BJP British Journal of Pharmacology

DOI:10.1111/bph.13205
www.brjpharmacol.org

REVIEW

Constrictor prostanoids and uridine adenosine tetraphosphate: vascular mediators and therapeutic targets in hypertension and diabetes

Takayuki Matsumoto[1], Styliani Goulopoulou[2], Kumiko Taguchi[1], Rita C Tostes[3] and Tsuneo Kobayashi[1]

[1]*Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo, Japan,* [2]*Department of Integrative Physiology and Anatomy, Obstetrics and Gynecology, University of North Texas Health Science Center, Fort Worth, TX, USA, and* [3]*Department of Pharmacology, Ribeirao Preto Medical School University of Sao Paulo, Ribeirao Preto, SP, Brazil*

Correspondence
Takayuki Matsumoto, Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan. E-mail: t-matsu@hoshi.ac.jp

Received
21 January 2015
Revised
16 April 2015
Accepted
19 May 2015

Vascular dysfunction plays a pivotal role in the development of systemic complications associated with arterial hypertension and diabetes. The endothelium, or more specifically, various factors derived from endothelial cells tightly regulate vascular function, including vascular tone. In physiological conditions, there is a balance between endothelium-derived factors, that is, relaxing factors (endothelium-derived relaxing factors; EDRFs) and contracting factors (endothelium-derived contracting factors; EDCFs), which mediate vascular homeostasis. However, in disease states, such as diabetes and arterial hypertension, there is an imbalance between EDRF and EDCF, with a reduction of EDRF signalling and an increase of EDCF signalling. Among EDCFs, COX-derived vasoconstrictor prostanoids play an important role in the development of vascular dysfunction associated with hypertension and diabetes. Moreover, uridine adenosine tetraphosphate (Up_4A), identified as an EDCF in 2005, also modulates vascular function. However, the role of Up_4A in hypertension- and diabetes-associated vascular dysfunction is unclear. In the present review, we focused on experimental and clinical evidence that implicate these two EDCFs (vasoconstrictor prostanoids and Up_4A) in vascular dysfunction associated with hypertension and diabetes.

Abbreviations

AA, arachidonic acid; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; CDK2, cyclin-dependent kinase 2; $cPLA_2$, cytosolic PLA_2; DOCA, deoxycorticosterone-acetate; E2, 17β-oestradiol; ECs, endothelial cells; EDCF, endothelium-derived contracting factor; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; ET-1, endothelin-1; GK, Goto-Kakizaki; GPER, G protein-coupled oestrogen receptor; HETEs, hydroxyeicosatetraenoic acid; HO-1, haem oxygenase-1; HUVEC, human umbilical vein endothelial cells; L-NAME, N^G-nitro-l-arginine methyl ester; L-PGDS, lipocalin-type PGD synthase; MLC_{20}, myosin light chain 20; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; OLETF, Otsuka Long-Evans Tokushima Fatty; OPN, osteopontin; PDGFR, platelet-derived growth factor receptor; PGI_2, prostacyclin; PGIS, prostacyclin synthase; ROCK, Rho kinase; ROS, reactive oxygen species; S6K, S6 kinase; SHR, spontaneously hypertensive rats; SMCs, smooth muscle cells; STZ, streptozotocin; TP, TxA_2/endoperoxide receptor; TxA_2, thromboxane A_2; TxS, thromboxane synthase; Up_4A, uridine adenosine tetraphosphate; VP, vasopressin; WKY, Wistar-Kyoto rats

Table of Links

TARGETS	
Enzymes[a]	**GPCRs**[c]
Akt	AT1 receptor
AMPK, AMP-activated protein kinase	EP receptor
CDK2, cyclin-dependent kinase 2	DP receptor
COX-1	GPER (GPR30)
COX-2	IP receptor
$cPLA_2$, cytosolic PLA_2	$P2Y_2$ receptor
ERK1/2	$P2Y_4$ receptor
HO-1, haem oxygenase-1	$P2Y_6$ receptor
L-PGDS, lipocalin-type PGD synthase	TP receptor
NOS	**Catalytic receptors**[d]
PGIS, prostacyclin synthase (CYP8A1)	PDGFR, PDGF receptor
TxS, thromboxane synthase (CYP5A1)	**Nuclear hormone receptors**[e]
ROCK, Rho kinase	Erα oestrogen receptor (NR3A1)
Ligand-gated ion channels[b]	Erβ oestrogen receptor (NR3A2)
P2X1 receptor	

LIGANDS	
20-HETE	LY294002
AA, arachidonic acid	NO
ADP	Metformin
Angiotensin II	PD98059
Diclofenac	PGD_2
Clonidine	PGE_2
DOCA, deoxycorticosterone acetate	$PGF_{2\alpha}$
E2, 17β-oestradiol	PGI_2
EPA, eicosapentaenoic acid	Rapamycin
ET-1, endothelin-1	Suramin
L-arginine	Testosterone
Ip_5I	TxA2, thromboxane A_2
L-NAME	U46619
Losartan	VP, vasopressin

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d,e]Alexander *et al.*, 2013a,b,c,d,e).

The endothelium plays a pivotal role in the regulation of vascular tone (Vapaatalo and Mervaala, 2001; Pries and Kuebler, 2006; Flammer and Luscher, 2010; Toda *et al.*, 2010; Flammer *et al.*, 2012; Favero *et al.*, 2014). In response to mechanical forces (e.g. shear stress) and endogenous ligands, endothelial cells (ECs) release a diversity of factors that mediate or directly induce vascular smooth muscle contraction or relaxation (Vapaatalo and Mervaala, 2001; Flammer and Luscher, 2010; Flammer *et al.*, 2012). Accordingly, these factors are largely divided into relaxing and contracting factors [namely, endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) respectively]. Nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2) are well-established EDRFs (Furchgott and Zawadzki, 1980; Chen *et al.*, 1988; Feletou and Vanhoutte, 2004; Feletou, 2009; Garland *et al.*, 2011; Flammer *et al.*, 2012). On the other hand, vasoconstrictor prostanoids, endothelin-1 (ET-1), angiotensin II and more recently uridine adenosine tetraphosphate (Up_4A) have been described as major EDCFs (Yanagisawa *et al.*, 1988; Jankowski *et al.*, 2005; Vanhoutte *et al.*, 2005; 2009; Vanhoutte and Tang, 2008; Matsumoto *et al.*, 2014a).

Endothelial dysfunction is a hallmark of the pathophysiology of diabetes and arterial hypertension and contributes to macrovascular and microvascular complications associated with these disease states (De Vriese *et al.*, 2000; Kobayashi *et al.*, 2000; Vanhoutte *et al.*, 2005; 2009; Versari *et al.*, 2009; Ding and Triggle, 2010; Barton *et al.*, 2012; Bazi *et al.*, 2012; Campia *et al.*, 2012). An imbalance between EDRFs and

EDCFs plays a key role in the development of vascular dysfunction in hypertension and diabetes (Vanhoutte *et al.*, 2005; 2009; Michel *et al.*, 2008a; Vanhoutte and Tang, 2008; Ding and Triggle, 2010; Tang and Vanhoutte, 2010; Mukohda *et al.*, 2012). EDRF production, bioavailability and signalling in smooth muscle cells (SMCs) are decreased in vessels under diabetic and hypertensive conditions (Kamata *et al.*, 1989; De Vriese *et al.*, 2000; Vanhoutte *et al.*, 2005; 2009; Vanhoutte and Tang, 2008; Ding and Triggle, 2010). On the other hand, EDCF production and/or signalling are increased in vessels in the presence of those diseases (Vanhoutte *et al.*, 2005; 2009; Vanhoutte and Tang, 2008; Feletou *et al.*, 2009; 2010a; 2011; Tang and Vanhoutte, 2010; Matsumoto *et al.*, 2014a). Therefore, the fine regulation of this balance may be important in order to prevent the development of diabetes- and hypertension-associated vasculopathies.

EDRFs have attracted great attention and several excellent reviews have been written on EDRF-mediated vascular signalling in metabolic and cardiovascular diseases including diabetes and hypertension (De Vriese *et al.*, 2000; Griffith, 2004; Triggle *et al.*, 2005; Feletou *et al.*, 2008; 2009; Matsumoto *et al.*, 2008a; Grgic *et al.*, 2009; Barton, 2010; Ding and Triggle, 2010; Edwards *et al.*, 2010; Forstermann and Li, 2011; Campia *et al.*, 2012; Tousoulis *et al.*, 2012; Sena *et al.*, 2013; Toda *et al.*, 2013; Bruder-Nascimento *et al.*, 2014; Manrique *et al.*, 2014; Goulopoulou and Davidge, 2015). The role of EDCF in vascular dysfunction associated with these disease states, however, is not well understood. Constrictor prostanoids are EDCFs with potent and complex effects on vascular smooth muscle function and have been implicated in both diabetes and hypertension (Vanhoutte *et al.*, 2005; Feletou *et al.*, 2009; 2011; Versari *et al.*, 2009; Barton *et al.*, 2012). Up_4A, identified as an EDCF in 2005 (Jankowski *et al.*, 2005), also modulates vascular function (Matsumoto *et al.*, 2011a); however, the pathophysiological role of Up_4A is unclear.

