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Relazione 3° anno

Espressione del sistema degli endocannabinoidi (CB1-CB2) nel tessuto adiposo epicardico umano

Candidato: Cristiano Ariatti

***Responsabile del progetto*: Prof. Umberto Dianzani**

1.1 Introduzione e razionale

Le malattie cardiovascolari rappresentano la principale causa di morte nel mondo occidentale.

Il diabete di tipo 2, l'obesità e l'insulino-resistenza rappresentano i più importanti fattori di rischio cardiovascolare.

La nozione che l'infiammazione giochi un ruolo importante nello sviluppo dell'insulino-resistenza e del diabete è sostenuta da molteplici linee di evidenza.

Il concetto di infiammazione associato alle malattie metaboliche, come l'obesità e l'insulino-resistenza, risale alle osservazioni fatte da Hotamisligil agli inizi degli anni Novanta, il quale dimostrò che gli adipociti esprimono costitutivamente la citochina proinfiammatoria TNF α e che l'espressione di TNFα negli adipociti di animali obesi (topi *ob/ob*, e ratti *fa/fa* Zucker) è marcatamente aumentata. Inoltre, in questo modello sperimentale, la neutralizzazione di TNF α mediante recettori solubili del TNFα comportava la riduzione dell'insulino-resistenza. [(1)]

Tali osservazioni furono la prima dimostrazione del legame esistente tra aumentata espressione di citochine, livelli di citochine proinfiammatorie ed insulino-resistenza.

Successivamente veniva dimostrato che anche il tessuto adiposo umano era capace di esprimere costitutivamente TNFα e che la sua espressione si riduceva dopo perdita di peso. [(2)] Comunque, i tentativi per ridurre l'attività del TNFα nell'uomo, mediante infusione di anticorpi anti-TNFα non hanno avuto successo nel migliorare la sensibilità insulinica. [(3)]

Un'altra recente acquisizione riguarda la compartimentalizzazione del tessuto adiposo. Diversi studi hanno oramai dimostrato che solo alcuni distretti di tessuto adiposo umano sono in grado di determinare insulino-resistenza e tra questi vanno ricordati quello sottocutaneo, quello omentale e quello epicardico, viceversa il tessuto adiposo gluteo-femorale sembra avere un ruolo protettivo nei confronti delle malattie cardiovascolari. [(4-8)]

Il tessuto adiposo epicardio viene dunque considerato come un vero e proprio organo endocrino in grado di secernere, oltre al TNFα, diversi mediatori tra cui la leptina, l'adiponectina, la resistina, e il PAI 1. [(9-10)] Queste molecole, definite adipocitochine, giocano un ruolo importante nell'omeostasi metabolica e quando la loro produzione non è correttamente regolata possono contribuire alla genesi di disordini metabolici come la sindrome metabolica, il diabete di tipo 2 e l'aterosclerosi.

Il 1994 è stato l'anno in cui venne identificata per la prima volta la leptina, un ormone prodotto principalmente, anche se non esclusivamente, dall'adipocita. Da tale scoperta si è venuta a

generare nuova luce sui meccanismi deputati al controllo dell'introito alimentare e dell'omeostasi energetica.

L'ipotalamo infatti è prepotentemente emerso come centralina di segnali circa lo stato nutrizionale percepito in periferia che qui vengono integrati e quindi nuovamente direzionati verso gli organi periferici deputati al controllo energetico.

Tra i molteplici attori di questa comunicazione centro-periferia il sistema cannabinoide endogeno si è posto recentemente in luce per l'insolita capacità d'azione di interagire a livello di bilancio energetico sia a livello centrale che periferico. [11-13]

1.2 Gli endocannabinoidi e i loro recettori

I cannabinoidi endogeni o endocannabinoidi sono una famiglia numerosa di mediatori lipidici. Si tratta di molecole di piccole dimensioni e con proprietà lipofiliche, accomunate, dal punto di vista strutturale, dal fatto di essere delle amidi di acidi grassi. I principali cannabinoidi endogeni presenti nei tessuti e nei liquidi biologici sono l'arachidonil-etanolamide (AEA), noto anche come anandamide, ed il 2-arachidonoil-glicerolo (2AG). [14] Si tratta di due molecole entrambe derivate dall'acido arachidonico e che legano con diversa affinità i recettori CB1 e CB2. Numerose sono inoltre le molecole sintetiche in grado di legare i recettori CB1 e CB 2 con azione agonista o antagonista e con un potenziale sviluppo farmacologico nelle diverse funzioni biologiche in cui il sistema endoccanabinoide è coinvolto. [15-16]

Gli endocannabinoidi vengono sintetizzati "a domanda" per l'azione di specifiche vie enzimatiche che utilizzano, come substrato di partenza, dei fosfolipidi abbondantemente presenti nella struttura della membrana plasmatica. La fosfatidil-etanolamina è utilizzata per la sintesi dell'AEA mentre la sintesi del 2-AG utilizza il fosfatidil-inositolo.

