Critical Review

Signaling Pathways Involved in the Cardioprotective Effects of Cannabinoids

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Abstract. The aim of the present article is to review the cardioprotective properties of cannabinoids, with an emphasis on the signaling pathways involved. Cannabinoids have been reported to protect against ischemia in rat isolated hearts, as well as in rats and mice in vivo. Although these effects have been observed mostly with a pre-treatment of a cannabinoid, we report that the selective CB₂-receptor agonist JWH133 is able to reduce infarct size when administered either before ischemia, during the entire ischemic period, or just upon reperfusion. Little is known about the signaling pathways involved in these cardioprotective effects. Likely candidates include protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) since they are activated during ischemia-reperfusion and contribute to the protective effect ischemic preconditioning. The use of pharmacological inhibitors suggests that PKC, p38 MAPK, and p42/p44 MAPK (ERK1/2) contribute to the protective effect of cannabinoids. In addition, perfusion with JWH133 in healthy hearts caused an increase in both p38 MAPK phosphorylation level and activity, whereas the CB₁-receptor agonist ACEA was associated with an increase in the phosphorylation status of both ERK1 and ERK2 without any change in activity. During ischemia, both agonists doubled p38 MAPK activity, whereas ERK1/2 phosphorylation level and activity during reperfusion were enhanced only by the CB₂-receptor agonist. Finally, although nitric oxide (NO) was shown to exert both pro and anti-apoptotic effects on cardiomyocytes, with an apparently controversial effect on myocardial survival, our data suggest that NO may contribute to the cardioprotective effect of some cannabinoids.

Keywords: cannabinoid, ischemia, reperfusion, mitogen-activated protein kinase, preconditioning

Introduction

There has recently been marked interest in cannabinoids and their protective properties against ischemic damage. Considering their wide palette of effects, which includes anti-inflammatory as well as vasodilatatory properties, cannabinoids effectively represent an interesting family of drugs in regard to ischemic protection.

Abbreviations used are (in alphabetical order): 2-AG: 2-arachidonoylglycerol, an endogenous cannabinoid; ACEA: arachidonoyl-2’-chloroethylamide, a selective CB₁-agonist; AM251: a CB₁-receptor inverse agonist; AM630: a CB₂-receptor antagonist/inverse agonist; AP-1: activator protein-1; ATF-2: activating transcription factor 2; CP55940: a non-selective cannabinoid agonist; CXCL8: CXC chemokine ligand-8; DAG: diacylglycerol; ERK: extracellular signal-regulated kinase; FAAH: fatty acid amide hydrolase; HU-210: a non-selective cannabinoid agonist; IL: interleukin; IP₃: inositol trisphosphate; IPC: ischemic preconditioning; JNK: Jun amino-terminal kinase; JWH015: a selective CB₂-agonist; JWH133: a selective CB₂-agonist; L-NAME: N(G)-nitro-L-arginine methyl ester, a NOS inhibitor; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MAPKAP-K2: MAPK-activated protein kinase 2; NLA: N(6)-nitro-L-arginine, a NOS inhibitor; NO: nitric oxide synthase; PD098059: an inhibitor of the ERK1/2 cascade; PEA: palmitoylethanolamide, a cannabinomimetic; PKC: protein kinase C; PKG: protein kinase G; PPARα: peroxisome proliferator-activated receptor α; RACK: receptor for activated C kinase; SB203580: an inhibitor of p-38 MAPK; SNP: sodium nitroprusside, a NO donor; SR141716A: a CB₁-receptor inverse agonist; SR144528: a CB₂-receptor inverse agonist; TAN-67: a δ-opioid receptor agonist; TNFα: tissue necrosis factor α; TRPV1: transient receptor potential vanilloid-1; WIN55212-2: a non-selective cannabinoid agonist.
However, they can exert a direct cardioprotective effect through specific cannabinoid receptors and activation of key intracellular signaling pathways. The neuroprotective properties of those compounds in the central nervous system have been thoroughly reviewed by several authors to whom the readers are referred to for a deeper insight (1–3).

