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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).

2.5. Determination of COX-2 protein degradation half-life

b.end5 cells were preincubated with vehicle or NADA (2.5 μM) during 12 h and then cycloheximide (CHX, 10 μM) was added to inhibit further protein synthesis. Following incubation for 1, 2, 4 and 6 h, cells were harvested and lysed, and cell lysates were collected as described above. Western blotting was performed in the same way as described above. Normalized density ratio of COX-2 over α-tubulin was performed using the Quantity One program (Bio-Rad). Protein degradation rate is expressed as half-life ($t_{1/2}$), the time for degradation of 50% of the protein.

2.6. Determination of COX-2 mRNA half-life

b.end5 cells were preincubated with vehicle or NADA (2.5 μM) during 12 h and then Actinomycin D (Act-D, 10 μM) was added to inhibit general gene transcription. Following incubation for 1, 2, 4 and 6 h, cells were harvested and total RNA was isolated by using the kit RNAspin Min (GE Healthcare). Total RNA was used to study the expression of *COX-2* gene by qRT-PCR analysis as described subsequently and mRNA half-life ($t_{1/2}$) was calculated by linear regression analysis.

2.7. qRT-PCR

b.end5 cells were stimulated as indicated and total RNA was isolated by using the kit RNAspin Min (GE Healthcare) according to the manufacturer's instructions. One μg of total RNA served as the template for single strand cDNA synthesis in a reaction using Oligo (dT) primers and SuperScriptTM II RNase H- Reverse Transcriptase (Invitrogen Ltd., San Diego, USA) according to the manufacturer's protocol. For quantitative RT-PCR analysis expression we used the iCycler (Bio-Rad) system. Equal amounts of cDNA were used in triplicate and amplified with the specific primers. The PCR mixture consisted of 50 μl containing 1 μl of cDNA, 25 μl of 2× SYBR Green Supermix (100 mM KCl, 40 mM Tris–HCl pH 8.4, 0.4 mM of dNTP, 50 u/ml iTaq polimerasa, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein and stabilizers), and 0.2 μM concentration of each sequence-specific primer. The primers used were as follows:

COX-2 sense primer, 5′TGAGCAACTATTCCAAACCAGC3′;
COX-2 antisense primer, 5′GCACGTAGTCTTCGATCACTATC3′;
L-PGDS sense primer, 5′GCTCTTCGCATGCTGTGGAT3′;
L-PGDS antisense primer, 5′GCCCCAGGAACTTGTCTTGTT3′;
mPGES-1 sense primer, 5′AGCACACTGCTGGTCATCAA3′;
mPGES-1 antisense primer, 5′CTCCACATCTGGGTCCACTCC3′;
GAPDH sense primer, 5′TGGCAAAGTGGAGATTGTTGCC3′;
GAPDH antisense primer, 5′AAGATGGTGATGGGCTTCCCG3′.

The amplification profile consisted of an initial denaturation for 3 min at 95 °C and then 40 cycles of 30 s at 95 °C, annealing for 30 s at 50 °C, and elongation for 30 s at 72 °C. A cycle of 10 s at 83 °C and a final extension for 1 min was carried out at 72 °C. Amplification efficiencies were validated and normalized against GAPDH, and fold increases were calculated by using the comparative threshold cycle method for quantification.

2.8. Determination of PGE_2 and PGD_2 by enzyme immunoassay

Supernatants were harvested, centrifuged at 10,000 × *g* for 10 min and levels of PGE_2 or PGD_2 in the media were measured by enzyme immunoassay (EIA) (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions. For the PGE_2 assay the standards were used in the interval of 7.8 to 1.000 pg/ml (detection limit of 15 pg/ml). For the PGD_2 assay the standards were used in the interval of 78 to 10,000 pg/ml (detection limit approximately 200 pg/ml). To calculate concentrations of PGs outside the limit of detection, dilutions of the supernatants were used.

2.9. Statistical analysis

At least three independent experiments were used for data analysis. Statistical analysis was performed using one-way ANOVA analysis. In the case of a significant result in the ANOVA, Student's *t*-test was used for dose–response assays and Bonferroni's test was used for post-hoc analysis for all other experiments. Data are reported as mean values ± standard error of mean (S.E.M.).

3. Results

3.1. N-Arachidonoyl-dopamine induces the expression of COX-2 on brain endothelial cells

The murine brain endothelial cell line b.end5 is a recognized model to study blood–brain barrier cell function [41]. To study the effects of NADA on COX-1 and COX-2 protein expression we treated b.end5 cells with increasing concentration of NADA during 20 h and the steady state levels of COX-1, COX-2 and α-tubulin were investigated by western blots. COX-2/COX-1 optical density values were normalized to α-tubulin and represented in arbitrary units. In Fig. 1A, it is shown that NADA, at the concentration of 1 μM and higher, clearly increased the expression of COX-2, but not COX-1, in b.end5 cells. NADA is a mixed agonist of CB_1 and