La degradazione degli endocannabinoidi è altrettanto rapida e prevede la degradazione dell'AEA ad acido arachidonico ed etano lamina ad opera dell'enziam FAAH (fatty acids amide hydrolase) mentre la degradazione del 2-AG prevede l'idrolisi della molecola da parte dell'enzima monoacilglicerolo-lipasi (MAGL). [17] E' possibile pertanto ritenere che in condizioni normali il sistema degli endocannabinoidi non sia attivo, e che questi mediatori lipidici vengano prodotti acutamente su domanda, agiscano localmente e vengano rapidamente inattivati.

I cannabinoidi esercitano la loro azione farmacologica attraverso le interazioni con specifici recettori CB1 e CB2, che sono stati descritti e clonati tra gli anni '80 e '90. CB1 è espresso in maniera preferenziale a livello del SNC, del tessuto adiposo, muscolare scheletrico e miocardico, nel tratto gastrointestinale, nel fegato e a livello delle cellule endoteliali. Il recettore CB2 invece, è localizzato nelle cellule del sistema linfatico e nei macrofagi periferici.[18]

I recettori per gli endocannabinodi hanno un'elevata affinità per i due ligandi endogeni l'anandamide e il 2-AG che appartengono ad una vasta famiglia di composti lipidici (lipid mediators) con affinità variabile con i recettori CB1 e CB2.

1.3 Gli endocannabinoidi e la regolazione ipotalamica del bilancio energetico

Dopo la scoperta dei recettori per i cannabinoidi e la disponibilità di molecole di sintesi ad azione agonista ed antagonista, la ricerca sugli effetti del sistema cannabinoide endogeno subì una forte accelerazione ed il ruolo di questo sistema nel controllo centrale e periferico del bilancio energetico iniziò a chiarirsi.

Nel 2001 venne chiaramente dimostrato come i livelli di anandamide e 2-AG erano aumentati nell'ipotalamo di topi *ob/ob, db/db* e ratti Zucker, 3 modelli di obesità animale geneticamente determinati. Inoltre il trattamento con leptina, un ormone anoressante periferico prodotto dall'organo adiposo, era in grado di ridurre i livelli di endocannabinoidi ipotalamici sia nei topi normali che nei topi ob/ob che sono geneticamente privi di leptina circolante. [19]

Complessivamente i risultati di questi esperimenti hanno indicato nel sistema cannabinoide ipotalamico un importante regolatore del consumo di cibo, integrato con altre vie ipotalamiche di controllo del bilancio energetico già da tempo note, in particolare con il sistema leptina-neuropeptide Y. Nei topi *knockout* per il gene CB1 si osserva una lieve, ma significativa riduzione della percentuale di peso corporeo e di massa grassa. [20]

1.4 Gli endocannabinoidi e la regolazione del metabolismo periferico

Recentemente alcuni dati sperimentali, ottenuti in modelli animali ed in linee cellulari, hanno mostrato che il sistema cannabinoide è presente con i suoi recettori ed i suoi enzimi di sintesi e degradazione, a livello di numerosi tessuti periferici. [21-24] Il recettore CB1, inizialmente ritenuto espresso solo nel SNC, è presente anche negli adipociti. [25] Inoltre è stato dimostrato che ratti geneticamente obesi (Zucker *fa/fa*) hanno livelli aumentati di espressione di CB1 nel tessuto adiposo.

E' stato quindi ipotizzato che nell'obesità sia presente un'iperattivazione del sistema endocannabinoide anche nel tessuto adiposo e che questa iperattivazione sia anch'essa responsabile dell'eccessiva deposizione di tessuto adiposo. In linee cellulari di adipociti come la linea 3T3-F442A è stata evidenziata una up-regolazione del sistema cannabinoide nello stadio che precede la

differenziazione adipocitaria, responsabile probabilmente della differenziazione della cellula ad adipocita maturo. [26] Questi dati, però, non sono stati confermati nell'uomo, dove i livelli d'espressione sia del recettore CB1 che dell'enzima FAAH nel tessuto adiposo sottocutaneo sono risultati francamente down-regolati nell'obesità.

I recettori CB1 sono presenti anche nel fegato e sembrano coinvolti, almeno nel topo, nella lipogenesi e nello sviluppo della steatosi epatica. Infatti, Osei-Hyiaman et al. [27] hanno dimostrato nel modello murino che l'attivazione del recettore CB1 aumenta l'espressione epatica del fattore di trascrizione SREBP 1c e dei geni da esso regolati (acetil CoA carbossilasi, acido-grasso sintetasi). Il trattamento con un antagonista recettoriale del CB1 riduce la lipogenesi epatica.