The present article will review the cardioprotective properties of cannabinoids against myocardial ischemic damage, with an emphasis on the signaling pathways mediating such effects. There are numerous controversies regarding the signaling pathways activated in the ischemic and reperfused heart, due in part to a lack of proper timing in the assessment of the latter. Hence, we will discuss the role of the PKC and MAPK pathways during ischemia and reperfusion injury, with a special emphasis on the time-course of activation of those kinases. To sort out which pathways represent a natural defense mechanism from those that are deleterious, the effect of one of the strongest cardioprotective phenomenon, IPC, on the signaling pathways, will be reviewed. When it comes to the role of p38 MAPK in cardioprotective properties of IPC or other protective agents, however, there are strong contradicting evidences supporting equally a deleterious or a protective role of this kinase. Finally, signaling pathways activated by cannabinoids through their receptors, namely CB₁ and CB₂ receptors, will be discussed, and their effects on signaling during ischemia reperfusion will also be reviewed.

**Cannabinoid CB₁ and CB₂ receptors**

Although signaling in the cannabinoid system has been reviewed recently by Schmid et al. (4), most of the focus has been on the central nervous system. In fact, very little is known about the CB₁ and CB₂ receptor signaling pathways in the heart and the cardiovascular system. As a matter of fact, until recently, the mere presence of both CB₁ and CB₂ receptors in the heart was questioned. We have confirmed the presence of both CB₁ and CB₂ receptors mRNA and proteins (5) and their cardiac localization by immunohistochemistry (6).

Both cannabinoid receptors are members of the G-protein-coupled-receptor family and both are negatively coupled to adenylate cyclase via Gi (7, 8). It has been proposed that three states of cannabinoid receptors exist, namely, R₀, R¹, and R², and that binding of an inverse agonist could induce the sequestration of the Gi protein, preventing it from signaling with other Gi-coupled receptors (9). Both CB₁ and CB₂ receptors exert a constitutive basal activity that can be inhibited by inverse agonists (SR141716A and SR144528 for CB₁ and CB₂ receptors, respectively) (9, 10). CB₂ receptors can be phosphorylated following their activation, which leads to their inactivation and loss of agonist activity (11). Furthermore, CB₁ receptors can inhibit N-type and Q-type calcium channels and activate potassium channels in a pertussis toxin-dependent manner, although calcium channel inhibition was proposed to be mediated directly by a G-protein and independently of cyclic AMP (12). Constitutively active CB₁ receptors can activate the Na⁺/H⁺ exchanger via the p42/p44 MAPK (ERK1/2) pathway (13). The effect of Na⁺/H⁺ exchanger is to increase intracellular pH in response to acidification of the cytoplasm, as seen in ischemic episodes and anaerobic respiration. The latter would therefore be an interesting mechanism in the cardioprotective properties of cannabinoids against ischemia.

**Cardioprotective properties of cannabinoids**

The first observation suggesting that cannabinoid may be beneficial against an ischemic insult was published by Lagneux and Lamontagne, who reported the involvement of cannabinoid receptors in LPS-induced cardioprotection in rat isolated hearts (14). Almost concomitantly, Krylatov et al. reported the anti-arrhythmic properties of HU-210, a non-selective cannabinoid agonist (15), and later on, of anandamide, an endocannabinoid (16), in a model of coronary occlusion and reperfusion in rats.

In the study by Lagneux and Lamontagne (14), the CB₂-receptor inverse agonist, SR144528, could block the cardioprotection conferred by a 24-h pre-treatment with LPS. The NOS inhibitor, NNLA, could also block the aforementioned protection; whereas SR144528 was also able to block the SNP (NO donor) induced cardioprotection. The CB₁-receptor inverse agonist SR141716A was ineffective in blocking any of these protective effects. In a similar study, SR144528 and NNLA, but not SR141716A, perfused during ischemia, were able to block the cardioprotection conferred by heat stress inflicted 24 h prior to ischemia (17).

