

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Different expression and function of the endocannabinoid system in human epicardial adipose tissue in relation to heart disease

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/133266> since 2016-09-04T20:53:22Z

Published version:

DOI:10.1016/j.cjca.2012.06.003

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in CANADIAN JOURNAL OF CARDIOLOGY, 29 (4), 2013, 10.1016/j.cjca.2012.06.003.

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

- (1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.
- (2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.
- (3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>), 10.1016/j.cjca.2012.06.003

The publisher's version is available at:

<http://linkinghub.elsevier.com/retrieve/pii/S0828282X12002826>

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/2318/133266>

Different Expression and Function of the Endocannabinoid System in Human Epicardial Adipose Tissue in Relation to Heart Disease

Giuseppe Cappellano, Francesca Uberti, Philippe Primo Caimmi, Stefano Pietronave, David A.S.G. Mary, Chiara Dianzani, Ezio Micalizzi, Matteo Melensi, Renzo Boldorini, Gabriella Nicosia, Enrico Crosio, Annalisa Chiocchetti, Franco Aina, Maria Prat, Umberto Dianzani, Giovanni Vacca, Cristiano Ariatti, Elena Grossini

Abstract

Background

The endocannabinoid system reportedly plays a role in the pathogenesis of cardiovascular diseases. This system is expressed also in adipose tissue, which could thus be involved in cardiac disorders through modulation of metabolically triggered inflammation. The current study aims to determine the relevance of the endocannabinoid system in epicardial adipose tissue in heart disease.

Methods

Expression of the endocannabinoid receptors CB1 and CB2, and of the endocannabinoid-degrading enzyme, fatty acid amidohydrolase, and activation of protein kinase A (PKA), phospholipase C (PLC), protein kinase C (PKC), endothelial nitric oxide synthase (eNOS) and inducible (i)NOS, and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (a member of the reperfusion-injury salvage kinase pathway), were analyzed by Western blot in patients after coronary artery bypass surgery (ischemics; N = 18) or valve surgery (nonischemics; N = 15) and in preadipocytes isolated from epicardial adipose tissue.

Results

In ischemics, the CB1-to-CB2 expression ratio shifted toward CB1 and was accompanied by higher PKA activation. In contrast, in nonischemics, CB2, fatty acid amidohydrolase, PLC and PKC, and ERK1/2 were upregulated. Moreover, NO production and iNOS-to-eNOS ratios were higher in preadipocytes from ischemics.

Conclusions

These results show a different modulation and functioning of the endocannabinoid system in ischemics compared with nonischemics. Hence, while CB2, PLC and PKC, ERK1/2, and eNOS are more strongly expressed in patients without ischemic heart disease, high CB1 and PKA expression is associated with low survival intracellular pathway activation and high iNOS activation in ischemic heart disease patients. The changes in the endocannabinoid system in ischemics may contribute to cardiac dysfunction and therefore represents a potential therapeutic target.

Résumé

Introduction

Le système endocannabinoïde jouerait un rôle dans la pathogenèse des maladies cardiovasculaires. Ce système est aussi exprimé dans le tissu adipeux, qui pourrait par conséquent être impliqué dans les troubles cardiaques par la modulation de l'inflammation métaboliquement déclenchée. L'étude actuelle a pour but de déterminer la pertinence du système endocannabinoïde dans le tissu adipeux épicardique de la maladie du cœur.

Méthodes

L'expression des récepteurs CB1 et CB2 des endocannabinoïdes, et de l'enzyme responsable de la dégradation de l'endocannabinoïde, l'amidohydrolase des acides gras et l'activation de la protéine kinase A (PKA), la phospholipase C (PLC), la protéine kinase C (PKC), la synthase endothéliale de l'oxyde nitrique (eNOS) et (i)NOS inductibles et des kinases 1 et 2 régulées par les signaux extracellulaires (ERK1/2) (un élément de la voie RISK [*reperfusion-injury salvage kinase*]) ont été analysés chez les patients par le *Western Blot* après le pontage coronarien (ischémiques; N = 18) ou la chirurgie valvulaire (non ischémiques; N = 15), et dans les préadipocytes isolés du tissu adipeux épicardique.

Résultats

Chez les ischémiques, le ratio d'expression CB1 : CB2 s'est déplacé vers CB1 et a été accompagné d'une activation élevée de la PKA. En revanche, chez les non ischémiques, le CB2, l'amidohydrolase des acides gras, le PLC et PKC et l'ERK1/2 ont été régulés à la hausse. De plus, la production de NO et les ratios iNOS : eNOS ont été élevés dans les préadipocytes des patients ischémiques.

Conclusions

Ces résultats montrent une modulation et un fonctionnement du système endocannabinoïde différents chez les ischémiques comparativement aux non ischémiques. En conséquence, bien que le CB2, le PLC et PKC, le ERK1/2 et la eNOS sont exprimés de manière plus importante chez les patients sans maladie cardiaque ischémique, l'expression élevée de CB1 et de PKA est associée à une faible activation des voies de signalisation intracellulaire de survie et une activation élevée de la iNOS chez les patients ayant une maladie cardiaque ischémique. Les changements dans le système endocannabinoïde chez les ischémiques peuvent contribuer au dysfonctionnement cardiaque, représentant ainsi une cible thérapeutique potentielle.

The endocannabinoid system (ECS) comprises 2 types of G-protein-coupled cannabinoid (CB) receptors, CB1 and CB2, and their endogenous ligands, the endocannabinoid arachidonyl ethanolamide (anandamide) and 2-arachidonoyl-glycerol (2-AG), which are degraded by fatty acid amidohydrolase (FAAH) and monoacyl glycerol lipase.¹

CB1 and CB2 belong to the 7-transmembrane G-protein-coupled receptor family and share 68% homology within the transmembrane domains and 44% homology throughout the total protein. The main signal transduction includes cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), p38-mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2) and phospholipase C (PLC) and protein kinase C (PKC).² CB receptors are expressed in many tissues³ and are involved in several physiological and pathologic processes.^{4, 5 and 6} In the cardiovascular system, CB1 and CB2 are expressed in the atria, ventricles, cardiomyocytes, and vascular endothelial cells,^{7, 8 and 9} and have been implicated in cardiometabolic regulation and vasodilatation.¹⁰ Furthermore, they were reported to play a role in metabolically triggered inflammation, or "metaflammation," and vascular muscle cell proliferation involved

in cardiovascular diseases, as well as in metabolic disorders.^{11, 12^{and} 13} In particular, CB1 stimulation may support progression of metaflammation and cardiac dysfunction,¹¹ and CB2 stimulation may protect against cardiac ischemia and reperfusion injuries⁷ through a PKC, p38-MAPK, and ERK1/2-mediated signalling pathway.¹⁴ Moreover, various endocannabinoid agonists were shown to protect cardiac cells against ischemia and reperfusion injury or hypoxia through modulation of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) cardiac equilibrium and nitric oxide (NO) production.^{9^{and} 15}

Epicardial adipose tissue (EAT), which substantially increases with obesity, may play a key role in cardiac disease.¹⁶ Hence, EAT is a metabolically active organ and a source of several bioactive molecules that might substantially affect the myocardium and coronary arteries.^{16, 17, 18^{and} 19} Its proximity to the myocardial tissue suggests that factors released by EAT may affect coronary wall inflammation, cardiac function, and progression of atherosclerosis.^{14^{and} 20} Since adipocytes and preadipocytes are endowed with the full biochemical machinery to synthesize, degrade, and respond to endocannabinoids, our study analyzed the ECS in EAT from cardiac patients with and without ischemic heart disease in order to assess its involvement in cardiac diseases.

