brought forward by Liu *et al.*, (1998) may have far reaching physiological consequences.

EDHF

EDHF is defined as a mediator of vascular relaxation via a non-NO and non-prostaglandin mechanism, notably in resistance arteries where NO appears to have less prominence than in conduit vessels (McGuire *et al.*, 2001). There are several candidates that have been identified that may represent physiological 'EDHFs' viz., NO per se, L-citrulline, potassium ions, anandamide, epoxyeicosatrienoic acids (EETs) and H_2O_2. The EETs are potential candidates for endothelium-derived hyperpolarizing factor (EDHF) because they hyperpolarize and relax vascular smooth muscle cells by activating calcium-sensitive potassium channels. EETs are the product of CYP activity (see review by McGuire *et al.*, 2001). In situations of reduced endothelial NO availability (for example, in eNOS knock out mice), there is an up-regulation of EDHF-mediated relaxation in resistance arteries (Waldron *et al.*, 1999; Ding *et al.*, 2000). Reduced bioavailability of NO will remove the inhibitory action of NO on haem-containing enzymes such as cytochrome P450 (CYP) (and also nitric oxide synthase, NOS). In ongoing work, we have demonstrated that these products may be up-regulated in the db/db mouse (Pannirselvam *et al.*, 2003) (Fig. 3).

Regulation of EDHF by K-channels

Calcium-activated K-channels play an important role in the regulation of vascular tone and the actions of EDHF. Ca^{2+}-activated K^+ channels (K_{Ca}) can be sorted into three classes A to C:

(A) large-conductance (BK_{Ca}) channels, sensitive to iberiotoxin. These channels play an important role in vascular smooth muscle cells (VSMC) as a negative feedback, opposing the depolarization initiated by vasoconstrictor stimuli. BK_{Ca} channels are also inhibited by low concentrations (1 mM) of tetraethylammonium (TEA) and are the primary target for NO-mediated hyperpolarization via, in most instances, the activation of soluble guanylyl cyclase (sGC) although direct activation of BK_{Ca} channels by NO has been reported (Bolotina *et al.*, 1994). One of the candidate EDHF molecular families is the EET family and a number of EETs (5,6-; 8,9-; 11,12-; and 14,15-EET) have been reported to activate the BK_{Ca}, and are iberiotoxin-sensitive (Hu and Kim, 1993). However, BK_{Ca} channels have not been shown to be expressed in native endothelial cells (Triggle, 2001). (B) intermediate-conductance K_{Ca} (IK_{Ca}) channels, which are inhibited by the toxin charybdotoxin (which also blocks BK_{Ca} and certain K_V channels); however, IK_{Ca}, although present on endothelial cells, are not thought to be of importance in VSMC, at least under normal physiological conditions, and (C) small-conductance K_{Ca} (SK_{Ca}) channels, which are sensitive to the toxin apamin. Although apamin alone may inhibit responses to EDHF in some vessels, SK_{Ca} channels are not universally found in VSMC (Murphy and Brayden, 1995) such that their role in inducing hyperpolarization may be related to their endothelial cell location (Edwards *et al.*, 1998).

There are three subtypes of SK_{Ca} channels, namely the SK1, SK2 and SK3, which are voltage-independent but are activated by a calmodulin-dependent mechanism regulated by intracellular Ca^{2+} levels in the submicromolar ranges of Ca^{2+}. Their amino acid sequences are unique with no significant sequence similarity, except in the pore region, to other K^+-channels. However, there is structural conservation with other K^+-channels, such as K_V, in that the subunits contain six transmembrane domains (Jan and Jan, 1990), but are not voltage-dependent. Our lab has shown that the inhibitors apamin and tubocurarine in combination with the IK_{Ca} blocker ChTx block the Ach-induced EDHF-mediated relaxation. Tubocurarine also inhibits the relaxation in the absence of ChTx to the same extent as with the second blocker (Ding *et al.*, 2003). Apamin and tubocurarine have differing affinities for the SK_{Ca} isoforms. Apamin potently inhibits SK2 and SK3 channels more than SK1. On the other hand, curare potently blocked SK2 over SK1 channels (Kohler *et al.*, 1996). Immunocytochemistry data from our lab has shown an endothelial and smooth muscle cell plasma membrane location for SK3, while SK2 has been found in the nuclear region of smooth muscle and the plasma membrane of endothelial cells (Ding *et al.*, 2003). Based on these preliminary results and the effects of inhibitors, SK2 may be the likely isoform on the endothelial cells that contribute to the EDHF response.

In many vessels a combination of apamin and charybdotoxin inhibits EDHF activity. Of significance, iberiotoxin usually cannot substitute for charybdotoxin. Since SK_{Ca} and IK_{Ca} are not thought to reside universally on VSMC it has been suggested that SK_{Ca} and IK_{Ca} play a critical role in the regulation of either the synthesis and/or release of EDHF and that the channels are most likely located on the endothelial cells (Edwards *et al.*, 1998). Although the specific location of the SK_{Ca} and IK_{Ca} channels is not known it may be hypothesized that they should be located primarily ab-luminally and thus facilitate the release of EDHF towards the VSMC. We have begun to explore this hypothesis, and by using immunohistochemical techniques and RT-PCR studies, have examined the distribution of SK_{Ca}, IK_{Ca}, BK_{Ca} channels and

Table 1 Distribution of K_{Ca} channels in endothelial cells and vascular smooth muscle cells from small mesenteric arteries from C57BL/6J mice

	Endothelial cells	Smooth muscle cells
K_{Ca} Channel		
SK1	– (1)	
SK2	– (1); + (2)	+ (2)
SK3	+ (1); + (2)	+ (2)
IK_{Ca}	Inconclusive (1)	
BK_{Ca}	– (1); – (2)	+ (2)

1. RT-PCR
2. Immunocytochemistry

preliminary data from freshly dispersed ECs and VSMCs indicates a uniform distribution of $SK_{Ca}2$ and $SK_{Ca}3$ and these data are summarized in Table 1.

If iberiotoxin is not a universal blocker of EDH then which K-channel(s) on VSMC mediates EDH? What is the VSMC target for EDHF? These are unanswered questions. Furthermore, is there a novel channel K_{Ca} in endothelial cells that requires the presence of both apamin and charybdotoxin for inhibition? K_{Ca}-channels with unusual characteristics have been described in cell cultures and, for instance, in bovine aortic endothelial cell cultures. Cai *et al.* (1998) have described an IK_{Ca} that has some of the pharmacological properties expected of BK_{Ca} and $K_V1.2$ channels. Zygmunt and colleagues (1997) have also argued that a novel SK_{Ca} channel may exist that is only sensitive to apamin in the presence of charybdotoxin. Figure 4 depicts the distribution of the potassium channels that regulate the production, synthesis, release and action of NO, PGI_2 and EDHF.

