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Endogenous *N*-acyl-dopamines induce COX-2 expression in brain endothelial cells by stabilizing mRNA through a p38 dependent pathway

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ABSTRACT

Cerebral microvascular endothelial cells play an active role in maintaining cerebral blood flow, microvascular tone and blood brain barrier (BBB) functions. Endogenous *N*-acyl-dopamines like *N*-arachidonoyl-dopamine (NADA) and *N*-oleoyl-dopamine (OLDA) have been recently identified as a new class of brain neurotransmitters sharing endocannabinoid and endovanilloid biological activities. Endocannabinoids are released in response to pathogenic insults and may play an important role in neuroprotection. In this study we demonstrate that NADA differentially regulates the release of PGE_2 and PGD_2 in the microvascular brain endothelial cell line, b.end5. We found that NADA activates a redox-sensitive p38 MAPK pathway that stabilizes *COX-2* mRNA resulting in the accumulation of the COX-2 protein, which depends on the dopamine moiety of the molecule and that is independent of CB_1 and TRPV1 activation. In addition, NADA inhibits the expression of mPGES-1 and the release of PGE_2 and upregulates the expression of L-PGD synthase enhancing PGD_2 release. Hence, NADA and other molecules of the same family might be included in the group of lipid mediators that could prevent the BBB injury under inflammatory conditions and our findings provide new mechanistic insights into the anti-inflammatory activities of NADA in the central nervous system and its potential to design novel therapeutic strategies to manage neuroinflammatory diseases.

1. Introduction

The past decade has seen a sudden spurt of interest in the endocannabinoid system (ECs). This system regulates a plethora of biological effects and is composed of cannabinoid and vanilloid receptors, endogenous signaling molecules (called endocannabinoids) and metabolism-related enzymes [1,2]. Endocannabinoids are a class of lipid mediators found in several tissues and based on a polyunsaturated fatty acid amide or ester motifs [3]. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most characterized endocannabinoids acting in the brain and in peripheral tissues mainly through the activation CB_1 and CB_2 cannabinoid receptors, respectively. AEA can also interact with the vanilloid receptor type 1 TRPV1 [4,5]. This non-selective cation channel is activated by vanilloids, such as capsaicin, and also by endogenous ligands *N*-acyl-dopamines (neurolipins), such as *N*-arachidonoyl-dopamine (NADA) and *N*-oleoyl-dopamine (OLDA) [6–8]. While NADA binds TPRV1 [7,9], and CB_1 receptor [10], OLDA is a capsaicin-like lipid with full TPRV1 agonist activity but devoid of affinity for CB receptors [8]. NADA induces several biological activities such as hyperalgesia [8], smooth muscle contraction in the guinea pig bronchi and bladder [11], vasorelaxation in blood vessels [12], and also has immunomodulatory, neuroprotective and antiinflammatory properties [13–15]. Their saturated analogs *N*-palmitoyl-dopamine (PALDA) and *N*-stearoyl-dopamine (STEARDA) were also identified as endogenous substances not activating TRPV1, although they significantly enhanced the TRPV1-mediated effects of NADA [8].

Endocannabinoids may play a major role in the central nervous system (CNS), immune control and neuroprotection by regulating the cellular network of communication between the nervous and immune system during neuroinflammation and neuronal damage [16–18]. In addition, *N*-acyl-dopamines influence the lipoxygenase pathway of arachidonic acid cascade as substrates or inhibitors and may also be involved in the regulation of inflammation [19,20].

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Cyclooxygenases catalyse the first step in the synthesis of prostanoids, a large family of arachidonic acid metabolites, including prostaglandins (PGs), prostacyclins and thromboxanes. The inducible isoform COX-2 is involved in the mediation of inflammation, immunomodulation, blood flow, apoptosis and fever [21,22]. COX-2 is rapidly expressed on several cell types in response to growth factors, proinflammatory molecules and cytokines [23,24]. Proinflammatory cytokines such as IL-1β and TNF-α increase the expression of COX-2 in brain microvessel endothelial cells and this has been related to increases in permeability of the cerebral microvasculature [22,25]. The production of several PGs, secondary to induction of COX-2, by the cells lining the blood–brain barrier (BBB), which may diffuse to the brain parenchyma, may have important consequences in brain inflammatory processes by modulating blood flow and also the intracerebral immune responses. Prostaglandin E_2 (PGE_2), the major prostanoid produced by COX activity in the brain, produced marked BBB breakdown when administered intracerebrally in the rat [26]. Selective COX-2 inhibition with NS-398 reduced the effects of TNF-α on cerebromicrovascular permeability in a rat cranial window model [27]. Furthermore, COX inhibition with indomethacin significantly reduced BBB disruption induced by TNF-α *in vitro* [28].

