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The Anandamide Effect on NO/cGMP Pathway in Human Platelets

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ABSTRACT

In this study the effect of the endocannabinoid anandamide on platelet nitric oxide (NO)/cGMP pathway was investigated. Data report that anandamide in a dose-and time-dependent manner increased NO and cGMP levels and stimulated endothelial nitric oxide synthase (eNOS) activity. These parameters were significantly reduced by LY294002, selective inhibitor of PI3K and by MK2206, specific inhibitor of AKT. Moreover anandamide stimulated both eNOSser1177 and AKTser473 phosphorylation. Finally the anandamide effect on NO and cGMP levels, eNOS and AKT phosphorylation/activation were inhibited by SR141716, specific cannabinoid receptor 1 antagonist, supporting the involvement of anandamide binding to this receptor. Overall data of this report indicate that low concentrations of anandamide, through PI3K/AKT pathway activation, stimulates eNOS activity and increases NO levels in human platelets. In such way anandamide contributes to extend platelet survival. J. Cell. Biochem. 112: 924–932, 2011.

KEY WORDS: ANANDAMIDE; HUMAN PLATELETS; NITRIC OXIDE; eNOS ACTIVITY; AKT

Endocannabinoids are a class of lipid mediators which includes amide and esters of long chain polyunsatured fatty acids. Anandamide (AEA) and 2-arachidonoylglycerol are the main endogenous agonists of type 1 (CB1) and type 2 (CB2) cannabinoid receptors [Salzet et al., 2000]. AEA seems to interact with CB1 and CB2 or to other binding sites such as type 1 vanilloid receptor [Bari et al., 2006; Di Marzo, 2008]. AEA is synthesized from membrane precursors by a specific *N*-acyl-phosphatidylethanolamine hydrolyzing phospholipase D [Di Marzo et al., 1994; Sugiura et al., 1996] and metabolized by intracellular hydrolysis to ethanolamine and arachidonic acid catalyzed by the enzyme fatty acid amide hydrolase (FAAH) [Di Marzo et al., 1994; McKinney and Cravatt, 2005]. When administrated in vivo AEA induces hypothermia [Pertwee et al., 1993], hypomotility [Mackie et al., 1993], and catalepsy [Smith et al., 1994]. AEA may be involved in the control of pain initiation [Calignano et al., 1998] or sleep induction [Mechoulam et al., 1997] as well as in immune [Facci et al., 1995; Pestonjamasp and Burstein, 1998; Cencioni et al., 2010] and cardiovascular functions [Kunos et al., 2000]. AEA has been shown to produce hypotension in animal models and vasorelaxation in a number of vascular beds [Kunos et al., 2002]. Enhanced AEA levels seem to be protective against inflammatory disorders such as atherosclerosis [Bátkai et al., 2007]. However the effect of AEA on isolated cells implicated in these processes is poorly documented. In human platelets AEA-membrane transporter and FAAH have been demonstrated [Maccarrone et al., 1999]. Moreover it was shown that AEA was able to extend platelets survival through CB1-dependent AKT signaling [Catani et al., 2010a]. Recently the expression of CB1 and CB2 receptors has been proved in human platelets [Catani et al., 2010b]. Both CB1 and CB2 are coupled to G1/G proteins and can activate PI3K/AKT pathway [Gómez del Pulgar et al., 2000; Liu et al., 2000].

Nitric oxide (NO) is the most important endogenous vasodilator which inhibits platelet aggregation, secretion, and adhesion. These NO effects are known to be mediated by cGMP which is produced by NO activated soluble guanylyl cyclase. The intracellular cGMP increase affects various platelet cGMP effector systems including cGMP-dependent protein kinase I (PKG1). Even if Münzel et al. [2003] supported an inhibitory role of cGMP/PKG1 system in platelet activation and aggregation, other authors proposed that cGMP has biphasic effect in platelets, promoting platelet activation at early times, and platelet inhibition at later times [Li et al., 2003]. In human platelets is present an independent L-arginine transport, and an endothelial nitric oxide synthase (eNOS)/NO pathway that

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produces NO [Radomski et al., 1990; Signorello et al., 2003]. NO formation depends on eNOS activation. The regulation of eNOS is a complex mechanism. Although eNOS was originally reported as a strictly calcium/calmodulin-dependent enzyme, there is some evidence that eNOS can be also activated in a calcium/calmodulin-independent manner when stimulated with agonists such as adiponectin [Hattori et al., 2003], by insulin [Hartell et al., 2005; Fleming et al., 2003] or by shear stress [Boo, 2006]. Platelet eNOS is now largely considered a calcium-independent enzyme and the phosphorylation/dephosphorylation of ser1177 and/or thr495 residues play a vital role in the regulation of its activity. Phosphorylation of ser1177 residue activates eNOS, while phosphorylation of thr495 residue, being the negative regulatory site, inhibits the activity of the enzyme [Randriamboavonjy and Fleming, 2005]. This study has investigated the platelet response of AEA on NO/cGMP pathway and the molecular signaling mechanisms of AEA-mediated eNOS activation.

