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Themed Section: Conditioning the Heart – Pathways to Translation

REVIEW

Opioid receptors and cardioprotection – 'opioidergic conditioning' of the heart

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Ischaemic heart disease (IHD) remains a major cause of morbidity/mortality globally, firmly established in Westernized or 'developed' countries and rising in prevalence in developing nations. Thus, cardioprotective therapies to limit myocardial damage with associated ischaemia–reperfusion (I–R), during infarction or surgical ischaemia, is a very important, although still elusive, clinical goal. The opioid receptor system, encompassing the δ (vas *d*eferens), κ (*k*etocyclazocine) and μ (*m*orphine) opioid receptors and their endogenous opioid ligands (endorphins, dynorphins, enkephalins), appears as a logical candidate for such exploitation. This regulatory system may orchestrate organism and organ responses to stress, induces mammalian hibernation and associated metabolic protection, triggers powerful adaptive stress resistance in response to ischaemia/hypoxia (preconditioning), and mediates cardiac benefit stemming from physical activity. In addition to direct myocardial actions, central opioid receptor signalling may also enhance the ability of the heart to withstand I–R injury. The δ- and κ-opioid receptors are strongly implicated in cardioprotection across models and species (including anti-infarct and anti-arrhythmic actions), with mixed evidence for μ opioid receptor-dependent protection in animal and human tissues. A small number of clinical trials have provided evidence of cardiac benefit from morphine or remifentanil in cardiopulmonary bypass or coronary angioplasty patients, although further trials of subtype-specific opioid receptor agonists are needed. The precise roles and utility of this GPCR family in healthy and diseased human myocardium, and in mediating central and peripheral survival responses, warrant further investigation, as do the putative negative influences of ageing, IHD co-morbidities, and relevant drugs on opioid receptor signalling and protective responses.

LINKED ARTICLES

This article is part of a themed section on Conditioning the Heart – Pathways to Translation. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-8

Abbreviations

AMI, acute myocardial infarction; CGRP, calcitonin gene-related peptide; EGFR, epidermal growth factor receptor; eNOS, endothelial NOS; GSK3β, glycogen synthase kinase 3β; I–R, ischaemia–reperfusion; IHD, ischaemic heart disease; K_{Ca}, Ca^{2+}-activated K^+ channel; mK_{ATP}, mitochondrial K_{ATP} channel; mPTP, mitochondrial permeability transition pore; NOS, nitric oxide synthase; ROS, reactive oxygen species

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[d]
Adenosine A_1 receptors	12-lipoxygenase
β_1-Adrenoceptors	Adenylyl cyclase
β_2-Adrenoceptors	Akt
CGRP receptors	AMPK
DOR, δ opioid receptors	COX-2
KOR, κ opioid receptors	eNOS
MOR, μ opioid receptors	ERK1/2
Catalytic receptors[b]	GSK3β, glycogen synthase kinase 3β
EGFR, epidermal growth factor receptor	GPCR kinase
Toll-like receptor 4	iNOS
Ion channels[c]	JAK2
K_{ATP} ($K_{ir}6.x$) channels	MMP, matrix metalloproteinase
Ca^{2+}-activated K^+ channels (K_{Ca})	nNOS
L-type Ca^{2+} ($Ca_v1.x$) channels	p38
	PI3K
	PKC-δ
	PKC-ε
	PKG
	Src kinase

LIGANDS	
Aspirin	PGE_2
Atorvastatin	PGI_2
Bradykinin	Remifentanil
Calmodulin	TNF-α
cGMP	U50,488
CGRP, calcitonin gene-related peptide	
Connexin-43	
Dynorphin	
β-Endorphin	
Epoxyeicosatrienoic acid	
Fentanyl	
Glibenclamide	
Heat shock protein (HSP) 70	
Hexamethonium	
IL-1β	
Inositol 1,4,5-trisphosphate (IP_3)	
Lovastatin	
Morphine	
Naloxone	

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]Alexander *et al.*, 2013a,b,c,d).

Introduction

Although ischaemic heart disease (IHD) and resultant acute myocardial infarction (AMI) are leading causes of morbidity and mortality globally, the ability to effectively intervene and limit myocardial damage during ischaemia–reperfusion (I–R) (clinical cardioprotection) remains a largely unrealized therapeutic goal. A range of protective interventions have been identified as highly efficacious in the laboratory, with particular focus on ischaemic pre- and post-conditioning and related stimuli (Shi and Vinten-Johansen, 2012; Ovize *et al.*, 2013; Przyklenk, 2013; Ferdinandy *et al.*, 2014). Myocardial preconditioning describes induction of powerful resistance to I–R injury (and other forms of insult) in response to transient non-injurious ischaemia – a form of hormesis providing acute and more sustained stress resistance (Figure 1). Ischaemic post-conditioning, on the other hand, describes similarly potent protection arising from transient ischaemic episodes during the initial minutes of reperfusion following severe insult (Figure 1). It is now clear that both forms of protection can be induced directly or remotely (i.e. via transient I–R in the heart or an extra-cardiac organ/tissue), involve common mechanistic elements [e.g. mitochondrial protection via PK and/or reactive oxygen species (ROS) signals], and importantly appear to be mediated by the opioid receptor system, providing an opportunity to selectively engage such protection pharmacologically. The latter is important given mixed outcomes from ongoing trials of 'ischaemic' conditioning stimuli, which remain insufficient to recommend widespread clinical application (Peart and Headrick, 2009; Ovize *et al.*, 2013; Przyklenk, 2013). That said, there are some encouraging results from recent trials of ischaemic conditioning (Shi and Vinten-Johansen, 2012; Ferdinandy *et al.*, 2014), and those few trials of opioid receptor agonists undertaken to date have shown cardioprotective benefit in surgical ischaemia and angioplasty (Table 1). Moreover, there is evidence that opioid receptor agonists can augment the cardioprotective effects of ischaemic conditioning interventions (Rentoukas *et al.*, 2010).

The opioid receptor system represents an intriguing candidate for clinical cardioprotection: it appears mechanistically central to adaptive/protective responses to physiological and pathological stimuli (including ischaemic pre- and post-conditioning); it beneficially impacts all major determinants of I–R outcome (infarction/apoptosis, arrhythmogenesis, contractile dysfunction, inflammation); and it has been targeted for other purposes since pre-history (a background that can facilitate translation for novel clinical applications, including tissue protection). Indeed, use of opioid receptor-dependent analgesics and anaesthetics may already provide cardiac

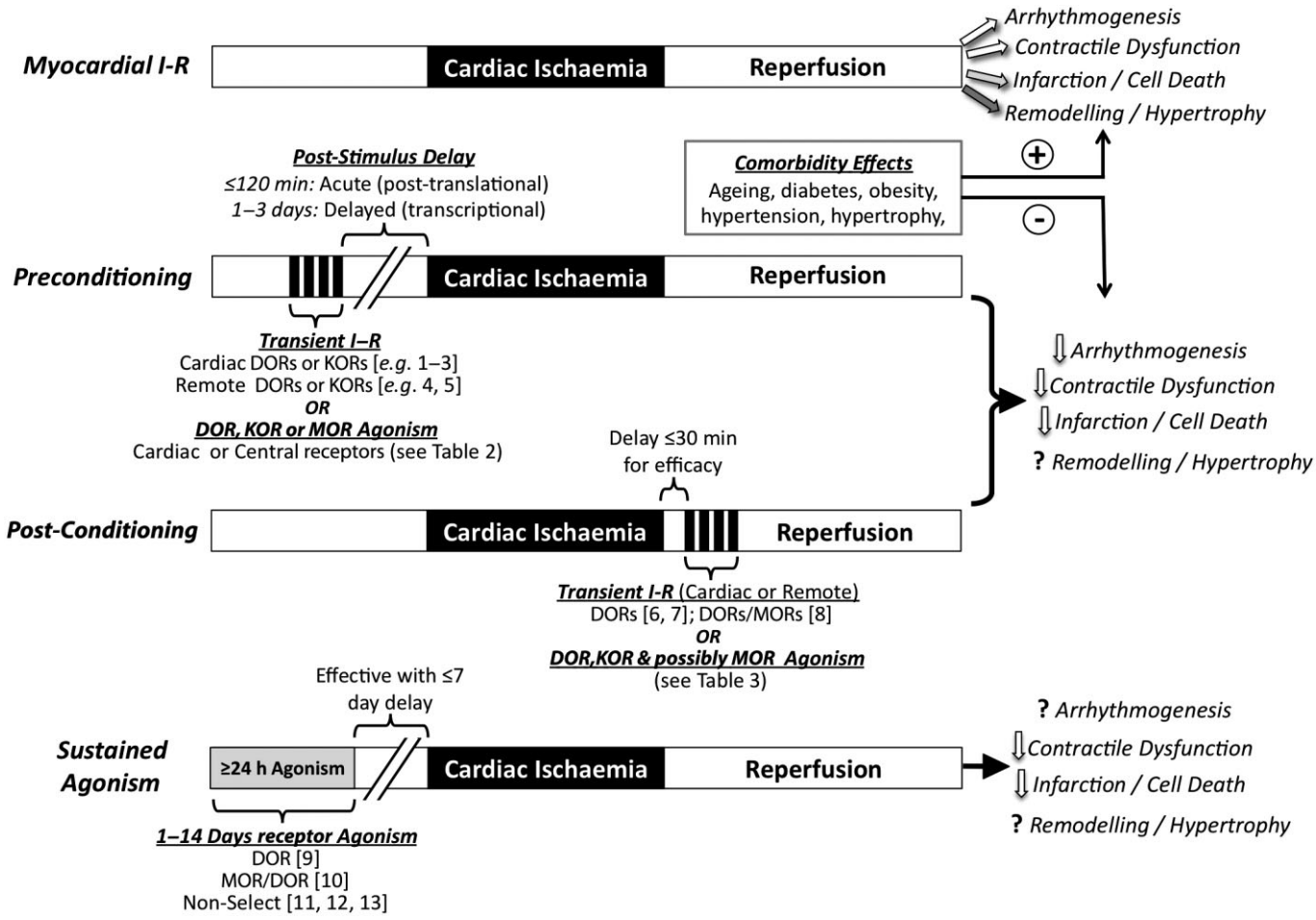

Figure 1

Diagram summarizing the temporal characteristics of pre- and post-conditioning stimuli (direct and remote), opioid receptor involvement, effects on myocardial I–R outcomes and the potential influences of common co-morbidities. References: 1, Schultz *et al.*, 1997; 2, Schultz *et al.*, 1998a; 3, Wang *et al.*, 2001; 4, Weinbrenner *et al.*, 2004; 5, Zhang *et al.*, 2006; 6, Wang *et al.*, 2008; 7, Jang *et al.*, 2008; 8, Zatta *et al.*, 2008; 9, Peart *et al.*, 2011; 10, Gross *et al.*, 2012b; 11, Wong & Lee, 1987; 12, Peart & Gross, 2004a; 13, Peart & Gross, 2006.

protection in patients suffering surgical or accidental I–R. This is supported by the ability of remifentanil to reduce cardiac injury markers in patients undergoing cardiopulmonary bypass (Xu *et al.*, 2009; Wong *et al.*, 2010a), while morphine can improve myocardial performance and reduces inflammatory responses (Murphy *et al.*, 2006; 2007) (Table 1). Despite promising observations there remain surprisingly few clinical trials of opioid receptor-targeted cardioprotection, with only morphine and partially μ-opioid receptor (MOR)-selective agents (fentanyl, remifentanil) assessed, and δ-opioid receptor (DOR) or κ-opioid receptor (KOR)-targeted approaches not tested (Table 1). Although opioid receptors can potentially induce cardiorespiratory depression, and prolonged agonism may lead to immunosuppressive or neuroendocrine changes (together with addiction), such outcomes are opioid receptor subtype specific, and experimental studies demonstrate cardioprotection can be effectively induced via very brief and also relatively low levels of subtype-specific opioid receptor agonism. Further trials of opioid receptor-based cardioprotection are needed, although several factors deserve attention in developing opioidergic (and also ischaemic) conditioning interventions: the powerful albeit poorly defined impacts of age and common co-morbidities (e.g. diabetes, obesity, hypertension); the intrinsic activation of opioid receptor and related protective pathways during I–R (potentially limiting scope for further protection); and the inadvertent induction or blockade of these responses via common clinical practice and pharmacological agents. A detailed mechanistic knowledge of intrinsic cardioprotective and cell death processes, together with an appreciation of the influences of critical factors (age, sex, disease, drugs) on these mechanisms, is crucial in implementing broadly effective clinical cardioprotection.

