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REVIEW

Endocannabinoids and cannabinoid receptors in ischaemia–reperfusion injury and preconditioning

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Ischaemia–reperfusion (I/R) is a pivotal mechanism of organ injury during stroke, myocardial infarction, organ transplantation and vascular surgeries. Ischaemic preconditioning (IPC) is a potent endogenous form of tissue protection against I/R injury. On the one hand, endocannabinoids have been implicated in the protective effects of IPC through cannabinoid CB_1/CB_2 receptor-dependent and -independent mechanisms. However, there is evidence suggesting that endocannabinoids are overproduced during various forms of I/R, such as myocardial infarction or whole body I/R associated with circulatory shock, and may contribute to the cardiovascular depressive state associated with these pathologies. Previous studies using synthetic CB_1 receptor agonists or knockout mice demonstrated CB_1 receptor-dependent protection against cerebral I/R injury in various animal models. In contrast, several follow-up reports have shown protection afforded by CB_1 receptor antagonists, but not agonists. Excitedly, emerging studies using potent CB_2 receptor agonists and/or knockout mice have provided compelling evidence that CB_2 receptor activation is protective against myocardial, cerebral and hepatic I/R injuries by decreasing the endothelial cell activation/inflammatory response (for example, expression of adhesion molecules, secretion of chemokines, and so on), and by attenuating the leukocyte chemotaxis, rolling, adhesion to endothelium, activation and transendothelial migration, and interrelated oxidative/nitrosative damage. This review is aimed to discuss the role of endocannabinoids and CB receptors in various forms of I/R injury (myocardial, cerebral, hepatic and circulatory shock) and preconditioning, and to delineate the evidence supporting the therapeutic utility of selective CB_2 receptor agonists, which are devoid of psychoactive effects, as a promising new approach to limit I/R-induced tissue damage.
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Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB, cannabinoid; IPC, ischaemic preconditioning; I/R, ischaemia–reperfusion

Introduction

Ischaemic–reperfusion (I/R) injury is the principal cause of tissue damage occurring in conditions such as stroke, myocardial infarction, cardiopulmonary bypass and other vascular surgeries, and organ transplantation, as well as a major mechanism of end-organ damage complicating the course of circulatory shock of various aetiologies. In all these conditions, the initial trigger of the damage is the transient disruption of the normal blood supply to target organs followed by reperfusion. Reperfusion of ischaemic tissues is the ultimate treatment to reduce tissue injury. Unfortunately, reperfusion itself inflicts additional tissue damage mediated by reactive oxygen (superoxide anion, hydrogen peroxide and hydroxyl radical) and reactive nitrogen species (for example, peroxynitrite) upon reperfusion, as well as to the rapid transcriptional activation of an array of proinflammatory genes (reviewed in Ferdinandy and Schulz, 2003; Podgoreanu *et al*., 2005; Pacher *et al*., 2005e, 2007; Ungvari *et al*., 2005). Direct consequences are the local sequestration and activation of polymorphonuclear leukocytes, leading to a rapid amplification of the initial inflammatory response and reactive oxygen species generation, so-called 'respiratory burst' (Lucchesi, 1990). Additional sources of increased reactive oxygen species generation during I/R can be xanthine and NAD(P)H oxidases, mitochondria, COX and uncoupled nitric oxide synthases (reviewed in Griendling *et al*., 2000; Ungvari *et al*., 2005; Pacher *et al*., 2006b). The burst of reactive oxygen and nitrogen species immediately upon reperfusion initiates a chain of deleterious cellular responses eventually leading to endothelial inflammatory response and dysfunction, adherence of neutrophils and

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lymphocytes to the endothelium, transendothelial migration of inflammatory cells, the release of various harmful mediators, cellular calcium overload, and eventually cell death and organ dysfunction.

Ischaemic preconditioning (IPC), first introduced by Murry *et al*. (1986) is a potent endogenous form of protection against I/R injury. In hearts and various other organs, IPC (brief episode(s) of ischaemia applied before the main I/R) reduces infarct size and enhances the recovery of organ function (Yellon and Hausenloy, 2005). Preconditioning can also be achieved with bacterial endotoxins and various other chemicals and when brief episodes of ischaemia are applied following the ischaemic period (the latter is called post-conditioning (for reviews see Yellon and Hausenloy, 2005; Bolli, 2007; Hausenloy and Yellon, 2007).

Circulatory shock classifies a syndrome precipitated by a systemic derangement in perfusion leading to widespread cellular hypoxia and vital organ dysfunction. Dependent on its initial pathophysiological mechanisms, shock is subdivided in three main categories, namely cardiogenic, haemorrhagic and septic shock. In the advanced stages, all shock states evolve to a common clinical picture characterized by profound tissue ischaemia, cardiovascular failure, the activation of cellular cytotoxic effectors (polymophonuclear leukocytes) and the upregulation of an array of proinflammatory genes, leading to systemic inflammation, organ dysfunction and death (Hotchkiss and Karl, 2003). From a clinical point of view, at present no therapy is available to limit reperfusion injury, which highlights the importance of a better understanding of its underlying mechanisms, to devise better future therapeutic approaches.