Methods

Patients

Patients were recruited at the Cardiac Surgery Unit, Azienda Ospedaliero-Universitaria Maggiore della Carità di Novara. They underwent either coronary artery bypass graft (N = 18) or valve replacement or repair (N = 15). Nonischemic patients showed no angiographic evidence of coronary disease. In each patient, 2 or 3 biopsies of EAT, close to the proximal portion of the right coronary artery, were taken immediately before the onset of extracorporeal circulation. Thoracic biopsies of subcutaneous adipose tissue (SAT) were taken as controls. Tissue specimens were flash frozen immediately for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis or immediately processed to prepare preadipocytes. All patients provided written informed consent. The study complied with the Declaration of Helsinki for investigation in human beings and was approved by the local ethical committee.

Isolation of preadipocytes

Samples were placed in a saline solution and processed as previously described.²¹ Tissue was cut into about 1 cm² sections and digested with type I collagenase (1 g/mL; Invitrogen Life Technologies, Carlsbad, CA) in Hanks balanced salt solution (Sigma, Milan, Italy) plus 1% penicillin-streptomycin (Euroclone, Pero, Milan, Italy) for 1 hour at 37°C. Samples were then filtered with sterile gauze and centrifuged at 190g for 10 minutes. The pellet containing preadipocytes was plated in a 1:1 mix of Dulbecco's Modified Eagle F12 medium supplemented with 10% fetal calf serum (Euroclone), 100 IU/mL penicillin, 100 µg/mL streptomycin, and glutamine, and incubated at 37°C in the presence of 5% CO₂. Cells were used between the third and sixth passage.

Western blot analyses

Tissue samples and preadipocyte s preparations were processed for sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in the Supplementary Material. Lysates were probed with antibodies directed against p-p44/42 MAPK (Thr202/Tyr204), p-eNOS (Ser1177), eNOS (Cell Signaling Technology Inc, Denver, CO), iNOS (Abcam, Cambridge, UK), p-PKA, p-PLC-γ, CB1, CB2, FAAH (Santa-Cruz, Tebu-Bio, Milan, Italy), and p-PKC (Abfrontier, Digital Valley, Seoul, South Korea).

Griess assay

NO production was measured by the Griess assay (Promega, Madison, WI), as previously described.²² Preadipocytes (1×10^4) were incubated in 24-well plates for 4 hours in Dulbecco's Modified Eagle's Medium without red phenol (Euroclone) and stimulated with the CB receptor agonists 2-AG (Cayman, Ann Arbor, MI), JWH015 (Sigma), or methanandamide (Cayman) for 60 seconds, 180 seconds, and 300 seconds. In a series of experiments, cells were pretreated for 30 minutes with the CB receptor antagonists AM630 (Cayman) or AM251 (Cayman), the cAMP-dependent PKA inhibitor H89 (Sigma), or the PKC blocker calphostin C (Tocris Biosciences, Ellisville, MO), before adding CB receptor agonists. Treatment with tumour necrosis factor α (Sigma) was used as positive control. Culture supernatants were then mixed with equal volumes of the Griess reagent and incubated in the dark at room temperature for 10 minutes; absorbance was measured by a spectrometer at 570 nm. NO production corresponded to the NO (μmol) produced after each stimulation by samples, each containing 1.5 μg of protein.

In another series of experiments, the diaminofluorescein fluorophore system (DAF-FM; Molecular Probes, Invitrogen, Carlsbad, CA) was used (see Supplementary Material).

RT-PCR

Tissue samples were treated with TRIzol reagent (Invitrogen, San Diego, CA) to extract RNA, and 0.5 μg total RNA was then reverse transcribed into complementary DNA with the ThermoScript RT-PCR System (Invitrogen) and Taq polymerase (Invitrogen) in a 25 μL final volume with primers: CB1: 5'-GAGCTCAGCCTAATCAAAG-3' (forward), 5'-TATGTACCTGTCGATGGC-3' (reverse); CB2: 5'-ACAAGCTCAGTGGAATCTG-3' (forward), 5'-ATAGTCACGCTGCCAATC-3' (reverse); and GA6DH: 5'-ACCACAGTCCATGCCATC-3' (forward), 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). Semiquantitative analysis was done through automated densitometry (VersaDoc; Bio-Rad Laboratories Ltd, Hercules, CA) in a double-blind manner.

Statistical analysis

The STATVIEW version 5.0.1 for Microsoft Windows (SAS Institute Inc, Cary, NC) provided statistical analysis. Continuous and categorical variables were respectively expressed as mean \pm SD and percentages. Two-way ANOVA followed by the Bonferroni post hoc test and the Mann-Whitney test were used to examine changes in NO production, protein phosphorylation, and messenger RNA levels. The chi-square or Fisher exact test was used to compare categorical variables. Statistical significance was set at $P < 0.05$.

Results

As reported in Table 1, ischemic and nonischemic patients differed only in left ventricular ejection fraction and acetylsalicylic acid intake.

Table 1.

Demographic data

	Ischemic patients (n = 18)	Nonischemic patients (n = 15)	<i>P</i>
Men-to-women (n)	15:3	12:3	> 0.05
Age (years)	66.1 ± 9	67.6 ± 8.2	> 0.05
Weight (kg)	76.1 ± 10	78.3 ± 18.6	> 0.05
BMI (Kg/m ²)	27.6 ± 3.8	27.2 ± 5	> 0.05
Dyslipidemia (n)	12	9	> 0.05
Hypertension (n)	15	13	> 0.05
Diabetes (n)	12	8	> 0.05
Previous myocardial infarction	11	0	< 0.001
History of heart failure	13	1	< 0.001
Left ventricular ejection fraction (mL)	48.6 ± 9.9	58.1 ± 7.4	0.008
Current smoker (n)	3	2	> 0.05
Former smoker (n)	9	8	> 0.05
β-blocker (n)	8	6	> 0.05
ACEI (n)	5	3	> 0.05
Sartans (n)	5	3	> 0.05
Nitrates (n)	8	4	> 0.05
Furosemide (n)	6	5	> 0.05
ASA (n)	13	3	0.003

	Ischemic patients (n = 18)	Nonischemic patients (n = 15)	<i>P</i>
Statins (n)	6	5	> 0.05
Insulin (n)	3	3	> 0.05
Oral hypoglycemic agents (n)	6	3	> 0.05
No. vessels revascularized:			
1 (n)	2		
2 (n)	4		
3 (n)	7		
4 (n)	5		
Mean	2.8 ± 0.9		
Mitral valve surgery (n)		7	
Stenosis		3	
Regurgitation		3	
Mixed		1	
Aortic valve surgery (n)		8	
Stenosis		4	
Regurgitation		3	
Mixed		1	

ACEI, angiotensin-converting enzyme inhibitors; ASA, acetylsalicylic acid; BMI, body mass index; Sartans, AT1-receptor antagonists.

Western blot showed that CB1 expression was higher in ischemics than in nonischemics, whereas CB2 and FAAH expression were higher in nonischemics (Fig. 1, A and B). Semiquantitative RT-PCR confirmed different CB1 expression, whereas CB2 showed no differences (Fig. 1, C and D). Moreover, activated PKA and iNOS levels were higher in ischemics than in nonischemics, whereas PLC, PKC, and ERK1/2 levels were higher in nonischemics than in ischemics (Fig. 2).