Questi dati, anche se necessitano di dimostrazioni dirette nell'uomo, indicherebbero che il sistema endoccanabinoide è profondamente coinvolto nella lipogenesi epatica ed è potenzialmente implicato nella patogenesi del patologico accumulo di trigliceridi nella steatosi epatica associata alla sindrome metabolica.

Studi condotti su modelli animali di obesità genetica hanno dimostrato che il recettore CB1 è up-regolato nei topi. [28] Inoltre, come è noto, l'espressione nel tessuto adiposo ed i livelli circolanti di adiponectina sono ridotti nell'obesità. Il trattamento con un antagonista CB1 è in grado di up-regolare l'espressione dell'adiponectina nel tessuto adiposo degli animali obesi. [28] Questi dati suggeriscono che gli effetti benefici del trattamento con l'antagonista CB1 possa essere, almeno in parte, mediato attraverso un meccanismo dipendente dall'adiponectina.

2.1 Scopo del lavoro

Scopo di questo lavoro è stato di dimostrare che il tessuto adiposo epicardico umano esprime il sistema degli endocannabinoidi (CB1 e CB2) sia come RNA messaggero che come proteina. L'immunoistochimica ha inoltre permesso di chiarire che i recettori CB1 e CB2 sono espressi dagli adipociti maturi. Inoltre, isolando dal tessuto adiposo la frazione vascolare stromale, frazione che contiene i pre-adipociti abbiamo dimostrato che quest'ultimi se seminati in un terreno di differenziamento adipogenico divengono adipociti maturi. Il fenotipo adipogenico è stato valutato osservando la morfologia delle cellule e colorando con un reagente commerciale i vacuoli; valutando poi negli adipociti maturi l'espressione mediante RT-PCR del messaggero dell'adiponectina, un noto marcatore di adipogenesi.

3.1 Risultati e Discussione

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CB1 AND CB2 RECEPTORS EXPRESSED IN HUMAN EPICARDIAL ADIPOSE TISSUE

Giuseppe Cappellano[1,2*] PHD, Stefano Pietronave[1*],PHD, Franco Aina[3],MD, Philippe Primo Caimmi[3,4]MD, Ezio Micalizzi[4] MD, Chiara Dianzani[5]PHD, Matteo Melensi PHD[1], Giovanni Teodori[4]MD, Renzo Boldorini[1]MD, Gabriella Nicosia[1]PHD, Enrico Crosio[1]MD, Annalisa Chiocchetti[1]MD, Maria Prat[1]MD, Umberto Dianzani[1]MD, Cristiano Ariatti[1]MD.

[1]Research Center of Autoimmune Diseases (IRCAD) and Department of Medical Sciences, "A. Avogadro" University of Eastern Piedmont, Novara, Italy;

[2]Div. of Clinical Immunology, Dept. of Laboratory Medicine, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden;

[3]Cardiac Rehabilitation Unit, University Hospital "Maggiore della Carità", Novara, Italy;

[4]Cardiac Surgery Unit, University Hospital "Maggiore della Carità", Novara, Italy;

[5]Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Torino, Italy.

[*]**These authors contributed equally to the work.**

§Corresponding author

Address correspondence to (corresponding author):

Giuseppe Cappellano, PHD

IRCAD-Department of Medical Sciences

Via Solaroli 17, 28100 Novara, Italy

phone:(+39)0321-660658;fax:(+39)0321-620421

giuseppe.cappellano@med.unipmn.it

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ABSTRACT

Background: Endocannabinoids (EC) regulate energy balance by modulating hypothalamic circuits controlling food intake and energy expenditure. Recent evidence indicates that they can also be produced by adipocytes and modulate adipogenesis, lipogenesis, and glucose uptake by acting on these cells. Two distinct mammalian cannabinoid receptors have been identified: the CB1 receptor which is primarily located in the central nervous system, but is also found in several other tissues and the CB2 receptor which is exposed in cells of the immune system, omental and subcutaneous adipocytes. The aim of this work was to evaluate the CB1 and CB2 expression in human epicardial adipose tissue (EAT) which is in close contact with the heart adventitia without interposition of a facial layer. ***Methods:*** The expression of CB1 and CB2 was assessed by RT-PCR and western blot analyses. Immuno-histochemistry was used to detect their expression at the surface of epicardial adipocytes. ***Results:*** RT-PCR and western blot detected a substantial amounts of both CB1 and CB2 in human epicardial adipose tissue. Moreover, RT-PCR detected CB1 and CB2 expression in the adherent fraction of stromal cells isolated from epicardial adipose tissue and expression was maintained upon their in vitro adipocytic maturation driven by an adipogenic tissue culture medium. ***Conclusion:*** The present study showed that CB1 and CB2 are expressed in human EAT and they may also play a role in EAT metainflammation which may be crucial for coronary and cardiac diseases because of the close proximity with these tissues.