As we will detail in this brief review, the endogenous opioid receptor system: (i) represents a well-conserved stress-sensitive cytoprotective system; (ii) is highly expressed in the heart (including endogenous ligands and protective opioid receptor subtypes), where it beneficially modulates function

Table 1
Clinical trials of opioid receptor-based cardioprotective interventions

Putative selectivity	Agent	Treatment/Cohort	Outcome measures	Effect	Study
Non-selective	Morphine versus fentanyl	Pretreatment, on-pump CAB	Post-operative contractile function/EF	Improved with morphine (not fentanyl)	Murphy *et al.*, 2006
	Morphine versus fentanyl	Pretreatment, on-pump CAB	Post-operative inflammation	Lower IL-8, CD11b, CD18 levels	Murphy *et al.*, 2007
	Morphine + PerC versus PerC	Prior to reperfusion, PCI	Post-operative ST-segment deviation, cTnI	Improved versus PerC alone (moderate reduction in TnI versus PerC)	Rentoukas *et al.*, 2010
	Morphine	Post-treatment, tetralogy of Fallot repair	Post-operative cTnI, cardiac ejection	Improved	Zhang *et al.*, 2013
MOR	Fentanyl versus diazepam	Pretreatment, PCI	Post-operative cTnI	Unaltered (versus Diazepam)	Abdel-Wahab *et al.*, 2008
	Remifentanil	Pretreatment, off-pump CAB	Post-operative cTnI, cardiac index	Improved	Xu *et al.*, 2009
		Pretreatment, On-pump CAB	Post-operative CK-MB, cTnI, IMA, hFABP	All improved	Wong *et al.*, 2010a

Outcomes are shown for clinical trials (in generally small cohorts) of opioid receptor agonist modulation of myocardial outcomes from surgical I–R (functional recoveries, cellular damage, inflammation). CAB, coronary artery bypass surgery; CD11b, integrin-αM (complement component 3 receptor 3 subunit); CD18, integrin-β2; CK-MB, creatine kinase-myoglobin; cTnI, cardiac TnI; EF, ejection fraction; hFABP, heart type fatty acid-binding protein; IMA, ischemia-modified albumin; PCI, percutaneous coronary intervention; PerC, perconditioning (conditioning during late ischaemia, prior to reperfusion).

and stress resistance; (iii) is essential to cardioprotection via ischaemic pre- and post-conditioning; (iv) can be pharmacologically engaged to selectively mimic these powerful conditioning responses (via cardiac and, potentially, nervous signalling); and (v) may be significantly influenced by age, disease or other drugs. While the latter is an important consideration, some experimental opioidergic stimuli can nonetheless confer protection independently of age and disease state (Peart and Gross, 2004b; See Hoe *et al.*, 2013), and opioid receptor agonists do appear cardioprotective in different clinical cohorts (Table 1).

The opioid receptor system orchestrates cellular to organism 'stress resistance'

The opioid receptor system is activated in response to varied forms of stress (emotional, physical, metabolic), and subsequently mediates systemic and organ-specific responses and adaptations. In terms of I–R, opioid receptors are key determinants of ischaemic/hypoxic tolerance: opioid levels rise in response to ischaemia or hypoxia in humans (Roth-Isigkeit *et al.*, 2000; Chang *et al.*, 2004) and animal models (Romano *et al.*, 2004b), with resultant opioid receptor activity inducing cardioprotection. Indeed, opioid receptor expression levels may govern stress resistance across species (e.g. elevated in anoxia-tolerant diving/hibernating animals), and between and within organs (e.g. higher expression in cerebral cortex vs. less tolerant subcortical regions; and in inner and outer vs. less tolerant middle cortical layers). Centrally and peripherally mediated resistance to injurious stressors, induction of metabolically efficient phenotypes, improved insulin sensitivity and glucose metabolism, sympathoadrenal activation, immunomodulation, increased pain tolerance, threat avoidance (aversion) and euphoria can all be considered elements of opioid receptor-orchestrated survival responses to extreme stress. Furthermore, the positive cardiovascular and nervous system effects of physical activity also appear to be mediated by opioid receptors, and feeding behaviour and caloric intake is under opioid receptor control. This regulatory system thus offers considerable potential for the targeted induction of myocardial I–R tolerance, together with beneficial modulation of systemic or metabolic phenotype.

The importance of the opioid receptor system in governing stress resistance from tissue to organism levels is highlighted by its role in mammalian hibernation, a profoundly protected state induced to limit energy consumption and tissue damage during prolonged periods of nutrient deprivation and hypothermia. Studies implicate an opioid-like agent in this powerful adaptation, the levels of which increase in hibernation to induce beneficial effects in a DOR-dependent manner, including myocardial tolerance to injurious insult (Bruce *et al.*, 1996; Bolling *et al.*, 1998; Kevelaitis *et al.*, 1999). Treatment with exogenous DOR-selective opioids can also induce hibernation states in multiple species, and enhance organ stress resistance (Oeltgen *et al.*, 1988; Chien *et al.*, 1994). As with cardiac protection via opioid receptor agonists and opioid receptor-dependent ischaemic preconditioning, the beneficial effects of hibernation involve activation of

ATP-sensitive K^+ (K_{ATP}) channels (Kevelaitis *et al.*, 1999). Despite essential involvement of the DOR subtype, several opioid receptors may contribute and play distinct physiological roles during specific hibernation or metabolic states. Romano and colleagues identify, for example, a role for KORs in mediating resistance to myocardial damage during combined hypothermia and ischaemia (Romano *et al.*, 2004a). Recently documented induction of prolonged and powerful protected states following several days of DOR agonist treatment (Peart and Gross, 2004a; 2006; Peart *et al.*, 2011) may reflect conserved aspects of the hibernation response in non-hibernating species.

Consistent with an orchestrating role in adaptive stress responses, the opioid receptor system is also essential to the positive cardiovascular and other adaptations arising with physical activity. Centrally, exercise modulates nervous system opioid and opioid receptor expression, and opioid receptor antagonists block the antinociceptive effects of physical activity (Galdino *et al.*, 2010). Within the heart, well-established myocardial protection arising from exercise has been shown to require opioid receptor-dependent signalling (Dickson *et al.*, 2008), including release of an opioid receptor ligand able to communicate a cardioprotective signal across species (Michelsen *et al.*, 2012). As will be detailed later (under The endogenous opioid receptor system underpins ischaemic pre- and post-conditioning responses), the opioid receptor system is also essential to widely studied preconditioning and post-conditioning responses, again supporting an overarching role for opioid receptors in cellular adaptation and tolerance to metabolic perturbation. This broad involvement of opioid receptors and opioids across diverse protective responses and phenotypes, including mammalian hibernation, exercise-dependent cardioprotection and ischaemic conditioning responses, highlights the potential value of opioid receptors in clinical cardioprotection – opioidergic conditioning of the heart.

Opioid and opioid receptor expression in the heart

The heart expresses particularly high levels of endogenous opioids, together with DORs and KORs across species (whereas myocardial MOR expression appears more species-specific). Within the heart, the opioid receptor system is an important regulator of β-adrenoceptor signalling and responses, excitation-contraction coupling, and cardiogenesis (Pugsley, 2002; Headrick *et al.*, 2012), among other actions. In terms of cardiac protection, all three opioid receptor subtypes are implicated in stress signalling and the induction of acute and prolonged protection in response to different pathophysiological stimuli.

Myocardial opioid expression

Cardiac cells are major sites of opioid peptide synthesis, storage and release, with myocytes possessing large stores of genes encoding the endogenous opioid precursors preproenkephalin, prodynorphin and pro-opiomelanocortin (Caffrey *et al.*, 1994; Barron, 2000). Indeed, ventricular myocardium may contain the highest levels of preproenkephalin mRNA in the body (Howells *et al.*, 1986), identifying the heart as an important neuro-endocrine organ. Myocardial synthesis and release of opioid peptides is variable, increasing with ischaemia (Chang *et al.*, 2004; Romano *et al.*, 2004b), exercise (Mougin *et al.*, 1987) and cardioprotective intervention (Zatta *et al.*, 2008), and is also influenced by ageing (Caffrey *et al.*, 1994) and disease state (Lendeckel *et al.*, 2005). Relative quantities of myocardial transcripts or peptides suggest a tendency to greater generation of endogenous DOR-selective ligands. The responsiveness of endogenous opioids to I–R potentially complicates clinical translation of opioid receptor-based interventions: increased cardiac and circulating β-endorphin levels with coronary angioplasty (Chang *et al.*, 2004) and on-pump coronary artery graft surgery (Roth-Isigkeit *et al.*, 2000) may engage opioid receptor signalling to intrinsically enhance I–R tolerance, as shown by animal studies (Romano *et al.*, 2004b). Such endogenous activation may limit further protection via exogenously applied opioid receptor agonists.

Myocardial opioid receptors

Endogenous opioid peptides – the endorphins, dynorphins and enkephalins – mediate cellular actions via engagement of MORs, DORs and KORs (Figure 2). The three receptors are $G_{i/o}$-coupled and possess a high degree of sequence homology, with variations occurring primarily within extracellular domains (Pradhan *et al.*, 2012). Stimulation of opioid receptors inhibits adenylyl cyclase activity, cAMP production and associated signalling in a *Pertussis* toxin-sensitive, $G\alpha_i$-dependent manner. At the sarcolemma, myocardial opioid receptors appear localized to and regulated by caveolae, with evidence of caveolar localization of both DORs and MORs, and caveolae/caveolin-3 dependence of cardiac DOR responses (Head *et al.*, 2005; Patel *et al.*, 2006; Tsutsumi *et al.*, 2010). The receptors mediate a range of myocardial actions via canonical PK signal cascades (Figure 2). From the viewpoint of pharmacological engagement, *in vitro* and *in vivo* evidence confirms biased agonism at all opioid receptor subtypes, with ligand-directed signalling resulting in distinct receptor–effector complexes (Pradhan *et al.*, 2012). This is relevant to the development of efficacious cardioprotectants, while avoiding induction of potentially untoward cardiac and systemic changes. The opioid receptors are internalized upon GPCR kinase-2 or -3 phosphorylation and β-arrestin-2 or -3 recruitment (Al-Hasani and Bruchas, 2011), with receptor trafficking integral to normal functionality. Receptor subtypes may be differentially trafficked following internalization. For example, DORs may be degraded after trafficking to lysosomes, whereas MORs are recycled to the cell surface (Ong *et al.*, 2014). However, specific details of opioid receptor trafficking in cardiomyocytes are lacking.