Proteinase-Activated Receptors (PAR) and Endothelial Function

Proteinase-Activated Receptors (PARs) constitute a recently discovered family member of the G protein coupled receptor superfamily. As their name implies their activation is mediated by serine proteases in their local environment (Hollenberg and Compton, 2002). These serine proteinases selectively cleave at specific sites for each PAR to unmask an extracellular N-terminal domain that then functions as a 'tethered ligand' to activate the receptor. These family members were named in chronological order of the discovery of their protein sequences (PAR_1, PAR_2, PAR_3, PAR_4). Importantly, PAR_1, PAR_3 and PAR_4 are targeted by the essential blood-clotting enzyme, thrombin, whereas PAR_2 is activated by trypsin and tryptase, but not by thrombin. In part because of this distinction in enzyme selectivity between PAR_2 and the other PARs, and despite some uncertainty in the nature of endogenous agonist(s), it is proposed that activation of PAR_2 may have an important role in the regulation of cardiovascular health.

Amongst the earliest reports regarding the activation of PAR_2 *in vitro* either by enzyme or peptide agonists, was the observation of an endothelium-dependent vasodilation of isolated blood vessels (Hollenberg *et al.*, 1996). The complete inhibition of vasodilation by an inhibitor of nitric oxide (NO) synthases, L-NAME, clearly indicates that, in most blood vessels, NO mediated the response to PAR_2 activation in many blood vessels (Glusa *et al.*, 1997; Moffatt and

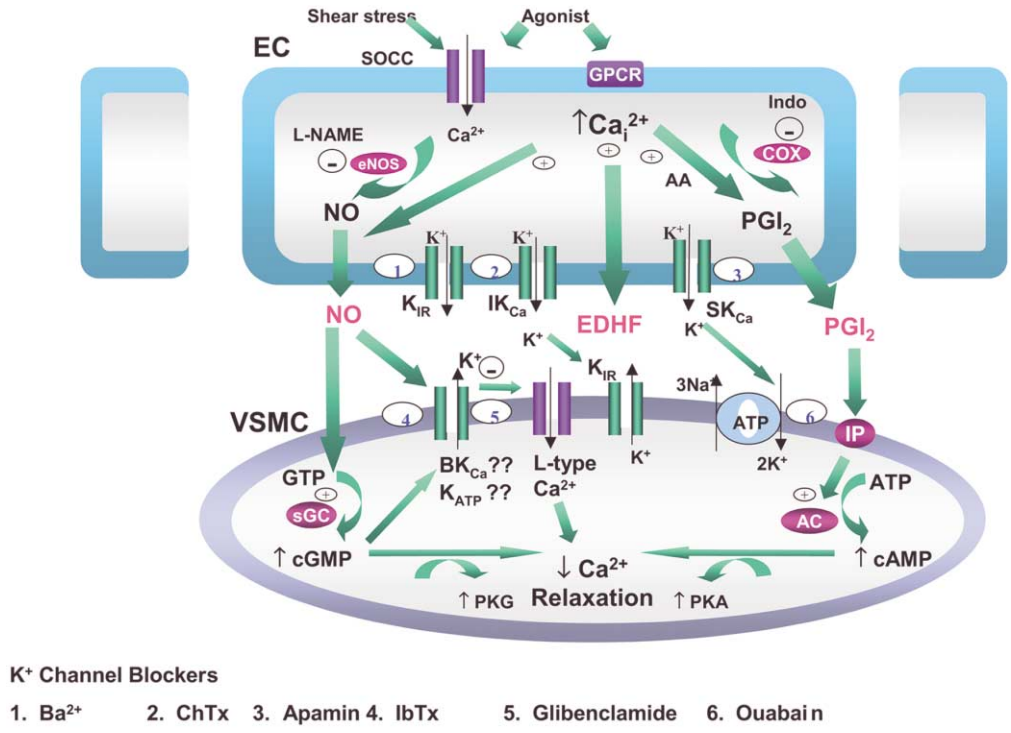

Fig. 4. Endothelial cell regulation of vascular smooth muscle cell relaxation and the putative location of the K-channels involved. A rise in endothelial calcium triggers the production of three relaxing factors: NO, PGI_2 and EDHF which diffuse to the smooth muscle cell leading to relaxation. The synthesis and release of the non-NO/PGI_2 relaxant, EDHF is thought to be regulated by K^+ efflux through the endothelial SK_{Ca} and IK_{Ca} channels K^+ Channel Blockers: 1) Ba^{2+}; 2) ChTx; 3) Apamin; 4) IbTx/ ChTx; 5) Glibenclamide; 6) Ouabain (Na^+-K^+ ATPase inhibitor).

Cocks, 1998; Sobey and Cocks, 1998). However, it was observed subsequently that NO produced by the endothelium could not account wholly for vasodilation or the blood pressure lowering effects that resulted from PAR_2 activation; nor did products of cyclo-oxygenases (Magazine *et al.*, 1996; Damiano *et al.*, 1999). Thus, it was proposed that unidentified endothelium-dependent hyperpolarization factor(s) (EDHFs) were involved as well as NO in the vascular responses to PAR_2 activation (Emilsson *et al.*, 1997; Hamilton and Cocks, 2000; McGuire *et al.*, 2002; McLean *et al.*, 2002; Trottier *et al.*, 2002). It should also be noted that the direct activation of PAR_2 expressed on the smooth muscle cells results in vasoconstriction of some blood vessels (Moffatt and Cocks, 1998; McGuire *et al.*, 2002). Therefore, the expression of PAR_2 on both ECs and VSMCs influences vascular reactivity.

