Autonomic & Autacoid Pharmacology

Broadley Festschrift 2008

The article below is published in Volume 29 Issue 3 following the Festschrift held from 16-17th September 2008 in honour of Professor Kenneth J. Broadley. Please refer to an autobiographical review of Professor Broadley's distinguished career on pages 51–62 of this issue. Professor Broadley has served as the Editor of Autonomic and Autocoid Pharmacology from 1987 until 2006 and remains active on the board as the current Deputy Editor.

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REVIEW

Purinergic regulation of vascular tone and remodelling

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Summary

1 Purinergic signalling is involved both in short-term control of vascular tone and in longer-term control of cell proliferation, migration and death involved in vascular remodelling.
2 There is dual control of vascular tone by adenosine 5′-triphosphate (ATP) released from perivascular nerves and by ATP released from endothelial cells in response to changes in blood flow (shear stress) and hypoxia.
3 Both ATP and its breakdown product, adenosine, regulate smooth muscle and endothelial cell proliferation.
4 These regulatory mechanisms are important in pathological conditions, including hypertension, atherosclerosis, restenosis, diabetes and vascular pain.

Keywords: atherosclerosis, adenosine 5′-triphosphate, hypertension, pain, purinergic, restenosis

Introduction

Purinergic signalling, i.e. adenosine 5′-triphosphate (ATP) acting as an extracellular signalling molecule, was proposed by Burnstock in 1972 when evidence was presented that ATP was the neurotransmitter in non-adrenergic, non-cholinergic neuromuscular transmission in the gut and urinary bladder (Burnstock, 1972). Later, it was recognized as a cotransmitter in sympathetic, parasympathetic, sensory-motor and enteric nerves (Burnstock, 1976). There was early resistance to this concept (see Burnstock, 2006a,b), but the roles of nucleotides and nucleosides as extracellular signalling molecules are now well established in both neural and non-neural tissues (Burnstock & Knight, 2004; Burnstock, 2007a). P1 receptors for adenosine, of which four subtypes (A_1, A_{2A}, A_{2B}

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and A_3) have been cloned and characterized, have been distinguished from P2 receptors for ATP, adenosine diphosphate (ADP) and uridine 5′-triphosphate (UTP) (Burnstock, 1978), and P2 receptors have been divided into P2X ligand-gated ion channel and P2Y G protein-coupled receptor families. Seven subtypes of P2X receptors and eight subtypes of P2Y receptors have been cloned and characterized (Ralevic & Burnstock, 1998; Burnstock, 2007b). The majority of studies involving purinergic signalling have been concerned with short-term events, such as neurotransmission or secretion. However, there is growing interest in the long-term trophic actions of extracellular nucleotides and nucleosides on cell proliferation and death (Neary *et al.*, 1996; Abbracchio & Burnstock, 1998).

Adenosine 5′-triphosphate and adenosine are involved in the mechanisms underlying local control of vessel tone (Burnstock, 1988; Burnstock & Ralevic, 1994) as well as cell migration, proliferation, differentiation and death during angiogenesis, atherosclerosis and restenosis following angioplasty (Erlinge & Burnstock, 2008).

Regulation of vascular tone

In the vascular system, short-term purinergic signalling events associated with the dual control of vascular tone by ATP released from nerves and endothelial cells have been clearly demonstrated (Burnstock, 1990a, 1999; Boarder & Hourani, 1998) (See Fig. 1).

Adenosine 5′-triphosphate released as a cotransmitter with noradrenaline (NA) from perivascular sympathetic nerves acts mainly on $P2X_1$ receptors on medial vascular smooth muscle to produce constriction, whereas ATP released as a cotransmitter from sensory-motor nerves during 'axon reflex' activity dilates some vessels (Burnstock, 2006c). There is transient constriction of cerebral arterioles via P2X receptors and sustained