MATERIALS AND METHODS

MATERIALS

Anandamide, L-arginine, BAPTA/AM, cadmium beads, Colorburst™ electrophoresis marker, DAF 2-DA, digitonin, Dowex AG 50W-X8, EGTA, β-mercaptoethanol, PGE_1, standard molecular weight markers and all chemicals were from Sigma–Aldrich, USA. FURA 2/AM and LY294002 were purchased from Merck Biosciences, Germany. MK2206 was from Selleck Chemicals USA. SR141716 (SR1) and SR144528 (SR2) were from Sanofi-Aventis Recerche, France. URB597 was from Alexis Biochemicals, USA. Inhibitors were diluted in saline from a stock DMSO solution immediately before each experiment. cGMP EIA kits was from Assay Design USA. Anti-eNOSser1177 antibody were from Millipore, USA. Anti-phospho-eNOSser1177 was from Cell Signalling Technology, USA. Anti-AKT, anti-phospho-AKTser473, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, USA. L-[2,3,4,5-3H] arginine was from PerkinElmer Life and Analytical Sciences, USA. ECL® system was from GE Healthcare, USA. Nitrocellulose membranes (pore size 0.45 μm) were purchased from Bio-Rad Laboratories, USA. Collagen was from Mascia Brunelli S.p.a., Italy.

BLOOD COLLECTION AND PREPARATIVE PROCEDURES

Freshly drawn venous blood from healthy volunteers of the ''Centro Trasfusionale, Ospedale San Martino'' in Genoa was collected into 130 mM aqueous trisodium citrate anticoagulant solution (9:1). The donors claimed to have not taken drugs known to interfere with platelet function during 2 weeks prior to blood collection, and gave their informed consent. Washed platelets were prepared centrifuging whole blood at 100 × *g* for 25 min. The obtained platelet-rich plasma was then centrifuged at 1,100 × *g* for 15 min. Pellet was washed once with pH 5.2 ACD solution (75 mM trisodium citrate, 42 mM citric acid, and 136 mM glucose), centrifuged at 1,100 × *g* for 15 min and then resuspended, if not otherwise reported, at 1.0×10^9/mL in pH 7.4 Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES) containing 2 mM $CaCl_2$. All the experiments, except for aggregation studies, were carried out in unstirring conditions.

AGGREGATION STUDIES

Platelet aggregation, performed in a Menarini Aggrecoder PA-3210 aggregometer, was monitored according to Born's method [1962, and quantified by the light transmission reached within 3 min. Washed platelets (3.0×10^8 platelets/mL) were preincubated with saline at 37°C before adding AEA.

INTRACELLULAR CA^{2+}MEASUREMENT

Washed platelets (3.0×10^8 platelets/mL) were incubated with 1 μg/mL FURA 2/AM for 45 min at 37°C. Two μM PGE_1 and 1 mM EGTA were added before centrifuging loaded platelets for 15 min at 1,100 × *g*. The pellet, resuspended at 2.0×10^8 platelets/mL in calcium-free Hepes buffer (pH 7.4), was preincubated at 37°C with saline then AEA was added. Fluorescence of FURA 2/AM-loaded platelets was monitored at 37°C for 3 min in a Perkin-Elmer fluorescence spectrometer model LS50B, with excitations at 340 and 380 nm and emission at 509 nm. The fluorescence of fully saturated FURA 2/AM (Fmax) was obtained by lysing the cells with 50 μM digitonin in the presence of 2 mM Ca^{2+}, while Fmin was determined by exposing the lysed platelets to 1 M EGTA. The fluorescence was fully quenched with 5 mM Mn^{2+}, in order to calculate the autofluorescence value. A software combined with the fluorescence spectrometer converted data into cytosolic Ca^{2+} concentration, yielding a Kd value for FURA 2/AM and Ca^{2+} of 135 nM.

NITRITE + NITRATE (NOX) MEASUREMENT

Washed platelets, prewarmed at 37°C with saline or additions, were incubated with 100 μM L-arginine and AEA as indicated. Incubation was stopped by sample sonication on ice. To measure NOx content suitable aliquots of supernatants were added to equal volumes of assay buffer (15 g/L glycine-NaOH, pH 9.7), containing activated cadmium beads and incubated for 2 h at room temperature under horizontal shaking. Cadmium beads, activated immediately before each experiment by 5 mM $CuSO_4$ in assay buffer, were washed three times with assay buffer. NOx formation, determined by the Griess reagent (l% sulphanilamide in 2.5% H_3PO_4 and 0.1% naphtylene-diamine dihydrochloride), was measured at 540 nm using a sodium nitrite calibration curve. Between experiments cadmium beads were stored in 0.1 M H_2SO_4.

cGMP DETECTION

Washed platelets were prewarmed at 37°C with saline or additions and then incubated for the indicated times with 100 μM L-arginine and AEA. The reaction was stopped by the addition of cold perchloric acid (2 M). Precipitated proteins were removed by means of a centrifugation at 12 000 × *g* for 2 min at 4°C. Obtained supernatants, neutralized with 2 M NaOH, were immediately analyzed by a cGMP specific EIA kit according to the manufacturer's protocol.