Figure 1.

Expression of endocannabinoid system in epicardial adipose tissue (EAT). **(A)** Example of Western blot performed from EAT from 4 ischemic (I) and 4 nonischemic (V) patients and from subcutaneous adipose tissue (SAT) of 1 ischemic patient (SI) and 1 nonischemic patient (SV). **(B)** Densitometric analysis for CB1 or CB2 receptors and FAAH on lysates from epicardial and SAT from ischemic (N = 18) and nonischemic (N = 15) patients. U937 = macrophage-like cells (negative control for CB1). **a:** $P < 0.05$ vs V, I, SV, and SI. **(C, D)** RT-PCR and densitometric analysis of messenger RNA for CB1 and CB2 receptor expression in EAT from ischemic (n = 4) and nonischemic (n = 4) patients. Samples included SAT in ischemic and nonischemic patients and negative control (Co), as well. Reported data are mean \pm SD. FAAH, fatty acid amidohydrolase; RT-PCR, reverse transcription–polymerase chain reaction.

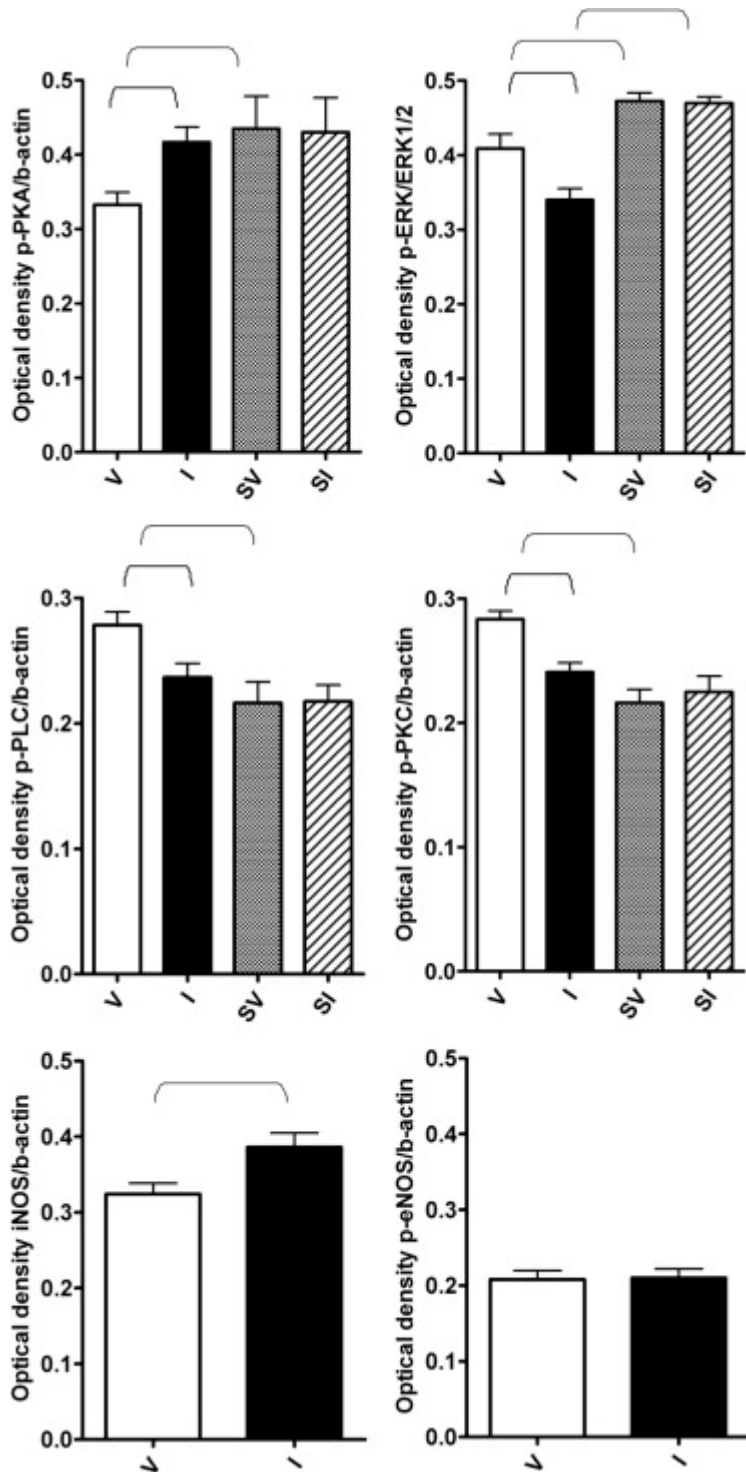


Figure 2.

Expression of activated PKA, ERK1/2, PLC, PKC, iNOS, and eNOS in epicardial adipose tissue (EAT). Densitometric analysis of Western blot performed in EAT and subcutaneous adipose tissue (SAT) obtained from 18 ischemic patients and 15 nonischemic patients. Only EAT shows a different activation of the PKA, ERK1/2, PLC/PKC, eNOS, and iNOS. Reported data are mean \pm SD. The assay was repeated twice. ERK1/2, extracellular signal-regulated kinases 1 and 2; eNOS, endothelial nitric oxide synthase; I, EAT from ischemic patients; iNOS, inducible nitric oxide synthase; PLC, phospholipase C; PKA, protein kinase A; PKC protein kinase C; SI, SAT from ischemic patient; SV, SAT from nonischemic patients; V, EAT from nonischemic patients.

Western blot analysis in preadipocytes confirmed the different CB receptor subtype expression in ischemics and nonischemics (Fig. 3). As shown in Figure 3, increasing concentrations of the CB1 agonist methanandamide (0.001 μ M-100 μ M) and of the CB1/CB2 agonist 2-AG (0.001 μ M-100 μ M) induced higher NO release in ischemics than in nonischemics ($P < 0.05$). In contrast, the CB2 agonist JWH015 (0.001 μ M-100 μ M) was more effective in nonischemics. Tumour necrosis factor α induced a high increase in NO production, similar to that elicited by methanandamide in ischemics (Fig. 4). Results obtained with the diaminofluorescein fluorophore method confirmed those obtained with the Griess assay (Supplemental Fig. S1).