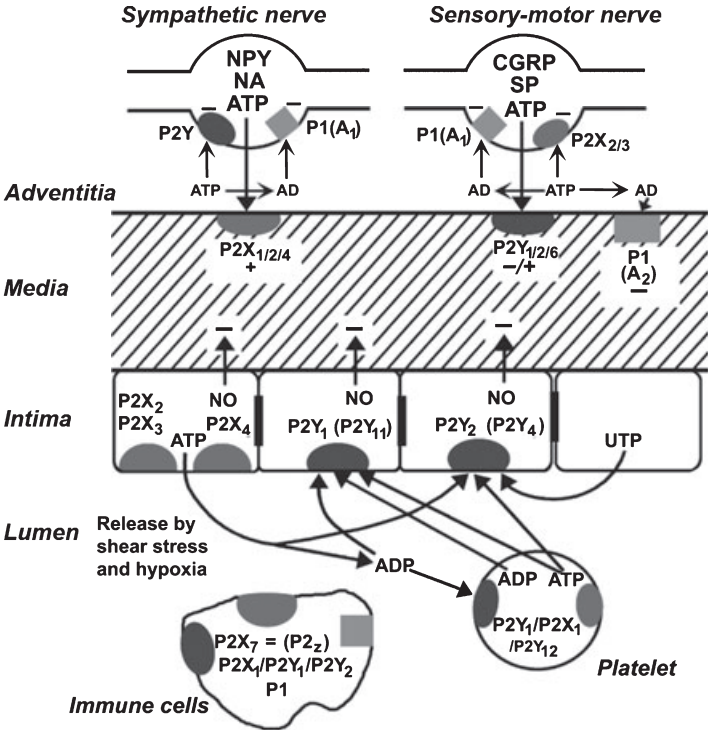

Figure 1 Schematic diagram illustrating the main receptor subtypes for purines and pyrimidines present in blood vessels involved in control of vascular tone. Adenosine 5′-triphosphate (ATP) is released as a cotransmitter with noradrenaline (NA) and neuropeptide Y (NPY) from sympathetic nerves in the adventitia to act at smooth muscle $P2X_1$ receptors and, in some vessels, $P2X_2$, $P2X_4$ and $P2Y_2$ and $P2Y_6$ receptors, resulting in vasoconstriction; ATP is released with calcitonin gene-related peptide (CGRP) and substance P (SP) from sensory-motor nerves during 'axon reflex' activity to act on smooth muscle P2Y receptors, resulting in either vasodilatation or vasoconstriction. P1 (A_1) receptors on nerve terminals of sympathetic and sensory nerves mediate adenosine (arising from ecto-enzymatic breakdown of ATP) modulation of transmitter release. $P2X_{2/3}$ receptors are present on a subpopulation of sensory nerve terminals. P1 (A_2) receptors on vascular smooth muscle mediate vasodilatation. Endothelial cells release ATP and uridine 5′-triphosphate (UTP) during shear stress and hypoxia to act on $P2Y_1$, $P2Y_2$ and sometimes $P2Y_4$, $P2Y_{11}$ and $P2X_4$ receptors, leading to the production of nitric oxide (NO) and subsequent vasodilatation. ATP, after its release from aggregating platelets, also acts, together with its breakdown product ADP, on these endothelial receptors. Blood-borne platelets possess $P2Y_1$ and $P2Y_{12}$ ADP-selective receptors as well as $P2X_1$ receptors, whereas immune cells of various kinds possess $P2X_7$ as well as P1, $P2X_1$, $P2Y_1$ and $P2Y_2$ receptors [figure is modified from Burnstock (1989b) with permission from Oxford University Press].

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constriction of large cerebral vessels, largely through $P2Y_2$ receptors (Sprague *et al.*, 2003).

Adenosine 5′-triphosphate released from endothelial cells during changes in blood flow (producing shear stress) or hypoxia acts on P2 receptors on endothelial cells to release nitric oxide (NO), resulting in relaxation (Bodin & Burnstock, 1996; Burnstock, 1999). $P2Y_1$ receptors are dominant in some vessels activated selectively by ADP (Ralevic & Burnstock, 1998), in other vessels $P2Y_2$ receptors are present that are activated equally by ATP and UTP (Motte *et al.*, 1995). UTP has been shown to be released from endothelial cells by shear stress (Saïag *et al.*, 1995). Endothelial cells in some vessels also express other types of purinergic receptors ($P2Y_4$ and $P2Y_6$) (Ralevic, 2001); and yet another P2Y receptor subtype ($P2Y_{11}$) is present on endothelial cells of human mammary artery and umbilical vein (Wang *et al.*, 2002). Endothelial cells of arteries and veins express different levels of $P2X_4$ receptors and it has been reported that, in some blood vessels, $P2X_4$ receptors induced vascular dilatation in an NO-dependent manner (Yamamoto *et al.*, 2006). In rat mesenteric arteries, $P2X_1$ receptors mediated endothelial-dependent vasodilation, but a NO synthase inhibitor had no effect on dilation (Harrington & Mitchell, 2004). $P2X_4$ and $P2X_6$ receptors (perhaps as heteromultimers) were reported to be associated with vascular endothelial-cadherin in human endothelial cells, suggesting a role for these receptors in modulating adhesion junctions between endothelial cells (Glass *et al.*, 2002). Adenosine produced after breakdown of released ATP from nerves and endothelial cells causes vasodilatation via smooth muscle P1 (usually A_2 subtype) receptors.