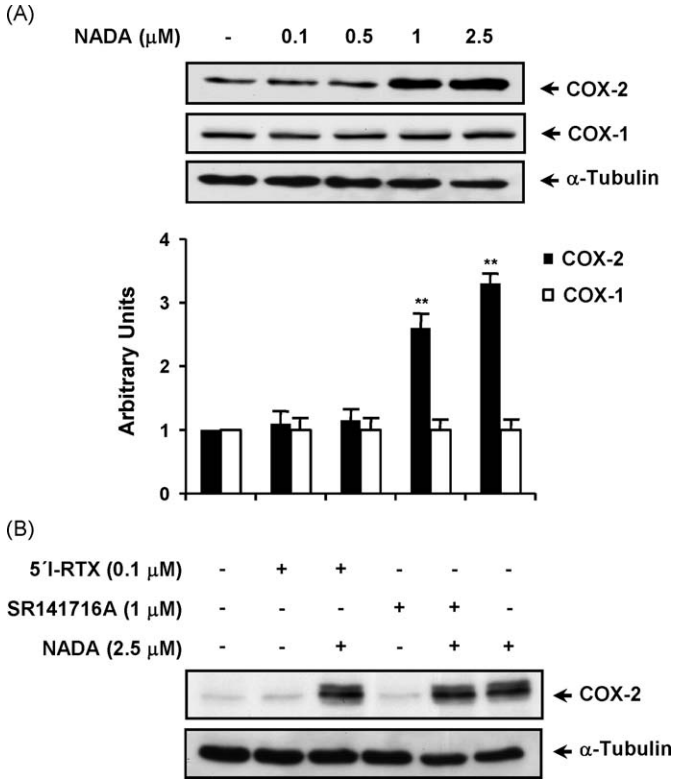

Fig. 1. NADA induces COX-2 protein expression in b.end5 cell line in a dose-dependent manner. (A) b.end5 cells were incubated with NADA at the indicated doses during 20 h. The expression of COX-1 and COX-2 protein was determined by immunoblot analysis of protein levels. We reported the representative blots and mean ± S.D. values of optical density of three independent experiments (one-way ANOVA with Student's *t*-test $^{**}P < 0.001$ and for NADA versus control). (B) b.end5 cells were pretreated with CB_1 antagonist, SR141716A (1 μM) and TRPV1 antagonist, 5′IRTX (0.1 μM) during 30 min before NADA (2.5 μM). Protein extracts were prepared after 20 h incubation with the compounds, and subjected to SDS-PAGE. We show a representative blot out of three independent experiments.

TRPV1 receptors [7], and it has been previously shown that b.end5 express the CB_1 receptor [42] and although the expression of TRPV1 has not been investigated in this cell line, it has been reported that TRPV1 is present on cerebromicrovascular endothelial cells [43]. Using specific chemical antagonists we investigated the involvement of CB_1 and TRPV1 receptors in NADA-induced COX-2 expression in b.end5 cells. We found that neither SR141716A (CB_1 antagonist) nor 5′-IRTX (TRPV1 antagonist) inhibited the effects of NADA on COX-2 expression in this cell line (Fig. 1B). Moreover, pre-incubation of b.end5 cells with a combination of both antagonist receptors (CB_1 and TRPV-1) or with butaclamol (Dopamine receptor antagonist) did not prevent NADA-induced COX-2 expression (data not shown).

3.2. The dopamine moiety of N-acyl-dopamines is required to upregulate COX-2 expression in b.end5 cells

N-Acyl-dopamines such as NADA are conjugates of fatty acids with dopamine via an amide bond. To investigate the role of the dopamine moiety in the induction of COX-2 expression in brain endothelial cells, we treated b.end5 cells with the four endogenous *N*-acyl-dopamines described so far [7,8], namely NADA, OLDA, PALDA and STEARDA. As depicted in Fig. 2A all the *N*-acyl-dopamines analyzed were able to upregulate COX-2 protein expression in b.end5 cells. We also tested the endocannabinoids AEA and 2-AG that share with NADA the arachidonic acid moiety and the synthetic hybrid CB_1/TRPV1 ligands, Arvanil and AM404, which contains a dopamine-like phenolic moiety. Interestingly, none of these compounds induced the expression of COX-2 in b.end5 cells. Altogether these results indicate that NADA and other *N*-acyl-dopamines upregulate COX-2 protein expression in brain endothelial cells through a mechanism independent of Dopamine, CB_1 and TRPV1 receptors. To further investigate the role of the dopamine moiety we treated b.end5 cells with increasing concentrations of dopamine and 20 h later the expression of COX-2 protein was analyzed by immunoblots. We show in Fig. 2C that dopamine was able to increase the steady state levels of the COX-2 protein in b.end5 cells, although NADA was at least 50-fold more potent than dopamine to up-regulate COX-2 expression.

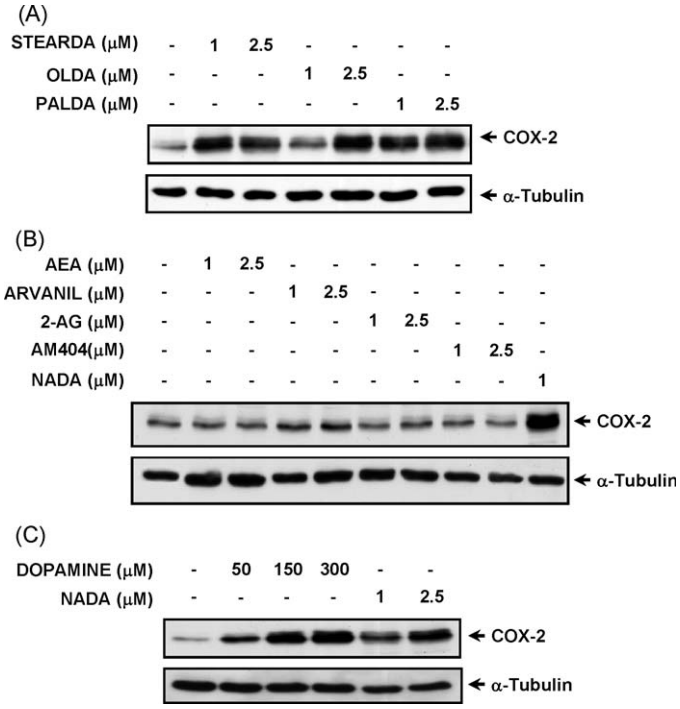

Fig. 2. The dopamine moiety of *N*-acyl-dopamines is required to induce COX-2 expression in b.end5 cells. (A) Cells were incubated during 20 h with STEARDA, OLDA, PALDA at the indicated concentrations. The expression of COX-2 protein was determined by immunoblot analysis. (B) Effects of AEA, Arvanil, 2-AG, AM404 and NADA on COX-2 protein expression were determined after 20 h of incubation with the compounds at the indicated concentrations. (C) b.end5 cells were incubated with dopamine and NADA during 20 h at the indicated concentrations. Protein extracts were prepared and subjected to SDS-PAGE. We show a representative blot out of three independent experiments.