Numerous studies have suggested that the endocannabinoid system may modulate I/R injury (reviewed in Lamontagne *et al*., 2006; Pacher *et al*., 2006a). Endocannabinoids have been implicated in the protective effects of IPC through cannabinoid (CB) receptor-dependent and -independent mechanisms; however, they may also contribute to the cardiovascular collapse associated with myocardial infarction and circulatory shock (reviewed in Pacher *et al*., 2006a). To date, two CB receptors have been identified by molecular cloning: the CB_1 and CB_2 receptors. The CB_1 receptor is abundantly expressed in brain tissue (Matsuda *et al*., 1990), but is also present in peripheral tissues including vasculature (Gebremedhin *et al*., 1999; Liu *et al*., 2000), heart (Batkai *et al*., 2004b; Pacher *et al*., 2005d) and liver (Batkai *et al*., 2001; Engeli *et al*., 2005; Osei-Hyiaman *et al*., 2005; Teixeira-Clerc *et al*., 2006). The CB_2 receptor was previously considered to be expressed primarily in immune and haematopoietic cells (Munro *et al*., 1993; reviewed in Pacher *et al*., 2006a). However, more recent studies have also found CB_2 receptors in brain (Van Sickle *et al*., 2005), myocardium (Mukhopadhyay *et al*., 2007), cardiomyoblasts (Shmist *et al*., 2006; Mukhopadhyay *et al*., 2007) and endothelial cells of various origins (Blazquez *et al*., 2003; Zoratti *et al*., 2003; Golech *et al*., 2004; Mestre *et al*., 2006; Rajesh *et al*., 2007a, b). The synthetic and natural ligands (the latter called endocannabinoids: arachidonoyl ethanolamide or anandamide (AEA) and 2-arachidonoylglycerol (2-AG)) of CB

Table 1 Ranges of K_i values for certain cannabinoid CB_1 and/or CB_2 receptor agonists or antagonists/inverse agonists for the *in vitro* displacement of [^{3}H]CP55940, [^{3}H]HU243 or [^{3}H]BAY38-7271 from CB_1- and CB_2-specific binding sites (based on Howlett *et al*., 2002; Pertwee, 2005a; for details see therein)

Agonist/ligand	*CB_1 K_i value (nM)*	*CB_2 K_i value (nM)*	*Reference*
CB_1-selective agonists			
ACEA	1.4–5.29	195 to >2000	Pertwee (2005a)
R-(+)-methanandamide	17.9–28.3	815–868	Pertwee (2005a)
Agonists without any marked CB_1/CB_2 selectivity			
Anandamide	61–543	279–1940	Pertwee (2005a)
BAY38-7271	1.85	5.96	Pertwee (2005a)
2-Arachidonoyl glycerol	58.3–472	145–1400	Pertwee (2005a)
HU-210	0.0608–0.1	0.17–0.524	Pertwee (2005a)
CP55940	0.5–5	0.69–2.8	Pertwee (2005a)
Δ^9-THC	5.05–53.3	3.13–75.3	Pertwee (2005a)
R-(+)-WIN 55,212-2	1.89–123	0.28–16.2	Pertwee (2005a)
CB_2-selective agonists			
JWH015	383	13.8	Pertwee (2005a)
JWH133	677	3.4	Pertwee (2005a)
HU-308	>10 000	22.7	Pertwee (2005a)
O-1966	5055 ± 984	23 ± 2.1	Wiley *et al*. (2002)
O-3853	1509 ± 148	6.0 ± 2.5	Zhang *et al*. (2007)
CB_1-selective antagonist/inverse agonists			
SR141716A	1.8–11.8	515–13 200	Pertwee (2005a)
AM281	12	4200	Pertwee (2005a)
AM251	7.49	2290	Pertwee (2005a)
LY320135	141	14 900	Pertwee (2005a)
CB_2-selective antagonist/inverse agonists			
AM630	5152	31.2	Pertwee (2005a)
SR144528	50.3 to >10 000	0.28–5.6	Pertwee (2005a)

receptors exert various anti-inflammatory and neuroprotective (Panikashvili *et al*., 2001, 2005, 2006) effects by inhibiting the generation and release of proinflammatory cytokines and mediators (reviewed in Mechoulam *et al*., 2002a, b; Klein, 2005; Pacher *et al*., 2006a). The pharmacological modulation of the endocannabinoid system represents a promising strategy in various cardiovascular, inflammatory, metabolic, gastrointestinal and liver disorders (reviewed in Di Marzo *et al*., 2004; Pacher *et al*., 2005a, 2006a; Pertwee, 2005b; Mackie, 2006; Mallat *et al*., 2007). The selectivity of the endocannabinoids and synthetic ligands used in various I/R studies towards CB_1/CB_2 receptors are summarized in Table 1 (for excellent detailed overviews on the subject and also on the development of CB_1/CB_2 receptor knockout mice, see Howlett *et al*., 2002; Pertwee, 2005a).

In this review, we will discuss the triggers and sources of endocannabinoid production during various forms of I/R injury (myocardial, cerebral, hepatic and retinal ischaemia, and circulatory shock) and preconditioning, as well as the diverse role of these novel mediators and their receptors in these processes. We will also overview the accumulating evidence obtained through the use of various synthetic CB_1/CB_2 receptor ligands, with particular focus on the novel role of CB_2 receptors, suggesting that the modulation of the endocannabinoid system can be therapeutically exploited in various forms of I/R injury.