In this review, we focus on the contribution of constrictor prostanoids and Up_4A to vascular dysfunction in diabetes and arterial hypertension. We address the mechanisms associated with the production and signalling of prostanoids and Up_4A in vascular SMCs and the interactions between EDCFs with EDRFs. Further, we discuss experimental evidence that implicates prostanoids and Up_4A in diabetes- and hypertension-associated vascular pathology. Potential therapeutic strategies to target the overproduction and actions of EDCFs and to decrease EDCF-mediated responses are also reviewed. The influence of sex and sex steroid hormones on vascular function have been previously described (Tostes *et al.*, 2003; 2008; Orshal and Khalil, 2004; Khalil, 2005; Arnal *et al.*, 2010; Huxley and Wang, 2010; Hart *et al.*, 2011; Nilsson *et al.*, 2011; Bubb *et al.*, 2012; Miao and Li, 2012; Kittikulsuth *et al.*, 2013) and considerable progress has been made in our understanding of the cellular and molecular mechanisms underlying the effects of sex hormones and their receptors in the vascular system (Tostes *et al.*, 2003; 2008; Orshal and Khalil, 2004; Khalil, 2005; Arnal *et al.*, 2010; Huxley and Wang, 2010; Hart *et al.*, 2011; Nilsson *et al.*, 2011; Bubb *et al.*, 2012; Miao and Li, 2012; Kittikulsuth *et al.*, 2013). In the present review, therefore, the effect of sex on EDCF vascular signalling in the context of diabetes and arterial hypertension is also discussed.

COX-mediated production of vasoconstrictor prostanoids

The production and signalling of vasoconstrictor prostanoids are increased in arterial SMCs of subjects with hypertension and diabetes (De Vriese *et al.*, 2000; Vanhoutte *et al.*, 2005; 2009; Vanhoutte and Tang, 2008; Feletou *et al.*, 2009; 2010a,b; 2011; Versari *et al.*, 2009; Ding and Triggle, 2010; Tang and Vanhoutte, 2010; Wong and Vanhoutte, 2010; Barton *et al.*, 2012; Figure 1). Increased constrictor prostanoid production and activity are attributable to the enhanced expression and/or activity of COXs (COX-1, COX-2) and to increased production of reactive oxygen species (ROS). ROS are considered EDCFs themselves as well as enhancers/modulators of EDCF responses (Tang *et al.*, 2007; Tang and Vanhoutte, 2010) in hypertensive arteries (Vanhoutte *et al.*, 2005; 2009; Vanhoutte and Tang, 2008; Feletou *et al.*, 2009; 2010a; 2011; Tang and Vanhoutte, 2010).

Stimulation of ECs (e.g. by ACh and ADP) leads to an increase in intracellular concentration of calcium (Ca^{2+}) and subsequent release of arachidonic acid (AA), which is then metabolized by COXs (Vanhoutte *et al.*, 2005; 2009; Vanhoutte and Tang, 2008; Feletou *et al.*, 2009; 2010a,b; 2011; Tang and Vanhoutte, 2009; 2010). PLA_2 significantly contributes to this process via Ca^{2+}-dependent and -independent mechanisms (Tang and Vanhoutte, 2009; Feletou *et al.*, 2010a,b). Elevated cytosolic concentration of Ca^{2+} is a trigger for endothelium-dependent contractions, probably by activating Ca^{2+}-dependent PLA_2 in ECs (Tang and Vanhoutte, 2009; 2010; Vanhoutte, 2009; Vanhoutte *et al.*, 2009; Feletou *et al.*, 2010a,b). Indeed, inhibition of Ca^{2+}-dependent PLA_2 suppresses EDCF-mediated contractions (Tang *et al.*, 2007; Ishida *et al.*, 2011). Endothelial ligands such as ACh, ET-1 and nucleotides lead to increased intracellular Ca^{2+} and then release of COX-derived EDCFs (Feletou *et al.*, 2010a,b; Tang and Vanhoutte, 2010). The calcium ionophore A23187 directly increases intracellular Ca^{2+} in ECs, and therefore, A23187 may act as an inducer of EDCF production (Shi *et al.*, 2007; Tang *et al.*, 2007). Excessive accumulation of intracellular Ca^{2+} in ECs is critical and triggers the production of COX-derived EDCFs. This abnormality has been reported in arteries from hypertensive and diabetic subjects (Shi *et al.*, 2007; Tang *et al.*, 2007; Tang and Vanhoutte, 2010). Ca^{2+}-independent PLA_2 inhibition also suppresses ACh-induced EDCF production and contraction in aorta of spontaneously hypertensive rats (SHR), a genetic rat model of hypertension (Wong *et al.*, 2010a). Thus, both Ca^{2+}-dependent and -independent mechanisms are involved in the vascular production of EDCF in hypertensive and diabetic conditions.

COXs (COX-1 and COX-2) are rate-limiting enzymes in the AA cascade (Rouzer and Marnett, 2009; Feletou *et al.*, 2011; Nakano, 2015). AA is enzymically cyclized and oxygenated to produce the endoperoxide PGG_2. COX, which catalyses this cyclooxygenation reaction, also reduces a hydroperoxyl in PGG_2 to a hydroxyl to form PGH_2 via a separate peroxidase site on the enzyme (Simmons *et al.*, 2004; Rouzer and Marnett, 2009; Salvemini *et al.*, 2013). COX-1 is constitutively expressed and is usually abundant in ECs, whereas endothelial COX-2 is mainly induced by inflammatory stimuli (Feletou *et al.*, 2009; 2010a,b; 2011; Tang and

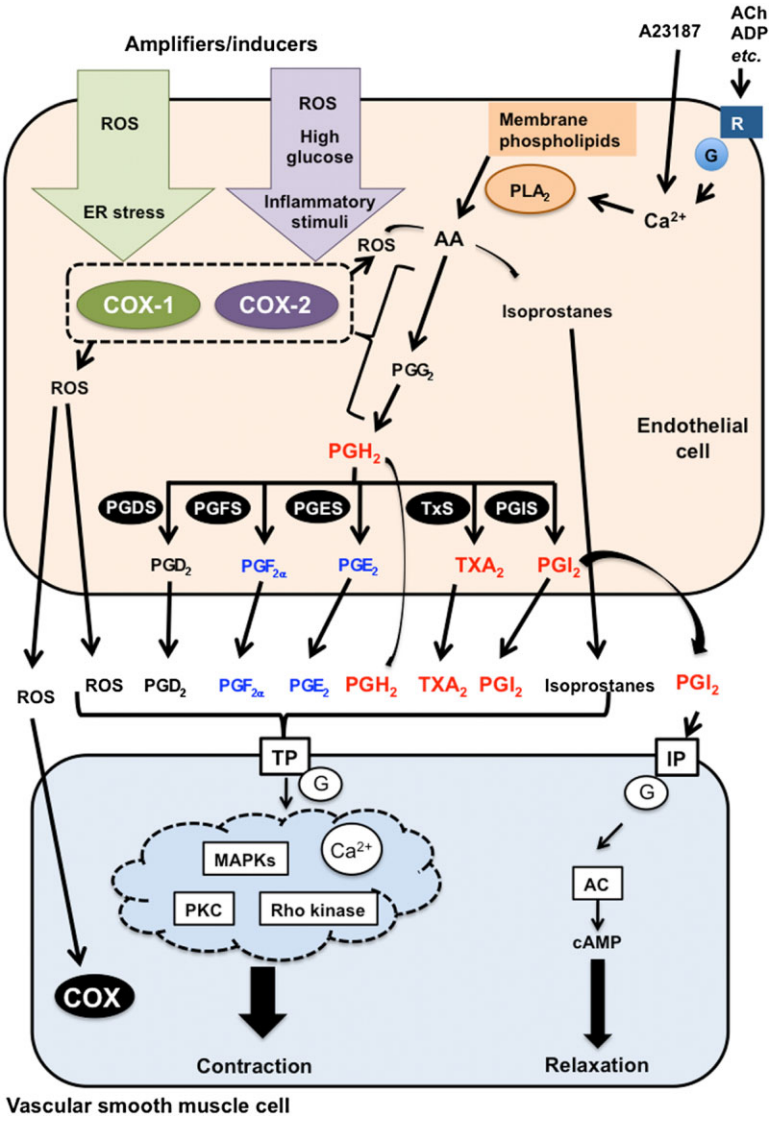

Figure 1

Major EDCF-mediated signalling pathways in hypertension and diabetes. When intracellular Ca^{2+} levels are increased via the activation of GPCR by, for example, ACh, ADP or Ca^{2+} ionophore (A23187), AA is produced from membrane phospholipids by PLA_2. AA is metabolized to endoperoxides [PGG_2 and PGH_2] through activation of COXs (COX-1 and COX-2). PGH_2 is further metabolized into PGD_2, PGE_2, $PGF_2\alpha$, PGI_2 and TxA_2 by specific synthases including PGDS, PGES, PGFS, PGIS and TxS. All these prostanoids and endoperoxides can activate TP receptors in vascular SMC leading to contraction via multiple pathways. Although PGI_2 usually leads to vasodilatation through activation of the IP receptor/adenylate cyclase (AC)/cAMP pathway, PGI_2 responses are impaired in some conditions, including hypertension and diabetes. Instead of abnormalities in the IP receptor/AC/cAMP pathway, other prostanoids such as PGE_2, $PGF_2\alpha$ and TxA_2, and endoperoxides may be increased and then stimulate TP receptors. In hypertensive arteries, mainly PGI_2, TxA_2 and PGH_2 mediate EDCF responses (in red). In addition to these prostanoids, PGE_2 and $PGF_2\alpha$ can also behave as EDCF in arteries from diabetic subjects (in blue). PGD_2 another EDCF has less significant effects compared with other PGs. COX generates ROS. ROS are considered EDCFs themselves as well as enhancers/modulators of EDCF responses, since ROS (1) further activate endothelial and smooth muscle COX and (2) increase production of isoprostanes via AA, leading to TP receptor activation. COX activity is regulated and further enhanced by several amplifier/inducers in hypertension and diabetes. Details are described in the text.