Purinergic signalling involved in proliferation of endothelial cells and smooth muscle

Endothelial cells

Administration of adenosine was reported to induce endothelial cell proliferation in the aorta, coronary vessels and human umbilical veins (see Burnstock, 2002). Adenosine has also been shown to stimulate canine retinal microvascular endothelial cell migration and tube formation (Lutty *et al.*, 1998). The action of adenosine in producing endothelial cell proliferation is mediated by A_{2A} and A_{2B} receptors partly by the modulation of vascular endothelial growth factor (VEGF) (Grant *et al.*, 1999). The selective A_{2B} receptor antagonists enprofylline and 3-isobutyl-8-pyrrolidinoxanthine inhibited 5′-(*N*-ethylcarboxamido)-adenosine stimulated proliferation of human retinal endothelial cells, extracellular signal-regulated kinase (ERK) activation, cell migration and capillary tube formation (Grant *et al.*, 2001).

Adenosine diphosphate was shown to be one of several agonists that induced cultured endothelial cell migration and proliferation (McAuslan *et al.*, 1983). Angiogenesis (or neovascularization) begins with the migration of endothelial cells, originating from capillaries, into the tissue being vascularized. ADP and to a lesser extent adenosine and adenine, showed strong chemotactic activity and were postulated to be angiogenesis factors *in vivo* (Teuscher & Weidlich, 1985). Adenine nucleotides were shown to have a mitogenic action on aortic endothelial cells, probably via P2Y receptors (Van Coevorden *et al.*, 1989). ATP has also been shown to produce proliferation of cultured bovine corneal endothelial cells (Cha *et al.*, 2000). The source of the purines involved in these trophic actions is largely from the endothelial cells, suggesting an autocrine mechanism. ATP and ADP released from aggregating platelets may also play a role. Stretch-induced changes in endothelial cell shape and changes produced by hypoxic stress may be mediated by the ATP (and adenosine after ecto-enzymatic breakdown) released from endothelial cells under both these conditions (see Bodin & Burnstock, 2001).

There is increasing evidence that cell proliferation and programmed cell death (apoptosis) are linked (Di Virgilio, 2000). For example, VEGF turns on cell proliferation, but inhibits apoptosis (Mallat & Tedgui, 2000). Distinct signal transduction cascades, composed of at least three protein kinases, mediate cell proliferation and differentiation, growth arrest and apoptosis (Neary, 1997). In diseases such as carcinogenesis, degenerative disorders and ischaemia/reperfusion injury, there is an imbalance between cell division and cell death. Extracellular ATP and adenosine have been shown to cause apoptosis of pulmonary artery endothelial cells (Dawicki *et al.*, 1997). It has been speculated that ATP released from cells undergoing cytolysis or degranulation may cause endothelial cell death, perhaps by inhibition of methyltransferase activity and that this may be important in acute vascular injury or in limiting angiogenesis (Rounds *et al.*, 1998). In a study of porcine aortic endothelial cells, extracellular ATP and ADP, probably acting through $P2X_7$ receptors, were shown to activate nuclear factor-κB (NF-κB), a transcription factor involved in induction of apoptosis (von Albertini *et al.*, 1998).

Smooth muscle

An early study reported that adenosine produces changes in cyclic adenosine monophosphate and DNA synthesis in cultured arterial smooth muscle cells leading to inhibition of cell proliferation and the authors speculated that adenosine could be one of several regulatory factors in the development of atherosclerosis and might also regulate the release of a smooth muscle mitogen, platelet-derived growth factor (PDGF), from platelets (Jonzon *et al.*, 1985). A selective A_2 receptor agonist,

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2-octynyladenosine, reduced neointimal thickening in a rat femoral artery injury model (Takiguchi *et al.*, 1995).