3.3. N-Arachidonoyl-dopamine stabilizes COX-2 mRNA in brain endothelial cells

It has been reported that COX-2 undergoes proteasomal degradation via the endoplasmic reticulum-associated degradation pathway [44]. To investigate the mechanisms by which NADA induces the expression of COX-2 in b.end5 cells firstly we studied the half-life of the COX-2 protein. The cells were treated with vehicle or with NADA (2.5 μM) during 12 h, then CHX (10 μM) was added and the cells were collected at the indicated times. Finally, the expression of COX-2 and α-tubulin protein was analyzed by immunoblots. As expected, NADA induced a clear increase in COX-2 expression (Fig. 3A). The specific bands were quantified and the ratio COX-2/α-tubulin calculated for every point and plotted to calculate the decay rates for COX-2 protein expression (Fig. 3B). We found that the half-life ($t_{1/2}$) for COX-2 protein in b.end5 cells was approximately 6 h and this half-life was not modified by the treatment with NADA. These results, together with the inability of NADA to induce COX-2 phosphorylation (data not shown), suggest that protein post-translational modifications are not involved in COX-2 stabilization in NADA-treated cells.

Next, we studied whether or not NADA had some effect on the *COX-2* gene at the transcriptional level. The cells were transiently transfected with the COX-2-Luc plasmid and 24 h later stimulated with either LPS or NADA during 12 h and the cell lysates tested for luciferase activity. We show in Fig. 4A that LPS clearly induced luciferase expression driven by the *COX-2* promoter (4.2-fold induction). In contrast, NADA did not affect the basal levels of luciferase when compared to untreated cells. However, qRT-PCR experiments demonstrated that NADA induced a clear induction of the *COX-2* mRNA levels in b.end5 cells (Fig. 4B). These results suggest that the effects of NADA on COX-2 expression could be mediated by a mechanism that involves *COX-2* mRNA stabilization. To address this point directly, b.end5 cells were pretreated during 12 h with vehicle or NADA and then placed in serum-free medium containing actinomycin D, a general inhibitor of the transcriptional machinery. Cultures were then harvested 1, 2, 4 and 6 h later for determination of *COX-2* mRNA levels by qRT-PCR (Fig. 4C). In these experiments, the *COX-2* message half-life ($t_{1/2}$) in control cells, as judged by decay in the presence of actinomycin D, was approximately 3.8 h. This half-life was clearly extended to a period of 13.7 h in the presence of NADA (Fig. 4D).

3.4. N-Arachidonoyl-dopamine stabilizes COX-2 mRNA through a p38 MAPK pathway

The *COX-2* mRNA is stabilized by p38 MAPK activation in different cell types [45–47]. To study the role of p38 MAPK on NADA-induced *COX-2* mRNA stabilization we transiently transfected b.end5 cells with the COX-2-3′-UTR-luciferase plasmid for 24 h and then the cells were stimulated with either LPS or NADA, in the presence or absence of SB203580, a chemical p38 MAPK inhibitor. After 12 h of treatment the cells were collected and the luciferase activity measured in the cell lysates. As depicted in Fig. 5A, both LPS and NADA clearly induced *COX-2* mRNA stabilization that was prevented in the presence of SB203580.