Vanhoutte, 2009; 2010; Vanhoutte, 2009; Grosser *et al.*, 2010). A growing body of evidence suggests that COX-1 is the primary isoform related to endothelium-dependent contractions (Tang *et al.*, 2005b; Tang and Vanhoutte, 2008; Vanhoutte, 2009; Wong *et al.*, 2009; Feletou *et al.*, 2010a,b; Rovati *et al.*, 2010). For instance, endothelium-dependent contractions are abolished by specific COX-1 inhibitors, but are relatively insensitive to specific COX-2 inhibitors (Yang *et al.*, 2003a; 2004a). In rat aorta, both COX-1 and COX-2 are detected; however, the amount of COX-2 transcripts in ECs or SMCs is markedly less than that of COX-1 (Tang and Vanhoutte, 2008). Moreover, endothelium-dependent con-

tractions are seen in aortae isolated from wild-type and COX-2 deficient mice, but not from COX-1-deficient mice, suggesting that COX-1 is necessary for endothelium-dependent contractions (Tang *et al.*, 2005b).

Although in healthy conditions the contribution of COX-2 to endothelium-dependent contractions is negligible, COX-2 is implicated in such contractions in hypertension, diabetes and aging (Matsumoto *et al.*, 2007a; Shi *et al.*, 2008). In arteries of SHR or diabetic rats, COX-1 expression is up-regulated, and COX-1 inhibitors block the augmented endothelium-dependent contractions (Yang *et al.*, 2002; Gluais *et al.*, 2006; Shi *et al.*, 2007). COX-1-derived PGI_2, thromboxane A_2 (TxA_2) or endoperoxides all contribute to endothelium-dependent contractions in hypertension (Gluais *et al.*, 2006; 2007). In hamster aortae, endothelium-dependent, TxA_2/endoperoxide (TP) receptor-mediated contraction induced by ACh is mediated by $PGF_2\alpha$. In this study, $PGF_2\alpha$ derived from COX-2 and not from COX-1, enhanced the COX-2/$PGF_2\alpha$/TP pathway (Wong *et al.*, 2009). Human renal arteries also exhibit TP receptor-mediated ACh- or $PGF_2\alpha$-induced contractions and COX-2-dependent release of $PGF_2\alpha$ (Wong *et al.*, 2009). Endothelial COX-2 rather than COX-1 seems to be responsible for enhanced generation of EDCF in aortae from rats under chronic treatment with the NOS inhibitor L-NAME (Qu *et al.*, 2010).

In diabetic animal models, COX-2-derived prostanoids have been suggested to induce abnormal vasoconstrictor responses or to account for the development of endothelium-derived vasoconstrictor activity (Quilley and Chen, 2003; Bagi *et al.*, 2005; Guo *et al.*, 2005; Nacci *et al.*, 2009; Lopez-Lopez *et al.*, 2011; Ramos-Alves *et al.*, 2012a,b; Vessieres *et al.*, 2013). Indeed, COX-2 expression and activity are increased in diabetic arteries (Bagi *et al.*, 2006; Sanchez *et al.*, 2010; Kassan *et al.*, 2013; Martinez *et al.*, 2014). Moreover, at the cellular level, high glucose, which is a characteristic of diabetes (De Vriese *et al.*, 2000; Rask-Madsen and King, 2007; 2013; Forbes and Cooper, 2013), increases COX-2 expression and activity in ECs (Cosentino *et al.*, 2003; Sheu *et al.*, 2005). Others, however, have reported up-regulation of COX-1 and increased production of COX-1-derived prostanoids in arteries of diabetic subjects (Matsumoto *et al.*, 2007a; 2009b; Shi *et al.*, 2007; Shi and Vanhoutte, 2008; Feletou *et al.*, 2011). Using COX-1-deficient mice fed with a high-fat diet and treated with streptozotocin (STZ; animal model of type 1 diabetes), Zhu *et al.* (2014) demonstrated that COX-1 remains the major contributor to the synthesis of endothelial PGI_2 that leads to vasoconstrictor activity in these conditions (Zhu *et al.*, 2014). Discrepancies about the contribution of COX-1 and COX-2 to endothelium-mediated contractions in hypertension and diabetes may be attributed to different species, vascular beds, disease models and disease duration.

COX is involved in the endothelial generation of ROS (Tang and Vanhoutte, 2009; Feletou *et al.*, 2010a; Sena *et al.*, 2013; Hernanz *et al.*, 2014). Increased COX-derived endothelial production of ROS contributes to the development of endothelial dysfunction in SHR aorta (Tang *et al.*, 2007). ROS affects NO bioavailability (Hattori *et al.*, 1991; Kamata and Kobayashi, 1996; Matsumoto *et al.*, 2007b) and COX activation (Korbecki *et al.*, 2013; Hernanz *et al.*, 2014) and thus, it may also affect the balance between EDCF and EDRF signalling. Indeed, excessive formation of superoxide eliminates the vasodilatory, anti-thrombotic and anti-adhesive effects of NO and PGI_2 and stimulates the potent vasoconstrictor, pro-thrombotic and pro-inflammatory actions of PGH_2 and TxA_2 (Zou, 2007). Upon diffusion into vascular SMCs, ROS activate COX and further stimulates the production of contractile prostanoids (Katusic and Vanhoutte, 1989; Yang *et al.*, 2002; Feletou *et al.*, 2010a,b; Tang and Vanhoutte, 2010). Moreover, ROS induce the production of isoprostanes such as 8-*iso*-$PGF_{2\alpha}$ and 8-*iso*-PGE_2, which can activate TP receptors on vascular SMC, leading to contraction (Bauer *et al.*, 2014). Thus, ROS are key players in EDCF-mediated signalling (Tang *et al.*, 2007; Tang and Vanhoutte, 2010) in hypertensive arteries (Vanhoutte *et al.*, 2005; 2009; Vanhoutte and Tang, 2008; Feletou *et al.*, 2009; 2010a; 2011; Tang and Vanhoutte, 2010).

Prostanoids: endothelium-derived contracting factors

In the prostanoid pathway, both COXs oxidize AA to form unstable PGH_2 via the intermediate PGG_2. PGH_2 is further metabolized into PGD_2, PGE_2, $PGF_2\alpha$, PGI_2 and TxA_2 by specific synthases including PGD synthase (PGDS), PGE synthase (PGES), PGF synthase (PGFS), PGI synthase (PGIS) and thromboxane synthase (TxS) respectively (Simmons *et al.*, 2004; Tang and Vanhoutte, 2009; Figure 1). Among these prostanoids, PGI_2 and TxA_2 are primarily implicated in EDCF-mediated responses; however, PGD_2, PGE_2 and $PGF_2\alpha$ may also contribute to EDCF responses (Vanhoutte *et al.*, 2009; Wong and Vanhoutte, 2010; Figure 1). Although endoperoxides have a very short life, they have contractile actions (Feletou *et al.*, 2009). Stimulus (i.e. ligand), type of vessel and disease state are all determinants of which prostanoids and what amount of each prostanoid (and combination) would contribute to endothelium-dependent contraction. For example, in SHR aorta, ACh-induced endothelium-dependent contractions are largely due to the release of PGI_2 rather than other PGs (Gluais *et al.*, 2005). However, TxA_2 contributes to EDCF-mediated contraction induced by other ligands such as ADP, A23187 and ET-1 (Gluais *et al.*, 2006; 2007; Vanhoutte *et al.*, 2009; Wong and Vanhoutte, 2010). ACh-induced increased production of TxA_2 and PGE_2, but not $PGF_2\alpha$ or PGI_2 has been reported in superior mesenteric arteries from type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Matsumoto *et al.*, 2007a; 2008b).

Prostacyclin is the major COX-derived metabolite of AA via PGIS in ECs (Tang and Vanhoutte, 2009; Wong and Vanhoutte, 2010; 2011). Gene expression of PGIS is by far the most abundant and averages more than 90% of the total expression of prostanoid synthases in EC of SHR aorta (Tang and Vanhoutte, 2008). Moreover, the extent of co-localization of PGIS with COX-1 in EC is greater than that with COX-2 (Kawka *et al.*, 2007) and PGIS expression is increased in the aortae of SHR [vs. control Wistar-Kyoto rats (WKY)] (Numaguchi *et al.*, 1999; Tang and Vanhoutte, 2008). Thus, the majority of the endothelial COX-1-derived endoperoxides are transformed into PGI_2 in hypertensive arteries (Gluais *et al.*, 2005; 2006; Tang and Vanhoutte, 2008; 2010; Feletou *et al.*, 2009). Prostacyclin-induced IP receptor activation usually results in endothelium-dependent vasodilation

via the activation of the adenylyl cyclase/cAMP pathway (Alfranca *et al.*, 2006; Feletou *et al.*, 2010b). However, in experimental hypertension, PGI_2 binds to TP receptors to evoke vasoconstriction (Gluais *et al.*, 2005; 2006). This may be due to dysfunction of IP receptors and the AC pathway (Gomez *et al.*, 2008). Indeed, the relaxation induced by PGI_2 or its stable analog iloprost is lost in aortae from SHR and aged WKY (Rapoport and Williams, 1996; Gluais *et al.*, 2005). Although high concentrations of PGI_2 led to vasoconstriction via the activation of TP receptors, PGI_2 may be only weakly effective at TP receptors, as PGI_2 is rapidly degraded into its inactive metabolite 6-keto-$PGF_1\alpha$ (Gluais *et al.*, 2005). In disease states including hypertension and diabetes, PGI_2 abnormalities refer to its interaction with the IP receptor and its downstream signalling as well as its synthesis by PGIS. In hypertension and diabetes, ROS interact with NO to form peroxynitrite (Zou *et al.*, 2004; Szabo, 2009; Forstermann and Li, 2011). Peroxynitrite inhibits PGIS activity via nitration of tyrosine residues (Zou *et al.*, 2002; Nie *et al.*, 2006; Zou, 2007). Thus, the regulation of PGIS activity (e.g. suppression of nitrosative stress) and of IP receptors are important to improve PGI_2 signalling in hypertension- and diabetes-associated vasculopathy (Szabo, 2009; Forstermann and Li, 2011; Vanhoutte, 2011).