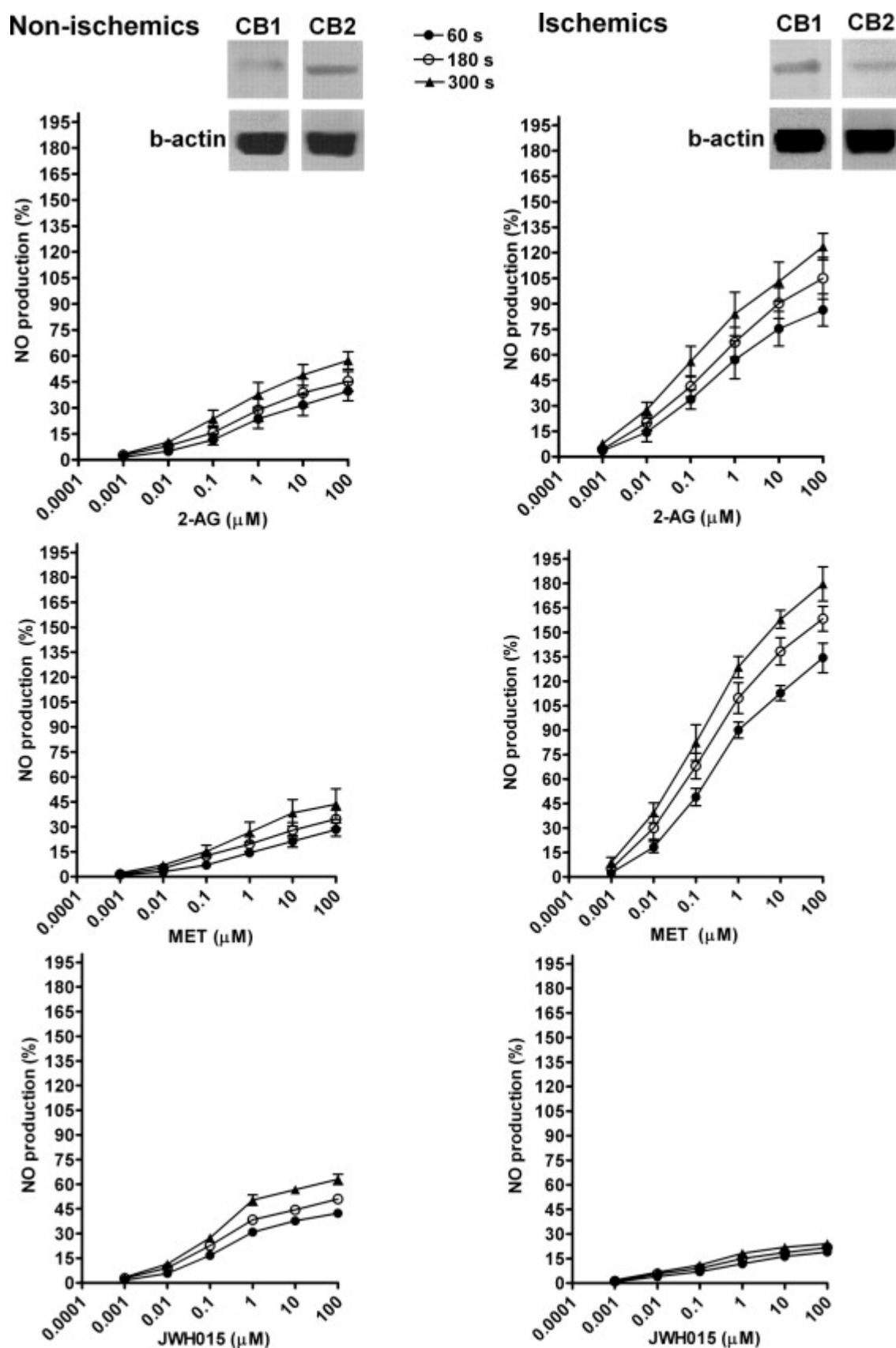


Figure 3.

CB agonists induce a dose-response and time-course production of nitric oxide (NO) in preadipocytes. NO production (%) was measured by Griess assay during preadipocyte stimulation with 2-arachidonoyl-glycerol (2-AG), methanandamide (MET), and

JWH015. Reported data are mean \pm SD of 5 independent experiments for each experimental protocol. Western blot performed in preadipocytes shows different CB expression in nonischemic and ischemic patients

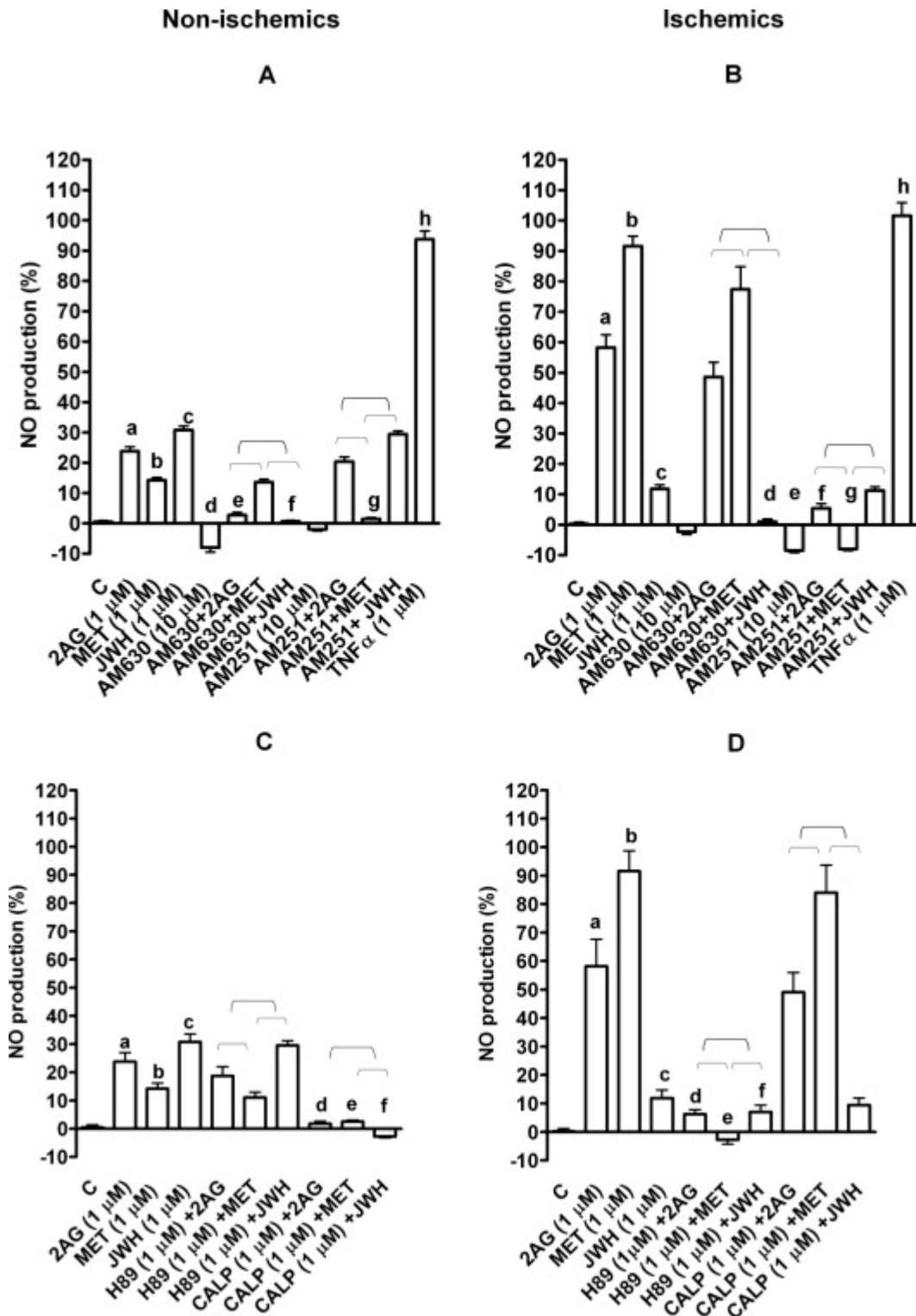


Figure 4.