CONFOCAL MICROSCOPY

Nitric oxide formation was further detected by confocal microscopy. Briefly washed platelets were loaded with 5 μM DAF 2-DA for 30 min at 37°C. Two μM PGE_1 and 1 mM EGTA were added before

centrifuging loaded platelets for 15 min at $1{,}100 \times g$. Pellet, resuspended at 1.0×10^9 platelets/mL in Hepes buffer (pH 7.4) containing 2 mM $CaCl_2$, was preincubated at 37°C with saline or additions and then stimulated for 1 min at 37°C with AEA or collagen. After a brief centrifugation, pellet was resuspended in the same buffer and suitable aliquots of the obtained suspension were mounted on coverslips. Images were taken using a Leica TCS SL2 confocal microscope (Leica Wetzlar, Germany) equipped with argon/He–Ne laser sources and anHCX PL APO CS 63.0×1.40 oil objective. During image acquisition, the 488 laser was set at 25% energy, the emission range was between 500 and 540 nm, and the photomultiplier voltage gain was set to eliminate platelets autofluorescence. Imaging settings were kept constant for all experiments to allow direct comparison between control and treated platelets and identical contrast adjustments were performed for all images.

eNOS ACTIVITY ASSAY

Endothelial nitric oxide synthase activity was measured by evaluating the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, according to the method already adapted to human platelets [Russo et al., 2004]. Briefly, aliquots of washed platelets, prewarmed at 37°C with saline or additions, were incubated with AEA as indicated in the presence of 1 μCi L-[^{3}H] arginine. Incubation was stopped by putting samples in ice. Platelets were then pelletted by centrifugation at $2{,}000 \times g$ for 4 min. After sonication, platelet lysates were mixed with Dowex AG 50W-X8 (Na^+-form) to absorb L-arginine and L-[^{3}H] citrulline was measured in supernatants by liquid scintillation counting (Packard Instruments).

IMMUNOBLOTTING ANALYSIS

Washed platelets, prewarmed with saline or additions at 37°C, were stimulated with AEA. Incubation was stopped by the addition of $2 \times$ Laemmli-SDS reducing sample buffer. Samples, heated for 5 min at 100°C, were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in 6% BSA (AKT) or 5% fat-free dry milk (eNOS) dissolved in TBST (Tris buffer saline, pH 7.6, containing 10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) at 37°C for 30 min and then incubated overnight at 4°C with anti-phospho-AKTser473 or anti-phospho-eNOSser1177 antibodies. Thus membranes were extensively washed and incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody. After further washings, blots were developed using the ECL® system and revealed by Bio-Rad Chemi-Doc instrument. Finally, nitrocellulose membranes, stripped by incubation with 62.5 mM Tris/HCl (pH 6.7), 2% SDS, and 100 μM β-mercaptoethanol for 30 min at 50°C, were reprobed with anti-AKT or anti-eNOSser1177 antibodies.

STATISTICAL ANALYSIS

Data are mean ± SD of at least five independent experiments, each performed in triplicate. Statistical comparisons between two groups were made through the unpaired Student's *t*-test. One-way ANOVA followed by Bonferroni's post hoc test was used to compare multiple groups. Statistical significance was defined as $P < 0.05$.

RESULTS

EFFECT OF AEA ON PLATELET AGGREGATION AND INTRACELLULAR CALCIUM ELEVATION

Anandamide (AEA) induced platelet aggregation and intracellular calcium elevation at non-physiological concentrations. In fact only at 1,000 μM the AEA effect on platelet aggregation was time-dependent and peaked after 2 min of platelet stimulation. Moreover 1,000 μM AEA gave rise to a gradual time-dependent increase of intracellular calcium concentration. On the contrary low AEA concentration (1–100 μM) were unable to induce aggregation as well as intracellular calcium elevation (Fig. 1).

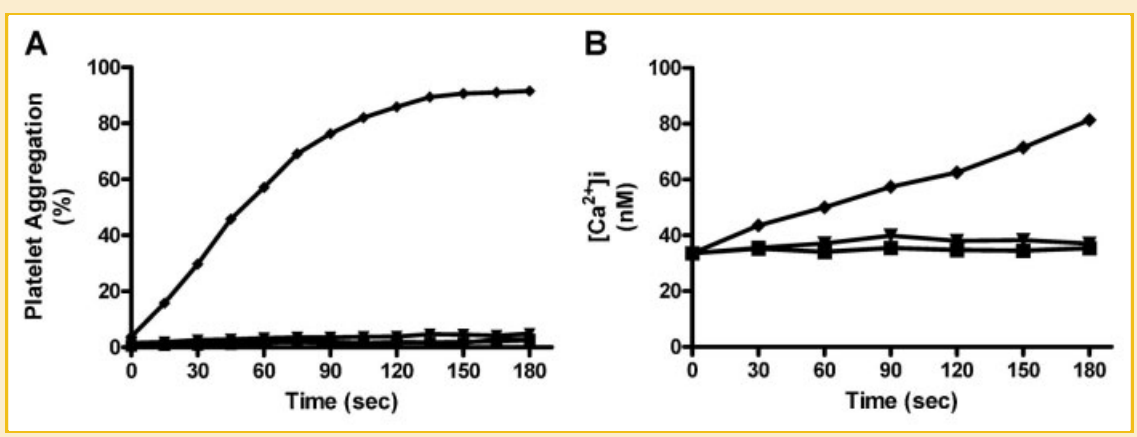

Fig. 1. Platelet aggregation and intracellular calcium elevation induced by AEA. Washed platelets (3.0×10^8 platelets/mL), preincubated at 37°C in the presence of saline, were challenged with AEA. Panel **(A)** reports platelet aggregation induced by varying concentrations of AEA (see below). Aggregation was quantified by the light transmission reached within 3 min. Panel **(B)** reports intracellular calcium elevation quantified in FURA 2-loaded platelets (2.0×10^8 platelets/mL), preincubated with saline at 37°C before the addition of varying AEA concentrations. Calcium elevation was monitored at 37°C in a fluorescence spectrophotometer, as detailed in Methods. Tracings of A and B are representative of five independent determinations. AEA was used at the following concentrations: 1 μM (■), 10 μM (▲), 100 μM (▼) or 1000 μM ().