TxA_2, a metabolite of AA through TxS enzymic activity, mediates a number of cellular responses including vasoconstriction and platelet aggregation (Narumiya *et al.*, 1999; Nakahata, 2008). Alterations in TxA_2 biosynthesis and actions have been investigated in a series of pathophysiological conditions such as atherosclerosis, myocardial ischaemia, asthma, diabetes, hypertension and pregnancy-induced hypertension (Meagher and FitzGerald, 1993; Hopkins, 2013; Taguchi *et al.*, 2014). The contribution of TxA_2 to endothelium-dependent contraction may be ligand, vessel and disease specific. ACh-induced endothelium-dependent contractions are not affected by TxS inhibitors in SHR aortae (Auch-Schwelk *et al.*, 1990; Tang and Vanhoutte, 2009) or canine basilar arteries (Katusic *et al.*, 1988). On the other hand, inhibitors of TxS suppressed ADP-, A23187- and AA-induced endothelium-dependent contraction in the same preparations (Katusic *et al.*, 1988; Gluais *et al.*, 2006; 2007). Treatment of type 2 diabetic OLETF rats with a TxS inhibitor suppressed ACh-induced TxA_2 production, partly improved ACh-induced endothelium-dependent, NO-dependent and EDHF-dependent relaxations, and inhibited ACh-induced endothelium-dependent contractions in superior mesenteric arteries. These data suggest that TxS inhibition normalizes endothelial dysfunction in type 2 diabetes (Matsumoto *et al.*, 2009c).

As mentioned above, PGD_2, PGE_2 and $PGF_2\alpha$ also induce endothelium-dependent contractions; however, the contribution of these prostanoids to EDCF-mediated contractions is considered marginal in most cases, including disease states (Vanhoutte and Tang, 2008; Tang and Vanhoutte, 2009).

Expression of PGDS is increased in response to fluid shear stress (Taba *et al.*, 2000). Studies using lipocalin-type PGDS (L-PGDS)-deficient mice indicate that L-PGDS plays an important role in regulating insulin sensitivity and the development of atherosclerosis in type 2 diabetes (Ragolia *et al.*, 2005). PGD_2 may act as an EDCF via activation of TP receptors rather than DP receptors (Gluais *et al.*, 2005; Feletou *et al.*, 2011).

Expression of PGES and PGFS has been detected in aortae from the SHR (Tang and Vanhoutte, 2008; Tang *et al.*, 2008) but their expression was lower than the expression of PGIS. Further, there was no difference in production of either PG between WKY and SHR aortae (Gluais *et al.*, 2005; 2006; 2007). Under normal physiological conditions, PGE_2 and $PGF_2\alpha$ are natural agonists of the EP and FP receptors, respectively (Alfranca *et al.*, 2006; Feletou *et al.*, 2011). In hypertension, however, PGE_2 and $PGF_2\alpha$ activate the TP receptor (Gluais *et al.*, 2005; Feletou *et al.*, 2011). PGE_2 and $PGF_2\alpha$ can act as EDCFs when PGIS is inhibited or the metabolism of PGH_2 is diverted (Gluais *et al.*, 2005), a phenomenon that may occur when severe oxidative stress leads to tyrosine nitration of PGIS (Zou *et al.*, 2002). Therefore, in the SHR aortae, TxA_2, PGH_2, PGI_2 and, depending on the situation, PGE_2 and $PGF_2\alpha$ can all act as EDCFs (Feletou *et al.*, 2010a).

Interactions between vasoconstrictor prostanoids and other endothelium-derived factors

In healthy ECs, the production and/or release of EDCFs is tempered by the presence of NO (Tang *et al.*, 2005a; Vanhoutte and Tang, 2008; Tang and Vanhoutte, 2009; Vanhoutte *et al.*, 2009) and EDHFs (Michel *et al.*, 2008a). Endothelium-dependent contractions induced by ACh and nucleotides were relatively weak under basal conditions, but NOS inhibition amplified these contractions (Yang *et al.*, 2003b; 2004a,b; Matsumoto *et al.*, 2007a; Ishida *et al.*, 2011). Accordingly, in cases of endothelial dysfunction (viz. impaired bioavailability of EDRFs), the production of EDCF predominates. Treatment with L-arginine, a substrate for NOS (Forstermann and Li, 2011), inhibited contractions and normalized relaxation responses to ACh in renal arteries from hypertensive Dahl salt rats, whereas U46619-induced contraction and nitroprusside-induced endothelium-independent relaxation were not affected by L-arginine treatment (Zhou *et al.*, 2001). Endothelium-dependent contractions were suppressed in arteries exposed to endogenous or exogenous NO (Tang *et al.*, 2005a; Feletou *et al.*, 2008). EDCF, on the other hand, leads to reduced NO bioavailability, suggesting a reciprocal relationship between NO and EDCF. ROS derived from stimulation of EC or TP receptor activation (Zhang *et al.*, 2008; Del Turco *et al.*, 2014) may reduce NO bioavailability. Activation of TP receptors in ECs has inhibitory effects on NO production, suppressing endothelium-dependent vasodilation (Liu *et al.*, 2009). EDCF can also suppress EDHF signalling. Michel *et al.* (2008a) found that EDCF-mediated contraction in rat renal arteries was amplified not only by NOS inhibition, but also EDHF inhibition. In contrast, TxA_2 can interact with signalling pathways associated with EDHF (Ellinsworth *et al.*, 2014). TP receptor stimulation is associated with loss of small-conductance Ca^{2+}-activated potassium (SK_{Ca}) channel activity and with decreased EDHF-mediated responses in the rat mesenteric artery (Crane and Garland, 2004).

ET-1 is an EDCF with an important role in the pathogenesis of hypertension and diabetes (Maguire and Davenport, 2014; Matsumoto *et al.*, 2014a; Sandoval *et al.*, 2014). Inter-

actions between ET-1 and vasoconstrictor prostanoids have been previously reported. Earlier studies showed that incubation of human aortic ECs with ET-1 led to production of the PGI_2 metabolite 6-keto-$PGF_1\alpha$, the TxA_2 metabolite TxB_2 and PGE_2 (Hollenberg *et al.*, 1994). In rat aorta, ET-1 induced the release of PGH_2 from ECs and this effect was greater in the hypertensive state (Asano *et al.*, 1994). Basal and ET-1-stimulated release of TxB_2 was found in endothelium-intact aortae from SHR, but not WKY rats. ET-1-induced contraction was reduced by denudation of the endothelial layer, inhibition of TxS, or antagonism of TP receptors in aortae from SHR, but not WKY (Taddei and Vanhoutte, 1993). Furthermore, exogenous ET-1-induced vasoconstriction was largely blocked by intrabrachial administration of indomethacin (Taddei *et al.*, 2000) in patients with essential hypertension, but not in normotensive subjects. Thus, ET-1/prostanoid interactions may be essential in the regulation of vascular tone.

Several studies in humans also suggest interactions between EDCF and other endothelium-derived factors. In healthy subjects, infusion of ACh into the brachial artery led to a concentration-dependent increase in flow-mediated dilation, which was diminished by NOS, but not by COX inhibition (Taddei and Vanhoutte, 1993; Taddei *et al.*, 1993; 1998). In contrast, ACh-induced vasodilatation was blunted in patients with essential hypertension and was resistant to NOS inhibition (Dohi *et al.*, 1990). COX inhibition enhanced ACh-induced vasodilatation in these patients, suggesting that COX-derived vasoconstrictors are largely responsible for the abnormal response to endothelium-dependent vasodilators in essential human hypertension (Vanhoutte *et al.*, 2005; Versari *et al.*, 2009; Virdis *et al.*, 2010).

Vasoconstrictor prostanoid-mediated signalling in vascular SMCs: focus on TP receptors

Prostanoids signal their constrictor effects on vascular SMC primarily via activation of the TP receptor, a member of the seven transmembrane GPCR superfamily (Nakahata, 2008; Woodward *et al.*, 2011; Alexander *et al.*, 2013). In humans, two distinct TP receptor isoforms have been found, namely TPα and TPβ, but these are not present in other species, such as non-human primates and rodents (Hopkins, 2013). TP receptors are expressed in endothelial and vascular SMC. Those located in vascular SMC are the primary contributors to the hypertensive phenotype (Sparks *et al.*, 2013) and to COX-dependent, endothelium-dependent vasoconstriction (Yang *et al.*, 2003a).

TxA_2-induced contractions in isolated bovine aortic SMC and isolated vascular tissue from various species involve both Ca^{2+}-dependent and -independent mechanisms. Various mouse arteries exhibit contractile responses to ACh (carotid > abdominal aorta > femoral) and these responses are abolished by TP receptor antagonism (Zhou *et al.*, 2005), suggesting that endothelium-dependent contractile responses in healthy vascular tissues are evoked through activation of TP receptors. Although TxA_2 is the preferred TP receptor ligand, high concentrations of endoperoxides, other PGs, as well as isoprostanes and hydroxyeicosatetraenoic acids (HETEs; Roman, 2002; Miyata and Roman, 2005) also activate the TP receptor (Feletou *et al.*, 2010a; 2011). 20-HETE has been shown to induce arterial contraction via TP receptor activation. The rate-limiting step of this action is the conversion of 20-HETE to 20-endoperoxides (20-OH-PGH_2, 20-OH-PGG_2) by COX (Escalante *et al.*, 1989; Schwartzman *et al.*, 1989). 20-HETE-induced contraction is partly dependent on the presence of the endothelium (Schwartzman *et al.*, 1989; Randriamboavonjy *et al.*, 2003) and is abolished by COX inhibition (Escalante *et al.*, 1989; Schwartzman *et al.*, 1989) and by TP receptor antagonists (Schwartzman *et al.*, 1989). In cerebral arteries, flow-mediated constriction involves increased production of ROS and COX activation, and is mediated by the interaction between 20-HETE and TP receptors (Toth *et al.*, 2011). These data suggest that TP receptors may be an integral part of all eicosanoid pathways.