Myocardial I/R and preconditioning

Initial studies used isolated heart preparations to study the role of endocannabinoid system in myocardial I/R and preconditioning. Lagneux and Lamontagne (2001) implicated for the first time the involvement of the endocannabinoid system in endotoxin (lipopolysaccharide (LPS))-induced preconditioning against myocardial I/R injury, based on the assumption that LPS increases endocannabinoid production in inflammatory cells (Varga *et al*., 1998; Maccarrone *et al*., 2001; Liu *et al*., 2003). They compared the effects of 90 min of low-flow ischaemia followed by 60-min reperfusion at normal flow in isolated hearts from rats pretreated with LPS or saline. LPS pretreatment reduced infarct size and enhanced functional recovery upon reperfusion compared to controls, which could be attenuated by the CB_2 antagonist SR144528, but not by the CB_1 antagonist SR141716, suggesting the involvement of myocardial CB_2 receptors in the observed LPS-induced cardioprotection (Lagneux and Lamontagne, 2001). In a consequent study, in which the preconditioning was triggered by heat stress, SR144528 but not SR141716 also abolished the infarct-size-reducing effect of heat stress (Joyeux *et al*., 2002). The conclusion of these early studies was that the protection afforded by LPS- or heat stress-induced preconditioning was mediated by endocannabinoids acting on CB_2 receptors. In contrast, in preconditioning induced by a brief period of ischaemia (5 min), either CB_2 or CB_1 receptor blockade could abolish the protection, and both CB_1 and CB_2 receptors were implicated in the preservation of the endothelium-dependent, 5-HT-induced vasodilation by IPC (Bouchard *et al*., 2003). Palmitoylethanolamide or 2-AG, but not AEA, added to the perfusion medium of isolated rat hearts afforded protection against ischaemia by decreasing myocardial damage and infarct size and by improving myocardial functional recovery (Lepicier *et al*., 2003). SR144528 completely blocked the cardioprotective effect of both palmitoylethanolamide and 2-AG, whereas SR141716 only partially inhibited the effect of 2-AG only (Lepicier *et al*., 2003). Similarly, CB_1 and CB_2 agonists ACEA and JWH015 also reduced infarct size in this model, and the CB_2 receptor-mediated cardioprotection by palmitoylethanolamide involved activation of p38/ERK (extracellular signal-regulated kinase)1/2 kinases and PKC (Lepicier *et al*., 2003). On the contrary, Underdown *et al*. (2005) have found that the infarct-size-reducing effect of AEA could be equally well antagonized by both CB_1 and CB_2 antagonists; however, it could not be mimicked by selective CB_1 or CB_2 agonists, suggesting the involvement of a site distinct from CB_1 or CB_2 receptors. Another recent study using a model of delayed preconditioning in rats induced by administration of the nitric oxide donor nitroglycerin for 24 h via transdermal application suggested that the protective effect of nitroglycerin against myocardial infarction is mediated via CB_1 receptors. Nitroglycerin increased the myocardial content of 2-AG, but not AEA (Wagner *et al*., 2006). The major limitation of the above-mentioned studies is the use of *ex vivo* models (for example, buffer-perfused isolated heart preparations) that could not address the question of whether endocannabinoids or synthetic agonists can modulate endothelial or immune cell activation and interactions, which are pivotal events in the sequel of reperfusion damage (reviewed in Pertwee, 2005a; Lamontagne *et al*., 2006; Pacher *et al*., 2006a). Despite the above-mentioned limitation, these pioneering studies importantly implied the possible contribution of functional CB_2 receptors in cardiomyocytes and/or endothelial cells responsible, at least in part, to the protective effects of preconditioning. Indeed, consequent studies have demonstrated the presence of CB_2 receptors in myocardium (Mukhopadhyay *et al*., 2007), cardiomyoblasts (Shmist *et al*., 2006; Mukhopadhyay *et al*., 2007) and endothelial cells of various origins (Blazquez *et al*., 2003; Zoratti *et al*., 2003; Golech *et al*., 2004; Mestre *et al*., 2006; Rajesh *et al*., 2007a, b). Consistently with the beneficial effect of CB_2 receptor activation on cardiomyocytes, a recent study demonstrated that delta(9)-tetrahydrocannabinol (THC) protected H9c2 cardiomyoblasts subjected to hypoxia *in vitro* presumably via CB_2 receptor activation and increased nitric oxide production (Shmist *et al*., 2006).

In a clinically more relevant rat model of I/R injury, both AEA and HU-210 decreased the incidence of ventricular arrhythmias and reduced infarct size, presumably through the activation of CB_2 but not CB_1 receptors (Krylatov *et al*., 2001, 2002a, b; Ugdyzhekova *et al*., 2001, 2002). In a mouse model of myocardial I/R induced by coronary artery ligation, the reduction of leukocyte-dependent second wave of myocardial damage subsequent to the initial IR injury was attributed to CB_2 receptor activation, since the protection afforded by WIN 55,212-2 could be prevented by AM630, but not by the CB_1 antagonist AM251 (Di Filippo *et al*., 2004). Two recent studies using rat models of acute and chronic

myocardial infarction demonstrated that endocannabinoids contribute to the hypotension and cardiodepression associated with acute cardiogenic shock, which could be attenuated by CB_1 antagonists (Wagner *et al*., 2001, 2003).

Collectively, although the role of CB receptors and endocannabinoids in protection afforded by preconditioning against myocardial I/R is still a very controversial issue requiring further clarification by using knockout mice and more selective ligands for CB_2 receptors, the findings implicating the importance of CB_2 receptor, presumably both on endothelial and inflammatory cells, and perhaps on cardiomyocytes, are very encouraging.

Cerebral I/R (stroke)

Ischaemic stroke, resulting from the reduction of cerebral blood flow in the territory of a major cerebral artery due to its transient or permanent occlusion by local thrombosis or embolus is the second leading cause of death in industrialized countries and the leading medical cause of acquired adult disability. One in six patients die in the first 4 weeks following ischaemic stroke, and half of the survivors are permanently disabled in spite of the best efforts to rehabilitate them to avoid complications (Klijn and Hankey, 2003). A cascade of complex molecular events is set in motion during cerebral ischaemia and culminates in neuronal cell death. Improving our understanding of these events might help to devise novel therapies to limit neuronal injury in stroke patients, a concept termed 'neuroprotection' (Lees *et al*., 2006).