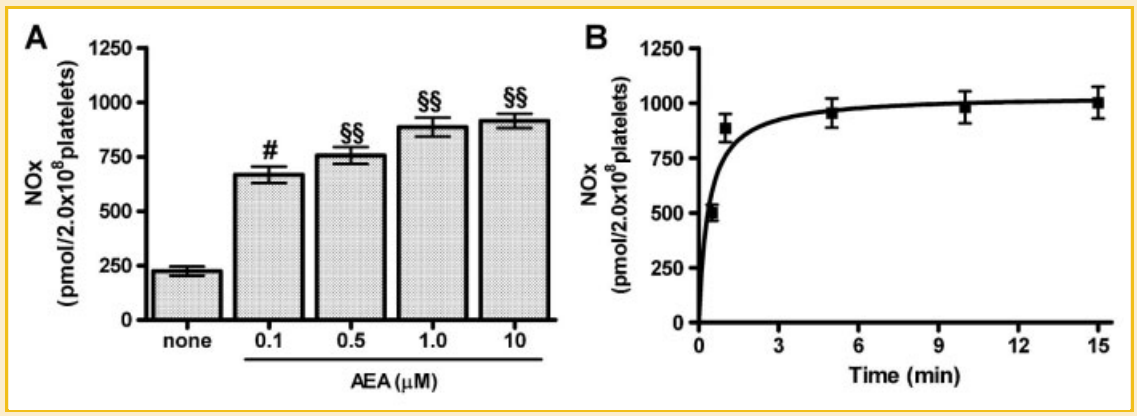

Fig. 2. The AEA effect on NOx formation. Washed platelets (1.0×10^9 platelets/mL) were prewarmed at 37°C with saline and then incubated in the presence of 100 μM L-arginine for 1 min with the indicated AEA concentrations (panel **A**). In panel **(B)** washed platelets were incubated in the presence of L-arginine with 1.0 μM AEA. NOx content was determined as detailed in Methods. Each bar of panel (A) represents the mean ± SD of five independent experiments carried out in triplicate. The curve reported in panel (B) is representative of five independent determinations. One way ANOVA-Bonferroni's post hoc test: #$P < 0.001$; §§$P < 0.05$.

EFFECT OF AEA ON NO AND cGMP LEVELS

Low concentrations of AEA (from 0.1 to 10 μM) induced a significant NO increase. The dose-response curve was dose-dependent with a peak at 1.0 μM AEA (Fig. 2A). Moreover the AEA effect was very rapid, peaked after 1 min of incubation and sustained for 15 min (Fig. 2B). NO signaling is often mediated by cGMP formation occurring through soluble guanylyl cyclase activation. Thus cGMP elevation upon platelet treatment with AEA was measured. As shown in Figure 3, the AEA effect on cGMP formation was rapid, dose-dependent and correlated with NO formation ($y = 0.002712 \times + 0.4902$; $r^2 = 0.9074$; $P = 0.0123$). On the basis of these results all experiments have been performed in the presence of 1.0 μM AEA that was incubated with platelets for 1 min. Since AEA activates different signaling pathways dependent on the receptor engaged, platelets were pretreated with 1.0 μM SR1 or 1.0 μM SR2, specific antagonists for CB1 or CB2 receptors, respectively. As shown in Figure 4 SR1 significantly reduced both NOx and cGMP formation, while SR2 failed to affect these parameters. Hydrolysis of AEA by FAAH produces arachidonic acid [Maccarrone et al., 1999]. Thus we wanted to verify whether the stimulatory effect of AEA on platelets could be mediated by arachidonic acid derived by AEA hydrolysis. We observed that the addition of URB597, inhibitor of FAAH, did not change NOx and cGMP formation in platelets stimulated by 1 μM AEA. Thus AEA acts by itself and not by arachidonic acid released. It is known that eNOS activation and the consequent NO formation can be dependent on PI3K/AKT pathway activation [Woulfe, 2010]. Thus the effect of specific inhibitors of PI3K and AKT was tested. It was found that LY294002, selective inhibitor of PI3K, and MK2206, specific inhibitor of AKT, strongly impaired both NO and cGMP formation induced by AEA.