It is widely reported that both KORs and DORs are expressed in adult myocardium from different mammalian species. In contrast, cardiac expression of the MOR appears to be dependent upon developmental stage and species. Expression of this receptor has been identified in neonatal rat myocardium (Zimlichman *et al.*, 1996), whereas ligand-binding and gene expression studies suggest no expression in adult tissue (Ventura *et al.*, 1989; Wittert *et al.*, 1996). Nonetheless, Head *et al.* (2005) document sarcolemmal and intracellular expression of MORs in adult rat cardiomyocytes, co-localized

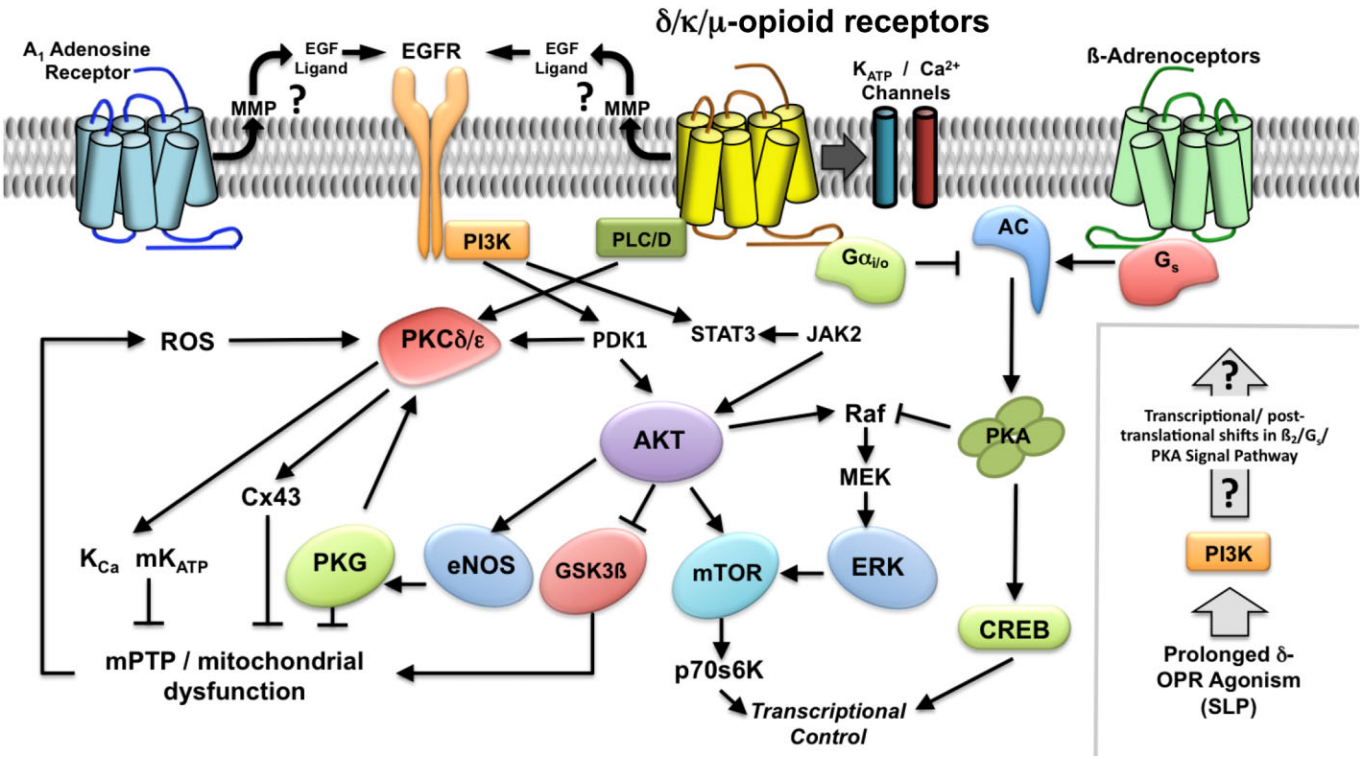

Figure 2

Intracellular signal paths coupled to the opioid receptors and implicated in cardiac stress signalling and cardioprotection. Receptor agonism leads to activation of PI3K/Akt and RISK pathway components (including PKs A, B, C and G), MAPKs (ERK, p38), ROS generation, JAK-STAT signalling, phospho-regulation of effector molecules such as GSK3β and connexin-43, and activation of mitochondrial K_{ATP} and K_{Ca} channels. These paths may converge on inhibition of the mPTP to preserve mitochondrial integrity and function. Not shown opioid receptor-coupled signal paths also affect expression/translocation of pro- and anti-apoptotic proteins, including Bax and Bcl-2, and opioid receptors may additionally participate in activation of the cardioprotective survivor activating factor enhancement (SAFE) pathway (involving TNF-α-dependent activation of STAT3 signalling).

with caveolin-3. Recent analyses of adult porcine myocardium identify DOR and KOR, but not MOR expression (Karlsson *et al.*, 2012; Theisen *et al.*, 2014), with the receptors primarily located on cardiomyocytes and vascular smooth muscle, evenly distributed between atria and ventricles, and both membrane-bound and intracellularly localized. While there are few studies in humans, one investigation applying PET, supports expression of both MORs and DORs in human heart (Villemagne *et al.*, 2002), and more recent analysis identifies all three subtypes on human myocardial cells (Sobanski *et al.*, 2014), with the DORs and MORs also expressed on sparse individual nerve fibres, and KORs on intrinsic cardiac adrenergic cell-like structures. This expression profile is consistent with beneficial responses to MOR and DOR agonists in isolated human myocardium (Bell *et al.*, 2000; Lemoine *et al.*, 2011; Fuardo *et al.*, 2013) and patients (Xu *et al.*, 2009; Wong *et al.*, 2010a) (Table 1). Myocardial expression of opioid receptors also appears modifiable under pathological conditions: I–R induces DOR mRNA and protein, together with KOR mRNA in myocardial area-at-risk in pigs (Karlsson *et al.*, 2012). Conversely, expression of KORs (and ligands for KORs and DORs) is repressed in fibrillating human atria (Lendeckel *et al.*, 2005). The cardiac consequences of such changes in opioid receptor expression remain unclear.

As reviewed by Tadevosyan *et al.* (2012), opioid receptors have also been localized to the nuclear membrane in cardiomyocytes. Functionally, receptor activation with dynorphin activates nuclear PKC and increases transcription of the dynorphin B precursor, prodynorphin (Ventura *et al.*, 1998). Although the cardiac roles of nuclear opioid receptors (presumably KORs) remain to be fully elucidated, they appear important in cardiac embryogenesis. Nuclear dynorphin B binding is also increased in myocytes from cardiomyopathic hamsters, suggesting potentially relevant shifts in this signalling in myopathies (Ventura *et al.*, 1998).

Opioid receptor crosstalk in the heart

Cardiovascular actions of opioid receptors involve interaction with and modulation of other receptors (Figure 2, Table 2), with crosstalk evident both within the opioid receptor family and with other GPCRs and the epidermal growth factor receptor (EGFR). This crosstalk may be indirect, for example co-regulation of downstream signalling paths, modulation of receptor agonist levels, or heterologous receptor sensitization/desensitization. On the other hand, crosstalk may arise more directly via physical receptor–receptor interaction and GPCR polymerization.

The DOR modifies myocardial β-adrenoceptor and calcitonin gene-related peptide (CGRP) signalling and responses (Pepe *et al.*, 1997; Nguyen *et al.*, 2012), and the anti-infarct effects of DOR activation can be countered by antagonism of β_2-adrenoceptors or CGRP receptors (Huang *et al.*, 2007a).

Table 2

Experimental studies of cardioprotection via opioid receptor agonists applied prior to ischaemia

Putative selectivity	Agent studied	Species, tissue	Primary outcome	Effect	Implicated effectors or targets	Study
Non-selective	Morphine	Rat, *in situ* heart	Infarct (TTC)	Improved	Neutrophil activity	Wang *et al.*, 1998
			Infarct (TTC)	Improved		Gross *et al.*, 2004a
		Rat, isolated heart	Infarct (TTC)	Improved	A_1R, mK_{ATP}, ROS	Peart & Gross, 2003
			Infarct (TTC)	Improved	AMPK	Li *et al.*, 2011
DOR	TAN-67 (δ/δ_1-receptor)	Rat, *in situ* heart	Infarct (TTC)	Improved	$G_{i/o}$, K_{ATP}	Schultz *et al.*, 1998b
			Infarct (TTC)	Improved	mK_{ATP}	Fryer *et al.*, 1999
			Infarct (TTC)	Improved	PKCδ	Fryer *et al.*, 2001
		Dog, *in situ* heart	Infarct (TTC)	Improved		Peart *et al.*, 2003
	DADLE (δ/δ_1 receptor)	Rat, isolated heart	CK leak, diastolic dysfunction	Improved	K_{ATP}	Kevelaitis *et al.*, 1999
			Infarct (TTC) Arrhythmogenesis	Improved Unaltered		Wang *et al.*, 2001
			Infarct (TTC)	Improved	PKCε, Cx43	Miura *et al.*, 2007
		Rat, *in situ* heart	Infarct (TTC)	Improved	PKCδ	Fryer *et al.*, 2001
			Infarct (TTC) Arrhythmogenesis Mortality	Improved Unaltered Improved	$G_{i/o}$, PKC, K_{ATP}	Valtchanova-Matchouganska & Ojewole. 2003
		Mouse, HL-1 cells	Necrosis	Improved	PKC, mK_{ATP}	Seymour *et al.*, 2003
		Rabbit, isolated heart	Infarct (TTC)	Improved	EGFR/RTK	Cohen *et al.*, 2007
		Human, atrial trabeculae	Contractility	Improved	mK_{ATP}	Bell *et al.*, 2000
	BW-373U86	Rabbit, *in situ* heart	Infarct (TTC)	Improved	δ_1 receptor, COX-2	Kodani *et al.*, 2002
		Dog, *in situ* heart	Infarct (TTC)	Improved		Peart *et al.*, 2003
		Rat, *in situ* heart	Infarct (TTC)	Improved		Gross *et al.*, 2004a
			Infarct (TTC) Arrhythmogenesis	Improved Unaltered		Peart *et al.*, 2004
		Mouse, isolated heart	Contractility	Improved		Peart & Gross 2004c
	DPDPE (δ/δ_1 receptor)	Pig, *in situ* heart	Infarct (TTC)	Improved		Sigg *et al.*, 2002
		Rat, *in situ* heart	Infarct (TTC)	Improved	β_2-adrenoceptor, intrinsic adrenergic cells	Huang *et al.*, 2007a
			Arrhythmogenesis	Unaltered		Maslov *et al.*, 2014
		Rat, neonate myocyte	Apoptosis	Unaltered		Shen *et al.*, 2012
		Rat, *in situ* heart	Infarct (TTC)	Improved	12-lipoxygenase	Patel *et al.*, 2003
		Mouse, *in situ* heart	Infarct (TTC, cTnI)	Improved	Caveolin-3	Tsutsumi *et al.*, 2010
	SNC-121	Rat, adult myocyte	Necrosis	Improved	Caveolae dependent	Patel *et al.*, 2006
	Deltorphin E (δ_2 receptor)	Rat, neonate myocyte	Apoptosis	Improved	MEK/ERK1/2	Shen *et al.*, 2012
		Rat, *in situ* heart	Infarct (TTC)	Improved	mKATP, sarcKATP	Patel *et al.*, 2002b
		Rat, *in situ* heart	Arrhythmogenesis	Improved	Peripheral δ_2 receptors	Maslov *et al.*, 2014
	Deltorphin-D (δ_2 receptor)	Pig, *in situ* heart	Infarction (TTC)	Improved		Sigg *et al.*, 2002
		Rat, *in situ* heart	Arrhythmogenesis	Improved	Peripheral δ_2 receptors	Maslov *et al.*, 2014
	Deltorphin-II (δ_2 receptor)	Rat, *in situ* heart	Infarction Arrhythmogenesis	Improved Improved	PKC, NOS, mK_{ATP} PKC, NOS, TK	Maslov *et al.*, 2009
	Fentanyl isothiocyanate (irreversible)	Rat, *in situ* heart	Infarction (TTC)	Improved	PI3K	Gross *et al.*, 2005
			Infarction (TTC)	Improved	JAK2, STAT3, Akt, GSK3β	Gross *et al.*, 2006