Activation of TP receptors mainly involves stimulation of Gq-dependent phospholipase C (PLC) β, leading to Ca^{2+}-dependent activation of myosin light chain (MLC) kinase and 20 kDa MLC (MLC_{20}) phosphorylation (Fukata *et al.*, 2001; Wilson *et al.*, 2005). Increases in Ca^{2+} influx due to opening of both receptor-operated and voltage-gated Ca^{2+}channels contribute to TP activation-induced Ca^{2+}-dependent effects (Okon *et al.*, 2002). One of the signalling molecules activated by TP receptors in smooth muscle is Rho kinase (Somlyo and Somlyo, 2003). The activation of Rho kinase leads to inhibition of myosin light chain phosphatase, which reduces MLC_{20} dephosphorylation (Somlyo and Somlyo, 2000; Nunes *et al.*, 2010). Activation of TP receptors involves receptor co-coupling to G_{12} and activation of RhoA/Rho kinase (ROCK) signalling, engaging Ca^{2+}-independent increases in overall levels of phosphorylated MLC_{20}, which leads to vascular contraction (Wilson *et al.*, 2005). To study the relationship between ROCK and EDCF signalling, Chan *et al.* (2009) investigated the expression of ROCK and the effect of Rho kinase inhibitors on endothelium-dependent contraction in aorta isolated from 1-year old SHR and WKY. They found that in the presence of an NOS inhibitor, ROCK inhibitors reduced endothelium-dependent contractions induced by ACh and the Ca^{2+} ionophore A23187 in aortae from both SHR and WKY. ROCK inhibitors did not affect the production of 6-keto $PGF_1\alpha$ (PGI_2 metabolite), but suppressed U46619- and $PGF_2\alpha$-induced contraction and ROCK expression in both groups (Chan *et al.*, 2009). These findings suggest that the suppression of EDCF-mediated contraction by ROCK inhibition is mainly due to direct suppression of EDCF-mediated signalling in vascular SMCs and not due to an effect on EDCF release (Chan *et al.*, 2009). Denniss *et al.* (2010) reported that increased ACh-induced contractions in SHR (vs. WKY) were abolished by endothelial denudation or COX-1 inhibition, and nearly eliminated by TP receptor antagonists or by ROCK inhibition (Denniss *et al.*, 2010). ACh-induced PGI_2 production was greater in carotid arteries from SHR (vs. WKY), but ROCK inhibition did not affect PGI_2 production. Protein expression of RhoA, but not ROCK-II, as well as Rho activation was increased in carotid arteries of SHR (vs. WKY artery). In addition, RhoA activation was increased by ACh stimulation. Quenching superoxide with tiron or NAD(P)H oxidase inhibition by apocynin reduced ACh-induced contraction in SHR, whereas the superoxide dismutase mimetic tempol

amplified the response. Exogenous H_2O_2-induced contractions in carotid arteries were greater in SHR than in WKY and these responses were abolished by COX-1 inhibition, and greatly reduced by TP receptor antagonism or ROCK inhibition (Denniss *et al.*, 2010). These results suggest that the RhoA/ROCK pathway may be a molecular switch, transducing signals from endothelium-derived PGs and ROS, to turn on vascular SMC contractile pathways (Denniss *et al.*, 2010). This evidence suggests that RhoA/ROCK is a key mediator of abnormal EDCF signalling in hypertension. In rat isolated mesenteric resistance and uterine arteries, inhibition of PKC, ERK1/2 and p38 MAPK (significantly reduced TP receptor-dependent contractions (Goulopoulou *et al.*, 2012). These data indicate that in addition to RhoA/ROCK, PKC and MAPKs are also important downstream effectors of activated TP receptors (Bolla *et al.*, 2002; Nakahata, 2008).

Activation of TP receptors significantly contributes to the pathogenesis of hypertension (Keen *et al.*, 1997; Francois *et al.*, 2004). TP receptor-deficient mice do not develop hypertension in response to chronic angiotensin II infusion and have a reduced BP response to chronic treatment with L-NAME (Keen *et al.*, 1997; Francois *et al.*, 2004; 2008). Spontaneously hypertensive rats have increased endothelium-dependent contractions in response to ACh and endoperoxides and these responses appear after the development of the hypertensive phenotype (Ge *et al.*, 1995; Yang *et al.*, 2002; 2003a). TP receptor antagonism reversed ACh-induced vasoconstriction to vasodilatory responses in cerebral arterioles and abolished isometric contractions in aortic rings from hypertensive rats (Okon *et al.*, 2002; Yang *et al.*, 2002). Moreover, contractions induced by the TP receptor agonist U46619 were increased in various arteries from SHR (vs. WKY; Gluais *et al.*, 2005; Chan *et al.*, 2007; Garcia-Redondo *et al.*, 2015). Thus, TP receptors are critical mediators of endothelium-dependent contractions in hypertension.

As described above, ROS, which are enhancers/modulators of EDCF responses, modulate TP receptor function, density and stability. Hydrogen peroxide prevents the translocation and degradation of TP receptors, increasing their density at the cell membrane (Valentin *et al.*, 2004), and TP receptor activation increases the stability of the receptors through a ROS-dependent post-transcriptional mechanism (Wilson *et al.*, 2009). Thus, it is reasonable to speculate that in hypertension, a condition characterized by increased production of ROS, an increase in TP density and stability contributes to augmented endothelium-dependent contractions. Interestingly, there are no differences in the mRNA and protein expression of TP receptors between aortae of WKY and SHR (Tang and Vanhoutte, 2008; Tang *et al.*, 2008), suggesting that an increase in TP receptor density is not the reason for the enhanced endothelium-dependent contractions found in the SHR aorta. Thus, an increase in EDCF production as well as alterations in signalling pathways downstream of TP receptors are more likely to contribute to the hyper-responsiveness to EDCF in hypertensive vessels.

Enhanced activation of TP receptors and augmented endothelium- and TP receptor-dependent vascular contractions are also observed in various metabolic diseases and experimental models of diabetes and hypercholesterolemia (Traupe *et al.*, 2002; Jerez *et al.*, 2008; Michel *et al.*, 2008b). Increased U46619-induced contractions are seen in arteries from obese and diabetic animals (Nobe *et al.*, 2008; Yang *et al.*, 2008; Baretella *et al.*, 2014; Matsumoto *et al.*, 2014b). Obesity and diabetes augment the responses to EDCF, possibly due to an increase in TP receptor gene expression (Traupe *et al.*, 2002). PG receptors other than TP are also involved in diabetes-associated increases in EDCF-induced contractions. In a rat model of type 1 diabetes (STZ-treated rats), PGE_2 promotes vascular contractions via EP receptor activation (Shi *et al.*, 2007). RhoA/ROCK signalling is central in diabetes-associated EDCF-induced contractions (Ishida *et al.*, 2012). However, TP receptor-induced augmented vasoconstriction in penile arteries from pre-diabetic obese Zucker rats is coupled to enhanced Ca^{2+} influx rather than ROCK-mediated augmentation of myofilament Ca^{2+} sensitization (Villalba *et al.*, 2011).

In summary, in hypertension and diabetes, endothelium-dependent contractions are mainly TP receptor-dependent. The molecular mechanisms downstream of TP receptors involve Ca^{2+}- dependent and -independent pathways, various kinases (ERK1/2, PKC, Rho kinase, p38 MAPK) and their predominance may be vascular bed and disease specific (Figure 1). TP receptors are a promising pharmacological target because their antagonism may suppress EDCF-induced contractions and restore the balance between EDRF and EDCF vasoactive actions (Belhassen *et al.*, 2003; Zuccollo *et al.*, 2005; Gelosa *et al.*, 2010).

Pharmacological targets for the control of EDCF signalling in diabetes and hypertension

Therapies aimed to correct the imbalance in the synthesis/release and signalling of vasoconstrictor and vasodilator prostanoids may be important in the treatment of hypertensive and diabetic subjects. Accordingly, putative targets for the control of EDCF signalling in hypertensive arteries have been suggested.

The regulation of COX activity plays an important role in the control of EDCF signalling. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit production of PGs by acting on COX-1 and/or COX-2 (Amer *et al.*, 2010). Non-selective NSAIDs, such as naproxen and ibuprofen, inhibit both COX-1 and COX-2 whereas selective NSAIDs act on COX-1 (aspirin) or COX-2 (celecoxib) isoenzymes (Amer *et al.*, 2010). Although COX inhibition seems a possible strategy to prevent COX-associated vascular complications, the incidence of serious adverse cardiovascular effects with COX-2 selective inhibitors (rofecoxib or Vioxx®) has greatly antagonized this concept (Bunimov and Laneuville, 2008; Wong *et al.*, 2010b). There is consensus, however, that low-dose aspirin exerts protective vascular effects. Further investigation of COX inhibitors is required, especially towards their specificity and/or direct inhibition of PGIS activity (Wong *et al.*, 2010b). Meanwhile, antagonism of TP receptors may emerge as a therapeutic alternative to reverse prostanoid-mediated vascular dysregulations (Chamorro, 2009; Jones *et al.*, 2009; Giannarelli *et al.*, 2010; Siller-Matula *et al.*, 2010; Wong *et al.*, 2010b; Davi *et al.*, 2012). Since the

Table 1

Potential pharmacological tools to suppress enhanced vascular EDCF-mediated signalling in hypertensive and diabetic models