Nitric oxide produced in platelets treated with AEA was also tested by confocal microscopy (Fig. 5). By this technique DAF 2-loaded platelets have been examined. Upon treatment with

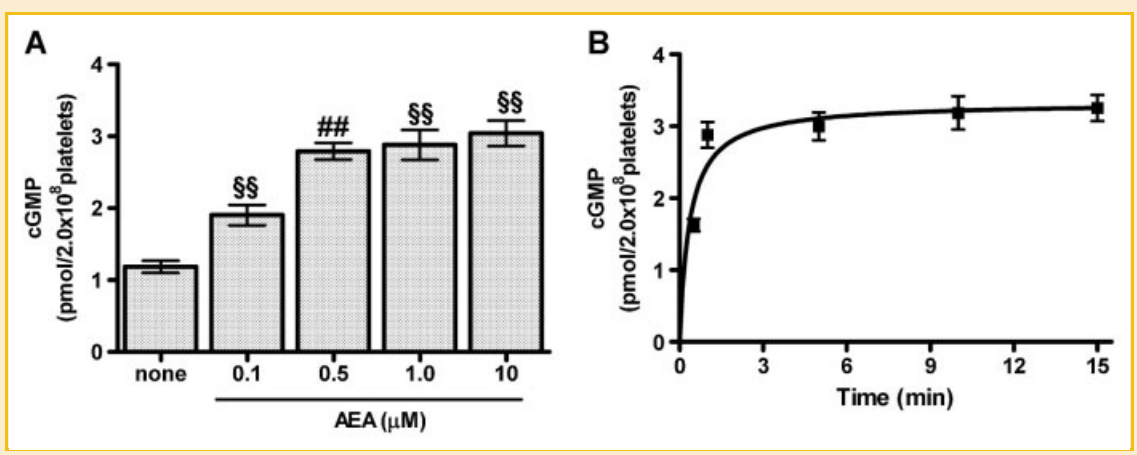

Fig. 3. The AEA effect on cGMP formation. Washed platelets (1.0×10^9 platelets/mL) were prewarmed at 37°C with saline and then incubated in the presence of 100 μM L-arginine for 1 min with the indicated AEA concentrations (panel **A**). In panel **(B)** washed platelets were incubated in the presence of L-arginine with 1.0 μM AEA. cGMP content was determined as detailed in Methods. Each bar of panel (A) represents the mean ± SD of five independent experiments carried out in triplicate. The curve reported in panel (B) is representative of five independent determinations. One way ANOVA-Bonferroni's post hoc test: ## $P < 0.01$; §§ $P < 0.05$.

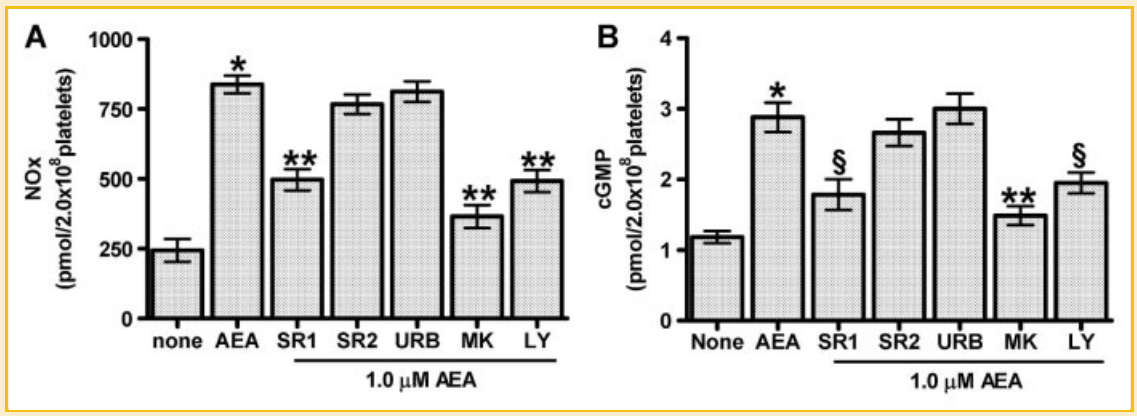

Fig. 4. Effect of selected agents on NOx and cGMP formation induced by AEA. Washed platelets (1.0×10^9 platelets/mL), prewarmed at 37°C with saline or 1 μM SR1, 1 μM SR2, 20 μM URB597 (URB), 1 μM MK2206 (MK) or 20 μM LY294002 (LY), were incubated for 1 min with 100 μM L-arginine in the presence of 1.0 μM AEA. NOx (panel **A**) and cGMP (panel **B**) content were determined as detailed in Methods. Each bar represents the mean ± SD of five independent experiments carried out in triplicate. Student's *t*-test: $^{*}P < 0.0001$ versus none; $^{**}P < 0.0005$; §$P < 0.005$ versus AEA

increasing concentrations of AEA from 0.1 to 10 μM (Fig. 5B–D) platelets were fully fluorescently marked as compared to control (Fig. 5A) and the increase in DAF 2 fluorescence intensity appears to be dose-dependent. In agreement with data shown in Figure 4, SR1 (Fig. 5E) and MK2206 (Fig. 5F) abolished fluorescence intensity induced by AEA, while LY294002 (Fig. 5G) was less potent. Collagen was used as positive control (Fig. 5G).

EFFECT OF AEA ON eNOS ACTIVITY

Increase of NOx levels by AEA could be related to eNOS activation. To verify this hypothesis in lysates of platelets incubated with AEA under varying conditions eNOS activity was assayed. AEA very rapidly (Fig. 6B) and in a dose-dependent manner increased eNOS activity (Fig. 6A,B). A good correlation between NOx and eNOS activity stimulated by AEA was found ($y = 0.004131 \times + 1.529$, $r^2 = 0.9668$, $P = 0.00026$). In agreement with above reported data on NO and cGMP formation SR1, MK2206, and LY294002 significantly decreased eNOS activity (Fig. 6C).