Soon afterward, we reported the direct cardioprotective properties of several cannabinoids, including 2-AG, an endogenous cannabinoid; PEA, an endogenous cannabinomimetic; and ACEA and JWH015, two synthetic cannabinoids selective for CB₁ and CB₂ receptors, respectively (18). When exogenously perfused into rat isolated hearts, these cannabinoids showed clear cardioprotective properties in terms of a reduction in infarct size and improved post-ischemic ventricular recovery (18). Even though the CB₂-agonist ACEA could reduce infarct size, only SR144528 and not SR141716A could block completely the cardio-
protective effects of the two endogenous compounds. Although we were not able to observe a cardioprotective effect with perfusion of anandamide, Underdown et al. have recently confirmed the cardioprotective effect of this endocannabinoid, which was susceptible to both SR141716A and SR144528, in slightly different experimental conditions (19). In our previous studies, cannabinoids were perfused throughout the entire ischemic period and stopped upon reperfusion. In order to test whether the cardioprotective effects of cannabinoids could be exploited in a more clinically relevant situation, we compared the effect of the CB2-receptor agonist JWH133 (10 nM) administered either before ischemia only, during the entire ischemic period, or at the onset of reperfusion. Infarct size was significantly lower in all three groups compared with that in the untreated hearts (Fig. 1). On the other hand, functional recovery was definitely faster when JWH133 was perfused either before or during ischemia (Fig. 2).

Di Filippo et al. have published interesting results with WIN55212-2, a synthetic non-selective cannabinoid agonist, which was cardioprotective in their in vivo mouse model of regional ischemia (20). They have furthermore demonstrated the immunomodulatory effect of WIN55212-2, with decreasing levels of IL-1β and CXCL8 in the infarcted myocardium: both effects were abolished by AM630 (CB2-receptor antagonist/inverse agonist) but not by AM251 (CB1-receptor inverse agonist). When administered alone, AM630 had a deleterious effect on infarct size and cytokine levels, which suggests inverse agonist properties and a basal activation of the CB2-receptor and confirms the hypothesis proposed by Bouaboula (11). We have recently confirmed that the non-selective cannabinoid CP55940 reduces infarct size in an in vivo rat model of regional ischemia (21).

Wagner et al. have studied the implication of endocannabinoids and CB1-receptors in the hypotension following myocardial infarction and have found that pre-treatment with SR141716A increased the mortality rate after 2 h of reperfusion (22). No CB2-receptor antagonists were included in that study. In their subsequent work, Wagner et al. showed a protective effect of a perfusion with the agonist HU-210 on endothelial function 12 weeks after myocardial infarction (23). They did not observe any protective effect on mortality or infarct size with HU-210, but the treatment with the cannabinoid agonist was started 24 h after surgery. Short term beneficial effect of cannabinoids on endothelial function has also been reported by Bouchard et al., where they showed that the protection of endothelial function afforded by ischemic preconditioning was completely blocked by either SR141716A or SR144528.
Such as calcium, DAG, and IP₃. Therefore, activation of PKC requires different stimuli such as calcium, DAG, and IP₃. The use of PEA or 2-AG, but not anandamide, could reproduce this protection via activation of CB₁- and/or CB₂-receptors.

Signaling pathways involved in ischemia reperfusion injury and ischemic preconditioning

PKC

PKC is a family of kinases divided into the following 3 main subfamilies: 1) Classical Ca²⁺-dependent, PKC-α, β, and γ; 2) Novel Ca²⁺-independent, PKC-ζ, η, δ, and ε; 3) Atypical DAG-independent, PKC-λ, μ, ζ, and τ. Therefore, activation of PKC requires different stimuli such as calcium, DAG, and IP₃. When activated, PKC translocates to the cell membrane, together with the proper RACK (receptor for activated C kinase), where it phosphorylates the appropriate substrate. Translocation of different PKC isoforms (α, δ, ε, and τ) have been reported during ischemia (24, 25).