Key words: CB1, CB2, epicardial adipose tissue, endocannabinoids

Introduction

The adipose tissue secretes several bioactive molecules, known as adipokines, that exert endocrine and paracrine functions influencing body weight, coagulation, fibrinolysis, insulin resistance, inflammation, and atherosclerosis [1]. Recently, adipocytes have been shown to produce endocannabinoids (EC) [2], which are generally envisaged as neuromodulatory molecules regulating neural excitability and involved in increasing food intake by interacting with the leptin-regulated central neural appetitive circuit [3-4]. They comprise the phospholipid anandamide (AEA) and the triglyceride 2-arachidonoylglycerol (2AG), that are produced from membrane lipids by the enzimatic activity of diacylglicerol lipase (DAGL) α and β, and *N*-acyl-phosphatidyl ethanolamine-hydrolysing phospholipase D (NAPE-PLD) respectively. Both AEA and 2AG are degraded by the enzymatic activity of fatty acid amidohydrolase (FAAH) and monoacyl glycerol lipase (MAGL) [4].

EC interact with cannabinoid receptor 1 (CB1) and 2 (CB2) expressed on the cell surface and exhibit about 44% homology. CB1 is expressed at high levels by both neurons and glial cells in the central nervous system (CNS), and at low levels in testicle, uterus, intestine, bladder, retinal and endothelial cells [5-6]. CB2 is primarily expressed by cells of the immune system, but it is also found in the lungs, uterus, and pancreas [6]. Recently, Roche et al. reported expression of both CB1 and CB2 in human omental and subcutaneous adipocytes [7]. CB1 and CB2 expression outside of CNS has been involved in feeding control, energy homeostasis, and metabolism modulation [1]. Dysregulation of their function is believed to contribute to obesity and type 2 diabetes [8]. In obese subjects, treatment with the CB1 antagonist Rimonabant induced significant reduction of body weight, waist circumference, insulin resistance, levels of insulin and glycosylated haemoglobin, adiponectin secretion from adipocytes, and also it displayed a positive effect on type 2 diabetes control [8-11]. In animal models, CB1 modulates the lipid and glucose metabolism in insulin-sensitive tissues, such as the adipose tissue [12] and the liver [13]. Part of the deleterious effects of obesity have been ascribed to metabolically-triggered inflammation, or "metaflammation", which might play a key role in the development of cardiovascular diseases, such as hypertension and

atherosclerosis, and metabolic disorders, such as type 2 diabetes. Metainflammation is caused by accumulation of adipose tissue macrophages (ATMs) and expression of inflammatory cytokines such as TNFα and IL-6 in the adipose tissue of obese mice and humans. The EC system may play a role in metainflammation as CB1 blockade significantly reduces the production of proinflammatory mediators of human macrophages in vitro [14-15]. Moreover, CB1 is expressed in the human coronary atherosclerosis plaques, especially in lesional macrophages [15]. Epicardial adipose tissue (EAT), which substantially increases with obesity, may play a key role in cardiometabolic disease, because it lies in close contact with the heart adventitia without the interposition of one fascial layers. This proximity of the adipose and myocardial tissues suggests that factors released by EAT may support coronary wall inflammation and stimulate progression of atherosclerosis from "outside to inside" of the vessels [15-16]. Moreover, excessive deposits of lipids within the myocardial tissue may be involved in non ischemic dilated cardiomyopathy by "cardiac lipotoxicity". These effects might theoretically involve the EC system, but expression of the EC receptors in EAT has never been investigated before. Therefore, this work investigated CB1 and CB2 expression in EAT and found that both are expressed in EAT adipocytes, which opens the way to the possibility that the beneficial effect exerted on cardio metabolic risk by pharmacological blockade of CB1 receptor might be partly ascribed to effects on EAT [17-18].

Materials and Methods

Tissue collection

Human EAT specimens (n=5) were collected from patients who underwent cardio-surgey intervention. Epicardial biopsies (average 0.5-1 g) were taken approximately 1 hour after anaesthesia, near to the proximal tract of the right coronary artery. Omental and subcutaneous biopsies were collected from control donors who underwent abdominal surgery. After removal, tissue specimens were flash frozen immediately for RT-PCR and Western Blot analysis or immediately processed with collagenase for ulterior studies on isolated cell populations.

All biopsies were collected in accordance with the local ethic committee guidelines and informed consent was obtained from all patients.

Isolation of stromal cells from human adipose epicardial and subcutaneous tissues

Human epicardial and subcutaneous adipose tissues were physically minced and then digested at 37°C in PBS containing 2% BSA and collagenase (1mg/ml, Worthington) for 40 min. Then, the debris was removed by filtering with a 70 μm cell strainer (Falcon), whereas mature adipocytes were removed as floating cells after low speed centrifugation. Cell pellets were re-suspended in complete medium (DMEM plus 10% FBS) and plated at a density of 50.000 cells/cm^2. The adherent fraction of stromal cells was then cultured in adipose commitment medium (Cambrex) and induction of the mature phenotype was detected by morphologic appearance of citoplasmic lipid vacuoles and RT-PCR detection of tissue specific mRNAs.