Table 2

Continued

Putative selectivity	Agent studied	Species, tissue	Primary outcome	Effect	Implicated effectors or targets	Study
KOR	Spiradoline	Rat, *in situ* heart	Arrhythmogenesis	Improved	Na^+ channel block	Pugsley *et al.*, 1998
	U50,488H	Rat, myocyte	Necrosis	Improved	HSP70	Liu *et al.*, 2004
		Rat, isolated heart	Infarct (TTC) Arrhythmogenesis	Improved Improved	PKC, mK_{ATP}	Wang *et al.*, 2001
			Infarct (TTC)	Improved	mK_{ATP}, PKC, ROS	Cao *et al.*, 2004
			Infarct (TTC)	Improved	PKC, K_{Ca}, mPTP	Cao *et al.*, 2005
		Rat, *in situ* heart	Infarct (TTC) Arrhythmogenesis	Improved Improved		Peart *et al.*, 2004
			Infarct (TTC)	Improved	HSP70	Qi *et al.*, 2004
			Infarct (cTnT, CK, LDH) Arrhythmogenesis	Improved Improved	K^+ current	Cheng *et al.*, 2007
			Arrhythmogenesis	Improved	κ_1 receptor, PKC, mK_{ATP}	Lishmanov *et al.*, 2007
			Arrhythmogenesis	Improved	Cx43	Zhang *et al.*, 2010
			Infarct (TTC)	Improved	TLR4/TNF-α	Lin *et al.*, 2013
		Rat, *in situ* and mouse, isolated heart	Infarct (TTC)	Improved	PI3K, mK_{ATP}	Peart *et al.*, 2008
		Mouse, isolated heart	Contractility	Improved		Peart & Gross 2004c
	Dynorphin B	Rabbit, myocyte	Necrosis	Improved	PKC, K_{ATP}	Cao *et al.*, 2003
MOR	DAMGO	Rat, *in situ* heart	Infarct (TTC)	Unaltered		Schultz *et al.*, 1998a
			Arrhythmogenesis	Unaltered		Maslov *et al.*, 2014
	Fentanyl	Rat, isolated heart	Contractility	Improved	mK_{ATP}, A_1 receptor	Kato *et al.* 2000
		Rabbit, *in situ* heart	Infarct (TTC) Arrhythmogenesis	Improved Improved	Peripheral opioid receptors Central opioid receptors	Lessa & Tibiriçá, 2006
	Remifentanil	Rat, *in situ* heart	Infarct (TTC)	Improved		Zhang *et al.*, 2004
			Infarct (TTC)	Improved	ERK1/2, Bcl2, Bax	Kim *et al.*, 2010
	Remifentanil Sufentanil	Human, atrial trabeculae	Contractile recovery	Improved		Lemoine *et al.*, 2011
MOR/DORs	Eribis peptide 94	Rat, *in situ* heart	Infarct (TTC)	Improved	Central MORs	Gross *et al.*, 2012a

Shown are the effects of pre-ischaemic opioid receptor agonists on outcomes from myocardial I–R in experimental animal models and *ex vivo* human tissue. Outcomes include infarction/cell death (via TTC staining, release of intracellular proteins, or markers of isolated cell viability), contractile recovery, and arrhythmogenesis. Also shown are the receptors, signalling elements or effector molecules implicated in protection (where assessed). AMPK, 5′ AMP-activated PK; BW373U86, 4-[(R)-[(2S,5R)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-hydroxyphenyl)methyl]-N,N-diethylbenzamide; CK, creatine kinase; Cx43, connexin-43; cTnI, cardiac Troponin I; cTnT, cardiac troponin T; DADLE, [d-Ala2, d-Leu5] enkephalin; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; DPDPE, D-penicillamine(2,5)-enkephalin; GSK3β, glycogen synthase kinase 3β; MEK, MAPK kinase; mK_{ATP}, mitochondrial KATP channel; $sarcK_{ATP}$, sarcolemmal K_{ATP} channel; SNC-80, 4-[(R)-[(2S,5R)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-N,N-diethylbenzamide; SNC-121, 4-((aR)-a-((2S,5R)-4-Propyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl)-N,N-diethylbenzamide; TAN-67, [(4aS*,12aR*)-4a-(3-hydroxyphenyl)-2-methyl-1,2,3,4,4a,5,12, 12a -octahydropyrido[3,4-b]acridine; TLR4, toll-like receptor 4; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride staining for infarction; U50,488H, [(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide].

Unique cardiac protection arising via sustained opioid receptor agonism (see Pharmacological induction of opioid receptor-mediated cardioprotection, later) is also sensitive to β_2-adrenoceptor antagonism (Peart and Gross, 2006). Although adrenoceptor/opioid receptor crosstalk is known to involve co-regulation of G-protein signalling, receptor dimerization could additionally contribute given the capacity of DORs and KORs to associate with β_2-adrenoceptors when co-expressed in model cells (Jordan *et al.*, 2001).

Crosstalk with adenosine receptors may be essential to opioid receptor-mediated cardioprotection, with opioid receptors in turn contributing adenosinergic protection. Kato and co-workers found the anti-stunning effects of fentanyl were abolished by adenosine A_1 receptor antagonism (Kato *et al.*, 2000). The anti-infarct effects of opioid receptor agonists can also be negated by A_1 receptor antagonism and *vice versa* (Peart and Gross, 2003), while protection via elevated endogenous adenosine is sensitive to DOR antagonism (Peart and Gross, 2005). Others report that adenosine receptors are essential to the cardiac protection arising with intrathecal (i.t.) morphine (Yao *et al.*, 2011), that remote IPC requires interaction between DORs or KORs and A_1 receptors (Surendra *et al.*, 2013), and that opioid receptors are also involved in beneficial effects of elevated endogenous adenosine in hearts with diabetes (Sharma *et al.*, 2013). Whether this essential interplay between adenosine and opioid receptor families involves receptor dimerization or indirect forms of crosstalk remains to be established. Interestingly, kinase signalling triggered by A_1 receptor or DOR agonists in cardiac myoblasts appears to be entirely EGFR-dependent (Williams-Pritchard *et al.*, 2008; 2011), with cardioprotective effects of adenosine and DORs also requiring EGFR and MMP activities (Cohen *et al.*, 2007; Förster *et al.*, 2007; Williams-Pritchard *et al.*, 2011). Thus, DORs and A_1 receptors may engage a common MMP/EGFR-dependent pathway to co-activate cardioprotective signalling, coupling the distinct GPCRs at the level of receptor transactivation and signal transduction (Figure 2).

The opioid receptor system also interacts importantly with lipid-derived mediators of tissue protection. Cardioprotective effects of epoxyeicosatrienoic acids, eicosanoids synthesized by cytochrome P450 epoxygenases, appear to be mediated via the DORs and KORs (Gross *et al.*, 2010), while DORs and MORs mediate their antinociceptive actions (Terashvili *et al.*, 2008). The basis of this functional interaction between epoxyeicosatrienoic acids and opioid receptors remains poorly defined. Antinociceptive effects of epoxyeicosatrienoic acids do not involve direct binding to either MORs or DORs (Terashvili *et al.*, 2008), and several GPCR families appear to be influenced by these lipid derivatives. As for crosstalk between A_1 receptors and opioid receptors, one possibility is co-activation of common signalling: the cardiovascular actions of opioids and epoxyeicosatrienoic acids are both dependent upon caveolae/caveolin (Patel *et al.*, 2006; Tsutsumi *et al.*, 2010; Chaudhary *et al.*, 2013) and the EGFR (Michaelis *et al.*, 2003; Förster *et al.*, 2007; Williams-Pritchard *et al.*, 2008), with the latter receptor also localized to caveolae (Liu *et al.*, 2003).

Receptor-independent actions of opioids

While the cardioprotective effects of opioids appear predominantly receptor-mediated, they may also influence cellular function independently of opioid receptors. β-endorphin, dynorphin A, nociceptin, endomorphins, hemorphins and some proenkephalin A-derived peptides have been reported to modify function in different cell types in an opioid receptor-independent manner. The basis of such responses awaits more detailed analysis, although they may be relevant to processes of cardiac injury and protection. For example, agonists for the KORs could afford protection via receptor-independent modulation of ion channel function: dromotropic and inotropic effects of some KOR agonists appear naloxone-insensitive, suggesting receptor-independent Na^+ and K^+ channel blockade (Pugsley *et al.*, 1993; 1998). It appears KOR agonists can also inhibit L-type Ca^{2+} channels in a KOR antagonist insensitive manner (Micol and Laorden, 1992). Opioids could thus modify ionic disturbances during I–R via non-receptor-mediated actions, consistent with evidence for receptor-independent effects of some opioid receptor agonists on post-ischaemic arrhythmogenesis (Peart *et al.*, 2004).

The endogenous opioid receptor system underpins ischaemic pre- and post-conditioning responses

Preconditioning reflects an intrinsic hormesis response, whereby sub-lethal metabolic stress (e.g. hypoxia or ischaemia) induces acute and delayed protection against more severe insult, providing adaptive resistance to injurious perturbations (Figure 1). In the heart, ischaemic stimuli induce short-term protection via post-translational signals paralleling those downstream of opioid receptors (Figure 2), together with a delayed second window of protection that persists for several days (associated with *de novo* synthesis of protective NOS and COX-2, among other proteins). Mechanisms implicated in ischaemic preconditioning have been intensely studied since its discovery, and extensively reviewed elsewhere (Peart and Headrick, 2009; Hausenloy *et al.*, 2011; Ferdinandy *et al.*, 2014). General mechanistic models of acute protection involve signal initiation by I–R-responsive GPCRs, with opioid receptors together with adenosine, bradykinin, adrenergic and CGRP receptors implicated. Receptor tyrosine kinase activity coupled to the EGFR may also participate in initiating protective signalling. As highlighted in the scheme in Figure 2, these upstream events are coupled to activation of multiple PKs, ROS generation and signalling, mitochondrial K^+ channel modulation, and inhibition of the mitochondrial permeability transition pore (mPTP). In addition, a survivor activating factor enhancement path triggered by I–R-responsive GPCRs (including opioid receptors) may also contribute to conditioning responses, involving TNF-α-dependent activation of STAT3 signalling (Hausenloy *et al.*, 2011; Shi and Vinten-Johansen, 2012). Finally, opioid receptors can influence cellular ion fluxes independently of second messengers, with evidence for direct DOR and MOR induction of K^+ channel opening, and KOR-dependent Ca^{2+} channel inhibition (Gross *et al.*, 1990; Schultz and Gross, 2001). Ultimately, mitochondrial preservation, repression of associated apoptotic signalling, and conservation of gap junction function via these signalling pathways improves

myocardial survival and function. The precise interactions between these varied signal pathways and effector mechanisms remain to be fully disentangled, and new details continue to emerge – for example, recent work suggests preconditioning may not actually require cytosolic signal transduction, but involve direct mitochondrial conditioning (Ruiz-Meana *et al.*, 2014). How this observation might be reconciled with evidence of essential sarcolemmal receptor and intracellular PK involvement is presently unclear.

Not strictly a hormesis response, although sharing mechanistic features of preconditioning, ischaemic post-conditioning (Figure 1) describes a marked reduction in post-ischaemic injury in response to brief cycles of I–R during early reperfusion (i.e. transiently 'stuttering' reperfusion). Whereas the clinical utility of *preconditioning* is primarily limited to planned surgical ischaemia, *post-conditioning* is highly relevant to the treatment of AMI, as this efficacious response can be initiated upon more predictable reperfusion. A potential limitation is that post-conditioning cannot prevent injury occurring during the ischaemic episode itself, although injury progression during reperfusion may be more critical as suggested by similar protective outcomes with post- and preconditioning.

Opioid receptors in ischaemic preconditioning

Experimental data from multiple species indicate intrinsic opioid receptor activity is essential to protection with ischaemic preconditioning. Antagonism of opioid receptors negates ischaemic preconditioning whether initiated prior to the conditioning stimulus (Schultz *et al.*, 1995), or during subsequent index ischaemia in the context of delayed preconditioning (Fryer *et al.*, 1999). Thus, intrinsic opioid receptor activity appears indispensable in both initially triggering the protected state and mediating later protection. This opioid receptor-dependence appears to extend to the human heart. Tomai and colleagues have reported that the effects of ischaemic preconditioning during coronary angioplasty (induced with repeated balloon inflations) are sensitive to the opioid receptor antagonist naloxone (Tomai *et al.*, 1999).

Both the DOR and the KOR subtypes are implicated in ischaemic preconditioning, including mediation of anti-infarct and anti-arrhythmic actions. The latter suppression of I–R-related arrhythmias is of considerable importance as they are a primary cause of death with AMI. Schultz *et al.* (1995) first demonstrated that endogenous opioid receptors are integral to the response, which was abolished by naloxone. Subsequent work by the group identified a role for peripheral DORs in preconditioning, but not MORs or KORs (Schultz *et al.*, 1997; 1998a). Conversely, Wang *et al.* (2001) found that intrinsic DOR activity reduced infarct development while KORs limited both infarct development and arrhythmogenesis in preconditioned rat hearts, consistent with later findings of Valtchanova-Matchouganska and Ojewole (2003). The DORs and KORs may thus each contribute protective roles in ischaemic preconditioning, with overlapping and potentially unique functions arising from common and distinct signalling. Consistent with essential roles in ischaemic preconditioning, pharmacological activation of DORs and KORs prior to ischaemia mimics this protection in many species via signalling implicated in ischaemic preconditioning (Table 2).