Adenosine 5′-triphosphate and ADP stimulate DNA synthesis and cell proliferation of cultured porcine artery vascular smooth muscle cells, an action mediated by P2Y receptors (Wang *et al.*, 1992). It was speculated that this mechanism was involved in the regulation of vascular smooth muscle cell proliferation during embryonic and early postnatal development, after injury and in arteriosclerosis. It was further suggested that the ATP released from endothelial cells causes not only autocrine mitogenic stimulation of the endothelial cells themselves but also paracrine stimulation of the smooth muscle cells that migrate to the intima after injury. Exogenous ATP and UTP also induce a limited cell cycle progression in arterial smooth muscle cells as well as having powerful mitogenic actions, probably via $P2Y_2$ or $P2Y_4$ receptors (Malam-Souley *et al.*, 1996; Miyagi *et al.*, 1996).

Sympathetic nerves exert a trophic influence on vascular smooth muscle (Southwell *et al.*, 1985; Bevan, 1989). As ATP as well as NA and neuropeptide Y are known to be released as cotransmitters from sympathetic nerves (Burnstock, 1990b), this was consistent with the possibility that ATP and/or its breakdown product, adenosine, might be involved in these trophic actions.

Adenosine diphosphate contributes significantly in synergy with the peptide growth factors PDGF, epidermal growth factor and transforming growth factor-β, to the platelet-induced proliferation of vascular smooth muscle (Crowley *et al.*, 1994). The mitogenic effect of ATP on vascular smooth muscle cells is synergistic with other mitogens, including insulin and insulin-like growth factor-1 (Wang *et al.*, 1992). ATP-stimulated vascular smooth muscle cell proliferation via P2Y receptors involves activation of protein kinase C, Raf 1 and mitogen-activated protein kinase (MAPK) and requires independent ERK and phosphatidylinositol 3-kinase-signalling pathways (Yu *et al.*, 1996; Wilden *et al.*, 1998). There are two phenotypes of smooth muscle: the contractile phenotype and the synthetic (proliferative) phenotype. In a study of cultures expressing these two phenotypes using quantitative reverse transcription-polymerase chain reaction, it was shown that $P2X_1$ receptors were strongly expressed in the contractile phenotype. In the synthetic (proliferative) phenotype, the mitogenic $P2Y_1$ and $P2Y_2$ receptor transcripts were up-regulated 342- and 8-fold, respectively, whereas the contractile $P2X_1$ receptor was totally down-regulated, while the $P2Y_4$ and $P2Y_6$ receptors were unchanged (Erlinge *et al.*, 1998). Furthermore, MAPK kinase-dependent growth factor induced the up-regulation of $P2Y_2$ receptors in vascular smooth muscle cells, which the authors suggested may be of importance in atherosclerosis and neointimal formation after balloon angioplasty (Hou *et al.*, 1999). Fig. 2 is a schematic summarizing these events.

Purinergic signalling in vascular diseases

The migration, proliferation and death of vascular smooth muscle and endothelial cells play an important role in the development of intimal thickening during arterial diseases, such as arteriosclerosis and restenosis after angioplasty, and in the growth of new vessels that takes place during wound healing and in tumours. ATP, ADP, UTP and adenosine play pivotal signalling roles in these long-term events (Erlinge, 1998; Neary & Abbracchio, 2001; Burnstock, 2002; Ralevic & Burnstock, 2003; Erlinge & Burnstock, 2008).

Atherosclerosis and restenosis

There is growing evidence that ATP signalling is involved in atherosclerosis (Burnstock, 2002, 2008; Di Virgilio & Solini, 2002; Seye *et al.*, 2006). Adenosine and ATP mediate endothelial and smooth muscle cell proliferation and an increase in the expression of VEGF mRNA, which plays an important role in the development of intimal thickening during atherosclerosis, in restenosis after angioplasty, and in the growth of new vessels that takes place during wound healing and in tumours.

It was speculated early on that adenosine could be one of several regulatory factors in the development of atherosclerosis and might also regulate the release of a smooth muscle mitogen, PDGF (Jonzon *et al.*, 1985). There is now supporting evidence that adenosine does indeed regulate smooth muscle cell proliferation in angiogenesis (Adair, 2005). Endothelial cell proliferation is mediated by A_{2A} and A_{2B} receptors.

It has been shown that ecto-5′-nucleotidase (CD73)-derived adenosine acts as an endogenous modulator protecting against vascular inflammation and monocyte recruitment, thus limiting the progression of atherosclerosis (Buchheiser *et al.*, 2007).