Compound name	General action	Main outcome (animal model)	References
PDTC	Low-molecular-weight thiol antioxidant and potent inhibitor of NF-κB (Cau *et al.*, 2011)	Suppresses EDCF- and AA (a source of EDCF)-mediated contraction, as well as the production of prostanoids (OLETF rat)	Matsumoto *et al.*, 2009a
Metformin	A biguanide derivative and one of the most commonly used drugs for the treatment of type 2 diabetes (Almabrouk *et al.*, 2014)	Reduces EDCF production and contraction induced by ACh and reduces oxidative stress (OLETF rat)	Matsumoto *et al.*, 2008b
AICAR	An activator of AMPK (AMP-activated protein kinase), a putative regulator of metformin signalling (Lempiäinen *et al.*, 2012)	Suppresses ACh-induced EDCF- mediated contraction and the production of vasoconstrictor prostanoids (TxA_2 and PGE_2; OLETF rat and SHR)	Matsumoto *et al.*, 2008b; Ford and Rush, 2011
Pravastatin	One of the statins which inhibit the enzyme HMG-CoA reductase and the production of cholesterol (Kobayashi *et al.*, 2000)	Pravastatin reduces EDCF-mediated responses by suppressing Rho kinase activity and by stimulating antioxidant activity (OLETF rat)	Ishida *et al.*, 2012
Losartan	Angiotensin AT_1 receptor antagonist (Kobayashi *et al.*, 2008)	Reduces ACh-induced production of prostanoids and EDCF(PGE_2)-mediated contraction in SMCs and normalizes oxidative stress (OLETF rat) Suppresses EDCF-mediated responses induced by purinergic receptor stimulation by suppressing COX-2 expression and $cPLA_2$ phosphorylation in arteries (GK rat)	Matsumoto *et al.*, 2010; Ishida *et al.*, 2011
TUDCA PBA	Chemical chaperones (Kraskiewicz and FitzGerald, 2012)	Suppresses ACh- and AA-induced vascular contractions and decreases expression of COX-1 and activities of $cPLA_2$ and ERK (SHR)	Spitler *et al.*, 2013
Haemin	HO-1 inducer (Ndisang *et al.*, 2001)	Suppresses ACh- and A23187-induced vascular contraction via a decrease in COX-1, but not COX-2 (SHR)	Li *et al.*, 2011
EPA	An omega-3 PUFA that modulates cell membrane fatty acid composition (Calder, 2012).	Suppresses EDCF- and AA-induced contractions and improves EDRFs (NO and EDHF)-mediated relaxation – beneficial effects that are associated with reduced COX-2 expression and activities of ERK and NF-κB (OLETF)	Matsumoto *et al.*, 2009b

PBA, 4-phenylbutyric acid; PDTC, pyrrolidine dithiocarbamate; PUFA polyunsaturated fatty acid; TUDCA, tauroursodeoxycholic acid.

actions of prostanoids are complex, the regulation of prostanoid signalling may need to be carefully planned, on a disease-by-disease basis.

Other molecular targets for the suppression of EDCF responses in hypertension and diabetes have been reported (Table 1). Ford and Rush reported that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an activator of AMP-activated protein kinase (AMPK; Lempiäinen *et al.*, 2012), suppresses EDCF-mediated responses in aortas from SHR and this was reversed in the presence of the AMPK inhibitor Compound C (Ford and Rush, 2011). Spitler *et al.* recently found that endoplasmic reticulum (ER) stress contributed to increased COX-1 expression and prostanoid release (Spitler *et al.*, 2013). In addition, ER stress increased phosphorylation of ERK1/2 and cytosolic PLA_2 ($cPLA_2$), contributing to enhanced EDCF-mediated responses. Treatment with chemical chaperones [ER stress inhibitor, taurourosodeoxycholic acid or 4-phenylbutyric acid] decreased ACh- and AA-induced contraction, COX-1 expression and phosphorylation of $cPLA_2$ and ERK1/2 in SHR aorta. Accordingly, pharmacological inhibition of ER stress suppressed EDCF-mediated responses in aortas and lowered BP in SHR (Spitler *et al.*, 2013). Li *et al.* (2011) found that treatment with haemin, an inducer of haem oxygenase-1 (HO-1; Ndisang *et al.*, 2001), decreased EDCF-mediated contractions induced by ACh or A23187 via suppression of COX-1, but not COX-2 in aortae from SHR (Li *et al.*, 2011). Moreover, induction of HO-1 with haemin improved endothelium-dependent vascular relaxation through suppression of ROS production and inhibition of COX-2 upregulation induced by diabetes (Wang *et al.*, 2014). Therefore, drugs aimed to increase AMPK or HO-1 activity to inhibit ER stress may represent therapeutic strategies for the suppression of EDCF-induced contractions and the improvement of treatment of vascular function in hypertension.

In OLETF rats, a model of type 2 diabetes associated with the metabolic syndrome, we found increased production and signalling of COX-derived vasoconstrictor prostanoids in mesenteric arteries (Matsumoto *et al.*, 2007a). Although the causal factors of enhanced vasoconstrictor prostanoids in OLETF rats remain unclear, potential pharmacological tools leading to suppression of EDCF-mediated signalling have been reported (Table 1). AICAR, an AMPK activator (Lempiäinen *et al.*, 2012), inhibited ACh-induced contraction and production of vasoconstrictor prostanoids (Matsumoto *et al.*, 2008b). Likewise, metformin (Almabrouk *et al.*, 2014) decreased ACh-induced contractions, as well as prostanoid superoxide production (Matsumoto *et al.*, 2008b). Treatment with pyrrolidine dithiocarbamate, an inhibitor of NF-κB (Cau *et al.*, 2011), suppressed ACh- and AA-induced contraction and ACh-induced prostanoid release (Matsumoto *et al.*, 2009a). Treatment with pravastatin [an hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor] attenuated endothelium-dependent contractions by inhibiting ROCK activity and up-regulating antioxidant activity (Ishida *et al.*, 2012). The angiotensin II AT_1 receptor antagonist, losartan (Kobayashi *et al.*, 2008), suppressed ACh-stimulated prostanoid production, ACh- and AA-induced contraction and reduced superoxide production (Matsumoto *et al.*, 2010). Treatment with losartan suppressed endothelium-dependent contraction by nucleotides and $cPLA_2$ phosphorylation in superior mesenteric arteries from type 2 diabetic Goto-Kakizaki (GK) rats at the chronic stage of the disease (Ishida *et al.*, 2011). The fish oil, eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid that modulates cell membrane fatty acid composition (Calder, 2012) has several beneficial effects against vascular dysfunction. EPA has anti-inflammatory, anti-platelet, anti-fibrotic, anti-oxidant and anti-atherogenic effects and also increases NO bioavailability (Balakumar and Taneja, 2012; Calder, 2012; Yates *et al.*, 2014). Treatment of OLETF rats with EPA suppressed ACh- and AA-induced contraction, improved ACh-, NO- and EDHF-mediated relaxation, reduced ACh-stimulated prostanoid release, COX-2 expression and activities of ERK1/2 and NF-κB (Matsumoto *et al.*, 2009b). A summary of the actions of these compounds on EDCF signalling is presented in Table 1. Collectively, these studies indicate that the beneficial effects of these drugs or dietary supplements may be partly attributable to their capacity to normalize EDCF-mediated signalling. In other words, the regulation of EDCF signalling may be a therapeutic target for diabetic and/or hypertensive vasculopathy.

Effects of sex hormones on prostanoid-induced vascular response and signalling

Sex hormones influence EC function and, consequently, EDRFs and EDCF synthesis and their signalling pathways in physiological and pathological conditions (Orshal and Khalil, 2004; Khalil, 2005; Miller and Mulvagh, 2007; Duckles and Miller, 2010). Oestrogen stimulates the release of various endothelium-derived factors (Tostes *et al.*, 2003; Hermenegildo *et al.*, 2006; Miller and Mulvagh, 2007). 17β-oestradiol (E2) stimulates NOS in human and animal ECs – ECs from human umbilical vein (HUVEC), bovine aortae and human aortae (Hayashi *et al.*, 1995; Hishikawa *et al.*, 1995). E2 also induces the production of PGI_2 in HUVEC (Mikkola *et al.*, 1995; Sobrino *et al.*, 2010) and ovine fetal pulmonary artery ECs (Sherman *et al.*, 2002). Moreover, oestrogen leads to increased EDHF-mediated signalling (Burger *et al.*, 2009). In cerebral arteries, E2 reduced vascular tone by shifting the primary end product of the endothelial COX-1 pathway from the constrictor PGH_2 to dilator PGI_2 (Ospina *et al.*, 2003).

Effects of oestrogens are mediated by multiple receptors such as the classic oestrogen receptors ERα and ERβ and the novel G protein-coupled oestrogen receptor (GPER, previously termed GPR30; Revankar *et al.*, 2005; Prossnitz and Barton, 2011) cloned from human ECs (Takada *et al.*, 1997). Natural oestrogens such as E2, a non-specific agonist of ERα, ERβ and GPER (Prossnitz and Barton, 2011), modulate vasoconstrictor prostanoid signalling (Miller and Vanhoutte, 1990; Dantas *et al.*, 1999; Zhang and Kosaka, 2002; Li and Stallone, 2005; Hermenegildo *et al.*, 2006; Li *et al.*, 2008). Moreover, inhibitory effects of E2 on COX-dependent responses to vasoconstrictors have suggested a role for oestrogen receptors (Meyer *et al.*, 1997) on endothelium-dependent contraction. However, the specific receptor(s) involved in this mechanism have not been described.

GPER activation is associated with beneficial and protective effects in the vasculature (Meyer *et al.*, 2011; Han *et al.*, 2013; Ferreira *et al.*, 2015). Meyer *et al.* (2012) showed that acute GPER antagonism enhanced endothelium-dependent contractions and reduced endothelial NO bioactivity. Chronic GPER deficiency was associated with increased endothelial prostanoid-induced vasoconstriction but had no effect on endothelial NO bioactivity, eNOS and TP receptor gene expression or vascular structure. GPER deletion also increased TP receptor-mediated contraction (Meyer *et al.*, 2012). Therefore, the regulation of GPER activity may have therapeutic potential for vascular complications associated with vasoconstrictor prostanoid signalling in hypertension and diabetes.