Paradoxically, in experimental models of both chronic hypertension (Spontaneously Hypertensive rats 46) and hypotension (sepsis, 47) the expression of PAR_2 has been observed to be up-regulated *in vivo*. Interestingly, PAR_2 expression was also observed to increase during arterial restenosis following balloon angioplasty in yet another animal model (Damiano *et al.*, 1999). Furthermore, PAR_2-mediated vasodilation of coronary arteries was preserved relatively better than that by other endothelial G protein coupled receptors in a model of global cardiac

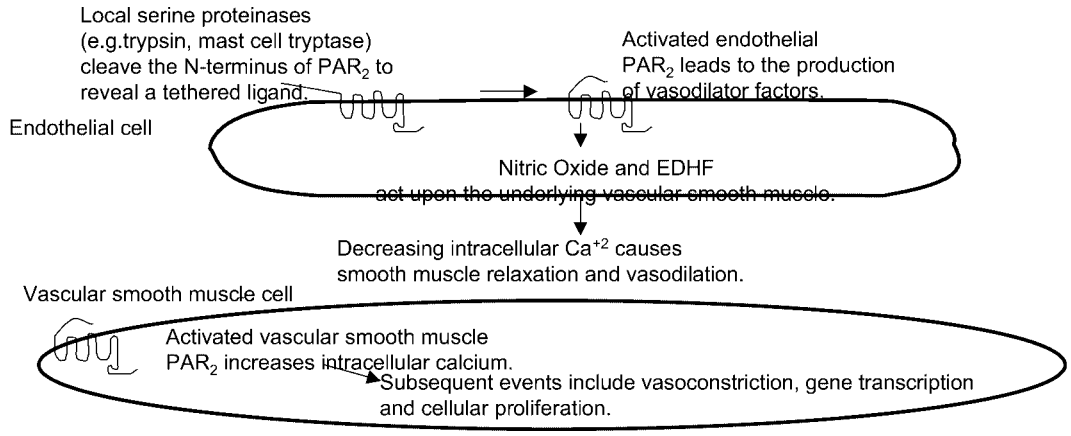

Fig. 5. Proteinase-Activated Receptor 2 (PAR_2) in the vasculature. A speculative cartoon summarizes the reported actions and putative consequences of activation of PAR_2 in the vasculature. The authors highlight the dichotomy of effects due to PAR_2 activation on endothelial versus vascular smooth muscle cell.

ischemia-reperfusion injury (McGuire *et al.*, 2002). These data indicate that PAR_2 expression increases following stress to the vasculature and this conclusion is consistent with cell culture experiments wherein various cytokines and pro-inflammatory substances induced changes to the cellular expression of PAR_2 in ECs and VSMCs (Nystedt *et al.*, 1996; Bono *et al.*, 1997; Molino *et al.*, 1997). We conclude that PAR_2 modulates, either directly or indirectly, vascular tone and blood flow; but for what purpose? And to what end? Figure 5 illustrates the role that PARs play in the regulation of vascular tone.

Endothelial Dysfunction in Diabetes

A reduced vasodilatory response to acetylcholine has been frequently reported in vascular tissue from both humans and animals with diabetes (De Vriese *et al.*, 2000; Pannirselvam *et al.*, 2003) and this observation defines "*endothelial dysfunction*". Endothelial dysfunction in human penile corpura cavernosa was first reported for type I and type II diabetic patients (Saenz *et al.*, 1989). Impaired endothelium-dependent relaxation has been reported in the peripheral circulation in type I (Johnstone *et al.*, 1993) and type II diabetic patients (Ting *et al.*, 1996), evaluated either *in vivo* or in isolated arteries from these individuals studied *in vitro*. Exceptions, however, have also been reported (Calles-Escandon and Cipolla, 2001). While hyperglycemia characterizes diabetes and is felt to play an important role in vascular disease, endothelial dysfunction is common in non-diabetics with conditions unrelated to diabetes, including hypertension and dyslipoproteinemias. In addition, it has been shown that endothelial dysfunction occurs in insulin-resistant obese individuals prior to the development of diabetes (Steinberg *et al.*, 1996).

Clinical studies support the hypothesis that both acute and chronic hyperglycemia plays an important role in the endothelial dysfunction that eventually leads to diabetic vascular complications (Williams *et al.*, 1997; Makimattila *et al.*, 1999). Several studies observed

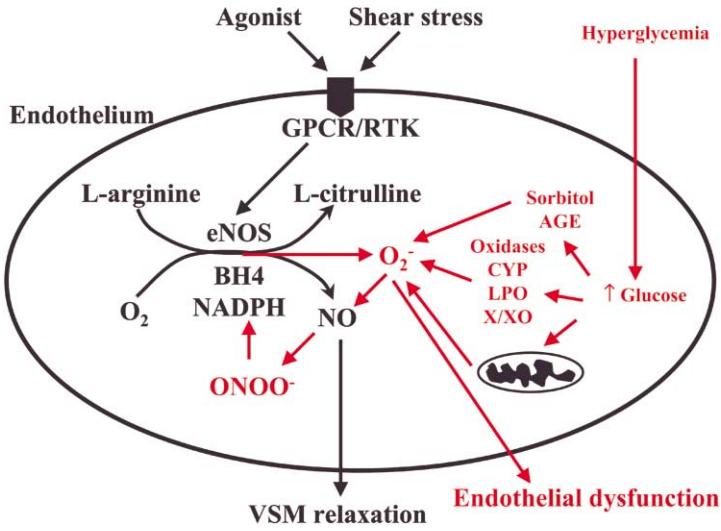

Fig. 6. Proposed scheme of "uncoupled" eNOS leading to endothelial dysfunction. Increased reactive oxygen species and oxidative stress from diabetes (mainly from mitochondria, AGE and sorbitol pathway and enzymes, such as NADPH oxidase, xanthine oxidase (X/XO), cytochrome P450 (CYP), lipoxygenase (LPO), increases the oxidation of tetrahydrobiopterin, an important co-factor for eNOS. Under sub-optimal concentration of BH_4, eNOS generates both NO and superoxide (O_2^-) anions resulting in the formation of peroxynitrite ($ONOO^-$) anions and hydrogen peroxide (H_2O_2).

improvement of endothelial function with anti-hyperglycemic agents supporting the hypothesis that hyperglycemia is the major risk factor in endothelial dysfunction in diabetes (Mather *et al.*, 2001). In human studies it is often difficult to separate hyperglycemic effects from those due to other metabolic modulating effects of pharmacological agents. Therefore to test this hypothesis further, we have compared endothelial function in three different mouse models of insulin resistance/type II diabetes viz., db/db (mutation in the gene encoding one of the isoforms of the leptin receptor), ob/ob (mutation in gene encoding for leptin secretion) and TallyHo mice (polygenic defect). Only db/db and Tallyho mice exhibit hyperglycemia & dyslipidemia whereas ob/ob mice have dyslipidemia, but are normoglycemia. Further, db/db and TallyHo mice, but not ob/ob mice show an impaired relaxation to acetylcholine. These data confirms that hyperglycemia is the major risk factor in the endothelial dysfunction and vascular complications. We believe that hyperglycemia increases oxidative stress from different sources, which in turn decreases the bio-availability of tetrahydrobiopterin, an important co-factor for nitric oxide synthase, leading to "uncoupled" eNOS. "Uncoupled" eNOS generates superoxide instead of nitric oxide resulting in endothelial dysfunction (Fig. 6).