The endocannabinoid system may represent a pivotal neuroprotective mechanism both in acute forms of neuronal injury (for example, stroke and traumatic brain injury) and in various chronic neurodegenerative disorders, including multiple sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease and amyotrophic lateral sclerosis (reviewed in Mechoulam *et al*., 2002b; Croxford, 2003; Sarne and Mechoulam, 2005; Mackie, 2006; Pacher *et al*., 2006a). Even though the exact mechanisms of these neuroprotective effects are not completely understood, numerous CB receptor-dependent as well as receptor-independent processes have been suggested to be involved, which include, but are not limited to: (1) modulation of immune responses and the release of inflammatory mediators by CB_1, CB_2 and non-CB_1/CB_2 receptors on neurons, astrocytes, microglia, macrophages, neutrophils and lymphocytes (Walter and Stella, 2004; Klein, 2005); (2) modulation of synaptic plasticity and excitatory glutamatergic transmissions via presynaptic CB_1 receptors (Freund *et al*., 2003; Piomelli, 2003); (3) activation of cytoprotective signalling pathways (for example, PKB/Akt, PKA or neurotrophic factors) (Pacher *et al*., 2006a); (4) modulation of calcium homoeostasis and excitability via interactions with Ca^{2+}, K^+ and Na^+ channels, gap junctions and intracellular Ca^{2+} stores, NMDA receptors (Freund *et al*., 2003; Piomelli, 2003; Pacher *et al*., 2006a); (5) CB_1 receptor-mediated central hypothermia, presumably by decreasing metabolic rate and oxygen demand; (6) antioxidant properties of CBs (Hampson *et al*., 2000); (7) modulation of endothelial activation and inflammatory response, leukocyte rolling, adhesion to the endothelium, transmigration and activation presumably by CB_2 receptors.

The first evidence for the neuroprotective effect of CBs came from the stroke research field from studies using synthetic non-psychotropic CB Dexanabinol/HU-211, which exerted its beneficial effects through CB_1/CB_2-independent mechanisms, in various rat and gerbil models of *in vivo* cerebral ischaemia (reviewed in Pacher *et al*., 2006a). Follow-up studies have also investigated the neuroprotective effects of CB_1 receptor stimulation using synthetic agonists. The synthetic CB WIN 55,212-2 attenuated hippocampal neuronal loss following transient global cerebral ischaemia in rats and reduced infarct size after permanent focal cerebral ischaemia induced by middle cerebral artery occlusion, when given 40 min prior or 30 min after the occlusion, in a CB_1-dependent manner, since the protective effect was preventable by SR141716 (Nagayama *et al*., 1999). WIN 55,212-2, as well as AEA and 2-AG, also protected cultured cerebral cortical neurons from *in vitro* glucose deprivation and hypoxia, but these effects were insensitive to CB_1 and CB_2 receptor antagonists (Nagayama *et al*., 1999; Sinor *et al*., 2000). In rat models of middle cerebral artery occlusion another synthetic agonist BAY38-7271 reduced infarct size even when given intravenously 4 h following the occlusion (Mauler *et al*., 2002). Similarly, HU-210 improved motor disability and decreased infarct size by up to 77% in a similar model (Leker *et al*., 2003). Pretreatment with SR141716 partially attenuated the protective effect of HU-210 indicating CB_1 receptor involvement. However, the protective effect of HU-210 could be abolished completely by warming the animals to the body temperature of controls, indicating that the CB_1-mediated hypothermia was responsible for the observed beneficial effects (Leker *et al*., 2003). Similarly, CB_1-mediated hypothermia was responsible for the neuroprotective effects of Δ(9)-tetrahydrocannabinol in a mouse ischaemic model of cerebral injury (Hayakawa *et al*., 2004) and, perhaps, also in a rat model of global cerebral ischaemia (Louw *et al*., 2000). Consistent with CB_1-mediated cerebroprotection, CB_1 knockout mice had increased neurotoxicity to NMDA and elevated mortality from permanent focal cerebral ischaemia, increased infarct size, more severe neurological deficits after transient focal cerebral ischaemia and decreased cerebral blood flow in the ischaemic penumbra during reperfusion, as compared to wild-type controls subjected to the same insult (Parmentier-Batteur *et al*., 2002).

In contrast, several more recent studies do not support the neuroprotective role of endocannabinoids and CB_1 receptor activation. In fact, the CB_1 antagonists SR141716 and LY320135 were found to reduce infarct size and to improve neurological function in a rat model of cerebral ischaemia induced by middle cerebral artery occlusion (Berger *et al*., 2004; Muthian *et al*., 2004; Sommer *et al*., 2006), whereas low doses of WIN 55,212-2 had no protective effect (Muthian *et al*., 2004).

Recent studies have also evaluated the effects of selective CB_2 agonists (O-3853, O-1966) in a stroke model. CB_2 agonists significantly decreased cerebral infarction and improved motor function after 1 h middle cerebral artery occlusion followed by 23 h reperfusion in mice, by attenuating the transient ischaemia-induced increase in leukocyte rolling and

adhesion to vascular endothelial cells (Zhang *et al.*, 2007). The role of CB_2 receptors in I/R injury was further supported by increased accumulation of CB_2-positive macrophages derived from resident microglia and/or invading monocytes following cerebral I/R (Ashton *et al.*, 2007).

Collectively, it appears that both CB_1 agonists and antagonists may afford neuroprotective effects against cerebral I/R. The reason for the contradictory effects of pharmacological blockade vs genetic knockout of CB_1 receptors is not clear, and may be related to CB_1 receptor-independent effects of antagonists, but this issue needs further clarification. In the case of CB_2 agonists, the most likely mechanism of protection is the attenuation of the transient I/R-induced increase in leukocyte infiltration, rolling and adhesion to vascular endothelial cells, and consequent activation.