Modulation of cannabinoid (CB)-mediated nitric oxide (NO) production by different agents in preadipocytes. **(A, B)** Effects of CB1 and CB2 receptor agonists on NO production measured through Griess assay in the absence or presence of CB receptors antagonists; 2-arachidonoyl-glycerol (2-AG), methanandamide (MET), JWH, and tumour necrosis factor α were administrated for 60 seconds. AM630 = CB2 receptor blocker; AM251= CB1 receptor blocker; C = control; JWH= JWH015. **(A) a, b, c, d, h:** $P < 0.05$ vs

control; **b, c, e, h:** $P < 0.05$ vs **a**; **c, g, h:** $P < 0.05$ vs **b**; **f, h:** $P < 0.05$ vs **c**. **(B)** **a, b, c, e, h:** $P < 0.05$ vs control; **b, c, f, h:** $P < 0.05$ vs **a**; **c, g:** $P < 0.05$ vs **b**; **h:** $P < 0.05$ vs **c**. **(C, D)** Effects of CB1 and CB2 receptor agonists on NO production in the absence or presence of PKA and PKC blockers; 2AG, MET, and JWH were administrated for 60 seconds; **a, b, c:** $P < 0.05$ vs control; **b, c, d:** $P < 0.05$ vs **a**; **c, e:** $P < 0.05$ vs **b**; **f:** $P < 0.05$ vs **c**. Reported data are mean \pm SD of 5 independent experiments for each experimental protocol. PKA, protein kinase A; PKC, protein kinase C; H89, PKA inhibitor; CALP, calphostin C, PKC inhibitor.

Cell response specificity to CB1 and CB2 receptor agonists was examined by performing the above cell-stimulation experiments with 2-AG, methanandamide, and JWH015 (60 seconds and 300 seconds) in the absence or presence of the CB1 or CB2 antagonist (AM251 and AM630, respectively; 10 μ M). In ischemics, AM251 noticeably inhibited the response to methanandamide and to 2-AG but not to JWH015, whereas AM630 decreased the response only to JWH015. In nonischemics, AM251 inhibited the response to methanandamide but had no significant effect on response to 2-AG and JWH015, whereas AM630 inhibited the response to JWH015 and 2-AG, but not to methanandamide (Fig. 4 and Supplemental Fig. S2).

Moreover, as shown in Figure 4 and Supplemental Figure S2, H89 (1 μ M) abolished the response to methanandamide and significantly reduced the response to 2-AG and JWH015 in ischemics only. By contrast, calphostin C (1 μ M) abolished the effect of JWH015 and 2-AG and significantly reduced that of methanandamide in nonischemics only.

Last, Western blot showed that 2-AG and methanandamide activated PKA (Fig. 5) and iNOS (Fig. 6) at higher levels in cells from ischemics than in those from nonischemics. In contrast, JWH015 activated PLC, PKC (Fig. 5), eNOS, and ERK1/2 (Fig. 6) at higher levels in cells from nonischemics than in cells from ischemics. These data confirmed those obtained in whole EAT, indicating predominant involvement of CB1 in ischemics and CB2 in nonischemics.

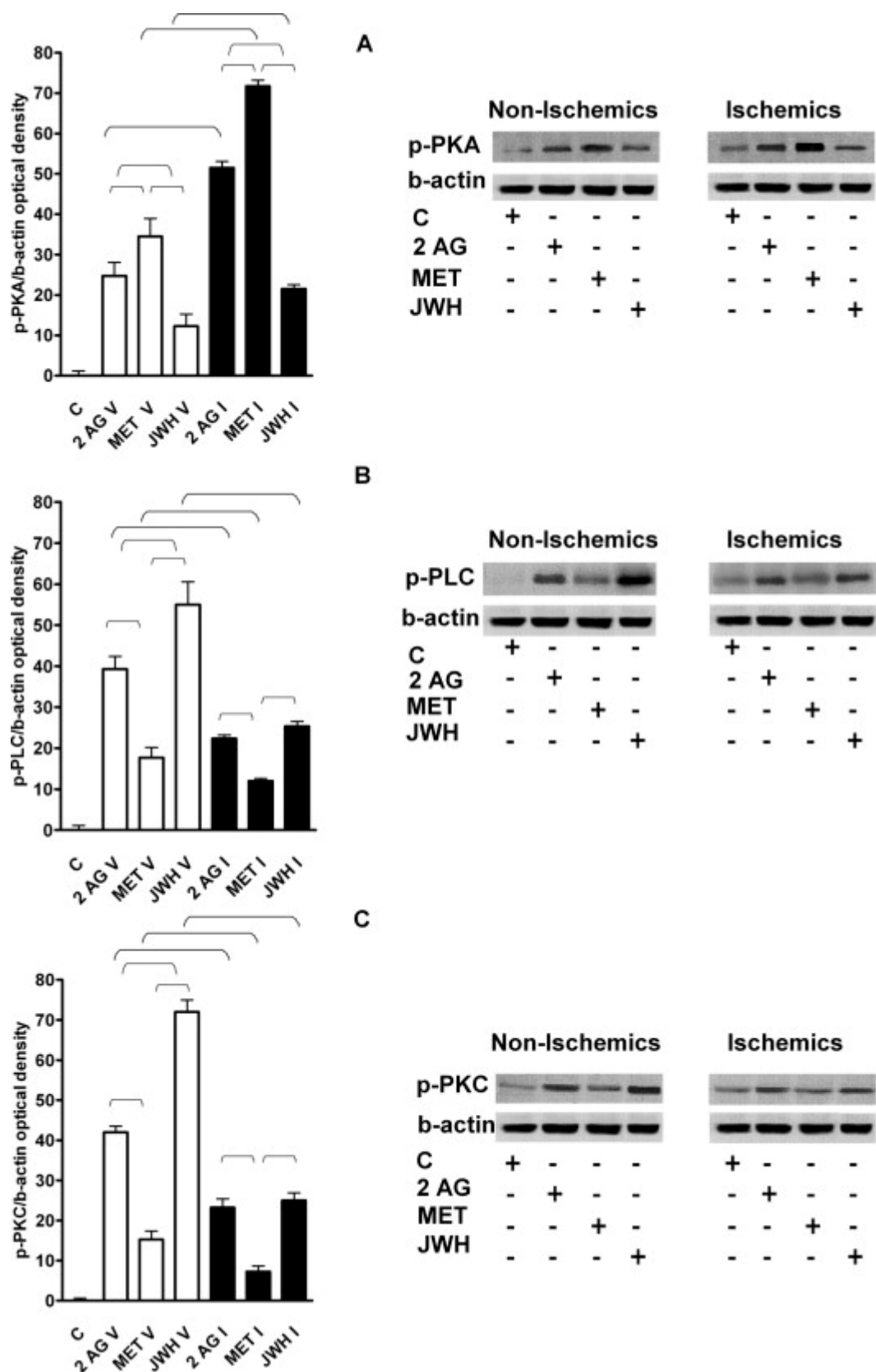


Figure 5.

CB agonists differentially activate PKA, PLC and PKC in preadipocytes. **(A, B, C)** Western blot and densitometric analysis of p-PKA, p-PLC, and p-PKC are reported. As shown, a different activation of p-PKA and p-PLC/p-PKC in response to CB agonists was found between ischemics and nonischemics. The abbreviations are the same used in previous Figures. Reported data are mean \pm SD of 5

independent experiments for each experimental protocol. 2-AG, 2 arachidonoyl-glycerol; C, control; I, ischemic patient; JWH, JWH015; MET, methanandamide; PLC, phospholipase C; PKA, protein kinase A; PKC protein kinase C; V, nonischemic patient.