EETs may also serve as ligands for PPARs (peroxisomal proliferator activated nuclear receptors) (Cowart *et al.*, 2002). This link with the PPARs is of importance since the expression of some CYP isoforms is under the control of PPARα. PPARγ agonists (such as rosiglitazone and pioglitazone) target insulin resistance and play a therapeutic role not only in the treatment of insulin resistance but also in modulating the inflammatory response associated with diabetes (Murphy and Holder, 2000). Glitazones have also been shown to improve endothelial function

in the OLETF rat model of type II diabetes and obesity (Yamagishi *et al.*, 2001). The synthesis of EETs may also be up-regulated in the db/db mouse.

Changes in Endothelial Function in Disease States and the Contribution of Reactive Oxygen Species (ROS)

Endothelial dysfunction appears to be a common, and early, feature of cardiovascular disease. The exact etiology of the cellular basis for this dysfunction is not clear, however, as discussed in conjunction with our data from mouse models of type II diabetes, an elevation of intracellular oxidative stress may well be the common denominator. Nonetheless, the trigger for this dysfunction may be disease specific. Although eNOS-derived NO is a key factor for maintaining a healthy endothelium, changes in the contribution and/or nature of other endothelium-derived vasodilators may also be an important consideration. In this regard, the contributions of H_2O_2 and C-Reactive Protein (CRP) merit consideration.

Reactive oxygen species (ROS) such as hydrogen peroxide appear to serve as physiological signalling molecules in the vasculature. Hydrogen peroxide (H_2O_2) is both a direct vasodilator itself (Wei *et al.*, 1996; Iida and Katusic, 2000) and also elicits endothelium-dependent vasodilatation in some arterial beds. Endothelium-dependent relaxations mediated by H_2O_2 have been reported in cat (Kontos *et al.*, 1984), rat (Yang *et al.*, 1991; Sobey *et al.*, 1997) and mouse cerebral arteries (Rosenblum, 1987), in canine coronary arteries (Rubanyi and Vanhoutte, 1986), in mouse mesenteric arteries (Matoba *et al.*, 2000) and in human vasculature (Matoba *et al.*, 2002; Miura *et al.*, 2003). Some of the mechanisms for the generation of H_2O_2 and the sites of action of H_2O_2 on vascular smooth muscle are depicted in Fig. 7.

Although these studies did not identify the enzymatic source of H_2O_2, ECs have been shown to generate significant amounts of ROS (Stroes *et al.*, 1998; Vasquez-Vivar *et al.*, 1998) and ECs express enzymes (e.g. eNOS, NADPH oxidase, CYP, COX) that can produce ROS in response to receptor activation or other cellular events that elevate intracellular calcium. NO is the principal endothelium-derived dilator operating in the vasculature and its activity can be governed by the amount of ROS in the vascular milieu, whereby superoxide anions can rapidly scavenge NO at a diffusion-controlled rate (Beckman *et al.*, 1990). NO displays high affinity for haem groups and many enzymes, including those noted above (NOS, COX, CYP etc) have haem groups, thus NO itself may inhibit the enzymatic production of superoxide and H_2O_2 (Griscavage *et al.*, 1994; Stadler *et al.*, 1994). The relative contributions of NO and ROS to vascular tone are inversely proportional to each other and the appearance of one could likely compensate for the absence of the other. Pathophysiological conditions such as diabetes and atherosclerosis display signs of oxidative stress and dysfunctions in the NO pathway, thus it may be valid to argue that endothelial ROS production could be compensating for impairments to normal relaxant mechanisms. If this hypothesis is correct then there should be an increased contribution of H_2O_2 in pathophysiological states where the normal production of NO is compromised. However, other than a study by Cosentino *et al.* (2001), which reported an increased contribution by H_2O_2 to endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice, there is little evidence to support this hypothesis. For example, Yang and others (1991)

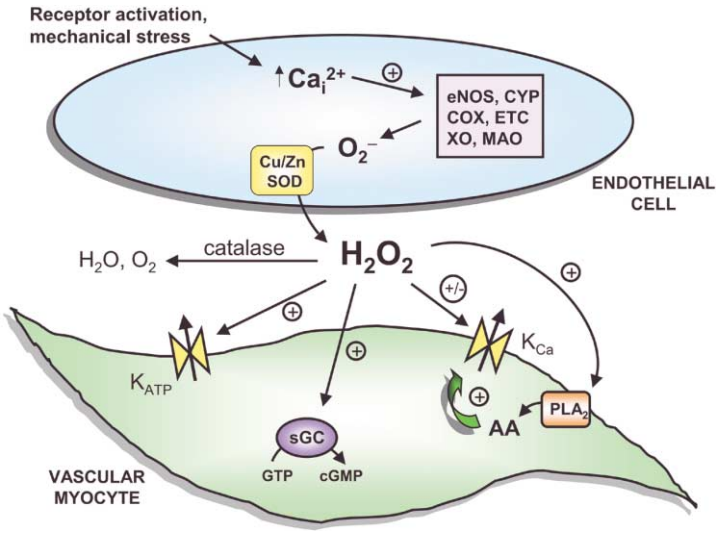

Fig. 7. A hypothetical scheme of how endothelium-derived hydrogen peroxide might mediate vasodilatation in arterial beds. Endothelial sources of ROS are activated when intracellular calcium levels increase, either in response to receptor activation or shear stress. Charged superoxide anions (O_2^-) reduced by superoxide dismutase (Cu/Zn SOD) form cell-permeable hydrogen peroxide (H_2O_2), which can diffuse across cellular membrane to act on cellular targets or it may undergo further metabolism (by catalase or transition metal cations). Putative cellular targets of H_2O_2 that could result in dilatation include potassium channel sulfhydryl groups (K_{ATP}, K_{Ca}, K_V) —resulting in increased or decreased channel permeability; guanylate cyclase-elevated cGMP levels; phospholipase A_2-increased arachidonic acid production available to activate K_{Ca} channels.

showed that endothelium-dependent relaxations to bradykinin in pial arteries from either normotensive or hypertensive rats were both similarly sensitive to catalase. In addition Matoba and colleagues (2000) identified H_2O_2 as an endothelium-dependent vasodilator in mesenteric arteries obtained from both eNOS-expressing and eNOS-deficient mice. It should also be noted that studies by other groups (Itoh *et al.*, 2003; Ellis *et al.*, 2003; Ellis and Triggle, 2003) have disputed the conclusions of Matoba *et al.* (2000). Nevertheless, additional studies should be performed in order to determine whether increased reliance on ROS as physiologically-relevant endothelium-derived relaxants occurs in selective vascular beds or in specific arterial diseases, and to correlate this to the level of oxidative stress under such conditions.