Circulatory shock (full organ/body ischaemia and/or I/R)

In addition to their well-known immunological and neurobehavioral actions, CBs and their endogenous and synthetic analogues exert complex cardiodepressive and vasodilatory effects, which have been implicated in the mechanism of hypotension associated with haemorrhagic (Wagner *et al.*, 1997; Cainazzo *et al.*, 2002), endotoxic (Varga *et al.*, 1998; Liu *et al.*, 2003, 2006; Batkai *et al.*, 2004a; Kadoi and Goto, 2006), septic (Kadoi *et al.*, 2005), and cardiogenic shock (Wagner *et al.*, 2001, 2003), advanced liver cirrhosis (Batkai *et al.*, 2001; Ros *et al.*, 2002), cirrhotic cardiomyopathy (Gaskari *et al.*, 2005; Pacher *et al.*, 2005c; Moezi *et al.*, 2006; Yang *et al.*, 2007; Batkai *et al.*, 2007a), doxorubicin-induced heart failure (Mukhopadhyay *et al.*, 2007) and the shock associated with necrotizing pancreatitis (Matsuda *et al.*, 2005). Importantly, these cardiovascular depressive effects could be prevented or reversed by pretreatment with CB_1 antagonists, and are subjects of numerous comprehensive recent overviews (Randall *et al.*, 2002; Hiley and Ford, 2004; Lamontagne *et al.*, 2006; Lepicier *et al.*, 2006; Mallat *et al.*, 2007; Mendizabal and Adler-Graschinsky, 2007; Pacher *et al.*, 2005a, b, 2006a). CB receptor antagonists (for example, SR141716, AM281, AM251 and SR144528) prolonged survival in endotoxic and septic shock or necrotizing pancreatitis (Varga *et al.*, 1998; Smith *et al.*, 2001; Cainazzo *et al.*, 2002; Kadoi *et al.*, 2005; Matsuda *et al.*, 2005; Kadoi and Goto, 2006), while increased mortality in haemorrhagic (Wagner *et al.*, 1997) and cardiogenic shock (Wagner *et al.*, 2001) despite the increase in blood pressure. One explanation for this puzzling controversy is that endocannabinoid-mediated vasodilation may have survival value through improving tissue oxygenation by counteracting the excessive sympathetic vasoconstriction triggered by haemorrhage or myocardial infarction, and this would be removed by CB_1 blockade. In contrast, CB_1 blockade may improve survival in endotoxic shock by preventing the primary hypotensive response to LPS (Randall *et al.*, 2002; Hiley and Ford, 2004; Mendizabal and Adler-Graschinsky, 2007; Pacher *et al.*, 2005a, b, 2006a). Complicating the picture, in haemorrhagic, cardiogenic and endotoxic shock, the CB agonists HU-210, WIN 55,212-2 and THC also improved endothelial function and/or survival (Wagner *et al.*, 1997, 2001; Varga *et al.*, 1998; Smith *et al.*, 2000, 2001). Since the cardiovascular dysfunction and failure in most of the above-mentioned conditions are triggered by overwhelming tissue ischaemia and/or I/R, and consequent oxidative/nitrosative stress and inflammatory response coupled with the activation of various downstream cell death pathways (reviewed in Evgenov and Liaudet, 2005; Ungvari *et al.*, 2005; Pacher *et al.*, 2005e, 2007), another explanation for the diverse beneficial effects of both agonists and antagonists in circulatory shock could lie in their various anti-inflammatory and/or antioxidant properties (reviewed in Walter and Stella, 2004; Klein, 2005), which may be attributed to their inverse agonistic properties or to $CB_{1/2}$ receptor-independent mechanisms (reviewed in Begg *et al.*, 2005; Pertwee, 2005a, b, 2006).

Overall, it seems that both CBs and antagonists of CB receptors may have various favorable effects in rodent shock models; however, the specificity of these effects and the relevance to human circulatory shock should be established by further studies.

Hepatic I/R

Hepatic I/R injury continues to be a fatal complication that can follow liver surgery or transplantation. It is well known that hepatic I/R injury is dependent on polymorphonuclear cell (PMN) infiltration, Kupffer cell activation and inflammatory cytokine responses (Jaeschke *et al.*, 1996, 1997, 2006; Ohkohchi *et al.*, 1999). Adhesion molecules mediate the initial attachment of neutrophils to the activated endothelium (Carlos and Harlan, 1994; Jaeschke, 1997). On reperfusion, tumour necrosis factor-α (TNF-α) acts as a continuous stimulator for neutrophil infiltration in the liver and it also upregulates the production of cell-type-specific leukocyte chemoattractants, known as chemokines, which have also been shown to cause upregulation of cell adhesion molecules and neutrophil activation (Jaeschke, 2006). The increased inflammatory response further aggravates oxidative stress and initiates a chain of deleterious events eventually culminating in cellular dysfunction and death.

In two recent studies, we have investigated the involvement of the endocannabinoid system in an *in vivo* mouse model of hepatic I/R injury using selective CB_2 agonists and CB_2 knockout mice (Batkai *et al.*, 2007b). Activation of CB_2 receptors by JWH133 prior to the insult protected against I/R damage (measured by serum transaminases activity) by decreasing inflammatory cell infiltration, tissue and serum TNF-α, chemokines macrophage-inflammatory protein-1α (MIP-1α) and macrophage-inflammatory protein-2 (MIP-2) levels, tissue lipid peroxidation, and expression of adhesion molecule intercellular adhesion molecule-1 (ICAM-1). JWH133 also decreased the TNF-α-induced ICAM-1 and vascular cell adhesion molecule-1 expressions in human liver sinusoidal endothelial cells and the adhesion of human neutrophils to human liver sinusoidal endothelial cells *in vitro*. In agreement with the protective role of CB_2 receptor activation, $CB_2^{-/-}$ mice developed increased I/R-induced tissue damage and proinflammatory phenotype (Batkai