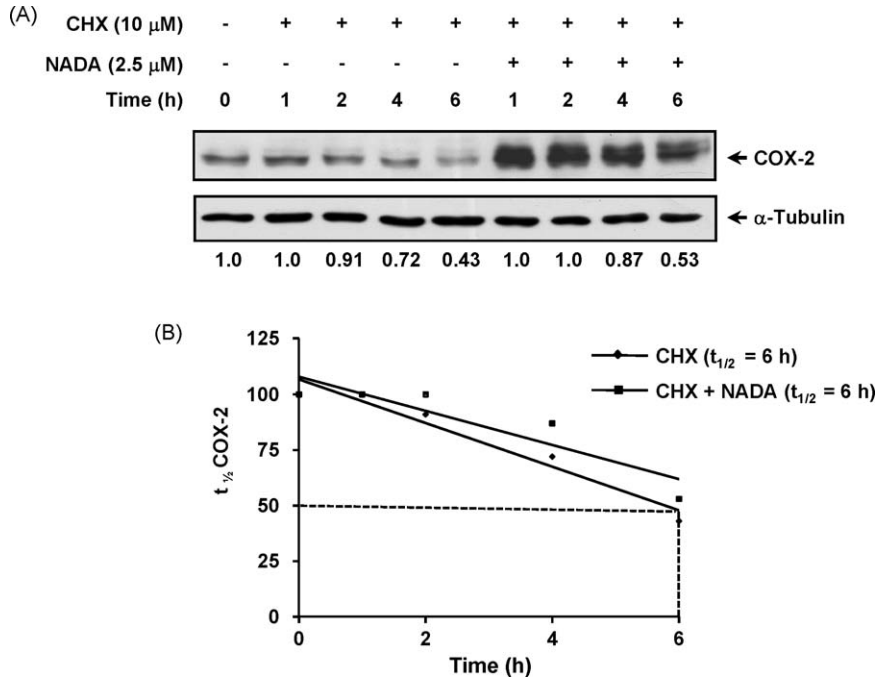

Fig. 3. NADA does not affect the half-life of COX-2 protein. (A) b.end5 cells were pre-incubated with vehicle or NADA (2.5 μM) during 12 h and then cycloheximide (CHX 10 μM) was added to inhibit further protein synthesis. Following incubation from 0 to 6 h, the cells were harvested and the expression of COX-2 protein was determined by immunoblot analysis. Normalized density ratio of COX-2 over α-tubulin is indicated for each band. A representative blot out of three independent experiments is shown. (B) The graph represents the COX-2 protein levels normalized to α-tubulin. Results from the densitometry analysis were expressed as arbitrary values and plotted relative to the zero time point.

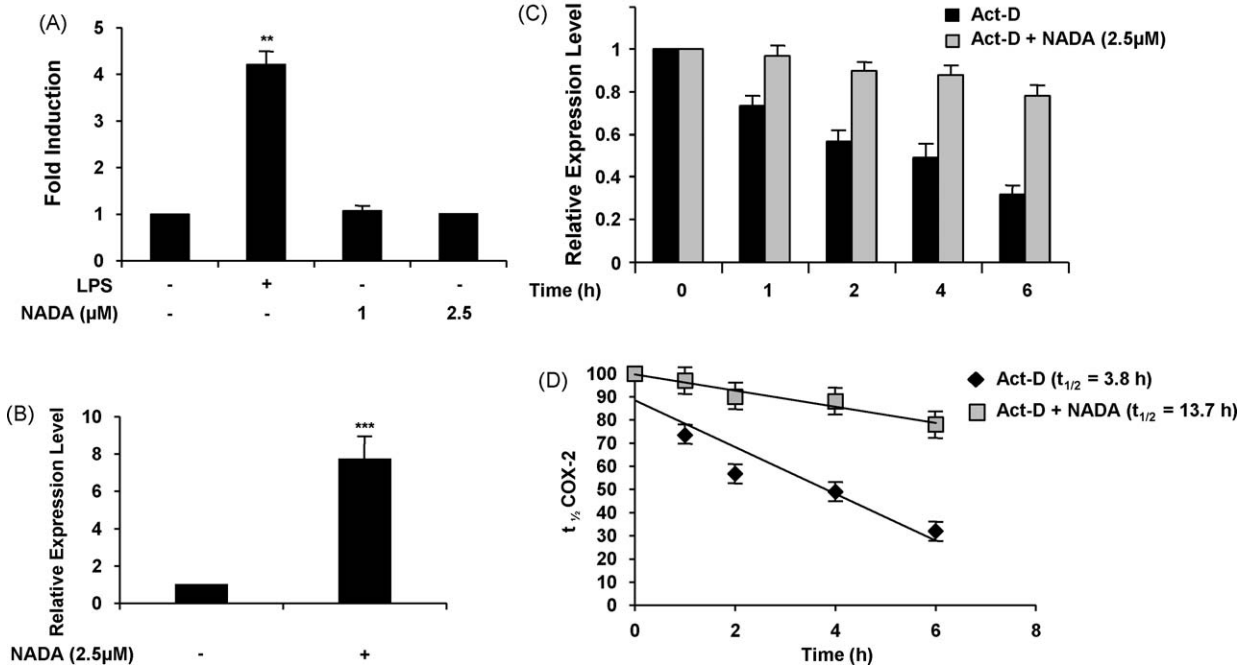

Fig. 4. NADA stabilizes *COX-2* mRNA. (A) b.end5 cells were transiently transfected with the luciferase reporter plasmid COX-2-Luc as described in Materials and Methods and 24 h after transfection the cells were incubated for 12 h with LPS or NADA, and the luciferase activity measured in the cell lysates. Results are the means ± S.D. of three determinations expressed as fold induction (observed experimental RLU/basal RLU in absence of any stimuli) (one-way ANOVA with Student's *t*-test $^{**}P < 0.001$ LPS versus control). (B) For the qRT-PCR analysis of *COX-2* expression, b.end5 cells were stimulated with NADA 2.5 μM for 12 h, total RNA extracted and qRT-PCR assay was performed. The graph shows the means ± S.D. of three experiments ($^{***}P < 0.0001$ NADA versus control). (C) b.end5 cells were pre-incubated with vehicle or NADA (2.5 μM) during 12 h and then Actinomycin D (Act-D 10 μM) was added. Following incubation from 0 to 6 h the *COX*-2 gene expression was determined by qRT-PCR analysis. Amplification efficiencies were validated and normalized against GAPDH expression. The graph shows the *COX-2* gene expression level when incubated with NADA (2.5 μM) and its quantification as compared to the level obtained with the vehicle. The graph shows the means ± S.D. of three experiments. (D) The graph represents the *COX-2* gene expression determined by qRT-PCR analysis expressed as arbitrary values. mRNA half-life ($t_{1/2}$) was determined using linear regression analysis. Data at each time point represent the means ± S.D. from three replicates.