Testosterone also influences the release of endothelium-derived factors by both inhibiting EDRF release and increasing EDCF expression (Farhat *et al.*, 1995; Hutchison *et al.*, 1997). Testosterone has been shown to stimulate TxS as well as COX-1 and COX-2 in rat thoracic aorta and mesenteric arteries (Cheuk *et al.*, 2000; Song *et al.*, 2004). Moreover, *in vitro* treatment with testosterone increased the number of functional TP receptors both in cultured rat aorta (Masuda *et al.*, 1991) and in vascular SMCs of the guinea pig coronary artery (Schror *et al.*, 1994). In addition to its effects on vascular tone under physiological conditions, testosterone also has pro-inflammatory effects. Accordingly, long-term treatment with testosterone, or with its non-aromatizable androgen receptor agonist dihydrotestosterone, exacerbates endotoxin-induced inflammation in the cerebral circulation by mechanisms that involve increased nuclear NF-κB activation and increased levels of COX-2 and inducible NOS (Gonzales *et al.*, 2009). Reinforcing a role for testosterone in vascular inflammation, data from our laboratory demonstrate that testosterone induces leukocyte migration by COX-2-dependent mechanisms (Chignalia *et al.*, 2015).

The modulatory effects of sex hormones on vascular tone may also be due to their influence on the production of vasoconstricor prostanoids. Miller and Vanhoutte (1990) found that AA-induced endothelium-dependent contractions of aortic rings were enhanced by treatment of ovariectomized rabbits with oestrogen for 2 weeks (vs. placebo treatment). In addition, oestrogen treatment augmented contractions induced by PGI_2 but not PGE_2, $PGF_2\alpha$ or U46619, whereas indomethacin suppressed noradrenaline-induced contraction in endothelium-intact aortae from estrogen-treated rabbits. These results suggested that chronic treatment with oestrogens could affect noradrenaline-induced contraction via an endothelium-dependent mechanism that may involve the metabolism of AA by COX, and that altered sensitivity of the SMC to PGI_2 may contribute in part to the enhanced contractions to AA upon oestrogen treatment (Miller and Vanhoutte, 1990). Oestrogen also potentiates vascular reactivity to vasopressin (VP), which releases TxA_2 and PGI_2 from both male and female rat aortae (Li *et al.*, 2008). Whereas ovariectomy attenuated, oestrogen therapy restored VP-stimulated release of TxA_2 and PGI_2, an effect mediated by upregulation of COX-2 and TxS expression in both ECs and vascular SMCs and up-regulation of TP expression in vascular SMCs (Li *et al.*, 2008). Moreover, sex differences in the endothelial regulation of vasoconstrictor responses due to modulatory effects on vasoconstrictor prostanoids have been described. Whereas the endothelium negatively modulates clonidine (α_2-adrenoceptor agonist)-induced contraction entirely via NO in female rats, an endothelial vasoconstrictor prostanoid contributes to clonidine responses in male animals (Tejera *et al.*, 1999).

Important sex differences in endothelial (dys)function have been reported in hypertensive and diabetic subjects (Kauser and Rubanyi, 1995; Hermenegildo *et al.*, 2006; Aloysius *et al.*, 2012). In hypertensive rats, E2 affects the release and/or action of endothelium-derived NO (Huang *et al.*, 1997; Costa *et al.*, 1998) and enhances endothelium-dependent relaxation in aortae of female SHR (Williams *et al.*, 1988). Further, it antagonizes the increased tone in renal arteries of female Dahl salt-sensitive rats by enhancing NO-dependent relaxation and suppressing EDCF-mediated responses via NO-independent mechanisms (Zhang and Kosaka, 2002). Regarding the effects of female sex hormones on the synthesis and the effects of EDCFs in the vasculature, Kahonen *et al*. demonstrated that diclofenac, a COX inhibitor, abolished sex differences in ACh vascular responses in SHR (Kahonen *et al.*, 1998). In addition, the removal of ovarian steroid hormones increased the generation of COX-derived vasoconstrictors, such as $PGH_2/PGF_{2\alpha}$ (Davidge and Zhang, 1998; Dantas *et al.*, 1999). Sex differences in renal prostanoid production have been reported in arterial hypertension, with female SHR exhibiting enhanced urinary excretion of PGE_2 and TxA_2 metabolites along with enhanced renal microsomal PGES and COX-2 expression, compared with male SHR (Sullivan *et al.*, 2005). In rabbit isolated carotid arteries, testosterone induces a concentration-dependent relaxation, which is increased in diabetic conditions by mechanisms that involve increased release of NO and COX-2-derived PGI_2 rather than the absence of COX-1-derived TxA_2 (Marrachelli *et al.*, 2010). An imbalance of prostanoid synthesis, with overproduction of vasoconstrictor prostanoids and reduced PGI_2 production has been observed in diabetes-associated vascular dysfunction in males (Bolego *et al.*, 2006; Du *et al.*, 2006; Nie *et al.*, 2006; Matsumoto *et al.*, 2007a; 2008b; 2009b; 2010; Ishida *et al.*, 2011). Using a model of diabetes in female rats, Akamine *et al*. (2006) reported that ACh-induced relaxation, which was decreased in arterioles of diabetic female rats, was ameliorated by diclofenac. In addition, improved production of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$, but not TxB_2, as well as superoxide generation were observed in ACh-stimulated arterioles from diabetic rats, and diclofenac normalized these alterations. These data suggest that increased release of constrictor prostanoids, most likely $PGF_{2\alpha}$, is involved in the reduced endothelium-dependent vasodilation in diabetic females and that enhanced COX activity may be a source of superoxide generation in this model (Akamine *et al.*, 2006).

The above-mentioned reports indicate that sex and sex hormones influence EDCF synthesis and EDCF-mediated signalling in physiological conditions and that EDCF-mediated responses are disrupted in diabetic and hypertensive conditions. While this adds further complexity to the regulation of EDCF signalling, it raises the possibility of using different therapeutic treatments to target EDCF-mediated responses in male and female patients.

Uridine adenosine tetraphosphate

Up_4A, a dinucleotide with purine and pyrimidine moieties, was identified by Jankowski *et al*. as a novel potent EDCF (Jankowski *et al.*, 2005). Up_4A is released from ECs in response to various stimuli such as mechanical stress, endogenous ligands (ACh, ET-1, ATP and UTP) and Ca^{2+} ionophore (A23187; Jankowski *et al.*, 2005). Although the molecular mechanisms underlying the production/release of Up_4A remain unclear, Jankowski *et al*. recently demonstrated that the intrinsic enzymic activity of the VEGF receptor 2 leads to the production of Up_4A in ECs (Jankowski *et al.*, 2013). Only two papers have been published on circulating Up_4A levels in disease states. Jankowski *et al*. found that circulating levels of Up_4A were increased in juvenile hypertensive patients compared with normotensives (Jankowski *et al.*, 2007) and Schuchardt *et al*. found that patients with chronic kidney disease had a higher plasma Up_4A concentration compared with healthy subjects (Schuchardt *et al.*, 2012).

The effects of Up_4A on vascular functions have been explored in various animal models (Matsumoto *et al.*, 2011a; Figure 2). Up_4A induces vascular calcification (Schuchardt *et al.*, 2012), proliferation and migration of vascular SMC (Gui *et al.*, 2011; Wiedon *et al.*, 2012). Because these events play important roles in the development of vascular dysfunction in diabetes and hypertension (Touyz and Schiffrin, 2004; Chen and Moe, 2012), the regulation of Up_4A-induced signalling in vascular SMCs may be a potential therapeutic target. Furthermore, Up_4A has been shown to modulate vascular tone in various arteries (Matsumoto *et al.*, 2011a). For example, Up_4A induces relaxation of rat aorta (Linder *et al.*, 2008) and swine coronary artery (Zhou *et al.*, 2013) and stimulates contraction in the rat pulmonary artery (Gui *et al.*, 2008), rat aorta (Linder *et al.*, 2008), mouse aorta (Hansen *et al.*, 2010) and rat perfused kidney (Jankowski *et al.*, 2005).

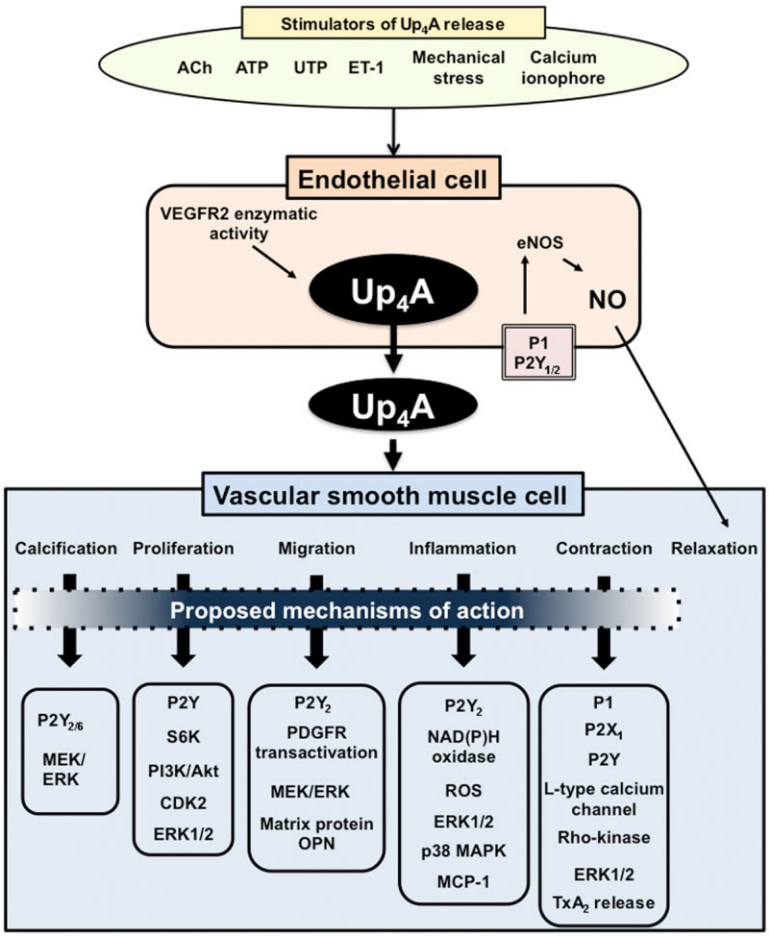

Figure 2

Effects of Up_4A on vascular smooth muscle functions. When EC are stimulated with, for example, ACh, ATP, uridine triphosphate (UTP), ET-1, mechanical stress or Ca^{2+} ionophore (A23187), Up_4A is generated. Released Up_4A acts in both ECs and vascular SMC. In ECs, Up_4A binds to P2Y1/2 or P1 receptor and activates endothelial nitric oxide synthase (eNOS); releasing NO and producing relaxation. In vascular SMCs, Up_4A leads to calcification, proliferation, migration, inflammation and contraction. Details are shown in text.