Up-regulation of vascular smooth muscle $P2Y_2$ receptors by MAPK-dependent growth factor may be important in atherosclerosis and neointimal formation after balloon angioplasty (Hou *et al.*, 2000). It has been proposed that up-regulation of $P2Y_2$ receptors may be a useful diagnostic indicator for the early stages of atherosclerosis (Elmaleh *et al.*, 1998). A novel role for $P2Y_2$ receptors in the development of atherosclerosis has been suggested, whereby UTP induces vascular cell adhesion molecule-1 expression in coronary artery endothelial cells that mediate the recruitment of monocytes (Seye *et al.*, 2003).

Vascular injury represents a critical initiating event in the pathogenesis of various vascular diseases. Large amounts of ATP are released from

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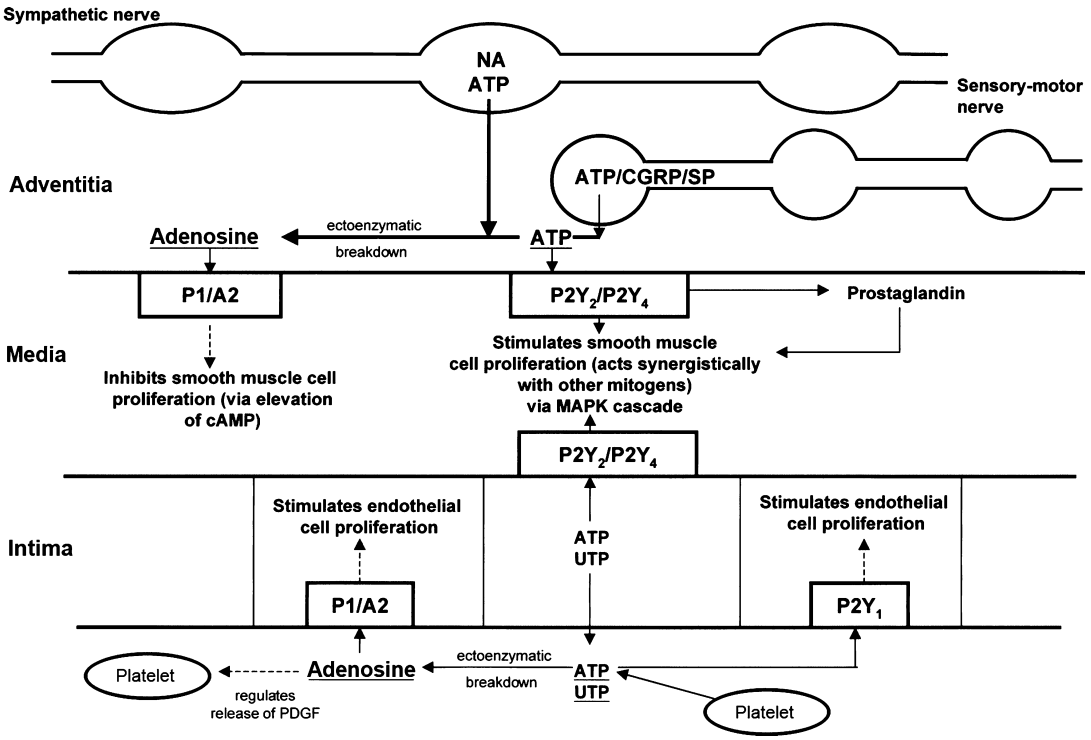

Figure 2 Schematic diagram of long-term (trophic) actions of purines released from nerves, platelets and endothelial cells [which also release uridine 5′-triphosphate (UTP)] acting on P2 receptors to stimulate or inhibit cell proliferation. Adenosine 5′-triphosphate (ATP) released as a cotransmitter from sympathetic nerves and sensory-motor nerves (during axon reflex activity) stimulates smooth muscle cell proliferation via $P2Y_2$ and/or $P2Y_4$ receptors via a mitogen-activated protein kinase (MAPK) cascade, whereas adenosine resulting from enzymatic breakdown of ATP acts on P1 (A_2) receptors to inhibit cell proliferation [via elevation of cyclic adenosine monophosphate (cAMP)]. ATP and UTP released from endothelial cells stimulate endothelial and smooth muscle cell proliferation via $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors. Adenosine resulting from ATP breakdown acts on P1 (A_2) receptors to stimulate endothelial cell proliferation and regulate the release of platelet-derived growth factor (PDGF) from platelets. NA, noradrenaline; CGRP, calcitonin gene-related peptide; SP, substance P (reproduced from Burnstock (2002) with permission from Lippincott, Williams and Wilkins).

