Contribution of endocannabinoids in the endothelial protection afforded by ischemic preconditioning in the isolated rat heart

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Abstract

The aim of the present study was to assess the contribution of endogenous cannabinoids in the protective effect of ischemic preconditioning on the endothelial function in coronary arteries of the rat. Isolated rat hearts were exposed to a 30-min low flow ischemia (1 ml/min) followed by 20-min reperfusion, after which the response to the endothelium-dependent vasodilator, serotonin (5-HT), was compared with that of the endothelium-independent vasodilator, sodium nitroprusside (SNP). In untreated hearts, ischemia-reperfusion diminished selectively 5-HT-induced vasodilatation, compared with time-matched sham hearts, the vasodilatation to SNP being unaffected. A 5-min zero-flow preconditioning ischemia in untreated hearts preserved the vasodilatation produced by 5-HT. Blockade of either CB1-receptors with SR141716A or CB2-receptors with SR144528 abolished the protective effect of preconditioning on the 5-HT vasodilatation. Perfusion with either palmitoylethanolamide or 2-arachidonoylglycerol 15 min before and throughout the ischemia mimicked preconditioning inasmuch as it protected the endothelium in a similar fashion. This protection was blocked by SR144528 in both cases, whereas SR141716A only blocked the effect of PEA. The presence of CB1 and CB2-receptors in isolated rat hearts was confirmed by Western blots. In conclusion, the data suggest that endogenous cannabinoids contribute to the endothelial protective effect of ischemic preconditioning in rat coronary arteries.

Keywords: Cannabinoids; Endothelial function; Ischemia; Preconditioning; Rat; Serotonin

Introduction

Ischemic preconditioning is well known for its potent cardioprotective effects, resulting in a smaller infarct size [1], a reduced risk of ischemia-reperfusion arrhythmias [2], and an improved recovery of
ventricular function [3]. The beneficial effect of preconditioning is not limited to cardiomyocytes, since it has been shown to protect the endothelium as well [4,5], in various experimental models including dog resistance coronary arteries in vivo [6], and rat conduit coronary arteries in vitro [7]. The mechanisms involved in the endothelial protective effect of preconditioning share several similarities with those involved in the cardioprotective effect: adenosine [8], des-Arg^9^-bradykinin [9], prostaglandin E_2 [10], and reactive oxygen species [11] have all been proposed as triggers of preconditioning, with ATP-sensitive potassium channels [8] and ICAM-1 [12] as end-effectors, activation of protein kinase C (PKC) being an obligatory intermediate [10,12].

Arachidonoylethanolamide (AEA, or anandamide) and sn-2 arachidonoylglycerol (2-AG) are natural constituents of the plasma membrane that act as CB_1 and/or CB_2 agonists and exhibit pharmacological activities comparable to cannabinoids [13]. Palmitoylethanolamide (PEA), although having low affinity for constitutive as well as transfected CB_1 and CB_2 receptors [14,15], exerts analgesic effects that are reversed by selective CB_2-receptor antagonists [16]. The presence of cannabinoid receptor mRNA in the heart [17] as well as the detection of both PEA and 2-AG in rat cardiac tissue [18] suggest a physiological role for these endogenous cannabinoids in the heart. The endocannabinoids exert a vasorelaxant effect which has been characterized as being either endothelium-dependent or independent, depending on the vascular bed [19,20]. Anandamide can also exert a vasorelaxant effect through activation of sensory nerves in a NO-dependent fashion [21]. It has been proposed that EDHF might be an endocannabinoid [22], whereas others have suggested that endocannabinoids may instead regulate the release of EDHF [23], indicating that endocannabinoids can exert major effects on the endothelium. In addition, endocannabinoids generated in monocytes and platelets have been shown to be involved in the hypotension following acute myocardial infarction in rats [24]. Furthermore, endocannabinoids are involved in the cardioprotective effect of LPS [25] and heat stress [26]. Therefore, the aim of the present study was to evaluate whether endocannabinoids can protect the endothelium against ischemia and reperfusion, and contribute to the endothelial protective effect of preconditioning.