THE AEA EFFECT ON eNOS PHOSPHORYLATION STATUS

The activity of eNOS has been shown to be tightly dependent on its phosphorylation status. In particular phosphorylation of ser1177

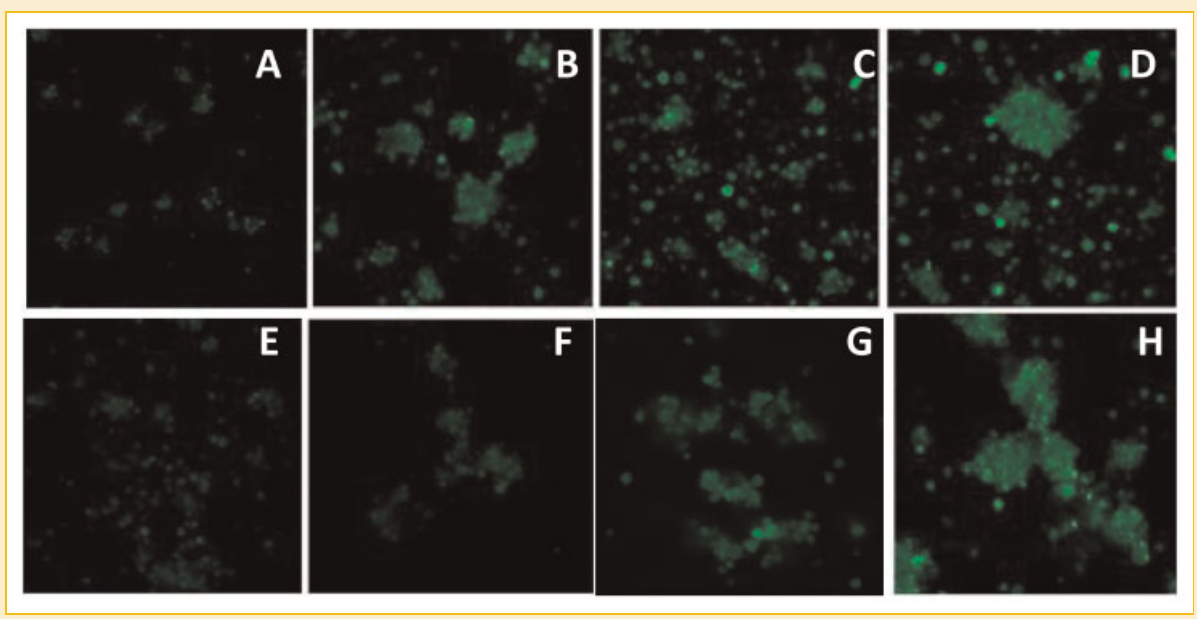

Fig. 5. Confocal microscopy images of NO formation. DAF 2 DA-loaded washed platelets (1.0×10^9 platelets/mL) were preincubated at 37°C with saline (**A**), and then stimulated for 1 min with 0.1 (**B**), 1.0 (**C**) or 10 (**D**) μM AEA. In Panel (**E–F–G**) washed platelets were preincubated with 1 μM SR1 (E), 1 μM MK2206 (F) or 20 μM LY294002 (G), and then stimulated for 1 min with 1.0 μM AEA. In panel (**H**) is reported the effect of 5 μg/mL collagen, used as a positive control. All the experiments were carried out in the presence of 100 μM L-arginine. NO formation was visualized by confocal microscopy as detailed in Methods.