Reverse Transcription Polymerase Reaction (RT-PCR) and Real Time PCR

The adipose tissues (epicardial, omental and subcutaneous), after mechanical mincing, as well as the cultures of adipose epicardial and subcutaneous stromal cells were treated with TRIzol reagent (Invitrogen) to extract RNA. Then, 0.5 μg total RNA was reverse by transcribed to cDNA using ThermoScript ^{TM} RT-PCR System (Invitrogen). To perform RT-PCR, 1 μL cDNA was amplified using the Taq Poymerase (Invitrogen) in a 25 μl final volume using the following primers: CB1: 5-GAGCTCAGCCTAATCAAAG-3 (forward), 5'-TATGTACCTGTCGATGGC-3' (reverse); CB2 5'-ACAAGCTCAGTGGAATCTG-3'(forward); 5'-ATAGTCACGCTGCCAATC-3' (reverse); GAPDH: 5'-ACCACAGTCCATGCCATC-3' (forward), 5'-TCCACCACCCTGTTGCTGTA-3'(reverse). PPARγ expression was assessed by Real Time PCR using the Taq-Man probe for PPARγ (assay ID: Hs01115512_m1) and GUSB (assay ID: Hs99999908_m1) purchased from Applied Biosystems, and an ABI Prism 7000 Sequence Detector (Applied Biosystems);

quantification was performed by the cycle threshold method, normalized to GUSB mRNA [GENBANK: NM_016083 (CB1); NM_001841 (CB2); NM_002046(GAPDH)].

Western Blot analysis

Minced tissues were treated with a RIPA lysis buffer (Tris Hcl pH 7.2 20mM, sodium deoxycholate 0.5%, Triton-X-100 1%, SDS 0.1%, NaCl 150 mM, EDTA 5 mM) for 20 minutes on ice. Lysates were then cleared by centrifugation for 20 min at 13000 rpm at 4°C. Proteins were quantified by the Bradford dosage assay. Then, 50 µg of proteins were dissolved in a SDS-PAGE loading buffer (63 mM Tris-HCl pH 6.8, 5% Glycerol, 1% SDS, 2.5% bromophenol-blue), separated on 10% SDS-PAGE, and transferred to nitrocellulose. Filters were blocked in a TBST buffer together 5% with non-fat milk for 1 hour and then incubated with antibodies to CB1 (Santa Cruz Biotechnology) and CB2 (Cayman Chemical) overnight at 4°C in TBST buffer plus 5% BSA. Signals were revealed with a HRP-conjugated anti-goat (for CB1) and anti-mouse (for CB2) Ig secondary antibody (Amersham, Arlington Heights, IL) and detected by enhanced chemiluminescence. Tubulin was detected with a mouse anti-tubulin antibody (Sigma-Aldrich, Milan, IT) followed by the same secondary anti-mouse Ig antibody.

Immunohistochemistry

Samples of fat tissue were fixed in 10% neutral formalin and embedded in paraffin; after which, 4 µm thick sections were obtained for histology and immunohistochemical analysis. Immunohistochemistry was performed using an automated staining machine (Dako Autostainer, Dako Co. Denmark); the sections were unmasked by treatment in sodium citrate buffer (10 mM) at 600 Watt on a microwave for 15 min and then incubated for 1 h with polyclonal antibody anti-CB1 (dilution 1:50; Affinity Bioreagents) and anti-CB2 (dilution 1:50 Affinity bioreagents). The subsequent reactions were performed using the EnVision detection System Rabbit/Mouse Kit (Dako Co. Denmark) with universal secondary antibody, and the signal was visualized using 3, 3'-

diaminobenzidine hydrochloride. Then, sections were slightly counterstained with hematoxylin; images were acquired using a microscope equipped with a digital camera (Zeiss, Germany).

Results

Expression of CB1 and CB2 in the EAT was evaluated using three different approaches.

Firstly, we evaluated their mRNA expression by RT-PCR in EAT from 4 subjects who had gone cardio-surgery intervention for a coronary by-pass. In all experiments, the mRNAs derived from omental and subcutaneous adipose tissues were used as controls. mRNAs for both CB1 and CB2 were found to be expressed in EAT as well as in one control tissues (**Fig. 1A**).