et al., 2007b). In a follow-up study, we have demonstrated that the potent CB_2 receptor agonist HU-308, given prior to the induction of I/R, significantly attenuated the extent of liver damage (measured by serum alanine aminotransferase and lactate dehydrogenase), decreased serum and tissue TNF-α, MIP-1α and MIP-2 levels, tissue lipid peroxidation, neutrophil infiltration, DNA fragmentation and caspase 3 activity. The protective effect of HU-308 against liver damage was also preserved when given immediately after the ischaemic episode. CB_2 receptor was expressed in human liver sinusoidal endothelial cells and its activation by HU-308 also attenuated the TNF-α-induced ICAM-1 and vascular cell adhesion molecule-1 expression and the adhesion of human neutrophils to human liver sinusoidal endothelial cells *in vitro*. These findings, coupled with recent results from myocardial (Di Filippo *et al*., 2004) and cerebral I/R models (Zhang *et al*., 2007), and antifibrotic effects of CB_2 receptor in the liver (Julien *et al*., 2005), suggest that selective CB_2 receptor agonists may represent a novel protective strategy against hepatic and other forms of I/R injury by attenuating endothelial cell activation/inflammatory response, chemotaxis of inflammatory cells, rolling and adhesion of inflammatory cells to the endothelium, transendothelial migration, adhesion to the parenchymal cells and activation, and interrelated oxidative/nitrosative stress/inflammatory response (Figure 1).

CB_2 receptor is also detectable in human coronary artery endothelial cells by western blotting, reverse transcription-PCR, real-time PCR and immunofluorescence staining, where its activation by JWH133 or HU-308 attenuates TNF-α-induced nuclear factor-κB and RhoA activation, upregulation of adhesion molecules ICAM-1 and vascular cell adhesion molecule-1, increased expression of monocyte chemoattractant protein, enhanced transendothelial migration of monocytes and augmented monocyte–endothelial adhesion (Rajesh *et al*., 2007a). CB_2 agonists also decreased the TNF-α- and/or endotoxin-induced ICAM-1 and vascular cell adhesion molecule-1 expression in isolated aortas and the adhesion of monocytes to aortic vascular endothelium (Rajesh *et al*., 2007a). Since the above-mentioned TNF-α- and endotoxin-induced phenotypic changes are critical in the initiation and progression of atherosclerosis, these findings suggest that targeting CB_2 receptors on endothelial cells may explain, at least in part, the previously observed beneficial effects of THC in a mouse model of atherosclerosis (Steffens *et al*., 2005).

Endocannabinoids in I/R: sources, triggers and roles

Previous pioneering studies hypothesized that circulating activated macrophages and platelets are the pivotal sources of endocannabinoids during haemorrhagic shock (Wagner *et al*., 1997), endotoxemia (Varga *et al*., 1998), myocardial infarction (Wagner *et al*., 2001) or liver cirrhosis (Batkai *et al*., 2001) both in experimental animals and in humans. When isolated and injected into normal rats, these activated cells elicited SR141716-sensitive hypotension, also pointing towards the involvement of CB_1 receptors in many of these conditions (Wagner *et al*., 1997; Varga *et al*., 1998; Batkai *et al*., 2001; Maccarrone *et al*., 2001; Ros *et al*., 2002; Liu *et al*., 2003).

Several studies have reported increased endocannabinoid levels following cerebral (Schmid *et al*., 1995; Panikashvili *et al*., 2001; Schabitz *et al*., 2002; Berger *et al*., 2004; Muthian *et al*., 2004) and hepatic and myocardial I/R injury (Wagner *et al*., 2001; Kurabayashi *et al*., 2005; Batkai *et al*., 2007b); however, the role of endocannabinoids and their sources in I/R injury remains to be a very controversial issue requiring further clarification. Interestingly, a recent study using a rat model of high intraocular pressure-induced retinal I/R found enhanced fatty acid amide hydrolase (FAAH) activity and downregulation of CB_1 and transient receptor potential vanilloid-1 ($TRPV_1$) receptors following I/R (Nucci *et al*., 2007). The I/R-induced cell death was attenuated either by the FAAH inhibitor URB597 or by the AEA stable analogue methanandamide (MetAEA), suggesting that endogenous AEA tone may play a protective role against injury (Nucci *et al*., 2007).

In a recent study, we attempted to identify cellular sources and triggers of endocannabinoid production using a mouse model of *in vivo* hepatic I/R (Batkai *et al*., 2007b). We found that I/R, but not ischaemia alone, triggered several-fold increases in the hepatic levels of the endocannabinoids AEA and 2-AG, which originated from hepatocytes, Kupffer and endothelial cells. Furthermore, these increases were positively correlated with the degree of tissue damage and serum TNF-α, MIP-1α and MIP-2 levels. Consistently, brief exposure of primary hepatocytes to various oxidants (H_2O_2, peroxynitrite) or inflammatory stimuli (TNF-α, endotoxin) (Pacher *et al*., 2006b, 2007) triggered marked increases in cellular endocannabinoid levels. Therefore, the important conclusions of this study are that not only inflammatory stimuli (for example, endotoxin) but also oxidative/nitrosative stress can modulate endocannabinoid levels in hepatocytes, and most likely in most other cell types too. The latter is also supported by recent findings demonstrating that the commonly used chemotherapeutic agent doxorubicin, which is known to mediate its cardiotoxicity by triggering oxidative/nitrosative stress (Pacher *et al*., 2003), increased endocannabinoid levels both in the myocardium *in vivo* and in cardiomyocytes *in vitro* (Mukhopadhyay *et al*., 2007). Similarly, up to sixfold increase in endocannabinoid AEA and/or 2-AG levels was observed in the hearts and livers of cirrhotic rats (notable cirrhotic cardiomyopathy is not associated with inflammatory cell infiltration of the myocardium; Batkai *et al*., 2007a). Therefore, parenchymal cells may also represent a very significant source of endocannabinoids produced in various pathological conditions associated with increased inflammation and/or oxidative tissue injury, in addition to the previously reported activated macrophages (Pacher *et al*., 2006a). The evidence on changes and possible regulation of endocannabinoid levels in various diseases was recently a subject of excellent overviews (Pertwee, 2005b; Di Marzo and Petrosino, 2007).