**Methods**

*Preparation of hearts*

The investigation was performed in accordance with the guidelines from the Canadian Council on Animal Care. The detailed methodology has been described earlier [8]. Briefly, male Sprague-Dawley rats (300–350 g) were exposed to a CO_2-enriched atmosphere until a complete loss of consciousness and rapidly decapitated. Hearts were rapidly excised and perfused at constant flow by means of a digital roller pump. A 20-ml compliance chamber along the perfusion line ensured a continuous flow (Langendorff perfusion). The flow rate was adjusted during the stabilisation period to obtain a coronary perfusion pressure of approximately 75 mm Hg and was held constant, with the exception of the ischemic periods during which flow was either stopped (zero-flow ischemia) or reduced to 1 ml/min (low-flow ischemia). A second adjustment of the flow rate was made at the end of the long reperfusion period, before the perfusion of drugs, to correct any deviation of the coronary perfusion pressure from 75 mm Hg, and was held constant thereafter. Perfusion pressure and flow rate were monitored to calculate coronary resistance. The perfusion solution (a modified Krebs-Henseleit buffer) contained (in mM): NaCl 118, KCl 4, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1, NaHCO_3 24, D-glucose 5, pyruvate 2, gassed with
95% O₂–5% CO₂ (pH 7.4), 37 °C. All drugs were administered through a Y connector in the aortic cannula with syringe pumps continuously adjusted to one hundredth of the coronary perfusion rate, which ensured a constant concentration despite the change in coronary perfusion rate during the ischemic periods. All concentrations mentioned in the text refer to the final concentrations after dilution.

Experimental protocols

The animals were assigned to one of five different experimental protocols (Fig. 1). The hearts in all groups were subjected to a 20-min stabilisation period. Drugs or vehicle infusion was then started, followed by an additional 15-min perfusion period. The ischemic groups (protocols 2 and 5, Fig. 1) were subjected to a 15-min sham period, followed by 30 min of low-flow ischemia (flow rate 1 ml/min) prior to a 20-min reperfusion period. In the preconditioned groups (protocol 3, Fig. 1), the hearts were exposed to 5 min zero-flow ischemia plus 10 min of reperfusion before the 30-min ischemia and 20-min reperfusion periods. The sham groups (protocols 1 and 4, Fig. 1) were not exposed to ischemia-reperfusion, but to a time-matched normal perfusion. After these periods, the vascular tone of the coronary arterial bed was increased with 0.1 μM U-46619, a thromboxane-mimetic agent, perfused throughout the rest of the experiment. Fifteen min after the beginning of U-46619 infusion, the endothelial function was evaluated by the vasodilatation produced by 10 μM serotonin (5-HT), whereas coronary smooth muscle function was evaluated with 3 μM sodium nitroprusside (SNP). These infusions were maintained for 10 min, which was long enough to reach a steady state. A washout period of 10 min was allowed between each infusion. Vasodilatation was evaluated by computing percent changes in coronary resistance (coronary perfusion pressure divided by coronary flow), measured immediately before each drug infusion, and after a new steady state. The concentrations of 5-HT and SNP were determined in preliminary dose-response experiments to produce near-maximal vasodilatation.

The contribution of endogenous cannabinoids in the endothelial protective effect of preconditioning was assessed using cannabinoid-receptor antagonists. Vehicle-treated sham, ischemic, and ischemic preconditioning hearts were compared with hearts treated with either 1 μM SR141716A or 1 μM SR144528, starting after the 20-min stabilisation period and lasting throughout the 30-min low-flow ischemia (protocols 1–3, Fig. 1). Antagonist infusion was stopped upon reperfusion.

In additional experiments, the endothelial protective effect of cannabinoids was evaluated (protocols 4 and 5, Fig. 1). After the initial 20-min stabilisation period, infusion of cannabinoid-receptor antagonists (1 μM SR141716A or 1 μM SR144528) or the vehicle was started. Fifteen min later, these hearts were perfused with either 2-AG (300 nM), PEA (300 nM), or AEA (10 nM–10 μM), and after an additional 15 min, exposed to a 30 min low-flow ischemia (1 ml/min) or a time-matched normal perfusion (sham hearts). All drugs were stopped upon reperfusion, and the endothelial and smooth muscle functions were evaluated as described above.