C-Reactive Protein (CRP) Effects on Endothelial and Vascular Smooth Muscle Cell Function

Arterial inflammation is now recognized as an indicator of the development and progression of atherothrombosis and C-reactive protein (CRP) has proved to be a well studied risk factor and indicator of the level of atherosclerosis (Bhatt and Topol, 2002; Libby *et al.*, 2002; Taubes, 2002). In addition to these actions CRP has also been shown to decrease NO production in human saphenous vein and umbilical vein endothelial cells via an effect on eNOS mRNA stability (Verma *et al.*, 2002) and thus, via decreased NO production, inhibited angiogenesis.

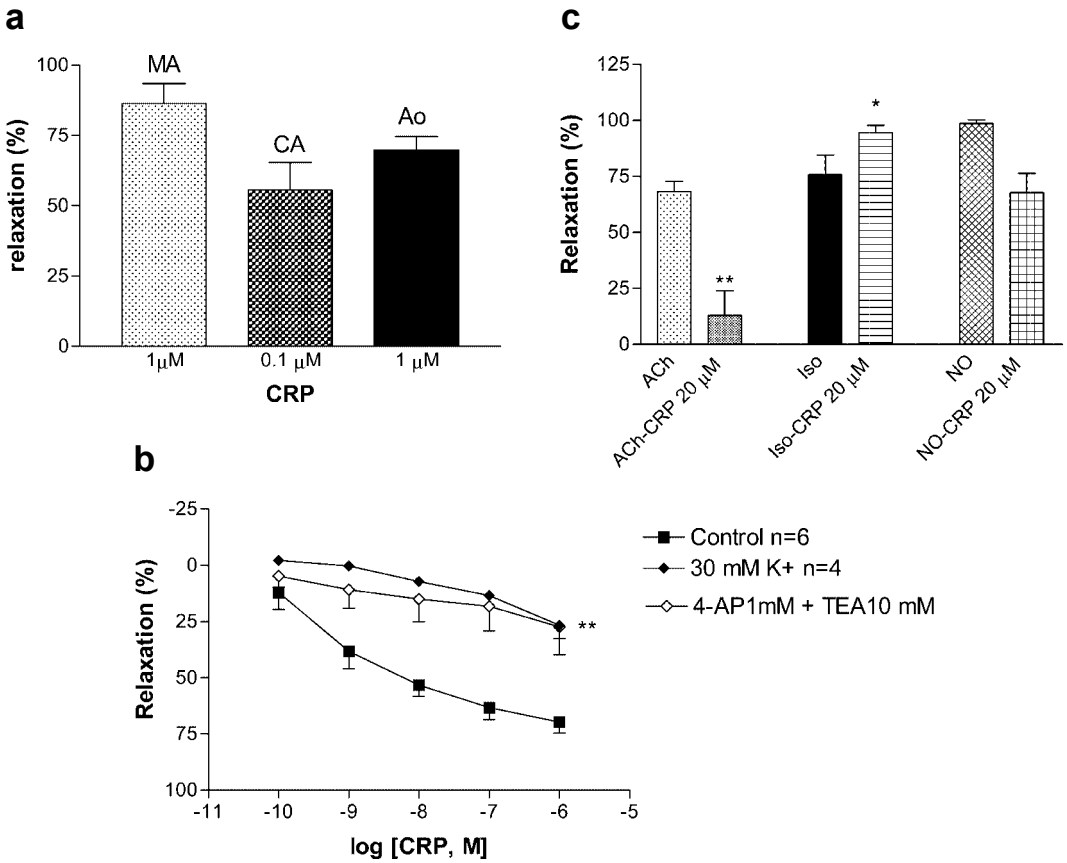

Fig. 8. Effects of C-Reactive Protein (CRP) on murine blood vessels. 8a. CRP -induced relaxation in C56BL/6 mice small mesenteric artery (MA), coronary artery (CA), and Aorta (Ao). 8b. CRP-induced relaxations were inhibited by 30 mM KCl or 1 mM 4-AP plus 10 mM TEA. $^{**}P<0.001$. 8c. ACh, but not isoproterenol- (Iso) or NO-, -induced relaxation of mouse thoracic aortae was inhibited by incubation with 20 μM CRP for 30 min. $^{**}P<0.001$, $^{*}P<0.05$.

Comparable data have also been reported for human aortic endothelial cells (Venugopal *et al.*, 2002). Furthermore, CRP has been shown to have an endothelium-independent vasorelaxation action, possibly via a direct action on potassium channels, in human internal mammary arteries obtained from patients undergoing coronary artery bypass surgery (Sternik *et al.*, 2002). Recent data from our laboratory indicate that CRP has both direct effects and indirect effects on vascular tone in blood vessels from the mouse. We have shown that CRP has endothelium-independent actions that lead to relaxation of vascular smooth muscle and this would appear to result from the opening of vascular K-channels (Fig. 8a and 8b). In addition the incubation of mouse aorta with CRP results in the reduction of acetylcholine (ACh), but not isoproterenol or NO,-mediated relaxation (Fig. 8c). These latter effects of CRP are clearly endothelium-dependent and are likely due to the effects of CRP on eNOS mRNA stability as already described for CRP on human endothelial cells by Verma *et al.* (2002).

Acknowledgements

The authors wish to acknowledge the Alberta Heritage Foundation for Medical Research (AHFMR), Canadian Diabetes Association (CDA), Canadian Institutes of Health Research (CIHR) and the Heart and Stroke Foundation of Canada (HSFC) for their financial support of the research and personnel.

References

Anderson, T.J. (1999). Assessment and treatment of endothelial dysfunction in humans. *J. Am. Coll. Cardiol.* **34**: 631–638.

Andrews, K.l., McGuire, J.J and Triggle, C.R. (2003). A photosensitive vascular smooth muscle store of nitric oxide in mouse aorta: No dependence on expression of endothelial nitric oxide synthase. *Br. J. Pharmacol.* **138**: 932–940.

Andrews, K., Triggle, C.R. and Ellis, A. (2002). NO and the vasculature: Where does it come from and what does it do? *Heart Failure Reviews* **7**: 423–445.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U. S. A.* **87**: 1620–1624.

Bhatt, D.L. and Topol, E.J. (2002). Need to test the arterial inflammation hypothesis. *Circulation* **106**: 136–140.