Our findings also imply that I/R-induced activation of hepatic endocannabinoids may limit hepatic injury by modulating the expression of adhesion molecules and the infiltration and activation of inflammatory cells by CB_2-dependent/-independent mechanisms, which is also

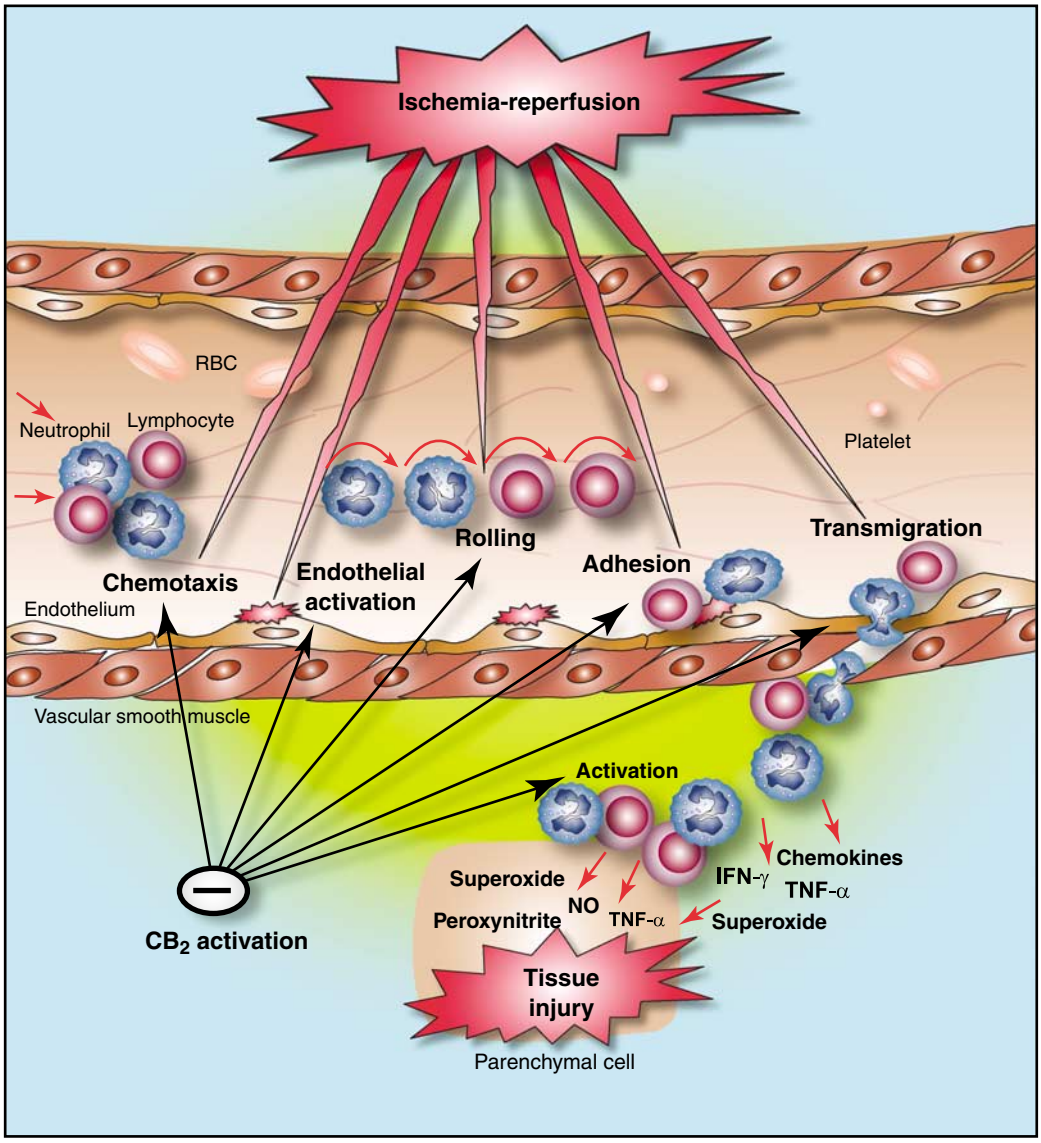

Figure 1 Mechanisms of CB_2 receptor-dependent protection in ischaemia/reperfusion (I/R). CB_2 receptor agonists may protect against I/R injury by attenuating endothelial cell activation/inflammatory response, chemotaxis of inflammatory cells, rolling and adhesion of inflammatory cells to the endothelium, transendothelial migration, adhesion to the parenchymal cells and activation, and interrelated oxidative/nitrosative stress/inflammatory response.

consistent with the emerging role of CB_2 receptors in regulating microglial cell function and neuroinflammation (Walter and Stella, 2004; Maresz *et al.*, 2005). Both mononuclear and polymorphonuclear leukocytes are known to express CB_2 receptors (Klein, 2005; Pacher *et al.*, 2006b), which could be activated on these infiltrating cells through a paracrine mechanism by endocannabinoids generated in and released from the various cell types in the liver. It is noteworthy that the endocannabinoid AEA can promote stellate cell and hepatocyte apoptosis *in vitro* by a mechanism not related to CB receptors (Siegmund *et al.*, 2005, 2006).