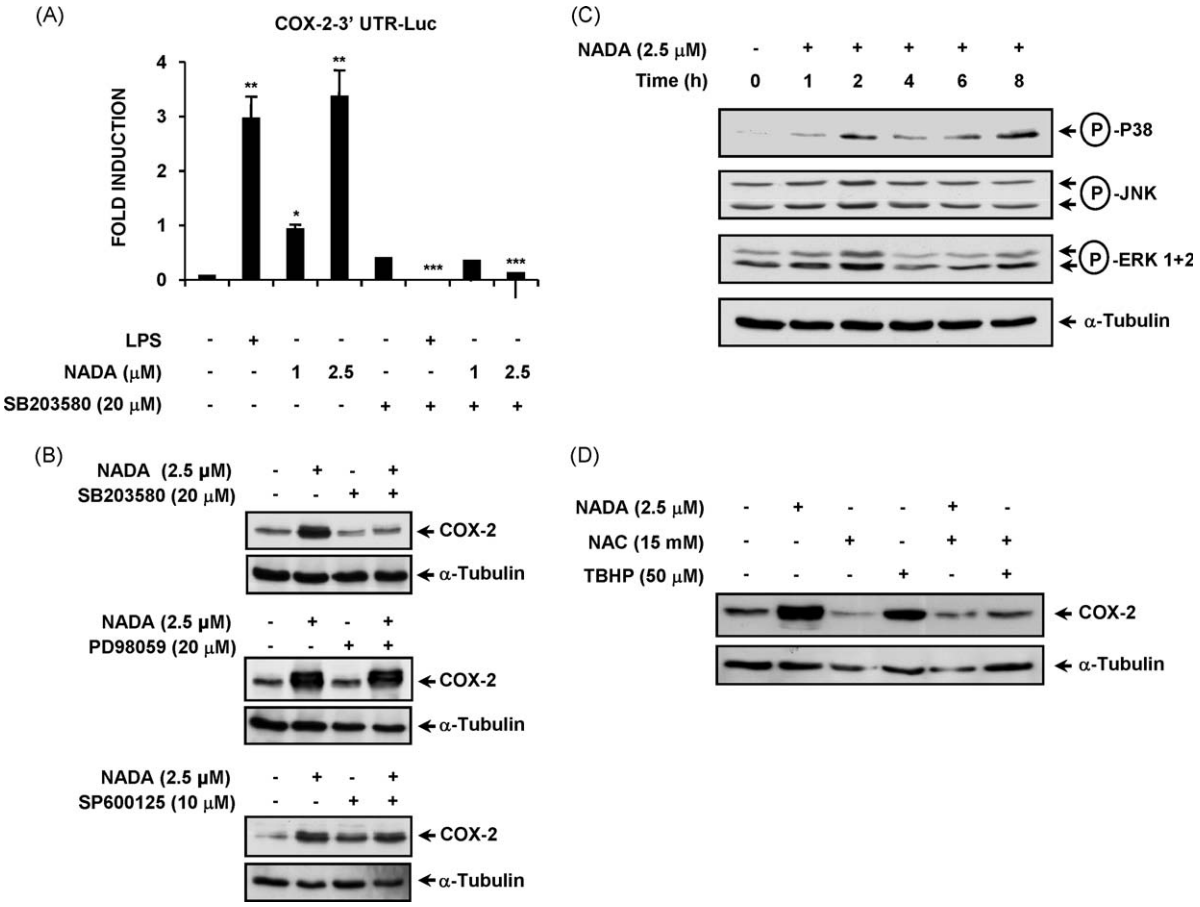

Fig. 5. NADA induces COX-2 expression by stabilizing mRNA through a p38 dependent pathway. (A) b.end5 cells were transiently transfected with the plasmid COX-2-3′UTR-Luc and 24 h later the cells were pre-incubated for 30 min with SB203580 (20 μM) and stimulated with LPS or NADA as indicated for 12 h. Luciferase activity was measured and the results are the means ± S.D. of three determinations expressed as fold induction (observed experimental RLU/basal RLU in absence of any stimuli) (one-way ANOVA with post-hoc Bonferroni's test $^{*}P < 0.05$ for NADA versus control and $^{**}P < 0.001$ for LPS/NADA versus control; $^{***}P < 0.0001$ for LPS + SB203580 versus LPS and for NADA + SB203580 versus NADA). (B) b.end5 cells were pretreated with SB203580 (20 μM), PD98059 (20 μM) or SP600125 (10 μM) for 30 min, and then incubated with NADA 2.5 μM for 20 h. The expression of COX-2 protein was determined by immunoblot analysis of protein levels. We reported the representative blots of three independent experiments. (C) The cells were incubated with NADA 2.5 μM at the indicated times and MAPKs activation detected by immunoblots using specific phospho-antibodies. (D) b.end5 cells were pretreated with NAC (15 mM) for 30 min and then treated with either TBHP or NADA for 20 h, and the expression of COX-2 protein and α-tubulin analyzed by immunoblot analysis. A representative blot out of three independent experiments is shown.

To further confirm the specific role of p38 on NADA-induced COX-2 protein expression we stimulated b.end5 cells with NADA for 12 h in the absence or the presence of SB203085. In Fig. 5B, it is shown that NADA-induced upregulation of COX-2 protein was completely prevented by the p38 MAPK inhibitor SB203085.

To study directly the effect of NADA on p38 MAPK activation, we incubated b.end5 cells with NADA (2.5 μM) for the indicated times, and the phosphorylation/activation status of p38, JNK, and ERK was investigated by western blots using specific mAbs that recognize the phosphorylated forms of these MAPKs. As depicted in Fig. 5C, NADA induced a sustained activation of the p38 MAPK and did not affect significantly the phosphorylation of the MAPKs JNK and ERK. Similarly, specific MEK and JNK chemical inhibitors (PD98059 and SP600125, respectively) did not show any effect on NADA-induced COX-2 protein expression (Fig. 5B). Since p38 is mainly activated by oxidative stress, we pre-incubated b.end5 cells with the antioxidant *N*-acetyl cysteine (NAC), which reduces oxidative-mediated cell damage, prior to treatment with either NADA or terbutyl-hydroperoxide (TBHP) for 8 h and COX-2 expression was analyzed by western blot. Interestingly, NAC completely inhibited both NADA- and TBHP-induced COX-2 protein expression (Fig. 5D). Altogether our results indicated that NADA induced a mild cellular oxidative stress that activated the p38 pathway that in turn stabilizes *COX-2* mRNA.