Opioid receptors in remote ischaemic preconditioning

Remote conditioning refers to induction of protection in a target organ (such as the heart) in response to transient ischaemic episodes in a remote organ or tissue (such as a limb) (Figure 1). Because of the potentially undesirable effects of direct cardiac conditioning versus the non-invasive, indirect and thus less stressful impact of remote conditioning (cuff inflation/deflation on a limb), the latter offers useful features for clinical translation. Remote conditioning appears effective across species, including evidence of efficacy in humans (Shi and Vinten-Johansen, 2012). However, outcomes from clinical trials remain mixed (Shi and Vinten-Johansen, 2012; Ferdinandy *et al.*, 2014), with a number reporting no clinical benefit or even worsening of outcome measures. Further research is needed in both understanding and refining the protective conditioning stimuli employed (e.g. optimizing conditioning algorithms; supplementation/replacement of ischaemic stimuli with opioid receptor agonism), and unravelling the impacts of co-morbidities and other clinically relevant factors on these responses.

The Przyklenk laboratory provided initial evidence that endogenous opioid receptor agonists released from a preconditioned organ could effectively communicate a protective signal to recipient myocardium (Dickson *et al.*, 2001). Subsequently, Patel and colleagues (Patel *et al.*, 2002a) identified a role for opioid receptors in remote preconditioning arising from mesenteric artery occlusion in anaesthetized rats. Both the DORs and KORs may participate in remote preconditioning responses (Weinbrenner *et al.*, 2004; Surendra *et al.*, 2013), although Zhang *et al.* (2006) report sensitivity of remote preconditioning to KOR, but not DOR blockade (with plasma levels of KOR-selective dynorphin, but not DOR-selective met-enkephalin, increased in remote preconditioning). On the other hand, there is also evidence the protective effects of remote preconditioning involve activation of spinal MORs (Wong *et al.*, 2012a). The relative roles of peripheral and central opioid receptor subtypes in remote ischaemic conditioning response awaits further delineation.

Consistent with conservation of a fundamentally important opioidergic stress response, remote conditioning is transferrable across species: human plasma dialysates obtained after transient limb ischaemia confer protection in rabbit cardiomyocytes (Shimizu *et al.*, 2009), and opioid receptor-dependent protection in rabbit (Michelsen *et al.*, 2012) and mouse hearts (Merlocco *et al.*, 2014). Mechanisms of protection via such a transferrable opioid may involve ROS generation (Weinbrenner *et al.*, 2004), ERK1/2 and PKC signals (Shimizu *et al.*, 2009), CGRP signalling (Zhang *et al.*, 2011) and inhibition of the mPTP (Zhang *et al.*, 2006) (Figure 2). Beyond the myocardium, the opioid receptor system is also involved in remote preconditioning of: kidney (Wever *et al.*, 2013); skeletal muscle, involving attenuated ATP depletion and neutrophil accumulation (Addison *et al.*, 2003); and the brain, where insulin and CGRP may contribute (Rehni *et al.*, 2007).

Opioid receptors in ischaemic post-conditioning

Endogenous opioids and opioid receptors also appear to mediate ischaemic post-conditioning, and exogenous opioid

receptor agonists applied immediately prior to or during reperfusion can effectively protect the heart (Table 3). The opioid receptor subtypes mediating ischaemic post-conditioning remain controversial. In isolated myocardium, the DOR appears crucial (Wang *et al.*, 2008), and DOR antagonism can also negate ischaemic post-conditioning *in vivo* (Jang *et al.*, 2008). Zatta *et al.* (2008) report elevations in cardiac enkephalins during ischaemic post-conditioning in rats (primarily the proenkephalin precursor), coupled with DOR and potentially MOR-dependent protection. Such findings implicate functional MORs in the heart, or alternatively, roles for extracardiac MORs *in vivo*. In contrast to studies implicating DOR and possibly MOR involvement, Guo *et al.* (2011) report increased cardiac content of the KOR peptide dynorphin during ischaemic post-conditioning in rats, in association with KOR-antagonist sensitive protection.

Mechanisms of ischaemic post-conditioning largely parallel those of preconditioning, with activation of the reperfusion injury salvage kinase (RISK) pathway (Shi and Vinten-Johansen, 2012; Ferdinandy *et al.*, 2014), NO/cGMP/PKG signalling (Jang *et al.*, 2008), and opening of Ca^{2+}-activated K^+ channel (K_{Ca}) (Wang *et al.*, 2008) and mitochondrial K_{ATP} channel (mK_{ATP}) channels to ultimately inhibit mPTP activity, energy disruption and associated cell death (Jang *et al.*, 2008; Shi and Vinten-Johansen, 2012; Ferdinandy *et al.*, 2014) (Figure 2). Opioids have also been shown to post-condition the brain via similar mechanisms, involving PI3K/Akt signalling (Wang *et al.*, 2011), and intrinsic opioid receptor activity is important in remote ischaemic post-conditioning of the brain, again involving PI3K/Akt-dependent mechanisms (Zhou *et al.*, 2011). In terms of clinical translation, DOR agonists have has been shown to effectively post-condition human myocardium (Fuardo *et al.*, 2013) (Table 1).

Pharmacological induction of opioid receptor-mediated cardioprotection: opioid receptor agonist-triggered preconditioning

Given opioid receptor involvement in mediating ischaemic preconditioning responses, these receptors are logical targets for enhancing myocardial I–R tolerance when ischaemia is planned or predictable (e.g. coronary artery bypass graft, valve repair, transplantation). Such preconditioning responses may also be of value in patients at particularly high risk of myocardial ischaemia. In terms of evidence for opioid receptor preconditioning in humans, studies in isolated atrial myocardium support DOR- and potentially MOR-mediated protection (Bell *et al.*, 2000; Lemoine *et al.*, 2011). While there are few clinical trials directly addressing the cardioprotective effects of opioid receptor-based preconditioning (Table 1), pretreatment with the MOR-selective agonist remifentanil reduced circulating troponin I (TnI) levels following off-pump coronary bypass (Xu *et al.*, 2009), and CK-MB levels following on-pump coronary bypass (Wong *et al.*, 2010a). Murphy and colleagues also provide evidence that morphine pretreatment reduced the inflammatory response and improved myocardial performance following cardiopulmonary bypass (Murphy *et al.*, 2006; 2007).

DOR-mediated responses. Preconditioning-like effects of selective DOR agonists have been confirmed in a variety of models (Table 2), including neonatal and adult rat cardiomyocytes (Patel *et al.*, 2006; Shen *et al.*, 2012), *in situ* and *ex vivo* rodent hearts (Fryer *et al.*, 2001; Peart and Gross, 2004c), rabbit (Kodani *et al.*, 2002), canine (Peart and Gross, 2003; Peart *et al.*, 2003) and swine models (Sigg *et al.*, 2002), together with human myocardium (Bell *et al.*, 2000). Which specific DOR is involved remains contentious, with evidence supporting δ_2- and not δ_1- opioid receptor engagement (Shen *et al.*, 2012) versus evidence for protective effects of selective δ_1-opioid receptor activation (Schultz *et al.*, 1998b; Fryer *et al.*, 2001). In terms of anti-arrhythmic effects, Maslov and colleagues provide evidence for specific δ_2-opioid receptor involvement in limiting I–R-related arrhythmogenesis (Maslov *et al.*, 2009, 2014).

At the level of the cell membrane, these DOR preconditioning effects may involve transactivation of the EGFR (Cohen *et al.*, 2007) and crosstalk with adenosine receptors (Peart and Gross, 2003; 2005), with the latter also EGFR-dependent (Williams-Pritchard *et al.*, 2011) (Figure 2). Caveolar function is also essential to DOR-dependent protection (Patel *et al.*, 2006), reflecting co-localization of receptor and signal elements within these microdomains. Mechanistically paralleling ischaemic preconditioning, pharmacological DOR preconditioning is *Pertussis* toxin-sensitive ($G_{i/o}$-dependent) and appears to engage a signal cascade involving: PKC (Schultz *et al.*, 1998b; Maslov *et al.*, 2009), with evidence for specific PKC-ε (Miura *et al.*, 2007) or PKC-δ involvement (Fryer *et al.*, 2001); ROS generation (Cohen *et al.*, 2007); NOS activation (Maslov *et al.*, 2009); PI3K/Akt, Src kinase and MAPK kinase/ERK1/2 signals (Gross *et al.*, 2004a; 2006; Cohen *et al.*, 2007; Shen *et al.*, 2012); JAK2/STAT3 signalling and phospho-inhibition of glycogen synthase kinase 3β (GSK3β) (Gross *et al.*, 2004a; 2006); mammalian target of rapamycin activation (Gross *et al.*, 2004a); and mK_{ATP} channel activity (Schultz *et al.*, 1998b; Kevelaitis *et al.*, 1999; Bell *et al.*, 2000) (Figure 2, Table 2). Recent work also supports some involvement of AMP-activated PK signalling (Li *et al.*, 2011). Distal mediation of protection with DOR preconditioning may involve reduced gap junction permeabilization via PKCε-dependent phosphorylation of connexin-43 (Miura *et al.*, 2007), together with preservation of mitochondrial function and inhibition of related cell death signalling. Connexin-43 may represent an important point of convergence linking control of gap junction and mitochondrial function (Ruiz-Meana *et al.*, 2014). The specific roles of the mPTP and mitochondrial versus sarcolemmal K_{ATP} channels in DOR-mediated preconditioning remain to be fully elucidated (Fryer *et al.*, 2001; Patel *et al.*, 2002b). The mK_{ATP} channel has been implicated in both acute and delayed opioid-mediated preconditioning (Schultz *et al.*, 1996; Fryer *et al.*, 1999; Peart and Gross, 2003), with sarcolemmal channels implicated in distinct DOR-mediated delayed protection (Patel *et al.*, 2002b) (Table 2).

Additional to survival-kinase/K_{ATP} channel-dependent signalling, DOR conditioning responses may also involve the arachidonic acid metabolism pathway (Table 2). DOR

Table 3

Experimental studies of cardioprotection via post-ischaemic opioid receptor agonism

Putative selectivity	Agent studied	Species, tissue	Primary outcome	Effect	Implicated effectors or targets	Study
Non-selective	Morphine	Rat, isolated heart	Infarct (TTC)	Improved	mK_{ATP}, KOR (DOR antagonist insensitive)	Chen *et al.*, 2008
			Infarct (TTC)	Improved	NO/PKG, mPTP	Jang *et al.*, 2008
			Infarct (TTC)	Improved	δ/δ_1 receptor, KOR, mPTP	Kim *et al.*, 2011
DOR	DADLE (δ/δ_1 receptor)	Rabbit, isolated heart	Infarct (TTC)	Improved	Akt, ERK1/2, EGFR	Förster *et al.*, 2007
		Human, atrial trabeculae	Contractility	Improved	mPTP	Fuardo *et al.*, 2013
	BW-373U86	Rat, *in situ* heart	Infarct (TTC)	Improved	mK_{ATP}, $sarcK_{ATP}$	Gross *et al.*, 2007a
		Rat, isolated heart	Infarct (TTC)	Improved	NO/PKG, mPTP	Jang *et al.*, 2008
	SNC-121	Mouse, *in situ* heart	Infarct (TTC)	Improved	ROS	Tsutsumi *et al.*, 2007
KOR	U50,488H	Rat, *in situ* and mouse, isolated heart	Infarct (TTC)	Improved	PI3K, mK_{ATP}	Peart *et al.*, 2008
		Rat, *in situ* heart	Infarct (TTC)	Improved	PI3K/Akt, eNOS	Tong *et al.*, 2011
MOR	Remifentanil	Rat, *in situ* heart	Infarct (TTC)	Improved	KOR, DOR	Wong *et al.*, 2010b
	Sufentanil	Rat, *in situ* heart	Infarct (TTC) Apoptosis	Improved	PI3K/Akt, GSK3β, Bax/Bcl-2	Wu *et al.*, 2012a
			Infarct (TTC)	Improved	Cx43	Wu *et al.*, 2012b
MOR/DOR	Eribis peptide 94	Pig, *in situ* heart	Infarct (TTC)	Improved		Karlsson *et al.*, 2012