injured cells and, as described above, ATP and adenosine have potent actions on smooth muscle and endothelial cell growth, migration, proliferation and death. Atherosclerotic damage modifies endothelium-dependent responses to ATP (Burnstock, 2006d). Clinical trials with clopidogrel and ticlopidine ($P2Y_{12}$ receptor antagonists) in patients with atherosclerotic disease have shown significant benefit compared with aspirin (Gachet, 2005). Natural killer cells represent the main source of interferon-γ that contributes to atherosclerotic plaque progression and instability. ATP and UTP released from endothelial cells during changes in blood flow resulting in shear stress is enhanced in inflammatory states (Bodin & Burnstock, 1998; Burnstock, 1999) and might represent a protective mechanism for killer cell-mediated plaque formation (Gorini *et al.*, 2007). Apoptotic cell death is recognized to occur in atherosclerosis and restenosis (Mallat & Tedgui, 2000).

Atherosclerosis is an inflammatory disease induced by hypercholesteraemia and increase in NTPDase (CD39, apyrase) and subsequent ATP and ADP hydrolysis has been shown in platelets of hypercholesteraemic patients (Duarte *et al.*, 2007). The authors suggest that this might be beneficial in reducing thrombus formation, but may also contribute to future fatal events such as unstable angina and myocardial infarction. ATP releases histamine from mast cells and releases inflammatory cytokines such as interleukin-1 from immune cells via $P2X_7$ receptors. In addition, occupation of P2Y receptors leads to prostaglandin and cyclooxygenase-2 (COX-2) synthesis, both involved in inflammatory processes. ATP-induced COX-2 expression is via p42/p44, MAPK, p38 and NF-κB in vascular smooth muscle cells and the authors suggest that nucleotides may promote atherosclerosis via these mechanisms (Wang & Yang, 2006).

The pattern of vascular connexins is altered during atherosclerotic plaque formation and in restenosis affecting gap-junctional communication between smooth muscle cells and genetically modified connexin expression alters the course of atherosclerosis and restenosis (Chadjichristos *et al.*, 2006). Connexin hemichannel formation has been shown to be regulated by ATP (Jiang *et al.*, 2005; Lurtz & Louis, 2007).

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In restenosis following balloon angioplasty, there is a peak in the proliferation and apoptosis of vascular smooth muscle at about 14 days (Han *et al.*, 1995). Saphenous vein, internal mammary and radial arteries have been used as grafts for coronary bypass surgery; the level of endothelial $P2Y_2$ receptors is comparable in all three vessels, but endothelial $P2X_4$ receptors vary from high in saphenous vein to significantly lower in the other two vessels. It has been suggested that $P2X_4$ receptors play a more significant role in intense proliferation in arteriosclerosis and restenosis than $P2Y_2$ receptors, as reflected by the susceptibility of saphenous vein grafts to atherosclerosis compared with internal mammary arteries (Ray *et al.*, 2002). In another study, $P2X_1$ and $P2Y_6$ receptors mediated more prominent contractions in the saphenous vein compared with the internal mammary artery; it has been suggested that selective antagonists to these receptors may prevent vasospasm and restenosis in the saphenous vein during and after revascularization surgery (Borna *et al.*, 2003). A study has shown that P2 receptors play an important role in homocysteine-induced atherosclerosis (Sharma *et al.*, 2007).

In conclusion, the long-term (trophic) roles of purinergic signalling in vascular smooth muscle and endothelial cell proliferation and death have been implicated in atherosclerosis and restenosis and there is exploration of novel therapeutic strategies in relation to these events (Ralevic & Burnstock, 2003; Burnstock, 2006d; Erlinge & Burnstock, 2008).