3.5. *N*-Arachidonoyl-dopamine inhibited PGE_2 release but increased PGD_2 release in b.end5 cells

COX-2 converts arachidonic acid to prostaglandin endoperoxide H_2 (PGH_2), which is the precursor of prostanoids and prostaglandins such as PGE_2 and PGD_2. Thus, we studied the effects of NADA on the release of these prostaglandins in b.end5 cells. Firstly, the cells were treated with NADA for 24 h and the content of these PGs measured in the supernatants by standard EIA assays. We found that NADA inhibited both basal and LPS-induced levels of PGE_2 released by b.end5 cells (Fig. 6A). In contrast, NADA clearly increased the amount of PGD_2 released by b.end5 cells and synergized with LPS to induce the release of this PG (Fig. 6B). Accordingly, we have recently shown that NADA also inhibited LPS-induced PGE_2 release in primary astrocytes and microglia cells. The mechanism by which NADA inhibited PGE_2 production in primary glia cells was due to inhibition of mPGES-1 expression, a terminal PGE synthase that produces PGE_2 from PGH_2 [15]. To study if this mechanism is also involved in the inhibitory ability of NADA in microvascular endothelial cells, we treated the b.end5 cells with LPS in the presence or absence of NADA and after 12 h we studied the expression of mPGES-1 protein and mRNA by western blot and qRT-PCR respectively. In Fig. 7A we show that NADA clearly inhibited the induction of mPGES-1 protein expression in

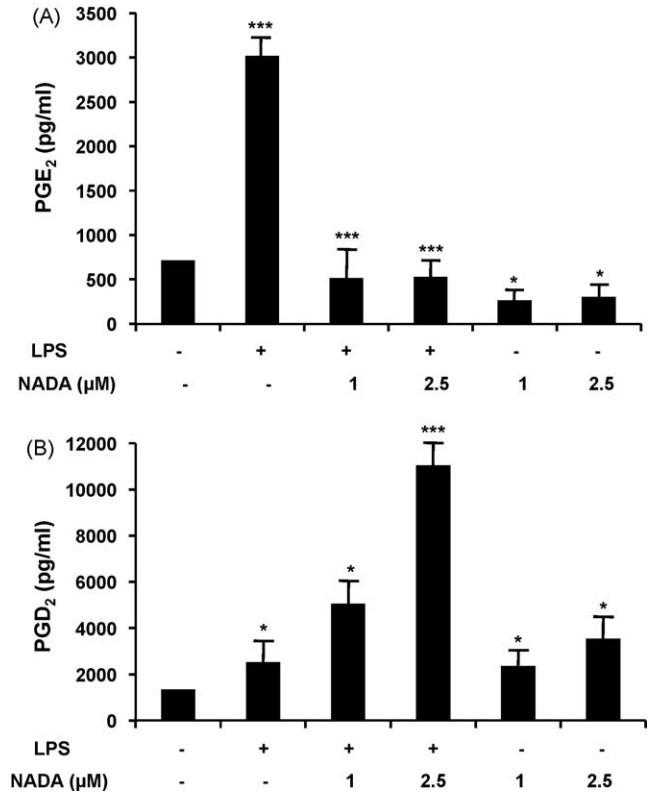

Fig. 6. NADA inhibits PGE_2 release and enhances PGD_2 formation in b.end5 cells. (A) b.end5 cells were preincubated with LPS either in the absence or the presence of NADA or with NADA alone for 24 h. The amounts of PGE_2 (A) and PGD_2 (B) in the culture medium were determined using an enzyme immunoassay. The graphs report the mean ± S.D. values of three independent experiments (one-way ANOVA with post-hoc Bonferroni's test $^{***}P < 0.0001$ (A) and $^{*}P < 0.05$ (B) for LPS versus control, $^{*}P < 0.05$ and $^{***}P < 0.0001$ for LPS + NADA versus LPS, $^{*}P < 0.05$ for NADA versus control).

LPS-activated cells. In contrast, the expression of COX-2 protein was co-induced by both LPS and NADA. To test the hypothesis that NADA inhibits mPGES-1 expression induced by LPS by a transcriptional mechanism, we carried out transient transfection experiments in b.end5 cells with the luciferase reporter plasmid mPGES-1-Luc. As shown in Fig. 7B, NADA was able to inhibit the luciferase expression induced by LPS in a dose-dependent manner. This result correlated with the inhibition by NADA at the concentration tested of the LPS-induced up-regulation of mPGES-1 at the mRNA level that we studied by qRT-PCR (Fig. 7C). Therefore, it is likely that NADA inhibited the inducible expression of mPGES-1 by acting at the transcriptional level. We also investigated the effects of NADA on the expression of the lipocalin PGD synthase (L-PGDS), which is expressed in the CNS and convert PGH_2 to PGD_2 [48]. b.end5 cells were stimulated with LPS, NADA or a combination of both for 12 h, the mRNA was isolated and the expression of L-PGDS mRNA analyzed by qRT-PCR. In Fig. 7D it is shown that LPS increased the mRNA levels for L-PGDS. NADA treatment also resulted in a weak induction of L-PGDS mRNA and an additive effect was found when the cells were treated with a combination of LPS and NADA.