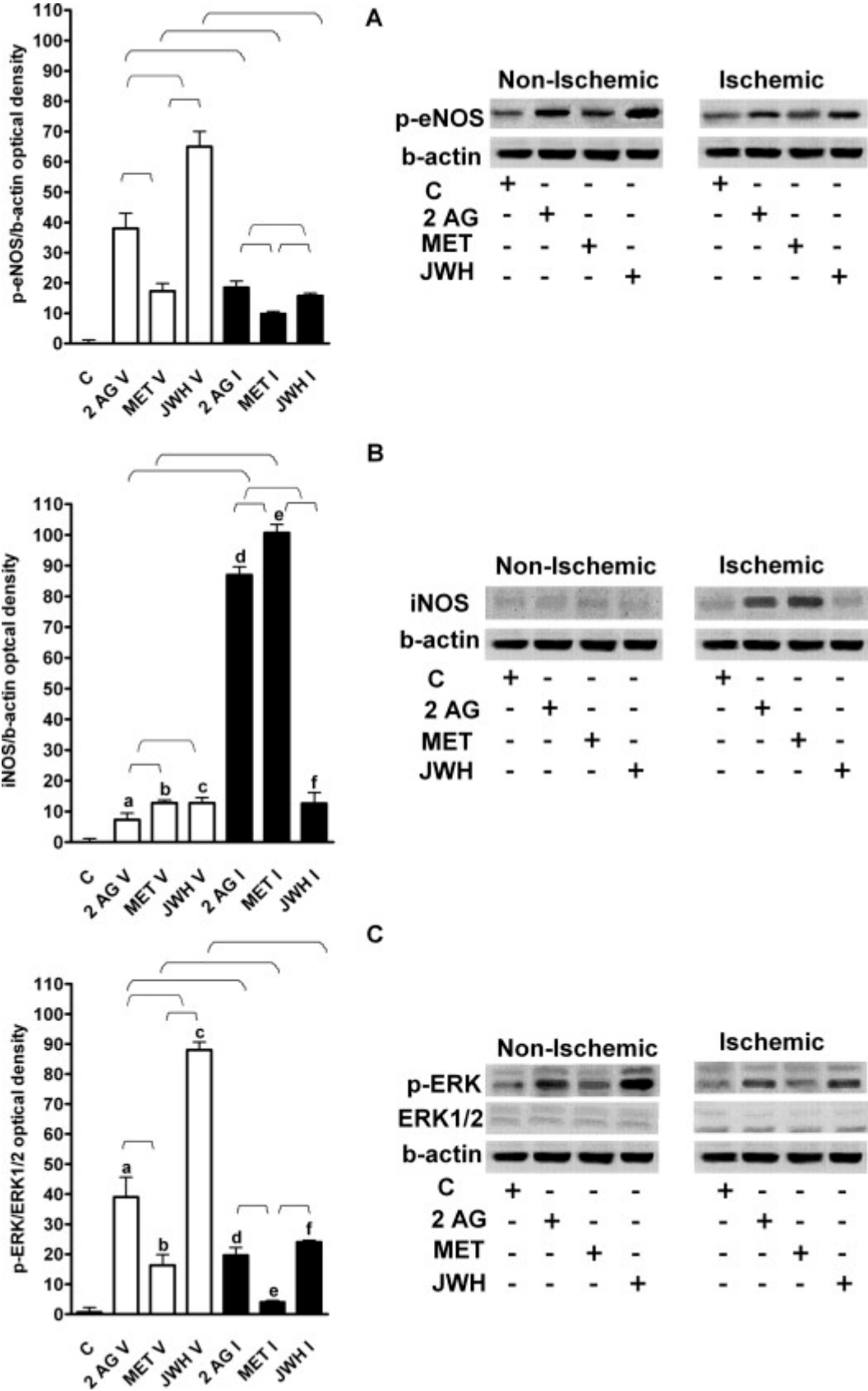


Figure 6.

CB agonists differentially activate eNOS, iNOS, and ERK1/2 in preadipocytes. **(A, B, C)** Western blot and densitometric analysis of p-eNOS, iNOS, and ERK1/2 are reported. CB agonists caused higher iNOS activation in preadipocytes from ischemics, whereas the activation of eNOS and ERK1/2 was higher in those from nonischemics. Reported data are mean \pm SD of 5 independent experiments for each experimental protocol. C, control; ERK1/2, extracellular signal-regulated kinases 1 and 2; eNOS, endothelial nitric oxide synthase; I, ischemic patient; iNOS, inducible nitric oxide synthase; JWH, JWH015; MET, methanandamide; V, nonischemic patient.

Discussion

This study showed different CB1 and CB2 expression and function in EAT between ischemics and nonischemics and suggests that these differences may play a role in the cardiac dysfunction developed by the former.

ECS expression and function in cardiovascular pathology

The ECS is reportedly involved in the physiological regulation of food intake and metabolism of energetic substances²³ and in the regulation of the cardiovascular system. Tonic activation of CB1 is implicated in the development of various cardiovascular risk factors in obesity, metabolic syndrome, and diabetes,²⁴ and furthermore, CB1 antagonists improve survival in rat models of myocardial ischemia²⁵ and cardiac function in cardiomyopathy.²⁶ Likewise, the protective effect of endocannabinoids via CB2 has been demonstrated in rat models of acute and chronic myocardial ischemia.¹⁵ CB2 activation can limit the endothelial inflammatory response, chemotaxis, and inflammatory cell adhesion and activation in atherosclerosis and reperfusion injury.²⁴ Moreover, increasing evidence implicates the ECS in the pathogenesis of various cardiovascular diseases²⁷ and in the cardiovascular risk factors associated with obesity, metabolic syndrome, and diabetes.^{13, 27, 28 and 29} Hence, pathologic overactivation of the ECS in various forms of shock and heart failure can contribute to the cardiodepressive state by activation of CB1 receptors.²⁴

Shift of CB1-to-CB2 ratio and FAAH expression in EAT in ischemics and nonischemics

The results obtained in the present study confirm these reports. Hence, comparison of EAT from ischemics and nonischemics showed a shift of the CB1-to-CB2 expression ratio detected through Western blot toward CB1 in the former, accompanied by downregulation of FAAH, which is expected to support the endocannabinoid function. It is interesting that the semiquantitative analysis of messenger RNA of CB1 also confirmed the above findings. Moreover, the different expressions were detected in EAT but not in SAT, which suggests that the direct interaction between EAT and myocardial tissue may influence the ECS in EAT, and the EAT profile may affect myocardium.

A dichotomous role has been attributed to EAT. Under physiological conditions, EAT could have a protective effect on coronary arteries and cardiac function, whereas possible hemodynamic abnormalities could turn it into an adverse proinflammatory organ. Additionally, since EAT and myocardial tissues share the same coronary arterial supply, it is reasonable to hypothesize that changes in local vascularization may influence the effects of EAT on cardiac functionality³⁰ also through a modulation of the biochemical and metabolic pattern and receptor expression involving the ECS.

Changes in NO release and related signalling examined in preadipocytes isolated from EAT taken from ischemics and nonischemics

We found that treatment of EAT-derived preadipocytes with the CB1 agonist, methanandamide,³¹ induced a higher time- and dose-dependent NO release in ischemics than in nonischemics, and this effect was specifically inhibited by the CB1 antagonist, AM251. Reciprocally, the CB2 agonist, JWH015, induced higher

NO production in nonischemics than in ischemics, and this effect was specifically inhibited by the CB2 antagonist, AM630.