Shown are the effects of post-ischaemic opioid receptor agonists on outcomes from myocardial I–R in experimental animal models and *ex vivo* human tissue. Agonists were applied in the late stages of ischaemia prior to reperfusion (perconditioning) or during the reperfusion phase. Outcomes include infarction or apoptosis, and contractile recovery. Also shown are the receptors, signalling elements or effector molecules implicated in protection (where assessed). BW373U86, 4-[(R)-[(2S,5R)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-hydroxyphenyl)methyl]-N,N-diethylbenzamide; Cx43, connexin-43; DADLE, [d-Ala2, d-Leu5] enkephalin; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; GSK3β, glycogen synthase kinase 3β; mK_{ATP}, mitochondrial K_{ATP} channel; $sarcK_{ATP}$, sarcolemmal K_{ATP} channel; SNC-121, 4-((aR)-a-((2S,5R)-4-Propyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl)-N,N-diethylbenzamide; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride staining for infarction; U50,488H, [(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide].

agonists increased 12-lipooxygenase transcription and expression, and accumulation of its product 12-hydroxyeicosatetraenoic acid during I–R, with associated cardioprotection abolished by 12-lipooxygenase inhibitors (Patel *et al.*, 2003). Moreover, DOR stimulation up-regulated myocardial expression of COX-2, PGI_2 synthase, PGE_2 and 6-keto-$PGF_{1\alpha}$, with COX-2 inhibitors abolishing the delayed phase of cardiac protection (Kodani *et al.*, 2002). The latter delayed protection also appears to require iNOS, with knockout eliminating the delayed effects of morphine treatment (Jiang *et al.*, 2004). Other studies support modulation of phosphoinositol signalling in response to DOR activation (Schultz and Gross, 2001). Ventura *et al.* (1992) identified a role for phosphatidylinositol turnover and intracellular Ca^{2+} depletion in myocardial responses to DOR and also KOR agonists, and opioid receptor-dependent effects on intracellular Ca^{2+} appear to be downstream of inositol 1,4,5-trisphosphate (Sheng *et al.*, 1996).

KOR-mediated responses. As for the DORs, pre-ischaemic activation of KORs limits I–R injury (Table 2), including both anti-infarct and anti-arrhythmic effects (Valtchanova-Matchouganska and Ojewole, 2003; Liu *et al.*, 2004; Peart and Gross, 2004c; Peart *et al.*, 2004; Cheng *et al.*, 2007). Also similar to the DOR, protection via pre-ischaemic KOR activation may mechanistically involve ROS and PKC (Wu *et al.*, 1999; Wang *et al.*, 2001; Valtchanova-Matchouganska and Ojewole, 2003; Cao *et al.*, 2004; Lishmanov *et al.*, 2007), with downstream modulation of mitochondrial (also sarcolemmal) K_{ATP} channels (Wang *et al.*, 2001; Valtchanova-Matchouganska and Ojewole, 2003; Cao *et al.*, 2004; Lishmanov *et al.*, 2007), together with increased high-conductance K_{Ca} and reduced mPTP activities (Cao *et al.*, 2005). There is also evidence for essential involvement of heat shock protein (HSP) 70 expression in KOR preconditioning (Liu *et al.*, 2004), with diabetes-dependent impairment of the KOR response potentially reflecting disease related reductions in HSP70 synthesis (Qi *et al.*, 2004). Protection via KOR signalling has also been linked to improvements in myocardial Ca^{2+} homeostasis (Liu *et al.*, 2004). In addition, recent evidence points to beneficial anti-inflammatory effects of KOR activation during I–R, with reductions in Toll-like receptor 4 and NF-κB expression coupled to reduced myeloperoxidase and myocardial TNF-α levels (Lin *et al.*, 2013). The anti-arrhythmic effects of KOR agonism appear to involve PKC and K_{ATP} channel-dependent action potential prolongation (Lishmanov *et al.*, 2007), together with modulation of K^+ currents (Cheng *et al.*, 2007) and connexin-43 function (Zhang *et al.*, 2010). There is also evidence that receptor-independent ion channel effects may contribute to the anti-arrhythmic effects of different KOR agonists in the rat (Peart *et al.*, 2004).

MOR-mediated responses. Despite evidence that MORs may be absent from adult myocytes in some species, the receptor is expressed in adult human hearts (Villemagne *et al.*, 2002; Sobanski *et al.*, 2014), and effects of partly selective MOR agonists (e.g. Xu *et al.*, 2009; Wong *et al.*, 2010a) functionally evidence cardiac expression (Table 2). That said, the identity of the receptor(s) mediating such effects remains unclear. Treatment of rats with the short-acting MOR agonist remifentanil affords cardiac protection equivalent to that with ischaemic preconditioning, and which is negated by antagonists at either MORs, KORs or DORs (Zhang *et al.*, 2004). Whether this reflects non-specific effects of the MOR agonists employed, involvement of multiple opioid receptor subtypes in the protective cascade, and/or roles for extracardiac MORs remains to be determined. Lessa and Tibiriçá (2006) provide evidence that the anti-infarct effects of partly selective MOR agonist fentanyl are mediated peripherally while anti-arrhythmic effects involve central opioid receptor signalling. The infarct-sparing effects of MOR agonists may involve attenuation of injurious inflammation (Wang *et al.*, 1998; Zhang *et al.*, 2004). Indeed, beneficial effects of morphine preconditioning are attenuated by co-administration of a neutrophil endopeptidase inhibitor (Wang *et al.*, 1998), and the MOR agonist fentanyl also represses TNF-α and IL-1β levels during cerebral I–R (Oh, 2002).

Opioid receptor agonist mediated post-conditioning

From a clinical perspective, the concept of opioidergic post-conditioning has broader potential than preconditioning, because of the timing of the stimulus and its applicability in both surgical ischaemia and AMI. The myocardium can be pharmacologically post-conditioned via opioid receptor agonism at the onset of reperfusion, with agonists at all three opioid receptor subtypes eliciting such protection (Table 3). Underlying mechanisms appear similar between receptors, and to those implicated in preconditioning responses. Indeed, cardioprotective effects of pharmacological pre- and post-conditioning are similar and non-additive, supporting common mechanistic elements (Chen *et al.*, 2008). Unfortunately, there are relatively few studies examining the signalling intermediates essential to opioidergic post-conditioning. There are also few studies of opioid receptor post-conditioning in human tissue. Nonetheless, there is evidence that isolated human atrial myocardium can be post-conditioned by DOR agonists (Fuardo *et al.*, 2013), and morphine post-conditioning also reduces cardiac TnI release and improves functional outcomes following surgical correction of tetralogy of Fallot (Zhang *et al.*, 2013) (Table 1). Additionally, morphine treatment prior to reperfusion appears to significantly augment cardioprotection achieved with ischaemic perconditioning in patients undergoing percutaneous coronary intervention (Rentoukas *et al.*, 2010).

In most studies, a post-conditioning effect is induced via DOR and/or KOR agonists, although agents with activity at MORs can also trigger post-conditioning (Table 3). Kim *et al.* (2011) found that morphine post-conditioning in isolated rat hearts was DOR dependent, whereas in an earlier study, Chen *et al.* (2008) reported morphine post-conditioning required KOR and not DOR activity in a similar model. Morphine post-conditioning of endothelium, on the other hand, is effectively abolished by antagonism of either KORs or DORs (Min *et al.*, 2011), although interestingly, the non-selective opioid receptor antagonist naloxone was without effect in these cells. Selective DOR activation affords protection via mechanisms involving ROS generation (Tsutsumi *et al.*, 2007), inhibition of GSK3β signalling (Gross *et al.*, 2004a; 2007a), sarcolemmal and mitochondrial K_{ATP} channel opening (Gross *et al.*, 2007a), and mPTP inhibition (Fuardo *et al.*, 2013). Post-conditioning with

the KOR agonist U50,488, limits infarct development and apoptosis (Peart *et al.*, 2008; Tong *et al.*, 2011), potentially involving PI3K, endothelial NOS (eNOS)/NO and mK_{ATP} channel-dependent mechanisms.

In vitro and *in vivo* post-conditioning effects of clinically relevant agonists possessing some selectivity for the MOR (Chen *et al.*, 2008; Wong *et al.*, 2010b; Kim *et al.*, 2011; Min *et al.*, 2011; Wu *et al.*, 2012a) suggest myocardial protection may be induced via this subtype (Table 3). Nonetheless, such protection can be countered by selective KOR or DOR, but not MOR, antagonists (Chen *et al.*, 2008; Wong *et al.*, 2010b; Kim *et al.*, 2011), linking more consistently expressed KOR or DORs to these responses. The mechanisms activated by MOR-selective agents also mirror those coupled to KOR and DOR responses, including PI3K/Akt signalling with associated inhibition of GSK3β and modulation of Bax and Bcl-2 expression (Wu *et al.*, 2012a), PKC activity and attenuation of intercellular adhesion molecule 1 expression (Min *et al.*, 2011), opening of mK_{ATP} channels (Chen *et al.*, 2008) and inhibition of the mPTP (Kim *et al.*, 2011). Connexin-43 may also be important in post-conditioning via sufentanil (Wu *et al.*, 2012b), paralleling involvement of the protein in DOR-mediated protection (Miura *et al.*, 2007). Whether post-conditioning via MOR agonists reflects a true MOR response versus engagement of DORs or KORs (consistent with common signalling and lack of effects of MOR antagonists) remains to be established.

Centrally mediated opioidergic conditioning

I.c.v. or i.t. application of morphine or fentanyl has been shown to significantly enhance myocardial resistance to I–R (Table 4), confirming the CNS as a remote regulator of cardiac stress resistance, and supporting capacity to centrally induce opioid receptor-dependent cardioprotection (Li *et al.*, 2009; Yao *et al.*, 2011; Zhang *et al.*, 2011; Lu *et al.*, 2014). However, some caution is warranted in interpreting cardiac effects of i.t. morphine, as circulating levels of morphine may be elevated with spinal application. Nonetheless, i.t. agonists modify protective NOS signalling in spinal tissue (Lu *et al.*, 2014), and Yao *et al.* (2011) observed specific effects of i.t. (vs. i.v.) adenosine receptor antagonism, supporting involvement of spinal receptor signals in protection with i.t. morphine. Moreover, autonomic nervous activity appears to be involved, with the ganglionic blocker hexamethonium blocking the protective action of i.t. morphine (Wong *et al.*, 2012b). Remote cardiac effects of intracerebral morphine are also blocked by intracerebrally applied DOR, MOR, and KOR antagonists (Zhang *et al.*, 2011), and are associated with changes in cerebral calmodulin levels. These studies collectively support roles for nervous system signalling in cardioprotection arising from spinal or cerebral opioids. Evidence supports involvement of all three opioid receptor subtypes in these responses (Li *et al.*, 2009; Zhang *et al.*, 2011), although Wong *et al.* (2012b) identified a specific role for MORs in the cardioprotection arising with spinal morphine. The latter is consistent with evidence for spinal MOR involvement in protection induced by remote ischaemic preconditioning (Wong *et al.*, 2012a).