The study of PKC isoforms becomes interesting when one considers the IPC phenomenon. Fryer et al. have observed a loss of the cardioprotection from IPC when hearts were perfused with chelerythrine, a non-selective PKC inhibitor (26). Ping et al. have furthermore demonstrated the importance of PKC in the activation of ERK 1/2 by IPC (27). Saurin et al., using knock-out mice lacking the cardiac PKC-ε, identified the latter isofrom as an important one (28). It is generally accepted that translocation and activation of PKC-ε induced by IPC is responsible for the cardioprotection associated with IPC (29). In another study, Fryer et al. demonstrated the importance of PKC-δ in the cardioprotective properties of opioids (30). It was suggested that different isoforms might be involved in different phenomena, with a different timing of activation, as will be the case with the p38 MAPK family.

ERK 1/2 (p42/p44 MAPK)

It is generally accepted that ERK1/2 is activated upon reperfusion and that it plays an important role in the ventricular recovery and protection of the reperfused heart (31 – 34). Omura et al. described an increase in the ERK1/2 kinase activity at reperfusion after IPC (5, 15, 30, and 90 min), using an in-gel kinase assay in an in vivo rat model of regional ischemia (32). The maximum increase in activity for p44 MAPK (4.1-fold) and p42 MAPK (2.9-fold) was observed 30 min after reperfusion and was maintained after 90 min of reperfusion. No difference was observed in activity level or phosphorylation status of the p44 MAPK during ischemia (30, 60, or 90 min), but the p42 MAPK activity was decreased during ischemia (respectively 40%, 49%, and 60% of the control for the same aforementioned time). In a Langendorff perfused model, Hausenloy et al. performed a 35-min regional ischemia with 15 min of reperfusion and did not observe any increase of ERK 1/2 phosphorylation (35). The reason of this discrepancy, given the methodological information available, remains unclear.

ERK1/2 is furthermore activated at reperfusion by protective stimuli such as IPC (27, 35), opioids (36), adenosine (37), and cannabinoids (18). Fryer et al. observed an increase in the phosphorylation status of both p44 and p42 MAPK at 5, 30, and 60 min of reperfusion with IPC and with a treatment with TAN-67, a δ₁-opioid receptor agonist (36). On the other hand, IPC, but not TAN-67, caused a significant but moderate increase in ERK1/2 phosphorylation during ischemia (36). Hausenloy et al. (35) reported a similar increase in the phosphorylation status of ERK 1/2 at reperfusion after IPC in a Langendorff model of regional ischemia. Armstrong has also reviewed the main studies on ischemia and IPC, in whole hearts and isolated cardiomyocytes, and gave a general assessment supporting a protective role for ERK 1/2 in reperfusion after an ischemic insult (29).

p₃⁸ MAPK

Yin et al. reported that p₃⁸ MAPK is transiently phosphorylated on its tyrosine residue after 20 min of ischemia, with no phospho-p₃⁸ MAPK detected at 45 min of ischemia (38). Phosphorylation reoccurred at reperfusion and was maintained for 3 h with a maximum at 15 min in their rat model of perfused heart. Gorog et al. also described an increase in the phosphorylation status of p₃⁸ MAPK using a monoclonal dual specific Thr180/Tyr182 antibody at 60 and 120 min of low or moderate flow ischemia in a mouse Langendorff perfused heart model (39). The availability of antibodies raised against the dual phosphorylated Thr180 and Tyr182 was a major development since phosphorylation at both sites is necessary for p₃⁸ kinase activity (40). On the other hand, Omura et al. did not find any change in either the phosphorylation status of p₃⁸ MAPK nor in its enzymatic activity in their in vivo rat model of regional ischemia (32). In the same animal model, Fryer et al., using a dual phospho-specific (Thr180/Tyr182) polyclonal antibody were not able to observe any significant change in phosphorylation status during ischemia or reperfusion (33).