Although evidence from vascular functional studies were all derived from non-disease models, we recently found that Up_4A-induced contraction is altered in various arteries isolated from deoxycorticosterone-acetate (DOCA)-salt hypertensive rats (Matsumoto *et al.*, 2011b; 2012) and type 2 diabetic GK rats (Matsumoto *et al.*, 2014b). In DOCA-salt hypertensive rats, Up_4A-induced contraction is increased in basilar, femoral and renal arteries and is decreased in small mesenteric arteries (vs. control uninephrectomized rats; Matsumoto *et al.*, 2011b; 2012). In renal arteries from hypertensive rats, increased contraction to Up_4A may be attributable to increased ERK1/2 activity in SMCs rather than alteration of P2Y receptors, which are putative Up_4A receptors (Jankowski *et al.*, 2005; Matsumoto *et al.*, 2011b). Moreover, we recently found that the Up_4A-induced contraction is increased in renal arteries from GK rats (vs. control Wistar rats), and this might be due to the enhanced activation of COXs/TP receptor signalling (Matsumoto *et al.*, 2014b). These findings provide the possibility of cross-talk between Up_4A and vasoconstrictor prostanoids in the presence of diabetes.

Uridine adenosine tetraphosphate-mediated signalling in vascular SMCs

Since Up_4A has purine and pyrimidine moieties, Up_4A may also exert its effects via activation of purinergic receptors in vascular SMCs. Purinergic receptors have been classified into two subtypes including P1 (i.e. adenosine receptor) and P2 [P2X (ionotropic) and P2Y (metabotropic)] receptors (Abbracchio *et al.*, 2006; Burnstock, 2007; Erlinge and Burnstock, 2008; Alexander *et al.*, 2013b,c; Burnstock and Ralevic, 2013). There are reports showing a link between Up_4A and purinoceptors in the vasculature. For instance, Jankowski's group demonstrated that Up_4A-induced vasoconstriction in perfused kidneys results from the activation of P2X1 receptors and probably also of $P2Y_2$ and $P2Y_4$ receptors (Jankowski *et al.*, 2005). More recently, the same group found that in addition to smooth muscle P2X1 receptor-mediated vasoconstriction in the rat perfused kidney, Up_4A also induces concentration-dependent $P2Y_2$ receptor-mediated, long-

lasting vasoconstriction (Tolle *et al*., 2010). Moreover, Up_4A-induced vasoconstriction is followed by vasodilation due to $P2Y_1$ and $P2Y_2$ receptor activation on ECs, which leads to NO release (Tolle *et al*., 2010). Using rat pulmonary arteries, Gui *et al*. (2008) found that Up_4A induces concentration-dependent contraction that is inhibited by suramin, a non-selective P2 receptor antagonist, but not by Ip_5I, an antagonist of P2X receptors, or by desensitization of P2X receptors with α,β-methylene-ATP (Gui *et al*., 2008). We also confirmed that Up_4A-induced rat renal arterial contraction is inhibited by suramin but not by Ip_5I (Matsumoto *et al*., 2011b). Linder *et al*. found that Up_4A-induced contraction in rat aorta is blocked by P1 and P2X receptor antagonists (Linder *et al*., 2008).

The downstream pathways of Up_4A-induced responses in vascular SMCs have been investigated (Matsumoto *et al*., 2011a; Figure 2). Linder *et al*. found that Up_4A-induced rat aortic contraction is suppressed by L-type Ca^{2+} channel blockade or by ROCK inhibition (Linder *et al*., 2008). On the other hand, Gui *et al*. suggested that Up_4A-induced contraction in rat pulmonary artery involves extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores, but not Ca^{2+} sensitization via the ROCK pathway (Gui *et al*., 2008). In rat renal arteries, we found that Up_4A-induced contraction was inhibited by the ERK1/2 pathway inhibitor PD98059, whereas the ERK1/2 activity (determined by phosphorylated ERK1/2 levels) was increased upon Up_4A stimulation in renal arteries from DOCA-salt hypertensive rats (vs. control uninephrectomized rat; Matsumoto *et al*., 2011b). These data suggest that Up_4A-induced responses rely on intracellular calcium-dependent and -independent (e.g. kinases) pathways in vascular SMCs.

Up_4A-induced vascular SMC migration dramatically depends on secretion of osteopontin (OPN), a multifunctional molecule associated with cell migration, adhesion, survival and tissue remodeling (Scatena *et al*., 2007). Simultaneous incubation with Up_4A and an OPN-blocking antibody suppressed vascular SMC migration (Wiedon *et al*., 2012). Using specific and non-specific purinoceptor antagonists and inhibitors of MEK/ERK pathway, Wiedon and colleagues demonstrated that Up_4A-induced vascular SMC migration was mediated by MEK/ERK pathway upon $P2Y_2$ receptor activation. Furthermore, Up_4A-induced migration was reduced by a platelet-derived growth factor receptor (PDGFR) antagonist and by PDGFR-β siRNA treatment, suggesting that transactivation of the PDGFR plays a role in vascular SMC migration mediated by Up_4A (Wiedon *et al*., 2012). Schuchardt *et al*. found that exogenous application of Up_4A increased mineral deposition in mouse and rat aortae and in rat vascular SMCs. In addition, Up_4A increased the expression of different genes specific for osteochondrogenic vascular SMCs such as Cbfa1 (Steitz *et al*., 2001; Naik *et al*., 2012), while decreasing the expression of SM22α, a specific marker for vascular SMCs (Steitz *et al*., 2001; Dong *et al*., 2012). The influence of different P2Y receptor antagonists on Up_4A actions indicated that $P2Y_{2/6}$ receptors might be involved. Mechanisms downstream of P2Y receptor signalling involved activation of the MEK/ERK1/2 pathway. Therefore, Up_4A activation of P2Y receptor could affect phenotypic transdifferentiation of vascular SMCs to osteochondrogenic cells, suggesting that purinergic signalling upon Up_4A stimulation may be involved in vascular calcification (Schuchardt *et al*., 2012). Gui *et al*. (2011) found that Up_4A-induced increase in bromodeoxyuridine incorporation was blocked by the mammalian target of rapamycin and the MEK/ERK1/2 inhibitor, PD98059, in human vascular SMCs. In addition, Up_4A-induced phosphorylation and activation of S6 kinase (S6K) and ERK1/2 were inhibited by PD98059, whereas S6K but not ERK1/2 activity was inhibited by rapamycin. Up_4A also increased Akt phosphorylation, which was inhibited by the PI3K inhibitor, LY294002. Up_4A-induced activation of S6K, but not ERK1/2, was also prevented by LY294002, whereas a P2 receptor antagonist, suramin, but not a P2X receptor antagonist, Ip_5I, inhibited Up_4A-induced phosphorylation and kinase activity of S6K and ERK1/2. Finally, Up_4A increased protein expression of cyclin-dependent kinase 2 (CDK2), which was prevented by rapamycin, PD98059 and suramin. These results demonstrate that the signalling mechanisms underlying Up_4A-induced proliferation of vascular SMCs are mediated by P2Y receptors and involve the PI3K/Akt and ERK1/2 pathways, leading to the independent activation of S6K and increased in CDK2 expression (Gui *et al*., 2011). In addition, Up_4A promotes the production of the chemokine CCL2 in vascular SMC (Schuchardt *et al*., 2011), suggesting a role for this EDCF in vascular inflammation. Up_4A induced ROS generation via NAD(P)H oxidase activation, which further stimulated CCL2 formation through ERK1/2 and p38 MAPK activation in vascular SMCs. This process by Up_4A requires the activation of $P2Y_2$ receptors (Schuchardt *et al*., 2011).

Intracellular signalling of Up_4A in the vasculature of hypertensive and diabetic subjects has not been fully characterized. The effects of sex and sex steroid hormones of Up_4A production and activity is also an unexplored area of study.

Conclusions and perspectives

In conclusion, vasoconstrictor prostanoids and Up_4A play an important role in vascular dysfunction associated with diabetes and arterial hypertension. The signalling pathways activated by these EDCFs may differ based on vessel type, stages of disease (e.g. early or chronic stage) and sex. The complexity of the intracellular signalling and the interactions between vasoconstrictor prostanoids and Up_4A provide an exciting area of investigation for the pursuit of new pharmacological targets for the management of vascular dysfunction in hypertension and diabetes. Sex and sex steroid hormones are essential mediators of these pathways and should be considered in the design of experimental studies and the development of therapeutic compounds.

Acknowledgements

This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan, by the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan, and by JSPS KAKENHI Grant Number 26460107 and by the American Heart Association (13SDG17050056, S. G.) and by Fundaçao

de Amparo a Pesquisa do Estado de Sao Paulo and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, Brazil.

Conflict of interest

There are no potential conflicts of interest among the authors regarding the publication of this manuscript.

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