Bolotina, V.M., Najibi, S., Palacino, J.J., Pagano, P.J. and Cohen, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**: 850–853.

Bono, F., Lamarche, I. and Herbert, J.M. (1997). Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). *Biochem. Biophys. Res Commun.* **241**: 762–764.

Cai, S., Garneau, L. and Sauve, R. (1998). Single-channel characterization of the pharmacological properties of the K(Ca^{2+}) channel of intermediate conductance in bovine aortic endothelial cells. *J. Membr. Biol.* **163**: 147–158.

Calles-Escandon, J. and Cipolla, M. (2001). Diabetes and endothelial dysfunction: a clinical perspective. *Endocr. Rev.* **22**: 36–52.

Cicala, C., Pinto, A., Bucci, M., Sorrentino, R., Walker, B., Harriot, P., Cruchley, A., Kapas, S., Howells, G.L., and Cirino, G. (1999). Protease-activated receptor-2 involvement in hypotension in normal and endotoxemic rats in vivo. *Circulation* **99**: 2590–2597.

Cocks, T.M. and Angus, J.A. (1990). Comparison of relaxation responses of vascular and non-vascular smooth muscle to endothelium-derived relaxing factor (EDRF), acidified sodium nitrite (NO) and sodium nitroprusside. Naunyn Schmiedebergs. *Arch. Pharmacol.* **341**: 364–372.

Cosentino, F., Barker, J.E., Brand, M.P., Heales, S.J., Werner, E.R., Tippins, J.R., West, N., Channon, K.M., Volpe, M. and Luscher, T.F. (2001). Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **21**: 496–502.

Cowart, L.A., Wei, S., Hsu, M.H., Johnson, E.F., Krishna, M.U. and Falck, J.R. (2002). The CYP4A isoforms hydroxylate epoxyeicosatrienoic acids to form high affinity peroxisome proliferator-activated receptor ligands. *J. Biol. Chem.* **277**: 35105–35112.

Damiano, B.P., Cheung, W.M., Santulli, R.J., Fung-Leung, W.P., Ngo, K., Ye, R.D., Darrow, A.L., Derian, C.K., de Garavilla, L. and Andrade-Gordon, P. (1999a). Cardiovascular responses mediated by protease-activated receptor-2 (PAR-2) and thrombin receptor (PAR-1) are distinguished in mice deficient in PAR-2 or PAR-1. *J. Pharmacol. Exp. Ther.* **288**: 671–678.

Damiano, B.P., D'Andrea, M.R., de Garavilla, L., Cheung, W.M. and Andrade-Gordon, P. (1999b).

Increased expression of protease activated receptor-2 (PAR-2) in balloon-injured rat carotid artery. *Thromb. Haemost.* **81**: 808–814.

Davisson, R.L., Possas, O.S., Murphy, S.P. and Lewis, S.J. (1997). Neurogenically derived nitrosyl factors mediate sympathetic vasodilation in the hindlimb of the rat. *Am. J. Physiol.* **272**: H2369–H2376.

De Vriese, An. S, Verbeuren, T.J., Van de Voorde, J., Lameire, N.H. and Vanhoutte, P.M. (2000). Endothelial dysfunction in diabetes. *Br. J. Pharmacol.* **130**: 963–974.

Ding, H., Jiang, Y. and Triggle, C.R. (2003). The contribution of d-tubocurarine and apamin-sensitive potassium channels to endothelium-derived hyperpolarizing factor-medaited relaxation of small mesenteric arteries from eNOS$^{-/-}$ mice. Proceedings of the Fourth International Workshop on Endothelium-Derived Hyperpolarizing Factor. EDHF 2002. Edited by Paul M. Vanhoutte, Taylor & Francis, London & New York, pp. 283–296.

Ding, H., Kubes, P. and Triggle, C.R. (2000). Potassium and acetylcholine-induced vasorelaxation in mice lacking endothelial nitric oxide synthase. *Br. J. Pharmacol.* **129**: 1194–1200.

Edwards, G., Dora, K.A., Gardener, M.J., Garland, C.J. and Weston, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* **396**: 269–272.

Ellis, A., and Triggle, C.R. (2003). Endothelium-derived reactive oxygen species: their regulationship to endothelium-dependent hyperpolarization and vascular tone. (A Review). *Canad. J. Physiol. Pharmacol.* **81**: 1013–1028.

Ellis, A., Pannirselvam, M., Anderson, T.J. and Triggle, C.R. (2003). Catalase has negligible inhibitory effects on endothelium-dependent relaxations in mouse aorta and small mesenteric artery. *Br. J. Pharmacol.* **140**: 1193–1200.

Emilsson, K., Wahlestedt, C., Sun, M.K., Nystedt, S., Owman, C. and Sundelin, J. (1997). Vascular effects of proteinase-activated receptor 2 agonist peptide. *J. Vasc. Res.* **34**: 267–272.

Feelisch, M., Te, P.M., Zamora, R., Deussen, A. and Moncada, S. (1994). Understanding the controversy over the identity of EDRF. *Nature* **368**: 62–65.

Furchgott, R.F, Sleator, W.J., Mcaman, M.W. and Elchlepp, J. (1955). Relaxation of arterial strips by light and the influence of drugs on this photodynamic effect. *J. Pharmacol. Exp. Ther.* **113**: 22–23.

Glusa, E., Saft, A., Prasa, D. and Sturzebecher, J. (1997). Trypsin- and SLIGRL-induced vascular relaxation and the inhibition by benzamidine derivatives. *Thromb. Haemost.* **78**: 1399–1403.

Griscavage, J.M., Fukuto, J.M., Komori, Y. and Ignarro, L.J. (1994). Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. *J. Biol. Chem.* **269**: 21644–21649.

Halcox, J.P, Schenke, W.H, Zalos, G, Mincemoyer, R, Prasad, A and Waclawiw, M.A. (2002). Prognostic value of coronary vascular endothelial dysfunction. *Circulation* **106**: 653–658.

Hamilton, J.R. and Cocks, T.M. (2000). Heterogeneous mechanisms of endothelium-dependent relaxation for thrombin and peptide activators of protease-activated receptor-1 in porcine isolated coronary artery. *Br. J. Pharmacol.* **130**: 181–188.

Hollenberg, M.D., Saifeddine, M. and Al Ani, B. (1996). Proteinase-activated receptor-2 in rat aorta: structural requirements for agonist activity of receptor-activating peptides. *Mol. Pharmacol.* **49**: 229–233.

Hollenberg, M.D. and Compton, S.J. (2002). International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol. Rev.* **54**: 203–217.