Weinbrenner et al. have observed a transient increase in the phosphorylation of Tyr 182 of p₃⁸ MAPK between 10 and 20 min of ischemia in a Langendorff perfused rabbit heart model after IPC (41). Marais et al., in a Langendorff perfused rat heart model, have found an increase in both the phosphorylation status and kinase phosphorylation of p₃⁸ MAPK (38). In a Langendorff perfused model, Hausenloy et al. performed a 35-min regional ischemia with 15 min of reperfusion and did not observe any increase of ERK 1/2 phosphorylation (35). The reason of this discrepancy, given the methodological information available, remains unclear.
activity (via phosphorylation of ATF-2) of p38 MAPK after 3 periods of IPC (42). Interestingly, they have found that p38 MAPK was activated after the first IPC episode and at a lesser extent but still significantly after the second episode, while no activation was observed after the third episode. Activation of p38 MAPK significantly declined after the first reperfusion episode following the first IPC. During ischemia, both preconditioned and non-preconditioned hearts showed an increase in p38 MAPK activation at 5 min of ischemia, reaching a maximal activation at 15 min. It is important to point out that the preconditioned group showed afterward a significantly less potent activation of p38 MAPK at 15 and 25 min of ischemia, when compared with the non-preconditioned group. Similarly, both groups showed an increase in p38 MAPK activity within 10 min of reperfusion, but the latter was only maintained in the non-preconditioned group. Lochner et al., in a similar model, studied the phosphorylation status of p38 MAPK and also found the same increase in p38 MAPK phosphorylation after one episode of IPC as well as with anisomycin, a p38 MAPK activator (43). Furthermore, p38 MAPK phosphorylation was assessed after 25 min of global ischemia, and they found that despite p38 MAPK being phosphorylated in all groups, IPC and anisomycin pre-treatment both provoked a significantly lower phosphorylation compared with untreated hearts. In the same study, the use of SB203580 during IPC abrogated the beneficial effect of one IPC episode, while it could not inhibit the protective effect of three IPC episodes. The use of SB203580 at the beginning of ischemia in the non-preconditioned group also decreased infarct size. Mocanu et al. found contradictory results in that the use of SB203580 during the IPC protocol did not abrogate the beneficial effect of IPC on infarct size, while its use just before ischemia did abrogate this beneficial effect (44). They found no effect of SB203580 when it was perfused just prior to ischemia in the non-preconditioned group. Another group, working with a Langendorff perfused rabbit heart model, also found an increase in p38 MAPK activity in early ischemia (5 and 10 min), which was less important in preconditioned hearts than non-preconditioned hearts, although they did not measure activity right after the IPC episode (45). Interestingly, treatment with the p38 inhibitor SB203580 early during ischemia decreased p38 MAPK activation during ischemia and also decreased infarct size following reperfusion. It is interesting to note that Gysembergh et al. used MAPKAP-2 for their kinase assay. Iliodromitis et al. studied the dual phosphorylation of p38 MAPK at 20 min of ischemia in a rabbit in vivo model of regional ischemia (46). They found an increase in p38 MAPK phosphorylation in the preconditioned group compared to the control group.

There may be several possible explanations for the apparent discrepancies regarding p38 MAPK activation following ischemia and reperfusion. Phosphorylation status of the different kinases involved in the signaling pathway of myocardial ischemia and reperfusion injury is an indication of their enzymatic activity, but cannot be considered a true measurement of what is going on, inasmuch as a phosphorylated kinase is not necessarily an active one. Yet, most data available in the literature provide the phosphorylation status because it is a much faster and convenient way to proceed and because it has been suggested to reflect the reality. Therefore, information on phosphorylation status of different MAPK at a precise time within an ischemia-reperfusion protocol must be interpreted with caution. On the other hand, in vitro kinase assays, despite providing an acute measurement of the enzymatic activity of the kinase, can sometimes be non-specific if the antibody used for the immunoprecipitation step is not specific enough. Both techniques then should be conducted together with highly specific antibodies.