Mechanistically, the cardiac effects of cerebral or spinal morphine have been linked to adenosine release and both central and peripheral adenosine receptor activity (Yao *et al.*, 2011), central changes in calmodulin together with increased extracellular CGRP (Zhang *et al.*, 2011), and neuronal NOS activity (Lu *et al.*, 2014) (Table 4). Wong *et al.* (2012b) also report involvement of bradykinin receptors in the cardiac protection afforded by i.t. morphine, together with shifts in myocardial Akt, eNOS and K_{ATP} channel activity. These signal paths may play important roles in other cardiac conditioning responses. Lim *et al.* (2010) report that protective effects of femoral artery occlusion require intact neural and humoral pathways, and Gross *et al.* (2012a) report central MOR involvement in the anti-infarct effects of the MOR and DOR-selective enkephalin derivative EP94. These studies collectively implicate central pathways in remotely induced cardioprotection, potentially involving the release and

Table 4

Experimental studies of cardioprotection via i.t. or intracerebral opioid receptor agonists

Putative selectivity	Agent – delivery	Species, tissue	Primary outcome	Effect	Implicated effectors or targets	Study
Non-selective	Morphine – i.t.	Rat, *in situ* heart	Infarct (TTC)	Improved	DOR, KOR and MOR	Li, *et al.*, 2009
				Improved	Central and peripheral adenosine receptors	Yao, *et al.*, 2011
				Improved	ANS, bradykinin and CGRP receptors, K_{ATP}, Akt, NOS	Wong *et al.*, 2012b
	Morphine – intracerebral	Rat, *in situ* heart	Infarct (TTC)	Improved	DOR, KOR and MOR, cerebral calmodulin, CGRP	Zhang *et al.*, 2011
MOR	Fentanyl – i.t.	Rat, *in situ* heart	Infarct (TTC)	Improved	Spinal nNOS	Lu *et al.*, 2014

Shown are effects of i.t. or i.c.v. opioid receptor agonists on myocardial outcomes from I–R in experimental models. In all cases agonists were applied prior to the ischaemic insult. The primary outcome in all studies was myocardial infarction. Also shown are the receptors, signalling elements or effector molecules implicated in protection. ANS, autonomic nervous system; nNOS, neuronal NOS; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride staining for infarction.

actions of central and peripherally active mediators including opioid, adenosine, CGRP and bradykinin receptor agonists.

Prolonged cardioprotection via opioid receptors

As in hibernation, opioid receptor activity has the capacity to induce long-term shifts in cardiac stress resistance, particularly in response to sustained opioid receptor agonism (Table 5). Generation of prolonged protected states via sustained opioid treatment (Peart and Gross, 2004a,b; Peart *et al.*, 2011) or irreversible opioid receptor agonism (Gross *et al.*, 2005) has distinct clinical advantages. Timing of prophylactic treatment relative to insult becomes less critical, and the need for ongoing therapy is reduced. Prolonged protection might also be particularly useful in prophylactic therapy in high-risk patients, and for limiting time-dependent progression of injury during or after surgery/transplantation. Temporal properties of classic preconditioning may be suboptimal in this regard: the powerful initial window is brief (1–2 h), and the less powerful second window of protection persists for as little as 2–3 days (Hausenloy *et al.*, 2011; Ovize *et al.*, 2013; Przyklenk, 2013) (Figure 1). The irreversible DOR agonist fentanyl isothiocyanate can confer significant and long-lasting cardioprotection in animal models, for up to 5 days following administration (Gross *et al.*, 2005). As for sustained opioid receptor agonism (Peart and Gross, 2006; Peart *et al.*, 2011), resultant cardiac protection appears PI3K-dependent during the treatment or 'trigger' phase, while this signalling is only partially responsible for mediating protection during subsequent I–R (Table 5).

Unique and prolonged cardioprotection has been observed following sustained opioid receptor activation (Figures 1 and 2, Table 5), initially dubbed chronic morphine preconditioning (Peart and Gross, 2004a), but subsequently broadened to sustained ligand-activated protection (SLP) (Peart *et al.*, 2011). The SLP response affords greater protection than acute opioid treatment (Peart and Gross, 2004a; 2006; Peart *et al.*, 2011) or conventional preconditioning responses (Peart *et al.*, 2011). Protection is triggered by DORs lacking untoward systemic effects of other opioid receptor subtypes, may be induced in as little as 48 h, and persists for up to 7 days after stimulus withdrawal (Peart *et al.*, 2011). Opioidergic SLP also augments protection via acute adenosine receptor activation (Peart *et al.*, 2011) and caveolin-3 overexpression (See Hoe *et al.*, 2014b). Importantly, SLP is effective in aged (Peart and Gross, 2004b) and diabetic myocardium (See Hoe *et al.*, 2013) refractory to conventional protective stimuli, and also retains efficacy following 4 weeks of β-adrenoceptor blockade whereas ischaemic preconditioning is lost under these conditions (See Hoe *et al.*, 2014a).

Protective SLP is mechanistically distinct from acute opioid receptor stimuli and ischaemic pre- and post-conditioning (Peart and Gross, 2006). The SLP response appears to involve initial PI3K-dependent phenotype induction and subsequent β_2-adrenoceptor/G_S/PKA-dependent protection (Peart and Gross, 2006). However, the full mechanistic details of this response remain to be determined, and divergent findings have been reported (Table 5). For example, Skrabalova *et al.* (2012) report no infarct-sparing effects of 10

Table 5

Experimental studies of cardioprotection via sustained periods of opioid receptor agonism (from 24 h to 14 days)

Putative selectivity	Agent/duration	Species, tissue	Primary outcome	Effect	Implicated effectors or targets	Study
Non-selective	Morphine (14 days)	Rat, isolated heart	Arrhythmogenesis	Improved		Wong & Lee, 1987
	Morphine (5 days)	Mouse, isolated heart	Infarction (LDH)	Improved		Peart & Gross, 2004a
			Contractility	Improved	PKA, β_2-adrenoceptors, G_s	Peart & Gross, 2006
	Morphine (10 days)	Rat, *in situ* heart	Infarction (TTC)	Unaltered		Skrabalova *et al.*, 2012
			Arrhythmogenesis	Improved		
DOR	BW-373U86 (5 days)	Mouse, isolated heart	Contractile recovery	Improved	PI3K (Induction)	Peart *et al.*, 2011
MOR/DOR	Eribis peptide 94 (24 h)	Rat, *in situ* heart	Infarction (TTC)	Improved	NO, mK_{ATP}	Gross *et al.*, 2012b
KOR	U50,488H (5 days)	Mouse, isolated heart	Contractile recovery	Unaltered		Peart *et al.*, 2011

Shown are effects of sustained opioid receptor agonism (24 h to 14 days) on myocardial outcomes from I–R in experimental models. Agents were applied to rodents and myocardial outcomes subsequently assessed in *in situ* or *ex vivo* hearts. Primary outcome include myocardial infarction, contractile recovery and arrhythmogenesis. Also shown are the receptors, signalling elements or effector molecules implicated in protection. BW-373U86, 4-[(R)-[(2S,5R)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-hydroxyphenyl)methyl]-N,N-diethylbenzamide; mK_{ATP}, mitochondrial K_{ATP} channels.

day treatment with a lower level of morphine (though anti-arrhythmic effects were evident), with no changes in myocardial β_2-adrenoceptor, $G_{i/o}$ or G_s expression. However, myocardial adenylyl cyclase V/VI was significantly augmented. These different outcomes may reflect differing dosages and timing of treatments, although this deserves further attention.

Myocardial transcriptomic responses to 5 days of morphine treatment in mice (Ashton *et al.*, 2013) reveal that the stress-resistant phenotype is primarily associated with repression of inflammatory and immune pathways, and induction of natriuretic peptides and sarcomeric elements. Pathways involved in cell stress, growth and development were also modified, whereas conventional protective molecules are generally unaltered (Ashton *et al.*, 2013). Interestingly, recent proteomic analysis reveals that the 10 day morphine treatment in rats selectively modifies expression of only 6–7% of the ~1100 myocardial proteins assessed (Drastichova *et al.*, 2012). Of note was a pronounced pattern of HSP induction, phosphorylation and/or mitochondrial translocation (for HSP10, 27, 60 and 70, together with αβ-crystallin), which may be highly relevant to I–R tolerance. On the other hand, and consistent with the transcriptional effects of morphine treatment (Ashton *et al.*, 2013), few signal transduction molecules were modified, although PLCδ1, p38α-MAPK and ADP-ribosyltransferase 3 were induced, and a number of enzymes involved in substrate metabolism significantly repressed. Caveolin-1, -2 and -3 and flotillin-1 were unaltered, suggesting no modulation of caveolar/lipid raft domains by morphine. This is consistent with recent data further distinguishing morphine-dependent SLP, which appears insensitive to caveolin-3 deletion, and thus caveolar control in otherwise healthy mice (See Hoe *et al.*, 2014b). This contrasts with the caveolae/caveolin-3 dependence of acute opioid responses (Patel *et al.*, 2006; Tsutsumi *et al.*, 2010), and has important implications deserving further interrogation. As myocardial DORs appear caveolin-3 dependent, the caveolin-3 independence of SLP implies extra-cardiac opioid receptor signalling (e.g. that engaged in remote/central conditioning responses) and/or a pool of caveolin-3-independent myocardial opioid receptors. Curiously, our unpublished findings (data not shown) nonetheless support up-regulation of caveolar density and caveolin-3 with SLP in aged or hearts with diabetes, which may be of specific benefit in these settings. While awaiting more complete delineation, these novel mechanistic features of SLP are likely to contribute to its preserved efficacy in senescent (Peart and Gross, 2004b) and diseased myocardium (See Hoe *et al.*, 2013).

Other groups have studied the cardiac effects of sustained opioid treatment. Prolonged morphine exposure enhances the vasodilating effects of morphine and β-endorphin (Koo and Wong, 1983), and reduces post-ischaemic arrhythmias (Wong and Lee, 1987). Gross *et al.* (2012b) have also shown that 24 h exposure to the enkephalin derivative EP94 limits infarct size through NO and sarcolemmal and mitochondrial K_{ATP}-dependent mechanisms. Cytoprotective effects of sustained opioid receptor activation are not limited to the heart, with morphine dependence also resulting in significant protection against renal I–R injury (Habibey and Pazoki-Toroudi, 2008).

Confounding impacts of ageing, co-morbidities and drugs

Despite the existence of powerful experimental stimuli, practical cardioprotection has yet to be achieved clinically. As already noted, while a number of clinical trials have identified moderate improvements in surrogate markers of myocardial injury with pre- and post-conditioning interventions (Shi and Vinten-Johansen, 2012; Ovize *et al.*, 2013; Ferdinandy *et al.*, 2014), other studies report little to no protection. Similarly, while effects of opioid receptor agonists are encouraging in the small clinical trials undertaken to date (Table 1), protective outcomes remain well below those reported in different experimental models. These modest clinical outcomes may be attributable to a number of factors. Conservation of conditioning responses (together with opioid receptor-mediated protection and implicated signalling) across species including rodents, rabbits, dogs, sheep, pigs, and healthy human cells and tissues *in vitro*, together with evidence of conditioning efficacy in younger human subjects (Abete *et al.*, 1997) and some clinical trials (Ferdinandy *et al.*, 2014), indicates poor translation is not a species-specificity issue. Rather, impaired responsiveness may stem from inhibitory impacts of clinical conditions prevalent in target 'ischaemic' cohorts, including hypertension, diabetes, obesity and dyslipidaemia (elements of the so-called metabolic syndrome), together with advancing age (Peart and Headrick, 2009; Przyklenk, 2011; 2013; Ferdinandy *et al.*, 2014). Such factors may well reduce myocardial capacity to both resist injury and respond to protective intervention. Moreover, given the positive influences of physical activity and caloric limitation in optimizing myocardial survival-signalling and stress resistance, the hypo-activity and hyper-caloric diets prevalent in modernized societies may further impair myocardial phenotype, repressing I–R tolerance and therapeutic responsiveness. A crucial avenue of strategic research must be the impacts of major co-morbidities and ageing, together with (in)activity and caloric intake, on myocardial capacity to withstand ischaemic insult and to respond to protective stimuli, including acute and sustained opioid receptor agonism. Greater benefit with cardioprotective interventions may ultimately be achievable in select cohorts, for example in paediatric cardiac patients who lack the negative influences of ageing and other co-morbidities (Peart and Headrick, 2009), or more broadly via the optimization of interventions based on an improved understanding of the effects of disease and age on protective signalling (e.g. development of more robust conditioning algorithms or opioid receptor stimuli; strategies to bypass/correct dysfunctional survival signalling).

Inhibitory influences of ageing

IHD and particularly AMI is predominant in mature to elderly populations – coronary artery disease affects 50% of those >65, with 80% of deaths occurring in this age group. We and others have documented age-related reductions in ischaemic tolerance in rodent hearts (Headrick, 1998; Peart and Gross, 2004b; Peart *et al.*, 2014), mirroring changes in human tissue (Liu *et al.*, 2012; Peart *et al.*, 2014). Intrinsic protective responses conferring I–R tolerance may also fail with age (Peart and Headrick, 2009; Peart *et al.*, 2014). Considerable

evidence indicates ageing reduces or eliminates opioid receptor-dependent responses, including ischaemic preconditioning in human (Abete *et al*., 1997) and animal tissue (Schulman *et al*., 2001), post-conditioning (Przyklenk *et al*., 2008), remote conditioning (Hu *et al*., 2002), and responses to direct opioid receptor agonism (Peart and Gross, 2004b; Peart *et al*., 2007, 2014).