Western blotting

Hearts used to perform western blots were snap frozen in liquid nitrogen, after being perfused in the Langendorff setup to remove any remaining blood, and kept at −80 °C until crushed in a mortar with dry ice in liquid nitrogen. The samples were homogenized on ice with a polytron for 10 sec in a lysis buffer containing Tris (pH 7.5) 20 mM, EDTA 1 mM, EGTA 1 mM, β-glycerophosphate 1 mM, NaCl 150 mM, sodium pyrophosphate 2.5 mM, MgCl₂ 4.5 mM, 1,4-dithiothreitol (DTT) 0.5 mM, phenyl-
methylsulfonyl fluoride (PMSF) 1 mM, Triton X-100 1% and leupeptin 1 µg ml⁻¹. They were then incubated on ice for 30 min and centrifuged at 12000 g for 30 min at 4 °C. Protein extracts were aliquoted for further experiments and kept at −80 °C. Protein quantification was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Protein extracts (20 µg) were separated on a 10% SDS polyacrilamide gel for 90 min and transferred overnight at 4 °C to a supported nitrocellulose membrane. After the membrane was blocked for 2 hours at room temperature with 5% non-fat dry milk solution in Tris-buffered saline containing 0.1% Tween 20 (TBST), it was probed overnight with either
rat CB1-receptor polyclonal antibody 1:1000 in BSA 5% solution in TBST (Calbiochem no. 209550) or mouse CB2-receptor polyclonal antibody 1:1000 in BSA 5% solution in TBST (Calbiochem no. 209554). Membranes were washed in TBST. They were incubated with anti-rabbit IgG horseradish peroxidase-linked antibody (1:1000 dilution in 5% non-fat dry milk solution) for 1 hour, washed and incubated with Amersham ECL Western blotting detection reagent. Membranes were analysed directly on a Chemilmager 5500 from Alpha Innotech Corporation (San Leandro, CA).

**Statistical analysis**

Values represent the mean ± SEM. Statistical significance of differences between means was evaluated by a two-way analysis of variance with Scheffé post-hoc test. In the presence of an interaction between the different groups, one-way analyses of variance were used for each group. A commercially available software (Systat® for Windows® version 6.1) was used. Only probability values (p) smaller than 0.05 were considered to be statistically significant.

**Drugs**

SR144528 and SR141716A were a kind gift from Sanofi Recherche (Montpellier, France). PEA was provided by Tocris Cookson (Ballwin, MO, USA). All other drugs were obtained from Sigma-Aldrich (Mississauga, ON, Canada). SR144528 and SR141716A (10 mM) were prepared in 1 ml 100% dimethylsulphoxide (DMSO). All these stock solutions were diluted in bidistilled water to obtain the desired final concentrations. Anandamide (1 mM) was diluted in 1 ml propylene glycol and 9 ml of Krebs-Henseleit buffer. 2-AG (13.2 mM) and PEA (16.7 mM) were dissolved in anhydrous ethanol and diluted in Krebs-Henseleit buffer to obtain the desired final concentration. U-46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin F2α, 28.5 mM) was dissolved in 100% ethanol and diluted in Krebs-Henseleit buffer to obtain the desired final concentration. Ethanol (0.003%) and DMSO (0.02%), at the concentration obtained in the final dilution, had no effect on any of the hemodynamic variables studied and on the dilator responses to 5-HT and SNP. All the other drugs were dissolved directly in Krebs-Henseleit buffer.