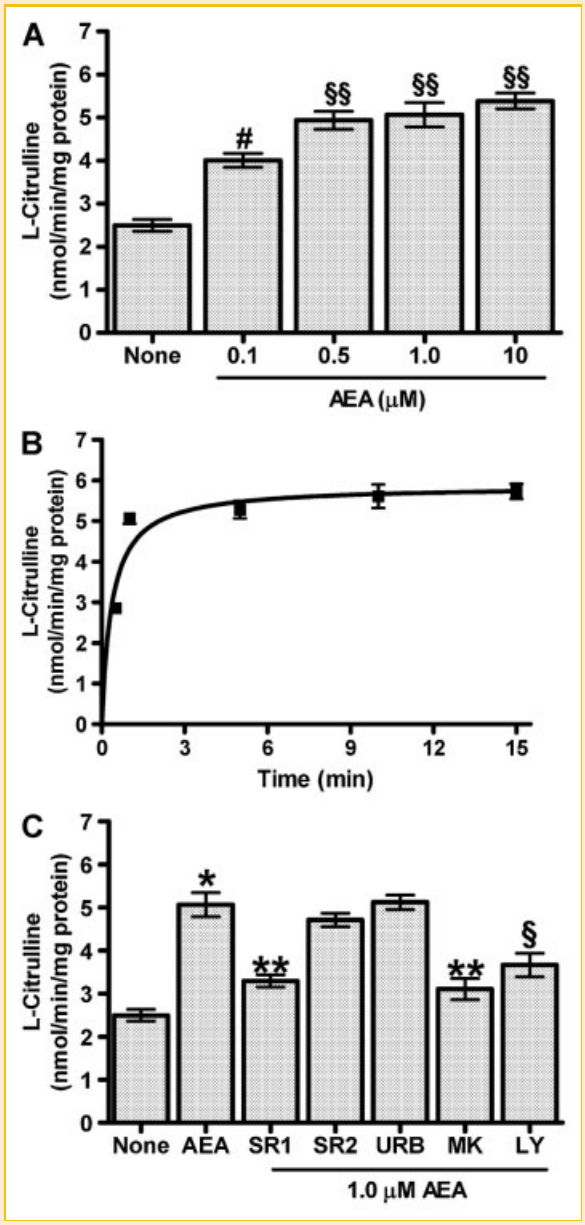

Fig. 6. Effect of AEA on eNOS activity. Washed platelets (1.0×10^9 platelets/mL) were prewarmed at 37°C with saline and then incubated in the presence of 100 μM L-arginine for 1 min with the indicated AEA concentrations (panel A). In panel (B) washed platelets were incubated in the presence of L-arginine with 1.0 μM AEA. In panel (C) washed platelets, preincubated with saline, 1 μM SR1, 1 μM SR2, 20 μM URB597 (URB), 1 μM MK2206 (MK), or 20 μM LY294002 (LY), were stimulated for 1 min with 1.0 μM AEA in the presence of 100 μM L-arginine. eNOS activity was assayed in platelet lysates as detailed in Methods. Each bar of panel (A) and (C) represents the mean ± SD of five independent experiments carried out in triplicate. The curve reported in panel (B) is representative of five independent determinations. One way ANOVA-Bonferroni's post hoc test: #$P < 0.001$, §§$P < 0.05$; Student's *t*-test: *$P < 0.0001$ versus none; **$P < 0.0005$; §$P < 0.005$ versus AEA.

activates the enzyme. Thus eNOSser1177 phosphorylation status was checked. Platelet treatment with AEA dose-dependently increased eNOS phosphorylation on ser1177 residue. The effect was very rapid and peaked after 1 min of platelet incubation with

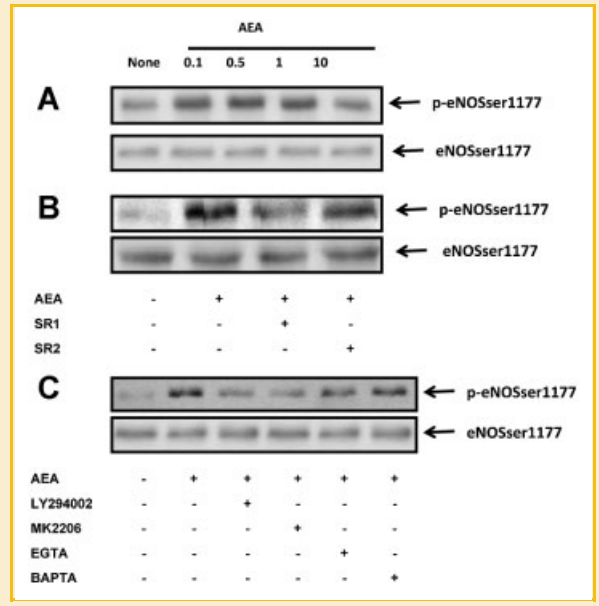

Fig. 7. The AEA effect on eNOS phosphorylation. Washed platelets (1.0×10^9 platelets/mL), preincubated at 37°C with saline, 1 μM SR1, 1 μM SR2, 20 μM LY294002, 1 μM MK2206, 1.0 mM EGTA, or 30 μM BAPTA/AM, were stimulated for 1 min with 1.0 μM AEA. At the end of incubation suitable aliquots were immunoblotted with anti-p-eNOSser1177 as detailed in Methods. Blots are representative of five independent experiments.

1 μM AEA (Fig. 7A). Moreover the CB1 receptor antagonist SR1 greatly decreased eNOSser1177 phosphorylation induced by AEA, whereas SR2 had a minor effect (Fig. 7B). On the other hand eNOSser1177 phosphorylation was greatly reduced by LY294002 and cancelled by MK2206 but it was not modified by platelet pretreatment with EGTA or BAPTA/AM (Fig. 7C). Since MK2206 greatly impaired eNOSser1177 phosphorylation, it was assumed that AKT could be involved in the eNOS phosphorylation/activation. Thus in platelets treated with AEA the AKT phosphorylation status was assayed. It was found that AEA stimulated AKTser473 phosphorylation in a dose-dependent manner from 0.1 to 1 μM (Fig. 8A). Moreover in agreement with the above reported data on other parameters, SR1 abolished AKT phosphorylation, while SR2 was poorly effective (Fig. 8B).