Angiogenesis

The growth of new blood vessels takes place in pathological events such as tumour growth, wound healing, psoriasis and the ischaemic retinopathies that occur in diabetes and sickle cell disease. In the adult, the development of new blood vessels, or neovascularization, occurs by budding from existing blood vessels and is referred to as angiogenesis (as distinct from vasculogenesis, which occurs in embryogenic development by vessel formation from mesenchyme precursor cells or angioblasts). Peptide growth factors such as fibroblast growth factor, transforming growth factor-α and VEGF are clearly involved in angiogenesis, but purines and pyrimidines also contribute to this process (Satterwhite *et al.*, 1999). Newly developing vascular endothelia express very high levels of the ectonucleotidase, NTPDase1, also seen under hypoxic conditions (Eltzschig *et al.*, 2003). Angiogenesis requires the dynamic interaction of endothelial cell proliferation and differentiation with orchestrated interactions between extracellular matrix and surrounding cells (such as vascular smooth muscle and/or pericytes). Such interactions could be coordinated by interplay between nucleotide release, P2 receptor modulation and altered NTPDase expression (Goepfert *et al.*, 2001). In rheumatoid arthritis, new capillary blood vessels invade the joint leading to destruction of the cartilage. In diabetes, new capillaries in the retina invade the vitreous body, bleed and cause blindness, and tumour growth and metastasis are angiogenesis-dependent (Folkman & Shing, 1992). Anginal patients treated chronically with dipyridamole to increase adenosine levels showed an increase in coronary angiogenesis (Picano & Michelassi, 1997).

Hypertension

Adenosine 5′-triphosphate plays a significant co-transmitter role in sympathetic nerves supplying hypertensive blood vessels. The purinergic component is increased in spontaneously hypertensive rats (SHR) (Vidal *et al.*, 1986; Brock & Van Helden, 1995). The increase in sympathetic nerve activity in hypertension is well established and there is an associated hyperplasia and hypertrophy of arterial walls (Julius & Nesbitt, 1996). Also, sympathetic neurons innervating the vasculature are dependent on nerve growth factor (NGF) in development, and an increase in NGF gene expression and protein has been described in SHR (Zettler & Rush, 1993). α,β-Methylene ATP has been shown to increase NGF secretion by vascular smooth muscle cells in SHR (Spitsbergen *et al.*, 1995). ATP is a rapidly acting hypotensive agent that compares favourably with sodium nitroprusside (Kien *et al.*, 1987). ATP-$MgCl_2$ is a safe, effective and preferential pulmonary vasodilator in children with pulmonary hypertension secondary to congenital heart defects; it has also been used for treating pulmonary hypertension after cardiac surgery (Brook *et al.*, 1994). ATP exerts mitogenic actions on human pulmonary artery smooth muscle cells, which may be relevant to the pathophysiological basis of severe pulmonary hypertension (Kääpä *et al.*, 1997; Zhang *et al.*, 2004). Eicosapentaenoic acid, one of the active components in fish oil that has antihypertensive effects, increases the release of ATP from vascular endothelial cells, leading to reduction of the blood pressure rise characteristic of ageing (Hashimoto *et al.*, 1998). $P2X_4$ receptors are localized in the syncytiotrophoblast, stroma and foetal capillary endothelial cells of human placenta. Placental $P2X_4$ receptors are significantly up-regulated in mild preeclampsia (Roberts *et al.*, 2007).

Vascular pain

$P2X_3$ receptors are found on nociceptive sensory nerve fibres and local release of ATP activates these fibres sending messages to the pain centres in the brain (see Burnstock, 2001). Burnstock speculated

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in 1996 (Burnstock, 1996) that vascular pain, such as angina, migraine, ischaemic muscle pain, lumbar pain and pelvic pain, may involve purinergic signalling. It was suggested that following local vasospasm, ATP released from microvessel endothelial cells during the reactive hyperaemia that follows and is known to be associated with pain, reaches $P2X_3$ receptors on the nociceptive sensory fibres in the adventitia to initiate pain. This concept was first proposed for the pain occurring in migraine (Burnstock, 1981, 1989a). In anginal pain, cardiac myocytes as well as coronary microvessel endothelial cells may be the source of ATP and adenosine reaching purinergic nociceptors (Crea *et al.*, 1990; Sylvén, 1993).

Diabetes

Along with its stimulating effect on bovine retinal capillary endothelial cells, adenosine has been shown to have an inhibitory effect on retinal pericytes, and it has been hypothesized that this dual function plays a role in the pathological neovascularization process that takes place in diabetes (Jackson & Carlson, 1992). Diabetic microangiopathy has been implicated as a fundamental feature of the pathological complications of diabetes, including retinopathy, neuropathy and foot ulceration (Kamal *et al.*, 1998).

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