Secondly, we evaluated expression of the CB1 and CB2 proteins by western blot in the same samples as described above. The Jurkat T cell line was used as a further control because of the known expression of CB2 in the immune cells. Both CB1 and CB2 proteins, at lower levels, were expressed in EAT, as well in the control adipose tissues, whereas Jurkat cells expressed only CB2 **(Fig. 1B).**

Thirdly, we investigated the expression of the CB1 and CB2 proteins in EAT and omental adipose tissue by immunohistochemistry. All the specimens described above were stained and displayed immunoreactivity for both CB1 and CB2 on the surface of mature adipocytes **(Fig. 2).** Moreover, even the abundant cells detectable in the interstitial spaces of epicardial connective tissue were positive for both CB1 and CB2 expression. These cells are rare in omental adipose tissue and comprise both an inflammatory infiltrate and a stromal cell fraction. Expression of EC-receptors in the inflammatory infiltrate was not surprising since CB2 is known to be expressed in immune cells

[6] and CB1 has been recently shown to be expressed in human macrophages [15], whereas few data are available on their expression in stromal cells.

To investigate the expression of CB1 and CB2 in stromal cells and to follow their expression during adipogenic differentiation, stromal cells were purified from EAT and subcutaneous adipose tissue and then cultured for 2 weeks in adipogenic or control culture medium. Stromal cells which had been isolated from both tissues, differentiated into mature adipocytes characterized by large rounded cytoplasmic vacuoles filled with lipids **(Fig. 3A).** Their adipocyte phenotype was confirmed by evaluating the induction of PPARγ mRNA, which is typically expressed at low level in undifferentiated adipocytes [19] and upregulated in mature adipocytes. Real-time PCR experiments showed that differentiation in the adipogenic medium substantially upregulated the PPARγ mRNA in both cells derived from the epicardial (8-fold) and the subcutaneous adipose tissue (20-fold) **(Fig. 3B)**. By contrast, primary human dermal fibroblasts did not produce lipid droplets in the same culture conditions (data not shown).

RT-PCR assessment of expression of the CB1 and CB2 mRNA in undifferentiated stromal cells and in vitro differentiated adipocytes showed that both mRNA were expressed in undifferentiated and differentiated cells without substantial differences in the cells derived from the two adipose tissues **(Fig. 3C)**.

Discussion

This work shows that CB1 and CB2 are expressed in adipocytes and pre-adipocytes from human EAT, which suggests that EC may play a similar role in the EAT physiology as in the other adipose tissues. This possibility was not obvious because it has been reported that human coronary perivascular adipocytes display a lower level of adipocytic differentiation than adipocytes derived from subcutaneous and visceral adipose tissues [20]. A second point is that EC may also play a role in EAT metainflammation which may be crucial for coronary and cardiac diseases because of the close proximity with these tissues. This potential effect may be particularly important in EAT since

adipocytes from this tissue display marked unbalancement toward a proinflammatory phenotype and produce higher levels of the pro-inflammatory molecules IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) and lower levels of the anti-inflammatory molecule adiponectin than adipocytes from subcutaneous and visceral adipose tissues [20]. Recently, Sugamura et al. showed that CB1 receptors were expressed in human coronary atheromata, particulary in lesional macrophages, and receptor blockade with Rimonabant reduced the production of the proinflammatory mediators from human macrophages [15]. Our data extent the relevance of this observation by suggesting that Rimonabant might also exert a direct effect on adipocyte-driven metainflammation.

Rimonabant traced an innovative and effective path to counteract obesity and the related cardiovascular risk factors [18], but severe psychiatric side effects, such as depression and anxiety, caused its non approval by FDA (Food and Drug Administration) in the United States and its recent withdrawal from the world market. Therefore, additional studies are in progress searching for new CB1 antagonists with similar efficacy but with reduced side effects [21]. In line with a common EC-mediated regulation of these adipose tissues, we obtained preliminary data showing a substantial decrease of EAT thickness, as assessed by echocardiography, in patients (n=5) with abdominal obesity who displayed substantial weight loss upon therapy with Rimonabant.

The magnitude of this decrease of epicardial fat seemed to directly correlate with initial epicardial fat thickness (data not shown).

It is noteworthy that, similarly to omental and subcutaneous adipocytes, EAT adipocytes also express CB2, which is generally envisaged as an immune cell receptor mainly expressed by T and B cells, natural killer cells, and macrophages [6]. The function of CB2 on adipocyte is not known, whereas that expressed by T cells and macrophages has been suggested to modulate activation and migration of these cells [7, 21-25], which may play an important role in the initiation and perpetuation of adipose tissue inflammation.

