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2-Arachidonoylglycerol modulates human endothelial cell/leukocyte interactions by controlling selectin expression through CB_1 and CB_2 receptors

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ABSTRACT

Accumulated evidence points to a key role for endocannabinoids in cell migration, and here we sought to characterize the role of these substances in early events that modulate communication between endothelial cells and leukocytes. We found that 2-arachidonoylglycerol (2-AG) was able to initiate and complete the leukocyte adhesion cascade, by modulating the expression of selectins. A short exposure of primary human umbilical vein endothelial cells (HUVECs) to 2-AG was sufficient to prime them towards an activated state: within 1 h of treatment, endothelial cells showed time-dependent plasma membrane expression of P- and E-selectins, which both trigger the initial steps (*i.e.*, capture and rolling) of leukocyte adhesion. The effect of 2-AG was mediated by CB_1 and CB_2 receptors and was long lasting, because endothelial cells incubated with 2-AG for 1 h released the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) for up to 24 h. Consistently, TNF-α-containing medium was able to promote leukocyte recruitment: human Jurkat T cells grown in conditioned medium derived from 2-AG-treated HUVECs showed enhanced L-selectin and P-selectin glycoprotein ligand-1 (PSGL1) expression, as well as increased efficiency of adhesion and trans-migration. In conclusion, our *in vitro* data indicate that 2-AG, by acting on endothelial cells, might indirectly promote leukocyte recruitment, thus representing a potential therapeutic target for treatment of diseases where impaired endothelium/leukocyte interactions take place.

Abbreviations: ABHD6, α/β-hydrolase domain 6; APC, allophycocyanin; 2-AG, 2-Arachidonoylglycerol; DAPI, 4′,6-diamidino-2-phenylindole; CB_1, type-1 cannabinoid receptor; CB_2, type-2 cannabinoid receptor; eCBs, endocannabinoids; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FAAH, fatty acid amide hydrolase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAGL, monoacylglycerol lipase; PI3 K/PKB or Akt, phosphatidylinositol-3-kinase/protein kinase B; PBS, phosphate buffered saline; PE, phycoerythrin; PUFA, polyunsaturated fatty acid; PSGL-1, P-selectin glycoprotein ligand-1; RT-PCR, reverse transcriptase-polymerase chain reaction; sL-selectin, soluble L-selectin; TNF-α, tumour necrosis factor-α; TNFR, tumour necrosis factor receptor.

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1. Introduction

Cell migration is crucial in several pathophysiological processes, such as immune response, inflammation, angiogenesis and tumour metastasis (Ley et al., 2007; Polacheck et al., 2013). In particular, adhesiveness and migration of leukocytes on endothelial cells involve sequential steps, all orchestrated by specific adhesion molecules that include selectins, chemokines and integrins (Ley et al., 2007). Among these compounds, P-, E- and L-selectins trigger the initial recruitment of leukocytes to the activated endothelium, and their subsequent rolling (Langer and Chavakis, 2009; Ley, 2003). Following specific stimuli, P-selectin quickly translocates from Weibel-Palade bodies to the cell surface of endothelial cells (Ley et al., 1995). E-selectin, also expressed in endothelial cells, is transcriptionally activated by pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1 (Bevilacqua et al., 1987; Ley, 2003). L-selectin, constitutively expressed by leukocytes, is responsible for lymphocyte binding to endothelial venules in lymphonodes, and for invasion of neutrophils in outbreaks of infection and inflammation (Arbones et al., 1994). Following cellular activation, leukocytes increase expression of L-selectin, which is then shed by proteolytic cleavage into a soluble form that inhibits leukocyte adhesion, and hence modulates the speed of leukocyte rolling (Hafezi-Moghadam et al., 2001; Smalley and Ley, 2005). Selectins bind to specific ligands through weak interactions, which enable leukocytes to roll on endothelium; among them, the P-selectin glycoprotein ligand-1 (PSGL-1), constitutively expressed by leukocytes, binds all three members of the selectin family (Moore, 1998).

It is widely accepted that endocannabinoids (eCBs) play an important role in cell migration. In particular, the marked expression of type-2 cannabinoid (CB_2) receptor in tissues (tonsils, spleen) and cells (mast cells, B cells, macrophages, NK cells) of the immune system suggests its major implication in immune responses (Graham et al., 2010). Understanding the role of eCBs as immunomodulators is a rather complex task, because of the tight dose-dependence of cellular effects and of the heterogeneity of these endogenous compounds (Croxford and Yamamura, 2005; Gokoh et al., 2005; Kishimoto et al., 2006; Rajesh et al., 2007). The major endogenous ligand of CB_1 and CB_2 receptors, 2-arachidonoylglycerol (2-AG), stimulates polymerization of actin filaments (Malorni et al., 2004), thus promoting motility and chemotaxis of polymorphonuclear leukocytes, as well as leukocyte adhesion to fibronectin, overall facilitating infiltration of immune cells into inflamed tissues (Gokoh et al., 2005; Kishimoto et al., 2006). It should also be emphasized that available data are mainly based on studies carried out on single cells and/or at late stages of the adhesion cascade (Kishimoto et al., 2004; Kobayashi et al., 2001). However, it is well known that in real life different cell types need to cooperate with each other in a dynamic sequence of events to allow adhesion (Ley et al., 2007).