The dual phosphospecific p38 MAPK antibody often used (NEB9211S) is not specific for either isoform of p38 MAPK (α, β, γ, or δ). Therefore, we cannot conclude whether the same p38 MAPK goes through two different phases of phosphorylation or if two different isoforms of p38 MAPK go through a single specific phosphorylation phase. It is generally accepted that p38 MAPK-α is associated with apoptosis, and p38 MAPK-β is associated with hypertrophy (47). Court et al. have identified both isoforms in the adult rat heart as well as SAPK3, also known as p38 MAPK-γ (48). Liang et al. recently reviewed the roles of p38 MAPK in cardiac hypertrophy in cell lines and genetically-modified animals (49). Because IPC is a transient phenomenon, one must be careful in extrapolating facts from animals expressing a cardiac selective negative dominant form of either p38 MAPK isoforms, considering the precise timing of activation of any mediator involved in the beneficial effect of IPC.

Despite some contradictory results, it appears that p38 MAPK plays an important role in the IPC phenomenon and in the damage resulting from an ischemic insult. With regard to the studies previously cited, we propose that an increase in p38 MAPK activity prior to the insult, like in IPC protocols, will tend to abrogate the activation of a possibly different isoform of p38 MAPK at the beginning of the ischemia, which in turn will be responsible for reducing the severity of the damage resulting from the ischemic insult. The key to this issue would be to better know which isoform is
activated at what time and what the effector of that isoform is.

**Cellular signaling in the pharmacology of CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors**

Bouaboula et al. studied the activation of ERK1/2 in cell lines transfected with either CB<sub>1</sub> or CB<sub>2</sub>-receptors and found ERK1/2 activation following treatment with CP55940, a non-selective cannabinoid agonist (50, 51). Galve-Roperh et al. furthermore described a pro-survival effect of HU-210 (CB<sub>1</sub>-receptor agonist) on an astrocytoma cell line mediated by a CB<sub>1</sub>-induced activation of ERK1/2 (52). The endogenous cannabinoid 2-AG was reported to activate AP-1 via phosphorylation of ERK1/2, but not JNK or p38 MAPK, with both CB<sub>1</sub> and CB<sub>2</sub>-receptors involved in this response (53). Liu et al. observed ERK1/2, p38, and c-Jun MAPK phosphorylation following anandamide treatment of human vascular endothelial cells endogenously expressing CB<sub>2</sub>-receptors (54). Derkinderen et al. found activation of p38 MAPK without JNK activation in rat hippocampus following treatment with endogenous and exogenous cannabinoids acting on CB<sub>1</sub>-receptors (55). Cannabinoid CB<sub>2</sub>-receptors have also been associated with activation of p38 MAPK and induction of apoptosis in a human leukemia cell line (56). In a microglial cell line, 2-AG was reported to induce proliferation via activation of CB<sub>2</sub>-receptors and the ERK1/2 pathway (57). The MAPKs are a large family of signaling proteins implicated in several phenomena, and thus it is not surprising to see them involved in such disparate cannabinoid effects, including immunomodulatory (58) and aqueous humor outflow (59).