In the biosynthetic pathway leading to PGE_2 and PGD_2, arachidonic acid released from membrane phospholipids by phospholipases A_2 is converted to PGH_2 by COX-1 or COX-2 and is then isomerized to PGE_2 and PGD_2 by terminal prostaglandin E synthases (PGES) and prostaglandin D synthases (PGDS) respectively. To date, three PGESs (mPGES-1, mPGES-2 and cPGES) and two PGDSs (H-PGDS and L-PGDS) have been characterized [29–32]. mPGES-1 and L-PGDS are found predominantly in brain tissues. Interestingly, mPGES-1 knockout mice (mPGES-$1^{-/-}$) show no increase in PGE_2 or body temperature after injection of LPS [33], indicating that mPGES-1 is a key enzyme in the production of PGE_2 in the brain. A recent study showed the induction of mPGES-1 in neurons, microglia, and endothelial cells in the cerebral cortex after transient focal ischemia [34].

PGE_2 synthesis inhibition has been an important anti-inflammatory strategy in the last years but specific inhibition of COX-2 has been correlated to cardiotoxic side effects [35–37]. In contrast, specific inhibition of mPGES-1 is believed to be a potential therapeutic approach to prevent the synthesis of PGE_2 but not of other prostanoids [38]. We have recently shown that NADA inhibits mPGES-1 expression and PGE_2 release in LPS-activated primary glia cells [15] and therefore the purpose of this work was to study the effects of NADA and other *N*-acyl-dopamines on COX-2, mPGES-1 and L-PGDS expression and function in murine brain endothelial cells (b.end5), which are recognized to present brain endothelium-like properties.

2. Materials and methods

2.1. Cell culture

The murine brain endothelial cell line, b.end5, was obtained from Dr. Carmen Guaza (CSIC, Madrid, Spain). The cells were maintained in exponential growth at 37 °C and 5% CO_2 in supplemented DMEM medium containing 10% heat-inactivated FCS, 2 mM glutamine and antibiotics.

2.2. Reagents and antibodies

Anandamide, AM404, Arvanil and 2-Arachidonoylglycerol (2-AG) were obtained from Alexis Co. (Laussane, Switzerland). *N*-Arachidonoyl-dopamine (NADA), dopamine and LPS from *Escherichia coli* 0111:B4, were obtained from Sigma (St Louis, MO, USA). *N*-Oleoyl-dopamine (OLDA), *N*-palmitoyl-dopamine (PALDA), *N*-stearoyl-dopamine (STEARDA) and 5′ iodo resiniferatoxin (5′IRTX) were a gift from Prof. Giovanni Appendino (Università degli Studi del Piemonte Orientale, Novara, Italia). The CB_1 receptor antagonist SR141716A was obtained from Sanofi-Aventis (Paris, France). The compounds were dissolved in ethanol, and vehicle concentration in the culture media was maintained at less than 0.1% (v/v). LPS was dissolved in sterile water as 5 mg/ml stock, and used at a final concentration of 1 μg/ml in the cell cultures. Antibodies against COX-2 (M-19), COX-1 (M-20), and phospho-ERK 1 + 2 (sc-7383) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against anti-phospho-JNK 1 + 2 (9255S) and anti-phospho p38 (Thr180/Tyr182, 9211) were from Cell Signaling (Danvers, MA, USA). Rabbit polyclonal antibody against mPGES-1 was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). The monoclonal antibody against α-tubulin and all other reagents were from Sigma.

2.3. Plasmids, transient transfections and luciferase assays

The plasmid COX-2-Luc was a gift from Dr. M. Iñiguez (UAM, Spain); this plasmid contains the *COX-2* promoter followed by the luciferase gene. The COX-2 translational reporter COX-2–3′-UTR-luciferase contains the 3′-UTR of *COX-2* sequence (763 bp) fused to the 3′ end of the luciferase gene under control of the SV40 promoter and enhancer elements [39]. The plasmid mPGES-1-Luc was kindly provided by Dr. Terry J. Smith (University of California, Los Angeles) and contains the human mPGES-1 promoter (−538/−28) followed by the luciferase gene [40]. For luciferase assays the b.end5 cells (75×10^3 cells/well) were transiently transfected with the indicated plasmids using Roti-Fect (Carl Roth GmbH, Karlsruhe, Germany) according to the manufacturer's recommendations for 24 h. Transfected cells were stimulated as indicated during 6 h and then lysed in 25 mM Tris–phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 953 (EG&G Berthold, USA) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA) and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation or the luciferase mRNA stabilization expressed as a fold induction over untreated cells. For the *COX-2* mRNA stabilization experiments the luciferase activity was normalized using pRL-TK-luciferase activity in each sample. All the experiments were repeated at least three times.

2.4. Western blot

b.end5 cells (10^6 cells/ml) were stimulated with the indicated compounds. Cells were then washed with PBS and proteins extracted in 50 μl of lysis buffer (20 mM HEPES pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaF, 1 mM DTT, leupeptin 1 μg/ml, pepstatin 0.5 μg/ml, aprotinin 0.5 μg/ml, and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA) and 30 μg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% non-fat dry milk overnight at 4 °C, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (GE Healthcare, New Jersey, USA). Normalized density ratio of COX-2/mPGES-1 over α-tubulin was performed using the Quantity One program (Bio-Rad).