The signalling data in preadipocytes supported this scenario, since ischemics displayed predominant activation of the PKA pathway and iNOS induced via CB1 activation, whereas nonischemics displayed predominant activation of the PLC/PKC pathway and eNOS induced via CB2 stimulation. In line with these findings, the CB1-mediated production of NO in ischemics was specifically inhibited by PKA inhibitors, whereas that mediated by CB2 in nonischemics was specifically inhibited by PKC inhibitors. A potential cAMP/PKA and PLC/PKC pathway in CB receptor-coupled signal transduction is not a new finding.² Moreover, these data confirm previous observations obtained in human adipocytes and preadipocytes³ and those related to CB1- and CB2-coupled G_s or G_{i/o} protein function modulation.³² Hence, while CB1 was reported to modulate adenylyl cyclase activity and the cAMP/PKA pathway through its ability to couple to both G_s and G_{i/o} proteins, CB2 was found to interact only with G_{i/o} protein, thus reducing the cAMP/PKA pathway.²

The shift of the iNOS-to-eNOS ratio toward iNOS may be relevant for ischemics, since iNOS produces the highest NO amounts and has been involved in several pathologic conditions.¹⁵ NO has been described as a biological “double-edged sword,” playing roles in cellular protection but also in cytotoxicity.³³ In particular, low levels of NO were shown to reversibly inhibit mitochondrial transition pore opening, whereas high levels could be involved in oxido-nitrosative tissue damage³⁴ and would cause apoptosis.³⁵

The results obtained in preadipocytes are in line with observations on the different roles played by CB1 and CB2 in several systems. On one hand, CB1 activation in endothelial cells and cardiomyocytes is shown to amplify the reactive oxygen species–MAPK-dependent cell death pathway in inflammatory conditions and oxidative and nitrosative stress.^{36 and 37} On the other hand, the stimulation of CB2 has been found to protect the kidney against cisplatin-induced injury, to contribute to infarct size–limiting effects mediated by anandamide, and to limit atherosclerotic plaque progression by attenuating the inflammatory response.^{33, 38, 39 and 40} Moreover, the activation of CB2 was found to be involved in cardiac protection against ischemia and reperfusion injuries through the restoration of iNOS-to-eNOS equilibrium.¹⁵ ERK1/2 is a member of the so-called reperfusion injury salvage kinase pathway, which is essential for cardioprotection against ischemic injuries.⁴¹ Thus, the fact that ERK1/2 activation was higher in nonischemics than in ischemics would indicate a cell death–prone status in the latter. Furthermore, recent findings have shown that endocannabinoids could exert a strong cardioprotective effect mainly through CB2 with the involvement of p38, ERK1/2, and PKC activation.¹⁴ In addition, this study shows for the first time that changes in CB receptor expression can be observed in ischemic cardiac disease in EAT and human myocardium.⁷

Conclusions

At present, one cannot rule out the causal relationship between myocardial ischemia and the EAT CB1 bias, however recent findings showing that FAAH activity suppresses the myocardial cell death induced by endocannabinoid, which was partly mediated by CB1 stimulation.⁴² supports the idea that the setting of the ECS in EAT may indeed play a role in the myocardial damage in ischemics. Furthermore, and although not clearly demonstrated, the shift of CB1/CB2 ratio toward CB1 in ischemics could be related to changes in local vascularization affecting biochemical, metabolic pattern and receptors expression of EAT, involving the ECS, as well.

Our ischemic heart-disease patients differed from the patients without ischemic heart disease in 2 significant ways: (1) a much higher prevalence of prior myocardial infarction (MI) and (2) much more history of heart failure. The results of the present study do not allow us to determine whether the differences in the ECS might have contributed to the risk of MI or heart failure, whether prior MI or heart failure contributed to the endocannabinoid changes, or whether there is no causal relationship. Further work is needed to address the potential pathophysiological role of the ECS in epicardial fat on myocardial function and ischemia, and of myocardial ischemia and failure on the epicardial fat ECS.

In summary, our results suggest a different role played by the ECS in EAT in the presence or absence of ischemic heart disease. In cases of ischemia, CB1 receptor and PKA upregulation, accompanied by CB2 receptor and FAAH downregulation, lead to an increased iNOS-to-eNOS ratio and reduction of cell survival signalling. In nonischemic conditions, the CB2 receptor-related pathway is involved in cardioprotection through iNOS-to-eNOS ratio restoration and the involvement of PLC/PKC and ERK1/2 signalling.

Funding Sources

This work was supported by the Fondazione Comunità Novarese Onlus (Novara), Fondazione Cariplo (grant no. 2008-2459 to M.P.) and Regione Piemonte (Ricerca Finalizzata: grants no. 2008-2981 to M.P.).

Disclosures

The authors have no conflicts of interest to disclose.

References

1.

- D. Piomelli
- **The molecular logic of endocannabinoid signalling**
- Nat Rev Neurosci, 4 (2003), pp. 873–884

2.

- D.G. Demuth, A. Molleman
- **Cannabinoid signalling**
- Life Sci, 78 (2005), pp. 549–563

3.

- R. Roche, L. Hoareau, S. Bes-Houtmann, *et al.*
- **Presence of the cannabinoid receptors, CB1 and CB2, in human omental and subcutaneous adipocytes**
- Histochem Cell Bio, 126 (2006), pp. 177–187

4.

- V. Di Marzo
- **The endocannabinoid system in obesity and type 2 diabetes**
- Diabetologia, 51 (2008), pp. 1356–1367

5.

- A.C. Howlett, F. Barth, T.I. Bonner, *et al.*
- **International union of pharmacology: XXVII. Classification of cannabinoid receptors**
- Pharmacol Rev, 54 (2002), pp. 161–202

6.

- S. Galiègue, S. Mary, J. Marchand, *et al.*
- **Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations**
- Eur J Biochem, 232 (1995), pp. 54–61

7.

- F. Weis, A. Beiras-Fernandez, R. Sodian, *et al.*
- **Substantially altered expression pattern of cannabinoid receptor 2 and activated endocannabinoid system in patients with severe heart failure**

- J Mol Cell Cardiol, 48 (2010), pp. 1187–1193
- 8.
- P. Lépicier, C. Lagneux, M.G. Sirois, D. Lamontagne
 - **Endothelial CB1-receptors limit infarct size through NO formation in rat isolated hearts**
 - Life Sci, 81 (2007), pp. 1373–1380
- 9.
- Y.A. Shmist, I. Goncharov, M. Eichler, *et al.*
 - **Delta-9-tetrahydrocannabinol protects cardiac cells from hypoxia via CB2 receptor activation and nitric oxide production**
 - Mol Cell Biochem, 283 (2006), pp. 75–83
- 10.
- J.A. Wagner, Z. Járai, S. Bátakai, G. Kunos
 - **Hemodynamic effects of cannabinoids: coronary and cerebral vasodilation mediated by cannabinoid CB(1) receptors**
 - Eur J Pharmacol, 423 (2001), pp. 203–210
- 11.
- A.R. Baker, N.F. Silva, D.W. Quin, *et al.*
 - **Human epicardial adipose tissue expresses a pathogenic profile of adipocytokines in patients with cardiovascular disease**
 - Cardiovasc Diabetol, 5 (2006), p. 1
- 12.
- K. Sugamura, S. Sugiyama, T. Nozaki, *et al.*
 - **Activated endocannabinoid system in coronary artery disease and antiinflammatory effects of cannabinoid 1 receptor blockade on macrophages**
 - Circulation, 119 (2009), pp. 28–36
- 13.
- M. Rajesh, P. Mukhopadhyay, S. Bátakai, *et al.*

- **Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy**
- J Am Coll Cardiol, 56 (2010), pp. 2115–2125

14.