**Results**

**Ischemic preconditioning**

Coronary resistance measured just before 0.1 μM U-46619 perfusion (n = 24) was 5.92 ± 0.30 mm Hg min ml⁻¹, for a coronary flow rate of 6.70 ± 0.20 ml min⁻¹ g⁻¹ (mean heart weight of 1.90 ± 0.05 g). Infusion of U-46619 (0.1 μM, n = 24) induced a comparable and significant vasoconstriction in all groups of untreated hearts (11.07 ± 0.42 mm Hg min ml⁻¹, p < 0.05). Perfusion of 10 μM 5-HT produced a 26.4 ± 2.2% decrease in coronary resistance in sham hearts (n = 8, Fig. 2A), corresponding to a 64 ± 9% reversal of the pre-constriction to U-46619. Thirty min of low-flow ischemia (n = 8) significantly diminished the 5-HT-induced vasodilatation by more than half (Fig. 2A). One period of preconditioning (n = 9) prevented the deleterious effect of ischemia on endothelium-dependent vasodilatation: the vasodilatation produced by 5-HT in preconditioned hearts was comparable to that
observed in hearts not subjected to ischemia (Fig. 2A). The endothelium-independent vasodilator, SNP (3 μM), produced a 30.2 ± 3.9% decrease in coronary resistance in sham hearts (n = 8, Fig. 2B), corresponding to a 82 ± 9% reversal of the pre-constriction to U-46619. This dilator response was not affected by ischemia and was found to be comparable in the three groups of hearts (sham, ischemia, and preconditioning, Fig. 2B).

SR141716A (1 μM), a selective antagonist of CB1 receptors, produced no significant change in coronary resistance (data not shown). Infusion of U-46619 induced a significant vasoconstriction (11.73 ± 0.51 mm Hg min ml⁻¹, p < 0.05, n = 24) that was comparable with that of untreated hearts. Vasodilatation produced by 10 μM 5-HT (24.7 ± 2.6% decrease in coronary resistance in sham hearts, corresponding to 63 ± 11% reversal of the pre-constriction, n = 10) was markedly reduced in the ischemic group (n = 8, Fig. 2C). Preconditioning in SR141716A-treated hearts (n = 6) was unable to prevent the deleterious effect of ischemia on 5-HT-induced vasodilatation (Fig. 2C). Vasodilatation induced by 3 μM SNP was comparable in all groups treated with the CB1-receptor antagonist (sham, ischemic, and preconditioning, Fig. 2D).

The effects of SR144528 (1 μM), a selective antagonist of CB2 receptors, were similar to those observed with the CB1-receptor antagonist: no significant change in coronary resistance and a comparable vasoconstriction to U-46619 (12.09 ± 0.63 mm Hg min ml⁻¹, n = 24). As with the
CB₁-receptor antagonist, preconditioning in SR144528-treated hearts (n = 6) was unable to prevent the deleterious effect of ischemia on 5-HT-induced vasodilatation (Fig. 2E). Vasodilatation to 3 μM SNP among the three different experimental protocols (sham, ischemic, and preconditioning) was comparable in the SR144528-treated groups (Fig. 2F).

**Perfusion of cannabinoids**

PEA had no effect on coronary resistance when measured 5 min after the beginning of its perfusion (data not shown). AEA and 2-AG induced a weak vasorelaxation (−15%) that did not reach the statistical level of significance. None of the cannabinoids perfused affected the vasoconstriction to U-46619 (13.37 ± 1.14, 12.70 ± 1.27, and 13.65 ± 1.47 mm Hg min ml⁻¹ for AEA, PEA, and 2-AG-treated hearts, respectively).

Thirty min of low-flow ischemia significantly diminished the 5-HT-induced vasodilatation by more than half in untreated hearts (Fig. 3). Treatment with AEA, (from 10 nM to 10 μM) starting 15 min before ischemia and lasting 45 min, was unable to preserve the vasodilatation produced by 10 μM 5-HT in ischemic hearts (data not shown). In contrast, treatment with either PEA or 2-AG preserved the vasodilatation produced by 10 μM 5-HT in ischemic hearts (Fig. 3A,C). Vasodilatation to 3 μM SNP was comparable in all cannabinoid-treated hearts (Fig. 3B,D).