DISCUSSION

Not numerous are the information concerning the AEA effect on platelets. Previous studies [Maccarrone et al., 1999] and this report have shown that high concentrations of AEA (about 1.0 mM) are able to activate human platelets. In contrast, in rabbit platelets physiological concentrations of AEA (3–10 μM) caused activation and the endocannabinoid effect was sensitive to cyclo-oxygenase inhibition, but insensitive to the CB1 receptor antagonist rimonabant [Braud et al., 2000]. To gain some insight into the molecular mechanisms elicited by AEA in human platelets, we studied the effect of this endocannabinoid on NO/cGMP pathway. We found that low concentrations of AEA increase platelet NO and cGMP basal

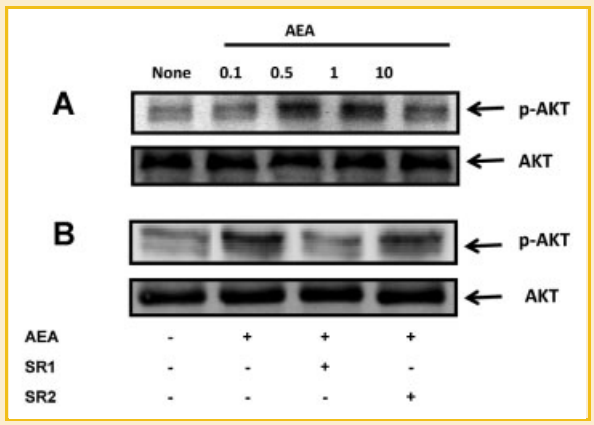

Fig. 8. The AEA effect on AKT phosphorylation. Washed platelets (1.0×10^9 platelets/mL), preincubated at 37°C with saline, 1 μM SR1 or 1 μM SR2, were stimulated for 1 min with 1.0 μM AEA. At the end of incubation suitable aliquots were immunoblotted with anti-p-AKT as detailed in Methods. Blots are representative of five independent experiments.

levels and stimulates eNOS activity, in agreement with data previously obtained in other cellular types such as monocytes [Stefano et al., 1996], human [Fimiani et al., 1999], and rabbit endothelial cells [McCollum et al., 2007]. Thus AEA, that cannot be considered a true agonist, can play other functions in platelets. For instance it may be one of the factors required for platelet survival and the elevation of endogenous NO can play an important role in these mechanisms, as NO can be considered a central effector in the regulation of both cell survival and apoptosis [Choi et al., 2002]. A key role of NO bioavailability in fatal arterial hypertension [Hu et al., 2010], in gestation [Suzuki et al., 2010], in liver fibrosis resolution [Mòdol et al., 2011], and in the regulation of erythrocytes survival was recently demonstrated [Nicolay et al., 2008; Chowdhury et al., 2010]. Varying kinases (PKA, AKT, calmodulin-dependent kinase II or AMP-activated protein kinase), dependent on the stimuli applied, appear to be involved in eNOS phosphorylation and activation. For instance adenosine and catecholamines stimulate platelet PKA activation, the phosphorylation of ser1177 residue and the consequent NO formation [Anfossi et al., 2002a; Anfossi et al., 2002b]. It was reported that phosphorylation of eNOSser1177 increases 2–3 fold NO basal level [McCabe et al., 2000]. Insulin increases platelet eNOS activity through eNOSser1177 residue phosphorylation [McCabe et al., 2000] and this effect is dependent on activation of AKT and partially on AMP-dependent protein kinase activation [Fleming et al., 2003]. Previously it was shown that the phosphorylation site on eNOSser1177 is a specific substrate of AKT [Woulfe, 2010]. In the present study it is shown that AEA increases eNOSser1177 phosphorylation (Fig. 7A) and stimulates eNOS activity (Fig. 6A). Since MK2206 significantly decreases eNOSser1177 phosphorylation, the kinase involved in this effect seems to be AKT. In addition AEA in a quite similar manner stimulates AKT phosphorylation and eNOSser1177 phosphorylation (Figs. 7 and 8). AKT is a ser/thr kinase that is critical for many functions such as cell survival, cell cycle progression, agonist-induced platelet activation, and the modulation of several target enzymes, including eNOS [Woulfe, 2010]. There are at least three different AKT isoforms identified in humans, which display more than 80% sequence homology and are named AKTα, AKTβ, and AKTγ. It has been shown that AKTα and AKTβ are expressed in human platelets, being AKTα major AKT subtype, whereas AKTγ is absent in human platelets [Kroner et al., 2000]. A key enzyme in AKT phosphorylation/activation is PI3K [Kroner et al., 2000]. AKT is activated by various agonists including platelet-derived growth factor, epidermal growth factor, insulin, nerve growth factor, U46619 (a thromboxane A_2 analogue), convulxin (a glycoprotein VI agonist), and thrombin [Kroner et al., 2000; Barry and Gibbins, 2002; Cho et al., 2002]. The AEA effect on NO, cGMP formation, and eNOS activation is significantly inhibited by SR1, indicating that AEA binds specifically to CB1.

In conclusion this study shows that AEA, through a CB1 receptor-mediated effect, stimulates eNOS activity and increases NO levels. The PI3K/AKT signaling pathway is involved in this activation. Thus the AEA capacity to increase NO level in human platelets could fit into many beneficial effects produced by this endocannabinoid during ischemic conditions [Wagner et al., 1998], as AEA produces vasodilatation in the arterial [Mukhopadhyay et al., 2002], isolated mesenteric [Kunos et al., 2000; Kunos et al., 2002], other vascular preparations [O'Sullivan et al., 2004], and in cultured rabbit aortic endothelial cells. Moreover AEA, stimulating platelet NO elevation, can contribute to extend platelet survival and can limit the self-amplification of platelet thrombus formation in vivo [McCollum et al., 2007].

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