These detrimental changes may involve dysfunctional signal transduction, with data supporting impaired activation of PKC (Tani *et al*., 2001) and p38-MAPK (Peart *et al*., 2007). Recent work supports age-related dysfunction in protective signal transduction from membrane GPCRs to mitochondrial targets in murine and human tissue, potentially involving caveolin-3/caveolae depletion (Peart *et al*., 2014). Age may thus impair opioid receptor-triggered signal transduction and protection despite potential increases in cardiac enkephalin mRNA and peptide levels (Boluyt *et al*., 1993). Importantly, the novel SLP response retains efficacy in aged hearts (Peart and Gross, 2004b), possibly as a result of its apparent insensitivity to caveolin-3 depletion (See Hoe *et al*., 2014b).

Inhibitory influences of disease

A variety of disease states, including diabetes, obesity and hypertrophy, may negatively influence opioid receptor responses and pre- and post-conditioning responses attributable to the opioid receptor system.

Diabetes. Cardiovascular disease is the major cause of premature death in the population with diabetes, representing > 300 million adults worldwide in coming decades. The risk of AMI increases with hyperglycaemia in both healthy subjects and subjects with diabetes, and two in three subjects with diabetes ultimately suffer myocardial or cerebral I–R. Unfortunately, diabetes also worsens outcomes, doubling mortality after AMI or coronary bypass. This dominant effect of cardiovascular disease demands cardioprotection for diabetics, yet the disease may also block conventional protective responses. Ghosh *et al*. (2001) reported the failure of preconditioning in human myocardium with diabetes, with others subsequently reporting impaired preconditioning-related responses in diabetic tissue (Hassouna *et al*., 2006). These data are consistent with evidence of failure of preconditioning responses in diabetic animal models (Kersten *et al*., 2000). Mechanistically, such changes may arise (as in ageing) from dysfunctional survival signalling (Hassouna *et al*., 2006; Gross *et al*., 2007b). Opioidergic conditioning is also abolished in hearts from streptozotocin-dependent diabetic models (Gross *et al*., 2007b; Kim *et al*., 2010; Chen *et al*., 2013), with diabetes negatively affecting opioid receptor modulation of GSK3β-associated signalling, including Akt, p70 ribosomal S6 kinase, ERK, JAK-2 and STAT3 (Gross *et al*., 2007b; Chen *et al*., 2013), and opioid receptor control of apoptotic signalling (Kim *et al*., 2010). Diabetic impairment of KOR preconditioning has also been linked to a reduction in protective HSP70 synthesis (Qi *et al*., 2004). Unfortunately, beneficial opioid responses to exercise may also be impaired in patients with diabetes (Wanke *et al*., 1996), which given the role of opioid receptors in exercise-dependent cardioprotection could limit the cardiac benefits of physical activity in such patients. Despite these negative impacts, our preliminary findings indicate that novel cardiac protection via sustained opioid receptor agonism may be effective in a setting of type II diabetes (See Hoe *et al*., 2013).

Obesity. Obesity is a leading risk factor for IHD, also increasing in global prevalence. The co-existence of obesity with other abnormalities, particularly components of metabolic syndrome (glucose-intolerance, hypertension, dyslipidaemia) complicates identification of the roles of component disorders. However, detrimental effects are apparent in diverse obesity models: preconditioning fails in obese insulin-resistant rats (Katakam *et al*., 2007) and in a model of metabolic syndrome (Wagner *et al*., 2008), while post-conditioning is impaired in leptin-deficient *ob/ob* hearts (Bouhidel *et al*., 2008). Dietary obesity also attenuates DOR protection in the rat, potentially through dysregulated RISK, GSK3β and NOS signalling (Donner *et al*., 2013).

Hypertension and hypertrophy. Effects of hypertension and hypertrophy on cardioprotection are less clear. Ebrahim *et al*. (2007) report that preconditioning is impaired in hypertensive rats, involving independent effects of age and hypertension. Riess *et al*. (2005) found that anaesthetic preconditioning is also limited in larger (or older) hearts, with other work supporting failure of ischaemic preconditioning in hypertrophied myocardium (Moolman *et al*., 1997).

Although preconditioning responses impaired with hypertrophy/hypertension are considered opioid receptor-dependent, the specific effects on opioid receptor-dependent signalling are unclear. There is evidence for significant changes in cardiac opioid receptor ligand expression, with pre-proenkephalin A mRNA elevated in spontaneously hypertensive rats (Dumont *et al*., 1991) and experimental hypertrophy (Weil *et al*., 2006), and myocardial met-enkephalin peptide also elevated in hypertrophy associated with angiotensin II overexpression (van den Brink *et al*., 2007).

The opioid receptor system is not only modified by hypertrophy, but appears important in modulating myocardial hypertrophy development. Stimulation of KORs inhibits hypertrophy and fibrosis induced by β_1-adrenoceptor stimulation (Wu *et al*., 2008; Yin *et al*., 2009; Jaiswal *et al*., 2010), reducing the amplitude and frequency of β_1-adrenoceptor induced Ca^{2+} transients and attenuating L-type Ca^{2+} current activation (Yin *et al*., 2009). Agonists of the KOR maintain expression of α- but not β-MHC, and reduce oxidative stress in β-adrenoceptor induced hypertrophy (Jaiswal *et al*., 2010). The effects of the KOR on hypertrophy and Ca^{2+} transients may involve modulation of CaMKIIδ expression (Wu *et al*., 2008).

Inhibitory influences of drugs

A number of studies raise concerns regarding inhibitory effects of common drugs on opioid receptor signalling and opioid receptor-dependent ischaemic conditioning responses. Additionally, opioid receptor-based analgesia or anaesthesia may also induce opioidergic cardioprotection, rendering hearts refractory to further opioid receptor-dependent stimuli (including ischaemic conditioning responses).

Sulphonylureas. Treatments to control diabetes/hyperglycaemia may interfere with conventional condition-

ing stimuli. The sulphonylurea glibenclamide is commonly employed to control hyperglycaemia in type II diabetics, and inhibits K_{ATP} channel activity. As activation of sarcolemmal and mitochondrial K_{ATP} channels is a key feature of archetypal conditioning responses, inhibition of these channels abolishes ischaemic and pharmacological conditioning responses (see Gross and Peart, 2003). However, non-sulphonylurea hypoglycaemic agents have been shown to be without effect on cardioprotective responses. Additionally, novel protection with sustained opioid receptor agonism appears to be relatively insensitive to K_{ATP} channel blockers (Peart and Gross, 2006).

Aspirin. Gross *et al*. (2004b) report blockade of opioid receptor-dependent protection with aspirin (but not ibuprofen, which may actually augment protection). Jancso *et al*. (2005) also found that aspirin eliminates delayed preconditioning, while Shinmura *et al*. (2003) suggest inhibition is dose-dependent – no inhibitory effects at low doses versus inhibition at high doses used in anti-rheumatic therapy.

Statins. Kocsis *et al*. (2008) report that acute and chronic administration of lovastatin interferes with infarct-limiting effects of post-conditioning, while Fan *et al*. (2012) recently showed that chronic atorvastatin may negate protection via post-conditioning (although somewhat paradoxically inducing protection alone).

Ca^{2+} *antagonists.* Ca^{2+} channel antagonism may interfere with protection via preconditioning, with Cain *et al*. (1999) reporting clinical antagonists eliminate preconditioning in human myocardium. Such data also suggest detrimental effects of Ca^{2+} blockade on patient mortality could stem from impairment of myocardial stress resistance.

Adrenoceptor blockers. The β-adrenoceptors are implicated in cardiac protection, with studies demonstrating induction of preconditioned states following receptor agonism (Penson *et al*., 2008), and impairment of cardioprotection following β-adrenoceptor blockade (Suematsu *et al*., 2004). The α-adrenoceptor also influences cardioprotection, with evidence of impaired preconditioning in patients treated with the α-adrenoceptor selective phentolamine (Tomai *et al*., 1997). Our pilot data indicate that chronic (28 day) treatment with the β_1-antagonist atenolol impairs intrinsic ischaemic tolerance and negates preconditioning-dependent protection in murine tissue (See Hoe *et al*., 2014a). Nonetheless, protection via sustained opioid treatment appears resistant to these inhibitory effects.

Influence of sarcolemmal makeup on opioid receptor protection

Ageing and disease states may negatively affect myocardial stress resistance and therapeutic responsiveness through shifts in membrane makeup (Peart *et al*., 2014; See Hoe *et al*., 2014b). Sarcolemmal caveolae provide signal scaffolds that enable and influence transmission of extracellular stimuli (via signalling receptors, ion channels, physical perturbation) to the intracellular milieu (Head *et al*., 2005, 2014). Cardiac opioid receptors are localized to caveolae, and many signal elements downstream of opioid receptors are also located within these domains (Head *et al*., 2005; Patel *et al*., 2006). Depletion of sarcolemmal cholesterol has been shown to modify opioid receptor-mediated signalling (Huang *et al*., 2007b), and disruption of caveolae via either cholesterol or caveolin-3 depletion impairs opioid receptor-mediated protection and I–R tolerance (Patel *et al*., 2006; Tsutsumi *et al*., 2010; See Hoe *et al*., 2014b). Other evidence suggests the inhibitory impacts of ageing on opioid receptor protection may involve repression of caveola-dependent stress signalling, via reductions in caveolae, caveolin-3 and cholesterol (Peart *et al*., 2014). As caveolin-3 expression is also reduced by saturated fats and in obesity (Knowles *et al*., 2013), metabolic disease and ageing may both negatively influence cardiac stress resistance and opioid receptor responsiveness via shifts in caveolar control. However, as noted earlier, chronic opioid receptor agonism appears to induce a unique form of cardioprotection independent of caveolin-3 expression (See Hoe *et al*., 2014b), and may thus be of specific utility in aged and diseased hearts.

Summary and future directions

Endogenous opioid peptides and their corresponding opioid receptors are implicated in evolutionarily conserved retaliatory and adaptive responses to cellular stress, and are integral to myocardial conditioning and other cardioprotective responses. Harnessing this intrinsic system to achieve clinically effective cardioprotection is thus an attractive option, with pharmacological targeting of opioid receptors able to induce powerful short- and long-term protection of cardiac tissue from experimental animal models and humans (Tables 2–5). While there have been relatively few clinical trials of cardioprotection using opioid receptor agonists, outcomes with morphine and remifentanil in surgical I–R and angioplasty are encouraging (Table 1). Further trials are clearly warranted, particularly in the setting of AMI, with none yet addressing effects of selective DOR agonists (which exhibit efficacy *in vitro* across species, including human tissue, and may lack untoward cardiorespiratory effects of other opioid receptor subtypes). However, there are important considerations in developing opioid receptor-based (and other) cardioprotective interventions: opioidergic and related ischaemic conditioning responses may be blunted or desensitized with ageing, common IHD co-morbidities, and relevant drugs; opioid receptor-mediated protection could represent an intrinsically active component of the cardiac response to I–R stress (limiting benefit via pharmacological intervention); and/or opioid receptor-mediated protection may be engaged by currently employed opioidergic analgesics and anaesthesia. Detailing the intrinsic protective roles and mechanisms of opioid receptor subtypes in human myocardium, and unravelling the basis of age-, disease- and drug-dependence of opioid receptor and ischaemic conditioning responses is critical in paving the way to efficacious opioid receptor-based cardioprotection. Evidence for the summative effects of ischaemic conditioning stimuli and opioid receptor agonists (Rentoukas *et al*., 2010) supports the potential to refine more robust protective stimuli, combining opioid receptor agonism with intrinsic activation via ischaemic

stimuli. Development of opioidergic or other protective stimuli that effectively activate cardioprotective signalling and effector mechanisms independently of age and disease (e.g. SLP) would also be of great value.

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Conflict of interest

The authors declare no conflicts of interest.

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