![Fig. 3. Effects of different cannabinoids on the change in coronary resistance (Δ%) induced by 10 μM serotonin (5-HT, top panels) and 3 μM sodium nitroprusside (SNP, bottom panels) after ischemia and reperfusion. The left and right panels show respectively the effect of palmitoylethanolamide (PEA, panels A and B) and 2-arachidonoylglycerol (2-AG, panels C and D), with the columns depicting (from left to right) sham hearts with the corresponding cannabinoid agonist, ischemic hearts without cannabinoid, and ischemic hearts with the corresponding cannabinoid either without antagonist, or in the presence of SR141716A, or SR144528. *p < 0.05, compared with sham hearts (one-way ANOVA).](image-url)
SR144528-pretreated hearts was unable to prevent the deleterious effect of ischemia on 5-HT-induced vasodilatation (Fig. 3A). In contrast, perfusion with 2-AG in SR141716A-pretreated hearts still prevented the deleterious effect of ischemia and reperfusion on 5-HT-induced vasodilatation, whereas the same perfusion in SR144528-pretreated hearts was unable to prevent this deleterious effect (Fig. 3C).

To confirm the presence of both subtypes of cannabinoid-receptors in the rat heart, Western blots were made from tissue extracts obtained from three different Langendorff-perfused hearts, using rabbit polyclonal antibodies raised against rat CB1 or mouse CB2-receptors. These blots showed strong signals for both subtypes of receptors (Fig. 4).

**Discussion**

In the present study, we have assessed the contribution of cannabinoids in the protective effect of preconditioning on the endothelial function in the rat heart. The major findings are 1) that the protection afforded by preconditioning against endothelial dysfunction following ischemia-reperfusion can be blocked by a pre-treatment with the cannabinoid-receptor antagonists SR141716A and SR144528, and 2) perfusion with the endocannabinoids 2-AG and PEA mimics the beneficial effect of preconditioning on endothelial function in the same model.

In the past, we have used the present experimental model extensively to study the mechanisms involved in the endothelial protective effect of preconditioning [8–10,27]. The coronary flow rate and duration of ischemia were selected in the beginning to inflict a selective endothelial dysfunction, which was confirmed by the blunted coronary vasodilatation to the endothelium-dependent dilator, 5-HT [28], contrasting with the full response to the endothelium-independent vasodilator SNP. One disadvantage of the isolated heart model is the unavoidable ischemia the heart is exposed to, from the excision to the time the Langendorff perfusion is initiated, that could theoretically induce a preconditioning. However, this period was limited to 30–60 s, which is probably to short to induce a measurable preconditioning [29].

To assess the contribution cannabinoids in the endothelial protective effect of preconditioning, selective cannabinoid-receptor antagonists were used. SR141716A is a potent and highly selective antagonist for CB1-receptors, with a Ki value of 2 nM for CB1-receptors and well above 1 μM for CB2-receptors [30]. Likewise, SR144528 is highly selective for CB2-receptors, with a Ki of 0.3 nM in cell lines expressing human CB2-receptors, and of 437 nM in cells expressing human CB1-receptors [31]. At the concentration used in the present study (1 μM), both antagonists blocked completely their targeted receptors. However, it is possible that SR144528 also blocked, at least partially, CB1-receptors. In the presence of either SR141716A or SR144528, ischemic preconditioning was unable to prevent the reduction in the vasodilatory response to 5-HT. This suggests that endogenously produced cannabinoids...
play a role in the endothelial protection afforded by preconditioning by acting on CB1 receptors, and possibly CB2 receptors. Whether cannabinoids are released during the preconditioning ischemia, a prerequisite for a true trigger of preconditioning, remains to be established. However, both PEA and 2-AG were found in rat cardiac tissue, anandamide being undetectable [18]. Moreover, cannabinoid-receptor mRNA has been detected in human cardiac tissue [17], and we have confirmed the presence of CB1 and CB2-receptors in isolated rat hearts in the present study. Therefore, the presence of endocannabinoids and cannabinoid-receptors in isolated rat hearts supports the data observed with the antagonists.