Conclusions, future directions

There is a marked increase of endocannabinoid production in various forms of I/R (myocardial, cerebral, hepatic and circulatory shock), which correlate with the degree of tissue injury and inflammation, and may originate from virtually any cell type involved (Figure 2). Both CB_1 agonists and antagonists may exert various neuroprotective effects against cerebral I/R, the specificity of which should further be studied by using knockout mice. Direct measurements should also confirm the increase of target tissue endocannabinoid levels following preconditioning (most recent evidence is based on assumptions of studies using pharmacological ligands), and experiments using knockout mice should determine the involvement of CB_1/CB_2 receptors. The latter is particularly important, since large body of evidence supporting the idea that CBs may mediate responses via interaction with other sites that probably represent novel CB receptor subtypes such as the putative endothelial CB receptor and GPR55 (Begg *et al.*, 2005; Mackie and Stella, 2006; Hiley and Kaup, 2007; Johns *et al.*, 2007),

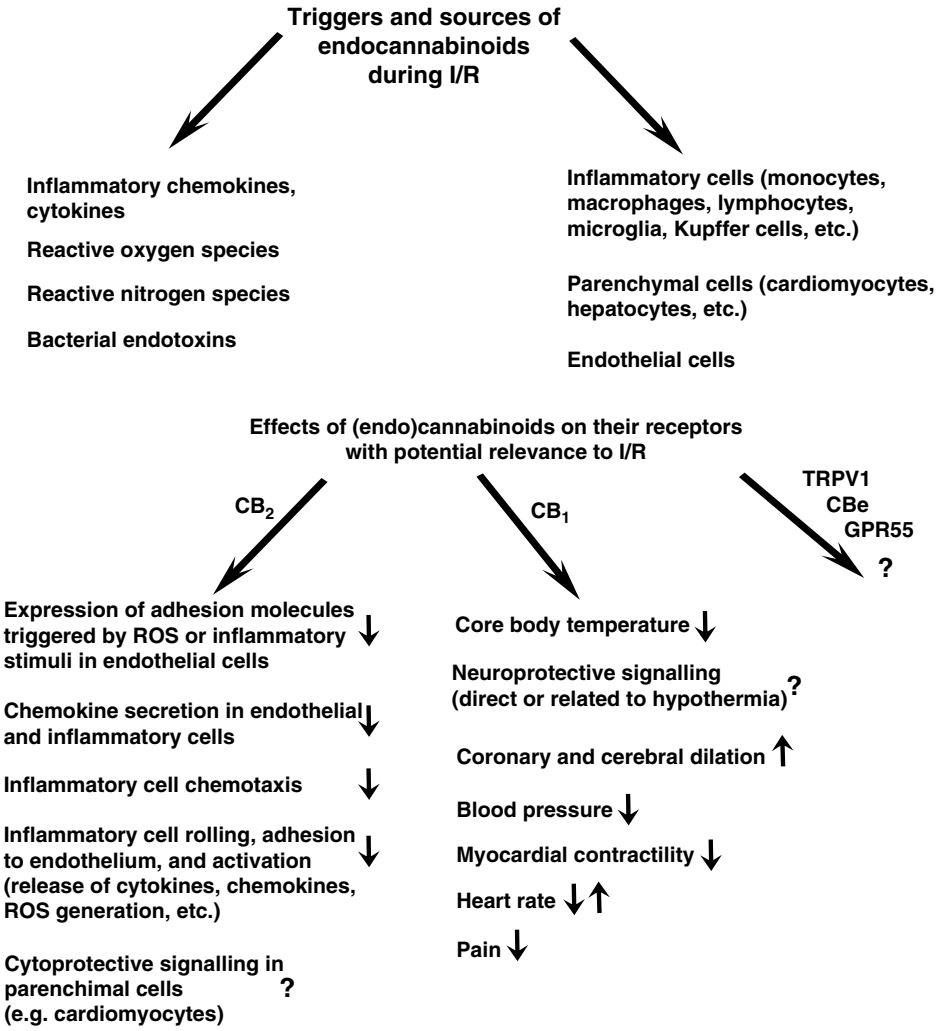

Figure 2 Triggers and sources of endocannabinoids during I/R, and effects mediated via their receptors with potential relevance to pathophysiology. I/R, ischaemia/reperfusion.

and that endocannabinoids may also behave as agonists of $TRPV_1$ receptors under certain conditions and are substrates for COX leading the generation of various biologically active metabolites (Pacher *et al*., 2005b).

Accumulating recent evidence suggests that selective CB_2 agonists may protect against myocardial, cerebral and hepatic I/R injuries by decreasing the endothelial cell activation/inflammatory response, the expression of adhesion molecules, inflammatory cytokines/chemokines levels, recruitment, adhesion and activation of inflammatory cells, and interrelated oxidative/nitrosative stress. There is considerable interest in the development of selective CB_2 receptor agonists, which are devoid of psychoactive properties of CB_1 agonists, for various inflammatory disorders. Further studies should also establish the therapeutic window of protection during the reperfusion phase with the currently available CB_2 receptor agonists, and new compounds should also be designed with better *in vivo* bioavailability, to devise clinically relevant treatment strategies against various forms of I/R. Nevertheless, the recently observed beneficial effects of CB_2 receptor agonists in hepatic and other forms of I/R, coupled with the absence of psychoactive properties, and antifibrotic effects of CB_2 receptor in the liver suggest that this approach may represent a novel promising strategy against various forms of I/R injury and other inflammatory disorders.

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Conflict of interest

The authors state no conflict of interest.

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