4. Discussion

The metabolism of the arachidonic acid by cyclooxygenases plays an important role both in physiological and pathological events in the CNS [49]. Prostanoids produced by vascular endothelium are important intercellular messengers that participate in the regulation of vascular tone. However, increased permeability of BBB induced by high levels of PGs is a pathological hallmark in several neurological disorders [50], and BBB disruption during neuroinflammation can be significantly reduced by COX inhibitors [28,51]. In this study we demonstrated that NADA, the archetypical *N*-acyl-dopamine endowed with CB_1 and TRPV1 agonistic activities, 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 pathway that stabilizes *COX-2* mRNA resulting in the accumulation of the COX-2 protein, an effect that depends on the dopamine moiety of the molecule. 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 integrity under inflammatory conditions.

We have observed previously that NADA did not affect the expression of COX-2 either in either primary astrocytes or microglia cells [15]. In addition NADA have no effects on COX-2 expression in tumour cell lines of neuronal origin such as C6.1 and BV2 (data not shown) indicating that the effect of N-acetyldopamines on COX-2 regulation could be specific for brain endothelial cells, at least for the b.end5 cell line. We are currently performing experiments in primary brain endothelial cells and in other primary endothelial cells to confirm our findings in b.end5 cells. In contrast, our results confirm our previous data showing that NADA inhibited mPGES-1 expression and PGE_2 release in primary glia cells [15]. These findings corroborate the notion that suggests that LPS-induced expression of mPGES-1 proceeds through molecular mechanisms that are different from those involved in COX-2 induction [51,52], providing evidence that the expression of mPGES-1 and COX-2 are not always coupled as suggested by other authors [53]. The uncoupled regulation of COX-2 and mPGES-1 could explain the effect of NADA on the release of PGD_2, which has been shown to exert neuroprotective effects in different *in vitro* and *in vivo* models [54–56]. Moreover, PGD_2 could be converted to the anti-inflammatory prostanoid 15-deoxy-PGJ_2, which is also a neuroprotector lipid mediator [57,58]. It has been hypothesised that resolvins such as PGD_2 and 15-deoxy-PGJ_2 resolve active inflammatory processes [59,60]. In accordance with this hypothesis, Gilroy et al., showed that COX-2 was pro-inflammatory (via PGE_2 expression) during the development of inflammation, but anti-inflammatory (via PGD_2 and 15-deoxy-PGJ_2) during resolution [61].

The pharmacological manipulation of the ECs has opened new research avenues for the development of novel therapies for the treatment of inflammatory and neurodegenerative diseases [62,63]. In this sense, it has been shown that both AEA and the synthetic cannabinoid WIN 55,212–2 induce COX-2 expression in brain microvascular endothelial cells [42,64]. However, and in spite of its structural similarity, AEA and NADA have opposite effects on PGE_2 synthesis. While AEA favours the production and release of PGE_2 through a CB_1-dependent mechanism [64], NADA inhibits PGE_2 and enhances PGD_2 release in a CB_1- and TRPV1-independent manner and provides another example of the complexity of the ECs.

Although the dopamine moiety is absolutely required to induce *COX-2* mRNA stabilization, NADA and OLDA do not signal through D_1 or D_2 receptors to induce vasorelaxation [10,12]. This result is of special interest since it has been shown that OLDA (and perhaps NADA) can penetrate the BBB [65], and therefore this group of lypophilic molecules has been proposed as pro-drugs to increase the levels of dopamine in the brain. Little is currently known about the synthesis and source of NADA, but the acylation of intracellular compounds has been previously described and its functional relevance demonstrated. It is possible that upon demand