- P. Lépicier, J.F. Bouchard, C. Lagneux, D. Lamontagne
- **Endocannabinoids protect the rat isolated heart against ischaemia**
- Br J Pharmacol, 139 (2003), pp. 805–815

15.

- C. González, E. Herradón, R. Abalo, *et al.*
- **Cannabinoid/agonist WIN 55,212-2 reduces cardiac ischaemia–reperfusion injury in Zucker diabetic fatty rats: role of CB2 receptors and iNOS/eNOS**
- Diabetes Metab Res Rev, 27 (2011), pp. 331–340

16.

- G. Iacobellis, A.C. Bianco
- **Epicardial adipose tissue: emerging physiological, pathophysiological and clinical features**
- Trends Endocrinol Metab, 22 (2011), pp. 450–457

17.

- T. Mazurek, L. Zhang, A. Zalewski, *et al.*
- **Human epicardial adipose tissue is a source of inflammatory mediators**
- Circulation, 108 (2003), pp. 2460–2466

18.

- A.R. Baker, N.F. Silva, D.W. Quinn, *et al.*
- **Human epicardial adipose tissue expresses a pathogenic profile of adipocytokines in patients with cardiovascular disease**
- Cardiovasc Diabetol, 5 (2006), pp. 1–7

19.

- J.N. Fain, H.S. Sacks, S.W. Bahouth, D.S. Tichansky, A.K. Madan, P.S. Cheema
- **Human epicardial adipokine messenger RNAs: comparisons of their expression in substernal, subcutaneous, and omental fat**
- Metabolism, 59 (2010), pp. 1379–1386

20.

- L.F. Van Gaal, A.M. Rissanen, A.J. Scheen, O. Ziegler, S. Rossner
- **Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study**
- Lancet, 365 (2005), pp. 1389–1397

21

- M.E. Fernyhough, J.L. Vierck, G.J. Hausman, *et al.*
- **Primary adipocyte culture: adipocyte purification methods may lead to a new understanding of adipose tissue growth and development**
- Cytotechnology, 46 (2004), pp. 163–172

22

- E. Grossini, C. Molinari, P.P. Caimmi, F. Uberti, G. Vacca
- **Levosimendan induces NO production through p38 MAPK, ERK and Akt in porcine coronary endothelial cells: role for mitochondrial K(ATP) channel**
- Br J Pharmacol, 156 (2009), pp. 250–261

23

- V. Di Marzo, I. Matias
- **Endocannabinoid control of food intake and energy balance**
- Nat Neurosci, 8 (2005), pp. 585–589

24

- P. Pacher, S. Steffens
- **The emerging role of the endocannabinoid system in cardiovascular disease**
- Semin Immunopathol, 31 (2009), pp. 63–77

25

- P. Caraceni, A.M. Pertosa, F. Giannone, *et al.*
- **Antagonism of the cannabinoid CB-1 receptor protects rat liver against ischaemia-reperfusion injury complicated by endotoxaemia**
- Gut, 58 (2009), pp. 1135–1143

26

- J.A. Wagner, M. Abesser, J. Harvey-White, G. Ertl
- **2-Arachidonylglycerol acting on CB1 cannabinoid receptors mediates delayed cardioprotection induced by nitric oxide in rat isolated hearts**
- J Cardiovasc Pharmacol, 47 (2006), pp. 650–655

27

- P. Pacher, P. Mukhopadhyay, R. Mohanraj, G. Godlewski, S. Batkai, G. Kunos
- **Modulation of the endocannabinoid system in cardiovascular disease: therapeutic potential and limitations**
- Hypertension, 52 (2008), pp. 601–607

28

- V. Di Marzo, M. Cote, I. Matias, *et al.*
- **Changes in plasma endocannabinoid levels in viscerally obese men following a 1 year lifestyle modification programme and waist circumference reduction: associations with changes in metabolic risk factors**
- Diabetologia, 52 (2009), pp. 213–217

29

- P. Pacher
- **Cannabinoid CB1 receptor antagonists for atherosclerosis and cardiometabolic disorders: new hopes, old concerns?**
- Arterioscler Thromb Vasc Biol, 29 (2009), pp. 7–9

|

30

- G. Iacobellis, A.E. Malavazos, M.M. Corsi
- **Epicardial fat: from the biomolecular aspects to the clinical practice**
- Int J Biochem Cell Biol, 43 (2011), pp. 1651–1654

31

- A. Barbonetti, M.R. Vassallo, D. Fortunato, S. Francavilla, M. Maccarrone, F. Francavilla
- **Energetic metabolism and human motility impact of CB₁ receptor activation**
- Endocrinology, 151 (2010), pp. 5882–5892

32

- M. Glass, C.C. Felder
- **Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor**
- J Neurosci, 17 (1997), pp. 5327–5333

33

- C. Lagneux, D. Lamontagne
- **Involvement of cannabinoids in the cardioprotection induced by lipopolysaccharide**
- Br J Pharmacol, 132 (2001), pp. 793–796

34

- S.S. Soskić, B.D. Dobutović, E.M. Sudar, *et al.*
- **Regulation of inducible nitric oxide synthase (iNOS) and its potential role in insulin resistance**
- Open Cardiovasc Med J, 5 (2011), pp. 153–163

35

- H.M. Razavi, J.A. Hamilton, Q. Feng
- **Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure**
- Pharmacol Ther, 106 (2005), pp. 147–162

36

- M. Rajesh, P. Mukhopadhyay, G. Haskó, L. Liaudet, K. Mackie, P. Pacher
- **Cannabinoid-1 receptor activation induces reactive oxygen species-dependent and -independent mitogen-activated protein kinase activation and cell death in human coronary artery endothelial cells**
- Br J Pharmacol, 160 (2010), pp. 688–700

37

- P. Mukhopadhyay, M. Rajesh, S. Bátkai, *et al.*
- **CB1 cannabinoid receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes**
- Cardiovasc Res, 85 (2010), pp. 773–784

38

- J. Liu, S. Batkai, P. Pacher, *et al.*
- **Lipopolysaccharide induces anandamide synthesis in macrophages via CD14/MAPK/phosphoinositide 3-kinase/NF-kappaB independently of platelet-activating factor**
- J Biol Chem, 278 (2003), pp. 45034–45039

39

- P.E. Szmitko, S. Verma
- **The endocannabinoid system and cardiometabolic risk**
- Atherosclerosis, 199 (2008), pp. 248–256

40

- P. Mukhopadhyay, M. Rajesh, H. Pan, *et al.*
- **Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress, and cell death in nephropathy**
- Free Radic Biol Med, 48 (2010), pp. 457–467

41

- D.J. Hausenloy, A. Tsang, M.M. Mocanu, D.M. Yellon
- **Ischemic preconditioning protects by activating prosurvival kinases at reperfusion**
- Am J Physiol Heart Circ Physiol, 288 (2005), pp. H971–H976

42

- P. Mukhopadhyay, B. Horváth, M. Rajesh, *et al.*
- **Fatty acid amide hydrolase is a key regulator of endocannabinoid-induced myocardial tissue injury**
- Free Radic Biol Med, 50 (2011), pp. 179–195