With the aim of shedding light on cell/cell interactions, here we focused on the early events of endothelium/leukocyte cross-talks. We show for the first time that 2-AG upregulates the expression of selectins. Indeed, after a short exposure to 2-AG, human umbilical vein endothelial cells (HUVECs) are activated and signal to Jurkat T lymphocytes, thus allowing an efficient endothelium/leukocyte cross-talk.

2. Materials and methods

2.1. Reagents

Chemicals were of the purest analytical grade. 2-AG, lipopolysaccharide (LPS), AM281, URB597, JZL184 and OMDM1 were from Sigma Chemical Co. (St. Louis, MO). ACEA, HU210 and JWH015 were from Tocris Bioscience (Bristol, UK). WWL70 and SR144528 were purchased from Cayman Chemical Co (Ann Arbor, MI). All compounds were endotoxin-free.

Anti-Akt, anti-phospho-Akt^{Ser473}, anti-PSGL-1, anti-tubulin, anti-ERK, anti-phospho-ERK^{Tyr204}, anti-L-selectin antibodies, as well as secondary antibodies conjugated to horseradish peroxidase and the enhanced chemiluminescence (ECL) kit, were from Santa Cruz Biotechnology (Santa Cruz, CA). Allophycocyanin (APC)-conjugated CD62P antibody was purchased from Miltenyi Biotec (Cologne, Germany), and phycoerythrin (PE)-conjugated CD62E antibody was from Biolegend (San Diego, CA). Anti-CB_1 and anti-CB_2 primary antibodies were from Cayman Chemical Co. Alexa-488 secondary antibody was from Invitrogen (Carlsbad, CA). Calcein-acetoxymethyl ester (calcein-AM) was from Life Technologies (Milan, Italy).

2.2. Cell cultures

Human Jurkat T cells (ATCC, Manassas, VA) were grown in DMEM:F12 (1:1) medium, supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen). HUVECs (Lonza Group Ltd, Basel, Switzerland) were grown in EGM-2 Bullet kit medium (BioWhittaker, Radnor, PA), as reported (Maccarrone et al., 2002). In order to remove serum, which is known to contain trace amounts of eCBs (Marazzi et al., 2011), cells were accurately washed three times in phosphate buffered saline (PBS), before setting each experiment.

Subconfluent cells were incubated in serum-free medium containing compounds to be tested, for the indicated periods of time. LPS, the endotoxin of Gram-negative bacteria, was used as positive control (Wright et al., 1990).

In conditioned medium (CM) experiments, HUVECs were treated with different compounds for 1 hour; after the incubation period, cells were washed three times with PBS, fresh serum-free medium was added, and the incubation was carried out for two additional hours. Finally, the culture medium was collected, and used to resuspend Jurkat T cells, that were further incubated for the indicated periods of time.

2.3. FACS analysis

Surface and intracellular P- and E-selectins, as well as CB_1 and CB_2 receptor expression, were quantified by flow cytometry in a FACSCanto instrument (Beckton Dikison, NJ). Briefly, 2-AG-treated HUVECs were collected and stained with APC-conjugated CD62P and PE-conjugated CD62E specific antibodies. For intracellular staining, cells were fixed with 4% paraformaldehyde for 15 min, and then stained intracellularly with the above-mentioned selectin-specific antibodies in 0.5% saponin, at room temperature. For CB receptor expression, HUVECs were stained with specific anti-CB_1 and anti-CB_2 primary antibodies for 15 min at 4 °C and then stained with Alexa-488 secondary antibody. Isotype-matched and secondary antibodies were employed to assess background staining and specificity. For each analysis 100,000 events were acquired and viable cells were analyzed using the Flowjo software (TreeStar, Ashland, OR).

2.4. Western blotting

Cells were lysed in 50 mM Tris–HCl (pH 7.4), containing protease and phosphatase inhibitors. Proteins (30 μg/lane) from whole lysates or membrane fractions were subjected to SDS-PAGE, electroblotted onto PVDF membranes, incubated with specific antibodies and detected with ECL, as reported (Catani et al., 2010).