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Legends to Figures

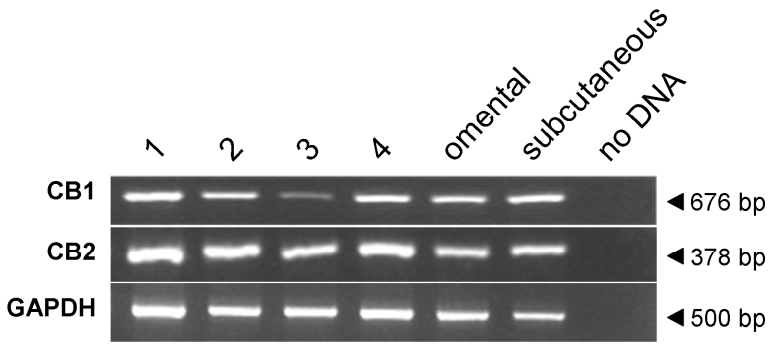
Figure 1. CB1 and CB2 are expressed in human epicardial adipose tissue.

A) RT-PCR was carried out using primers specific for CB1, CB2, and GAPDH. Samples included: epicardial adipose tissue (*lane 1-4*), omental adipose tissue (*lane 5*), subcutaneous adipose tissue (*lane 6*), and negative control (*lane 7*); B) Western blot was carried out using antibodies specific for CB1 or CB2 on lysates from epicardial adipose tissue (*lane 1-4*), omental adipose tissue (*lane 5*) and subcutaneous adipose tissue (*lane 6*) (positive control) and Jurkat cells (*lane 7*:negative control for CB1).

Figure 2. **Plasma membrane expression of CB1 and CB2 receptors by mature adipocytes isolated from omental and epicardial fat tissue**. Omental mature adipocyte (*panel A*) and epicardial mature adipocyte (*panel B*) staining with 1/50 anti-CB1 Ab. Omental mature adipocyte (*panel C*) and epicardial mature adipocyte (*panel D*) staining with 1/50 anti-CB2 Ab. Sections were counterstained with ematossilin.

Figure 3. CB1 and CB2 expression in stromal cells from the epicardial and subcutaneous adipose tissues differentiated or not in vitro with an adipogenic culture medium. *Panel A*: AdipoRed (Cambrex Biosciences) staining of lipid vacuoles in stromal cells from subcutaneous (a, b) and epicardial (c, d) tissues induced to differentiation (b, d) or not (a, c) in adipogenic culture conditions. *Panel B*: real-time PCR analysis of PPARγ mRNA expression in the same cell types shown in Panel A using the GUSB mRNA as a control; samples are marked with the same letter code as in *panel A*; results are showed as the fold increase of the PPARγ /GUSB mRNA expression ratio in cells cultured in adipogenic or control medium. *Panel C*: RT-PCR analysis of CB1 and CB2 mRNA expression in the same cell types shown in *panel A* using the GAPDH mRNA as a control; samples are marked with the same letter code as in *panel A*.