We also studied the signaling pathways associated with the cardioprotective properties of cannabinoids. In one of our previous reports, we examined the effects of pharmacological inhibitors of PKC, ERK1/2, and p38 MAPK (chelerythrine, PD98059, and SB203580, respectively) on the cardioprotective effect of PEA, as well as the phosphorylation status of ERK1/2, p38 MAPK, and JNK induced by PEA (18). Inhibition of PKC with chelerythrine had only a partial inhibitory effect on the cardioprotective properties of PEA (18). On the other hand, PEA enhanced phosphorylation of p38 MAPK during ischemia, and inhibition of the p38 MAPK pathway with SB203580 completely blocked the protective properties of PEA on both infarct size and functional recovery (18). PEA also enhanced ERK1/2 phosphorylation during reperfusion, but its inhibition with PD98059 only had a partial inhibitory effect on the cardioprotective properties of PEA (18). However, the contribution of ERK1/2 may have been underestimated under our experimental conditions, since perfusion of PD98059 was stopped during reperfusion when ERK was maximally activated. Since the cardioprotective effect of PEA was totally inhibited by the CB<sub>2</sub>-receptor inverse agonist SR144528 (18), the enhanced phosphorylation of p38 MAPK and ERK1/2 during ischemia and reperfusion, respectively, with PEA, along with the blunted cardioprotection observed with pharmacological inhibition of these kinases, suggest a contribution of the latter in the CB<sub>2</sub>-mediated cardioprotective effects.

To confirm that cannabinoid-receptor activation was associated with an increased MAPK activity, we measured both p38 MAPK and ERK1/2 phosphorylation and activity in the presence of selective CB<sub>1</sub> and CB<sub>2</sub> cannabinoid agonists (Fig. 3). We found that perfusion with the CB<sub>2</sub>-receptor agonist, JWH133, in healthy hearts caused a 1.5-fold increase in p38 MAPK phosphorylation (Fig. 3a) along with a 2-fold (although statistically non-significant) increase in its activity (Fig. 3b). On the other hand, perfusion with the CB<sub>1</sub>-receptor agonist ACEA in healthy hearts was associated with an increase in the phosphorylation status of ERK1/2 by more than 2-fold (Fig. 3: c and d, statistically significant for p-44 only), but without any change in the enzymatic activity of ERK1 (Fig. 3e). During ischemia, p38 MAPK activity significantly increased to double the level compared to that in untreated hearts, in the presence of both cannabinoid agonists (Fig. 3b). However, ERK1/2 phosphorylation levels and activity during reperfusion were enhanced only by the CB<sub>2</sub>-receptor agonist (Fig. 3: c – e). Thus, it appears that the relative contribution of p38 MAPK activation during ischemia and ERK1/2 activation during reperfusion in the cardioprotective effect of cannabinoids may vary according to the cannabinoid-receptor subtype.

**Involvement of NO and NOS in ischemia-reperfusion and IPC**

The three isoforms of NOS, namely, neuronal (nNOS, NOS-1), inducible (iNOS, NOS-2), and endothelial (eNOS, NOS-3), and the NO they produce, may play either a protective or deleterious role in cardiomyocyte survival by either worsening or preventing ischemic damage and cardiomyocyte apoptosis. Loss of cardiomyocytes during reperfusion is caused mainly by apoptosis rather than necrosis and is related to progression toward cardiac hypertrophy and heart failure (60). Both pro and anti-apoptotic properties have been associated with NO, as discussed in the review by Razavi et al. (61). Regarding the death-receptor pathway
and caspase-8 activation, it is known that TNFα induces cardiomyocyte apoptosis through iNOS expression and NO production (62). On the other hand, while low NO concentration can inhibit mitochondrial transition pores and cytochrome C release, supra-physiological NO concentration, that is, release rates above 2 µM/s, can accelerate their opening, thus facilitating the mitochondrial apoptotic pathway through release of cytochrome C and activation of caspase-9 (63). Inducible NOS is associated with inflammation and has been shown to increase oxidative stress when it produces high levels of NO (64).

NO also exerts several anti-apoptotic properties through different mechanisms, of which S-nitrosylation of key apoptotic executor proteins such as caspase-3 (Cys163) (65) or AP-1 (66) among others, seems to be the most relevant cyclic GMP-independent mechanism. Activation of the cyclic GMP/PKG pathway, though, can induce expression of Bcl-2, an anti-apoptotic protein.