Hu, S. and Kim, H.S. (1993). Activation of K^+ channel in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acid. *Eur. J. Pharmacol.* **230**: 215–221.

Iida, Y. and Katusic, Z.S. (2000). Mechanisms of cerebral arterial relaxations to hydrogen peroxide. *Stroke* **31**: 2224–2230.

Itoh, T., Kajikuri, J., Hattori, T., Kusama, N. and Yamamoto, T. (2003). Involvement of H_2O_2 in superoxide dismutase-induced enhancement of endothelium-dependent relaxation in rabbit mesenteric resistance artery. *Br. J. Pharmacol.* **139**: 444–456.

Jan, L.Y. and Jan, Y.N. (1990). A superfamily of ion channels. *Nature* **345**: 672.

Johnstone, M.T., Creager, S.J., Scales, K.M., Cusco, J.A., Lee, B.K. and Creager M.A. (1993). Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation* **88**: 2510–2516.

Karasu, C. (2000). Time course of changes in endothelium-dependent and -independent relaxation of chronically diabetic aorta: role of reactive oxygen species. *Eur. J. Pharmacol.* **392**: 163–173.

Kohler, M., Hirschberg, B., Bond, C.T., Kinzie, J.M., Marrion, N.V., Maylie, J. and Adelman, J.P. (1996). Small-conductance, calcium-activated potassium channels from mammalian brain. *Science* **273**: 1709–1714.

Kontos, H.A., Wei, E.P., Povlishock, J.T. and Christman, C.W. (1984). Oxygen radicals mediate the cerebral arteriolar dilation from arachidonate and bradykinin in cats. *Circ. Res.* **55**: 295–303.

Li, H. and Forstermann, U. (2000). Nitric oxide in the pathogenesis of vascular disease. *J. Pathol.* **90**: 244–254.

Libby, P., Ridker, P.M. and Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation* **105**: 1135–1143.

Lipton, A.J., Johnson, M.A., Macdonald, T., Lieberman, M.W., Gozal, D. and Gaston B. (2001). S-nitrosothiols signal the ventilatory response to hypoxia. *Nature* **413**: 171–174.

Liu, X., Miller, M.J., Joshi, M.S., Thomas, D.D. and Lancaster, J.R., Jr. (1998). Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. USA* **95**: 2175–2179.

Magazine, H.I., King, J.M. and Srivastava, K.D. (1996). Protease activated receptors modulate aortic vascular tone. *Int. J. Cardiol.* **53** (Suppl): S75–S80.

Makimattila, S., Liu, M.L., Vakkilainen, J., Schlenzka, A., Lahdenpera, S., Syvanne, M., Mantysaari, M., Summanen, P., Bergholm, R., Taskinen, M.R. and Yki-Jarvinen, H. (1999). Impaired endothelium-dependent vasodilation in type 2 diabetes. Relation to LDL size, oxidized LDL, and antioxidants. *Diabetes Care* **22**: 973–981.

Mather, K.J., Anderson, T.J. and Verma, S. (2001). Insulin action in the vasculature: Physiology and Pathophysiology. *J. Vasc. Res.* **38**: 415–422.

Matoba, T., Shimokawa, H., Nakashima, M., Hirakawa, Y., Mukai, Y., Hirano, K., Kanaide, H. and Takeshita, A. (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J. Clin. Invest.* **106**: 1521–1530.

Matoba, T., Shimokawa, H., Kubota, H., Morikawa, K., Fujiki, T., Kunihiro, I., Mukai, Y., Hirakawa, Y. and Takeshita, A. (2002). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem. Biophys. Res. Commun.* **290**: 909–913.

McGuire, J.J., Dai, J., Andrade-Gordon, P., Triggle, C.R. and Hollenberg, M.D. (2002a). Proteinase-activated receptor-2 (PAR2): vascular effects of a PAR2-derived activating peptide via a receptor different than PAR2. *J. Pharmacol. Exp. Ther.* **303**: 985–992.

McGuire, J.J., Ding, H. and Triggle, C.R. (2001). Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor(s). *Can. J. Physiol. Pharmacol.* **79**: 443–470.

McGuire, J.J., Hollenberg, M.D., Andrade-Gordon, P. and Triggle, C.R. (2002b). Multiple mechanisms of vascular smooth muscle relaxation by the activation of proteinase-activated receptor 2 in mouse mesenteric arterioles. *Br. J. Pharmacol.* **135**: 155–169.

McLean, P.G., Aston, D., Sarkar, D. and Ahluwalia, A. (2002). Protease-activated receptor-2 activation causes EDHF-like coronary vasodilation: selective preservation in ischemia/reperfusion injury: involvement of lipoxygenase products, VR1 receptors, and C-fibers. *Circ. Res.* **90**: 465–472.

Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M. and Currie, M.G. (1993). A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.* **214**: 11–16.

Miura, H., Bosnjak, J.J., Ning, G., Saito, T., Miura, M. and Gutterman, D.D. (2003). Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ. Res.* **92**: 31–40.

Moffatt, J.D. and Cocks, T.M. (1998). Endothelium-dependent and -independent responses to protease-activated receptor-2 (PAR–2) activation in mouse isolated renal arteries. *Br. J. Pharmacol.* **125**: 591–594.

Modin, A., Bjorne, H., Herulf, M., Alving, K., Weitzberg, E. and Lundberg, J.O. (2001). Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol. Scand.* **171**: 9–16.

Molino, M., Woolkalis, M.J., Reavey-Cantwell, J., Pratico, D., Andrade-Gordon, P., Barnathan, E.S. and Brass, L.F. (1997). Endothelial cell thrombin receptors and PAR-2. Two protease-activated receptors located in a single cellular environment. *J. Biol. Chem.* **272**: 11133–11141.

Murphy, M.E. and Brayden, J.E. (1995). Apamin-sensitve K^+ channels mediate an endothelium-dependent hyperpolarization in rabbit mesenteric arteries. *J. Physiol. (Lond.)* **489**: 723–734.

Murphy, G.J. and Holder, J.C. (2000). PPAR-gamma agonists: therapeutic role in diabetes, inflammation and cancer. *Trends Pharmacol. Sci.* **21**: 469–474.

Nagase, S., Takemura, K., Ueda, A., Hirayama, A., Aoyagi, K., Kondoh, M. and Koyama, A. (1997). A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine. *Biochem. Biophys. Res. Commun.* **233**: 150–153.

Nystedt, S., Ramakrishnan, V. and Sundelin, J. (1996). The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor. *J. Biol. Chem.* **271**: 14910–14915.