A


1
2
3
4
omental
subcutaneous
no DNA
CB1
CB2
GAPDH
676 bp
378 bp
500 bp

B

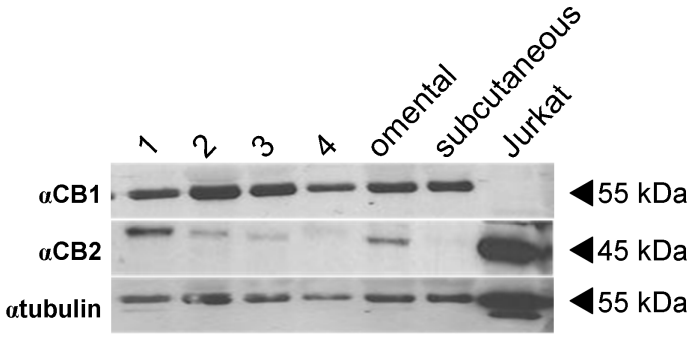

1
2
3
4
omental
subcutaneous
Jurkat
αCB1
αCB2
αtubulin
55 kDa
45 kDa
55 kDa

Fig. 1

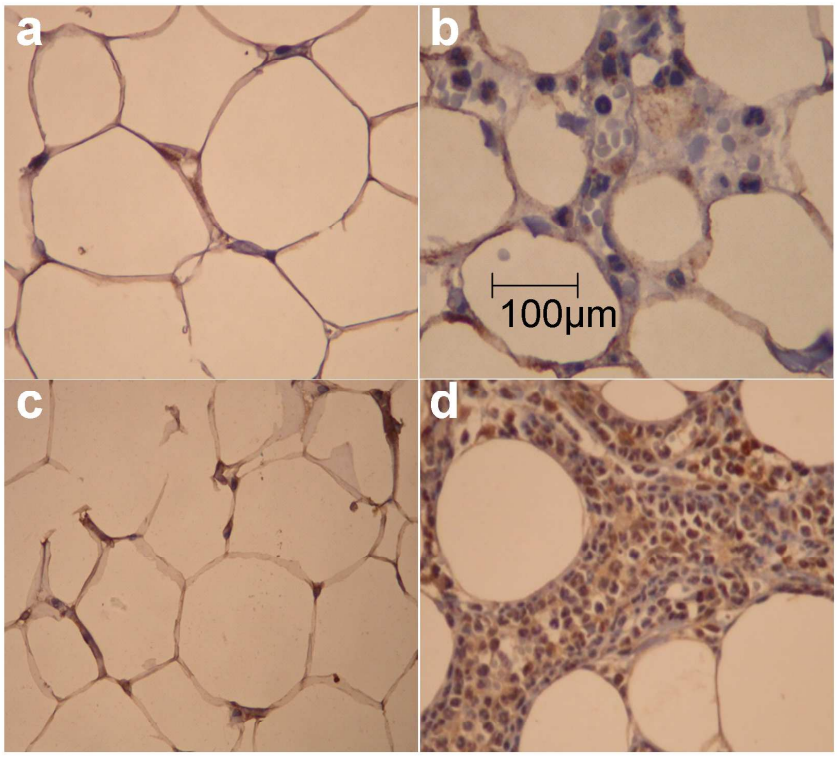


Fig. 2

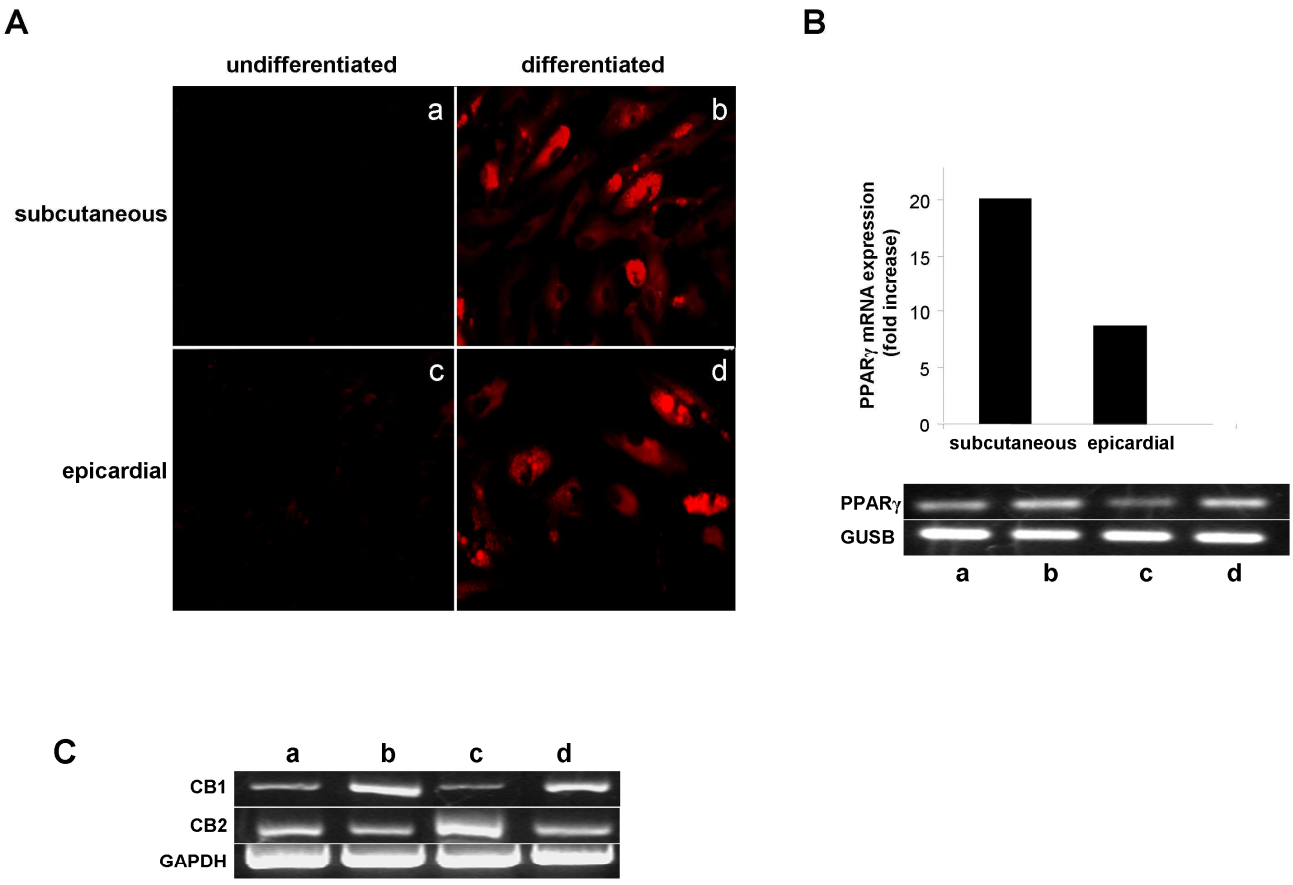


Fig. 3

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