The amount of NO produced, and its origin, seem to be important factors in the survival outcome for the cardiomyocytes. Studies on more reliable models like working hearts and in vivo ischemia are, though, critical for fully understanding the action of NO. Schulz et al. have thoroughly reviewed recent studies and analyzed their conclusions regarding the recovery of contractile function and irreversible tissue injury following ischemia and reperfusion (67). Most of them concluded
that there is a pro-survival effect of NO from an endogenous or exogenous source and a deleterious effect of NOS inhibitors on both functional recovery and irreversible damage, although many discrepancies have been reported (68).

The beneficial role of NO in IPC phenomenon is more widely accepted, specifically in late phase IPC, especially following the work of Guo et al. (69) who worked with iNOS−/− knock-out mice. In their work, they observed an induction of iNOS protein expression during late phase IPC, and the abrogation of the late phase IPC protective effect in the iNOS−/− mice, while acute IPC protection remained unchanged. Lochner et al. studied the role of NO in IPC and found that L-NAME (50 μM) perfusion during IPC, and its washout before onset of ischemia, could block the cardioprotection, while three short episodes of SNP perfusion could mimic IPC. Both IPC and SNP groups showed an elevation of p38 MAPK phosphorylation before ischemia and a decrease after ischemia when compared to the untreated group (70).

Wang et al. have studied the expression of iNOS in mouse hearts subjected to late IPC and found an increase in iNOS mRNA (2-fold), beginning 1 h after IPC and maintained for 24 h, along with an iNOS protein expression increase (by 2-fold) 24 h after IPC (71). This modest elevation was observed mostly in cardiomyocytes, and it was also present in endothelial cells and smooth muscle cells of large vessels, but absent in small vessels and fibroblasts. In contrast, a strong elevation of iNOS mRNA (6-fold) and protein (3.5-fold) in hearts subjected to permanent coronary occlusion for, respectively, 48 and 66 h, was found in inflammatory infiltrating cells but absent in necrotic and surviving cardiomyocytes. The elevation of iNOS protein was even higher (30-fold) when the animal was subjected to endotoxin for 8 h. Again, we are confronted with the fact that the amplitude of NOS expression, mostly iNOS, and the amount of NO produced is highly responsible for the protective or deleterious pro-inflammatory effect of NO in the heart. Reports of the involvement of cannabinoids in NO and NOS modulation appeared in the field of immunology, whether neuronal or systemic, they usually described an inhibitory effect of cannabinoids, acting on either CB1- or CB2-receptors (72–74). On the other hand, reports of vasodilatatory properties of anandamide were proposed to be mediated by activation of constitutive NOS (cNOS, nNOS) and CB1-receptors, as reviewed by Stefano et al. (75, 76).

Finally, one should not forget that from a clinical point of view, nitrates, either sublingual or transdermal nitroglycerine and long-acting nitrates, are still widely used drugs for ischemic heart diseases for their venodilatory properties.
References

3 Fowler CJ. Plant-derived, synthetic and endogenous cannabinoids as neuroprotective agents. Non-psychoactive cannabinoids, ‘entourage’ compounds and inhibitors of N-acetyl-ethanolamine breakdown as therapeutic strategies to avoid psychotopic effects. Brain Res Rev. 2003;41:26–43.
20 Di Filippo C, Rossi F, Rossi S, D’Amico M. Cannabinoid CB2 receptor activation reduces mouse myocardial ischemia-
37 Haq SE, Clerk A, Sugden PH. Activation of mitogen-activated protein kinases (p38-MAPKs, SAPKs/JNKs and ERKs) by adenosine in the perfused rat heart. FEBS Lett. 1998;434:305–308.


81 Jonsson KO, Vandevoorde S, Lambert DM, Tiger G, Fowler CJ. Effects of homologues and analogues of palmitoylethanolamide