Pannirselvam, M., Anderson, T.J. and Triggle, C.R. (2003a). Characterization of endothelium-derived hyperpolarizing factor-mediated relaxation of small mesenteric arteries from diabetic (db/db -/-) mice. Proceedings of the Fourth International Workshop on Endothelium-Derived Hyperpolarizing Factor. EDHF 2002, ed. by P.M. Vanhoutte, Taylor & Francis, London & New York, pp. 124–131.

Pannirselvam, M., Anderson, T.J. and Triggle, C.R. (2003b). Endothelial cell dysfunction in type I and II diabetes: The cellular basis for dysfunction. *Drug Dev. Res.* **58**: 28–41.

Rosenblum, W.I. (1987). Hydroxyl radical mediates the endothelium-dependent relaxation produced by bradykinin in mouse cerebral arterioles. *Circ. Res.* **61**: 601–603.

Rubanyi, G.M. and Vanhoutte, P.M. (1986). Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle. *Am. J. Physiol.* **251**: H815–H821.

Saenz, T., I., Goldstein, I., Azadzoi, K., Krane, R.J., and Cohen, R.A. (1989). Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *N. Engl. J. Med.* **320**: 1025–1030.

Sobey, C.G. and Cocks, T.M. (1998). Activation of protease-activated receptor-2 (PAR-2) elicits nitric oxide-dependent dilatation of the basilar artery in vivo. *Stroke* **29**: 1439–1444.

Sobey, C.G., Heistad, D.D. and Faraci, F.M. (1997). Mechanisms of bradykinin-induced cerebral vasodilatation in rats. Evidence that reactive oxygen species activate K^+ channels. *Stroke* **28**: 2290–2294.

Sobey, C.G., Moffatt, J.D. and Cocks, T.M. (1999). Evidence for selective effects of chronic hypertension on cerebral artery vasodilatation to protease-activated receptor-2 activation. *Stroke* **30**: 1933–1940.

Stadler, J., Trockfeld, J., Schmalix, W.A., Brill, T., Siewert, J.R., Greim, H. and Doehmer, J. (1994). Inhibition of cytochromes P4501A by nitric oxide. *Proc. Natl. Acad. Sci. U. S. A*, **91**: 3559–3563.

Stamler, J.S., Singel, D.J. and Loscalzo J. (1992). Biochemistry of nitric oxide and its redox-activated forms. *Science* **258**: 1898–1902.

Steinberg, H.O., Chaker, H., Leaming, R., Johnson, A., Brechtel, G. and Baron, A.D. (1996). Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *J. Clin. Invest.* **97**: 2601–2610.

Sternik, L., Samee, S., Schaff, H.V., Zehr, K.J., Lerman, L.O., Holmes, D.R., Hermann, J. and Lerman, A. (2002). C-reactive protein relaxes human vessels in vitro. *Arterioscler Thromb. Vasc. Biol.* **22**:

1865–1868.

Stroes, E., Hijmering, M., Van zandvoort, M., Wever, R., Rabelink, T.J. and Van Faassen, E.E. (1998). Origin of superoxide production by endothelial nitric oxide synthase. *FEBS Lett.* **438**: 161–164.

Taubes, G. (2002). Cardiovascular disease. Does inflammation cut to the heart of the matter? *Science* **296**: 242–245.

Ting, H.H, Timimi, F.K., Boles, K.S., Creager, S.J., Ganz, P. and Creager, M.A. (1996) Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J. Clin. Invest* **97**: 22–28.

Triggle, C.R. (2001) Endothelial cell K^+ channels, membrane potential and the release of vasoactive factors from the vascular endothelium. In: Potassium Channels in Cardiovascular Biology, ed. by N.J. Rusch and S. Archer, Plenum Publishing Corporation, New York, 2001, pp. 667–689.

Trottier, G., Hollenberg, M., Wang, X., Gui, Y., Loutzenhiser, K. and Loutzenhiser, R. (2002). PAR-2 elicits afferent arteriolar vasodilation by NO-dependent and NO-independent actions. *Am. J. Physiol.* **282**: F891–F897.

Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B.S., Karoui, H., Tordo, P. and Pritchard, K.A., JR. (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc. Natl. Acad. Sci. U. S. A*, **95**: 9220–9225.

Venugopal, S.K., Devaraj, S., Yuhanna, I., Shaul, P. and Jialal, I. (2002). Demonstration that C-reactive protein decreases ENOS expression and bioactivity in human aortic endothelial cells. *Circulation* **106**: 1439–1441.

Verma, S. and Anderson, T.J. (2002a). Fundamentals of endothelial function for the clinical cardiologist. *Circulation* **105**: 546–549.

Verma, S., Wang, C.H., Li, S.H., Dumont, A.S., Fedak, P.W., Badiwala, M.V., Dhillon, B., Weisel, R.D., Li, R.K., Mickle, D.A. and Stewart, D.J. (2002b). A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. *Circulation* **106**: 913–919.

Waldron, G.J., Ding, H., Lovren, F., Kubes, P. and Triggle, C.R. (1999). Acetylcholine-induced relaxation of arteries isolated from mice lacking endothelial nitric oxide synthase. *Br. J. Pharmacol.* **128**: 653–658.

Wei, E.P., Kontos, H.A. and Beckman, J.S. (1996). Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *Am. J. Physiol.* **271**: H1262–H1266.

Williams, B., Gallacher, B., Patel, H. and Orme, C. (1997). Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro. *Diabetes* **46**: 1497–1503.

Yamagishi, T., Saito, Y., Nakamura, T., Takeda, S., Kanai, H. and Sumino, H. (2001). Troglitazone improves endothelial function and augments renal klotho mRNA expression in Otsuka Long-Evans Tokushima Fatty (OLETF) rats with multiple atherogenic risk factors. *Hypertens. Res.* **24**: 705–709.

Yang, S.T., Mayhan, W.G., Faraci, F.M. and Heistad, D.D. (1991). Mechanisms of impaired endothelium-dependent cerebral vasodilatation in response to bradykinin in hypertensive rats. *Stroke* **22**: 1177–1182.

Zhang, Z., Naughton, D., Winyard, P.G., Benjamin, N., Blake, D.R. and Symons M.C. (1998). Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity. *Biochem. Biophys. Res. Commun.* **249**: 767–772.

Zygmunt, P.M., Edwards, G., Weston, A.H., Larsson, B. and Hogestatt, E.D. (1997). Involvement of voltage-dependent potassium channels in the EDHF-mediated relaxation of rat hepatic artery. *Br. J. Pharmacol.* **121**: 141–149.

(Received May 15, 2003; Accepted revised from October 31, 2003)