To confirm the contribution of cannabinoids to the endothelial protection afforded by preconditioning, the effect of exogenous perfusion with 2-AG, PEA, and anandamide on endothelial function following ischemia-reperfusion was studied. The concentration of these cannabinoids was selected from pilot experiments, in which concentrations from 10 nM to 10 µM were tested. No effect was observed with anandamide. However, perfusion with 2-AG and PEA prevented the ischemia-induced reduction in the vasodilatation to 5-HT, both at a concentration of 300 nM: no effect was observed at 100 nM or less, and no additional salutary effect resulted from an increase in concentration. Thus, both 2-AG and PEA can mimic the protective effect of preconditioning on the endothelial function. However, it appears to be an all-or-none phenomenon, with a threshold needed to be reached to observe the desired effect. The lack of effect of anandamide may be explained by the short half-life of this compound, which undergoes rapid uptake and inactivation in tissues [32,33]. Alternatively, it may be due to the fact that this cannabinoid is only a partial agonist of CB1-receptors [34].

The endothelial protective effect of 2-AG was blocked only by the CB2-receptor antagonist, whereas both CB1 and CB2-receptor antagonists abolished that of PEA. At first glance, these results may appear to be in contradiction with the current knowledge on cannabinoid receptor pharmacology. Although 2-AG has low affinity for transfected CB1 and CB2-receptors (Ki of 472 and 1400 nM, respectively) [35], it has been shown to increase intracellular free Ca\(^{2+}\) in cells expressing either CB1 [36] or CB2-receptors [37], with an EC\(_{50}\) of approximately 100 nM. On the other hand, PEA was found to be a much weaker agonist for both receptors [15]. At the concentration used in the present study (300 nM), perfusion with 2-AG resulted most probably in a full activation of both CB1 and CB2-receptors. Therefore, the capacity of SR144528 to block the effect of 2-AG suggests that CB2-receptors are the main receptors involved in the endothelial protective effect of 2-AG. However, it is conceivable that with a weaker agonist like PEA, CB1-receptors must be activated in addition to CB2-receptors, resulting in a synergistic action, in order to observe an effect. This could explain the inhibitory action of both SR144528 and SR141716A on the endothelial protective effect of PEA. Alternatively, one cannot rule out the contribution of cannabinoid-receptors distinct from CB1 and CB2 [38,39] in the endothelial protective effect of 2-AG and PEA.

Little is known about the signalling pathways involved in the endothelial protective effect of cannabinoids. In the brain, cannabinoids increase the activity of protein kinase C (PKC) [40], a key player in the endothelial protective effect of preconditioning [10,12,41]. Randall et al. reported that the anandamide-induced coronary relaxation in isolated rat hearts is blocked by a calcium-dependent potassium-channel blocker [22]. However, these channels are not involved in the endothelial protective effect of preconditioning, since the dilator response to 5-HT in preconditioned hearts perfused with 100 nM apamin and 10 nM iberiotoxin (−25.4 ± 6.8%, n = 6) was found comparable to that in untreated preconditioned hearts (−30.5 ± 3.0%, n = 8). Clearly, further experiments are needed to assess the pathways involved in the endothelial protective effect of cannabinoid.
We have recently reported that SR144528, but not SR141716A, blocked the infarct size-reducing effect of preconditioning with either a LPS injection [25] or a heat stress [26] 24 hours before ischemia in rats, suggesting an involvement of endocannabinoids, through CB2-receptors, in these effects. It appears therefore that endocannabinoids represent an important player in the protective effect of preconditioning, for both the myocardium and the endothelium, but through different receptors.

Conclusions

CB1 and CB2-receptor activation is required to observe a protective effect of ischemic preconditioning on the endothelial function of the isolated rat heart. Furthermore, exogenous perfusion of 2-AG and PEA can protect the endothelium against the deleterious effect of ischemia-reperfusion via activation of cannabinoid-receptors. These data suggest an important contribution of endocannabinoids in the endothelial protective effect of ischemic preconditioning.

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References