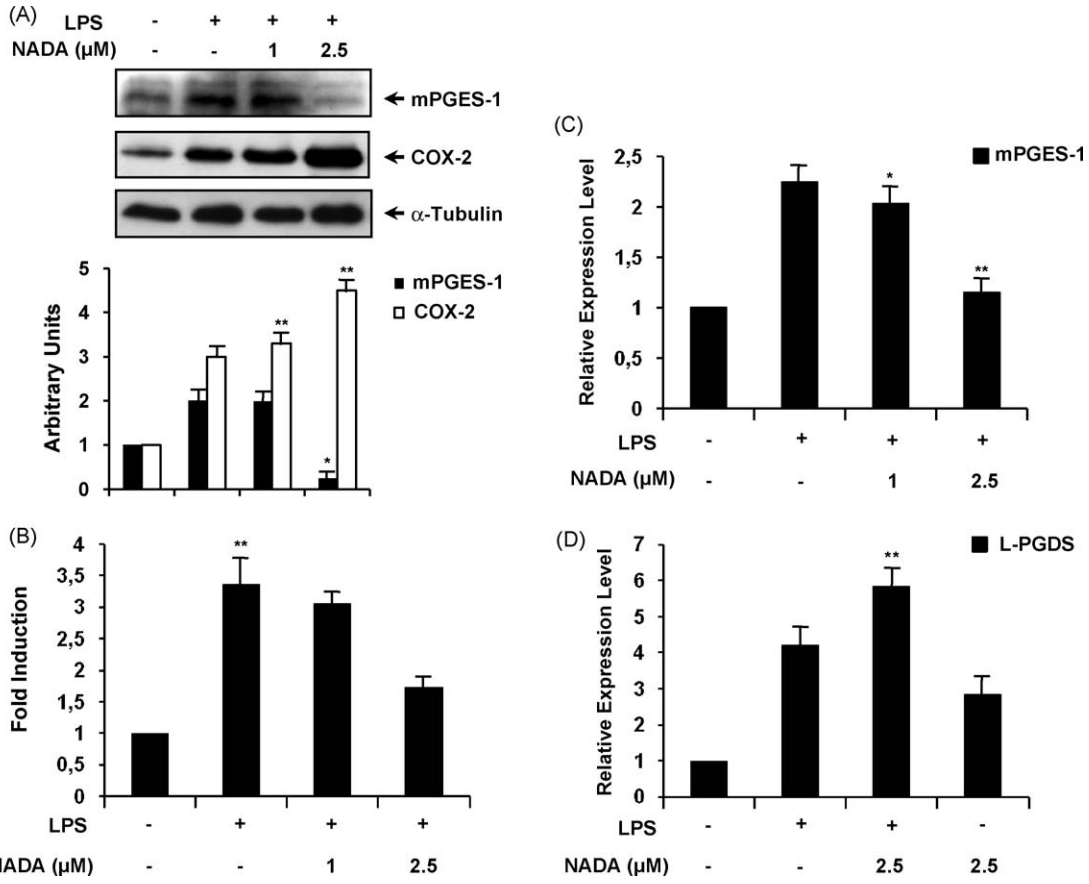

Fig. 7. Effects of NADA on mPGES-1 and L-PGDS expression in b.end5 cells. (A) b.end5 cells were preincubated during 30 min with NADA, and then stimulated with LPS for 12 h. The expression of mPGES-1 and COX-2 proteins was determined by immunoblot analysis of protein levels. We show a representative blot of three independent experiments and the means ± S.D. values of optical density (one-way ANOVA with Student's *t*-test $^{**}P < 0.001$ for LPS + NADA versus LPS). (B) b.end5 cells were transiently transfected with the luciferase reporter plasmid mPGES-1-Luc. Twenty-four h later they were preincubated during 30 min with NADA, stimulated with LPS for 12 h and the luciferase activity measured in the cell lysates. Results are the means ± S.D. of three determinations expressed as fold induction (one-way ANOVA with Student's *t*-test $^{**}P < 0.001$ LPS versus control). (C and D) Cells were preincubated during 30 min with NADA and then stimulated with LPS for 12 h and total RNA was extracted and the expression of mPGES-1 and L-PGDS mRNA was studied by qRT-PCR. The graphs represent the mPGES-1 (C) and L-PGDS (D) mRNA relative expression levels and show the means ± S.D. of three experiments (one-way ANOVA with Student's *t*-test $^{*}P < 0.05$ and $^{**}P < 0.001$ for LPS + NADA versus LPS).

dopamine acylation can take place in dopaminergic neurons from hypothalamus, nigra substance and chemo sensorial cells of the carotid body. For instance, under hypoxia conditions, carotid body cells increase the synthesis and release of dopamine [66], activate the arachidonic acid metabolism [67], and hydrolyse membrane phospholipids [68]. As a working hypothesis it could be possible that under hypoxia conditions these cells could synthesize NADA and other *N*-acyl-dopamines, which in turn could mediate two important functions; (1) transport dopamine to the brain and (2) induce COX-2 expression and PGD_2 release in cerebral microvascular cells allowing vasodilatation and the transport of nutrients and oxygen diffusion to the CNS.

At the CNS level it has been demonstrated that the hippocampus and the striatum are the regions of the brain containing higher concentrations of NADA [7]. Thus, the cells in these regions may also have a biosynthetic pathway of *N*-acyl-dopamines similar to the cells of the carotid body and under pro-inflammatory conditions CNS cells could release NADA in the brain and induce not only activities inherent to the activation of TRPV1 and CB_1 receptors but also neuroprotective activities by inhibiting the release of PGE_2 by activated glia and microvascular cells.

Since NADA is a very poor fatty acid amide hydrolase substrate, it has been proposed that NADA and perhaps other *N*-acyl-dopamines can be substrates of the enzyme catechol-O-methyl transferase (COMT) that is involved in the inactivation of catecholamines. COMT expression has been demonstrated in the cerebral cortex and neostriatum [69], and recently it has been observed that COMT co-localizes with TRPV1 in neurons for CA1 and CA3 areas and in Purkinje cells [70]. COMT transforms NADA into O-methyl-NADA, which is less active as a TRPV1 ligand [7]. Our results with Arvanil and AM404 suggest that O-methyl-NADA is probably inactive to stabilize *COX-2* mRNA and to inhibit mPGES-1 expression in b.edn5 cells and, therefore, COMT may be also the rate limiting enzyme for the anti-inflammatory activities of NADA. However, we can discard that the action of NADA and other *N*-acyl-dopamines are converted to the corresponding *N*-acyl-norepinephrines by the action of dopamine-β-hydroxylase.

Like many genes coding for mRNA short half-life (i.e. cytokines), the *COX-2* gene also contains ARE sequences (composed by six elements AUUUA), which regulate the stability of mRNA within the 3′-UTR region. Previous studies have shown that ARE sequences present in the 3′-UTR of *COX-2* gene are responsible for the mRNA stabilization [46,71,72]. Our results indicate that NADA, and probably other *N*-acyl-dopamines, increase the half-life of the *COX-2* mRNA in microvascular endothelial cells and this mechanism is dependent on the activation of p38. In other cell models it has been demonstrated that the activation of p38 allows the activation of MAPKAPK-2, which in turn activates a heat shock protein 27 (HSP27). This HSP27 has been implicated in the stabilization of *COX-2* mRNA and, therefore, it is very likely that the final mechanism by which NADA induces the stabilization of COX-2 is due to activation of HSP27.

All these results clearly show a potential role for NADA at the CNS highlighting the potential of endocannabinoids and *N*-acyldopamines as therapeutic agents for the treatment of inflammatory diseases.

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