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# Activation by 2-Arachidonoylglycerol of Platelet p38MAPK/cPLA$_2$ Pathway

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## ABSTRACT

The endogenous cannabinoid 2-arachidonoylglycerol (2-AG) is described as a platelet agonist able to induce aggregation and to increase intracellular calcium. In the present report we have confirmed these data and demonstrated that the inhibitor of p38MAPK SB203580 and the inhibitor of cPLA$_2$ metabolism ETYA affect both these parameters. Thus, we aimed to define the role of p38MAPK/cytosolic phospholipase A$_2$ (cPLA$_2$) pathway in 2-AG-induced human platelet activation. p38MAPK activation was assayed by phosphorylation. cPLA$_2$ activation was assayed by phosphorylation and as arachidonic acid release and thromboxane B$_2$ formation. It was shown that 2-AG in a dose- and time-dependent manner activates p38MAPK peaking at 10 μM after 1 min of incubation. The 2-AG effect on p38MAPK was not impaired by apyrase, indomethacin or RGDS peptide but it was significantly reduced by SR141716, specific inhibitor of type-1 cannabinoid receptor and unaffected by the specific inhibitor of type-2 cannabinoid receptor SR144528. Moreover, the incubation of platelets with 2-AG led to the phosphorylation of cPLA$_2$ and its activation. Platelet pretreatment with SB203580, inhibitor of p38MAPK, abolished both cPLA$_2$ phosphorylation and activation. In addition SR141716 strongly impaired cPLA$_2$ phosphorylation, arachidonic acid release and thromboxane B$_2$ formation, whereas SR144528 did not change these parameters. Finally platelet stimulation with 2-AG led to an increase in free oxygen radical species. In conclusion, data provide insight into the mechanisms involved in platelet activation by 2-AG, indicating that p38MAPK/cPLA$_2$ pathway could play a relevant role in this complicated process. J. Cell. Biochem. 112: 2794–2802, 2011. 

**KEY WORDS:** 2-ARACHIDONOYLGLYCEROL; HUMAN PLATELETS; p38MAPK; PHOSPHOLIPASE A2; ARACHIDONIC ACID

Endogenous cannabinoids are biologically active lipids that include anandamide and 2-arachidonoylglycerol (2-AG) as prototype members of fatty acid amides and monoacylglycerols, respectively [Petrosino et al., 2009]. It was proposed that anandamide and 2-AG are the main endogenous agonists of type-1 (CB1) and type-2 (CB2) cannabinoid receptors [Salzet et al., 2000], both of which belong to the superfamily of G-protein-coupled receptors. Apparently, 2-AG is the true physiological agonist of CB1 and CB2 [Sugiura and Waku, 2002]. 2-AG is a derivative of arachidonic acid conjugated with glycerol which can be formed from membrane phospholipids or lysophosphatidic acid and elicits a variety of biological responses "in vitro" and "in vivo" by targeting cannabinoid receptors in an autocrine and/or paracrine manner [Sugiura et al., 2006]. 2-AG is rapidly metabolized to arachidonic acid and glycerol mainly through the activity of a monoacylglycerol lipase [Di Marzo et al., 1999] and, to a lesser extent, by fatty acid amide hydrolase [Di Marzo et al., 1998]. Remarkably, endocannabinoids play major roles within the cardiovascular system [Randall, 2007; Mach et al., 2009]. Human vascular endothelial cells and human platelets generate 2-AG upon stimulation [Sugiura et al., 1998; Maccarrone et al., 2001]. Human platelets have the biochemical tools to metabolize endocannabinoids [Maccarrone et al., 2001], and are activated by micromolar concentrations of 2-AG through a CB1/CB2-dependent mechanism that leads to increased intracellular calcium and inositol-1,4,5 trisphosphate, and decreased cAMP [Maccarrone et al., 2001]. 2-AG has been shown to commit the bi-potential HEL cell line to the megakaryoblastic lineage [Catani et al., 2009]. The expression of CB1/CB2 receptors in human platelets recently has been demonstrated [Catani et al., 2010].

Arachidonic acid is released from membrane phospholipids by cytosolic phospholipase A$_2$ (cPLA$_2$) in response to a wide spectrum of extracellular stimuli including cytokines, hormones, and growth factors or by agents such as oxidation, hyperglycemia, UV light, and shear stress that mediate non-receptor stimulation of cells. cPLA$_2$ is regulated by an increase in intracellular calcium, which stimulates

Abbreviations used: 2-AG, 2-arachidonoylglycerol; CB1, type-1 cannabinoid receptors; CB2, type-2 cannabinoid receptor; cPLA$_2$, cytosolic phospholipase A$_2$; ROS, reactive oxygen species; SR1, SR141716; SR2, SR144528.

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its translocation from the cytosol to membranes through a calcium-dependent phospholipids binding domain [Clark et al., 1991; Nalefski et al., 1994; Schievella et al., 1995; Kramer and Sharp, 1997; Perisic et al., 1998] and by phosphorylation [Lin et al., 1992]. A role in the platelet $cPLA_2$ phosphorylation for p38MAPK has been proposed. Platelet p38MAPK has been found to be phosphorylated in response to thrombin [Kramer et al., 1995; Kramer et al., 1996], collagen [Saklatvala et al., 1996], thromboxane analogue [Saklatvala et al., 1996], low-density lipoproteins [Hackeng et al., 1999], and von Willebrand factor [Canobbio et al., 2004]. p38MAPK has been shown to phosphorylate $cPLA_2$ on ser505, an event associated with the activation of the enzyme [Kramer et al., 1996; Borsch-Haubold et al., 1997, 1998a].

Platelet activation is a complicated process involving distinct but related pathways. In this study, we examined whether or not 2-AG could induce the activation of p38MAPK/$cPLA_2$ pathway, which is known to play an essential role in the intracellular signal transduction of stimulated cells. We have demonstrated that p38MAPK is rapidly phosphorylated and activated by 2-AG. Activated p38MAPK phosphorylates and participates to the $cPLA_2$ activation, leading to arachidonic acid release and thromboxane (TX) $A_2$ formation. These results indicate that the p38MAPK/$cPLA_2$ pathway could play an important role in 2-AG induced platelet activation/aggregation.

## MATERIALS AND METHODS

### MATERIALS

2-AG, apyrase, Colorburst™ electrophoresis weight markers, ETYA (inhibitor of $cPLA_2$ activation and arachidonic acid metabolism), DCF (2′,7′-dichlorofluorescin), digitonin, diphenyleneiodonium (DPI, inhibitor of NADPH oxidase), GF109203X (inhibitor of protein kinase C), indomethacin, β-mercaptoethanol, *N*-acetyl-L-cysteine (NAC, antioxidant), PD98059 (inhibitor of MEK/ERK1,2 pathway), prostaglandin $E_1$ ($PGE_1$), RGDS peptide (inhibitor of fibronectin), RHC80267 (inhibitor of diacylglycerol lipase), theophylline, thrombin, and all chemicals were from Sigma-Aldrich, Co., St. Louis, MO. SB203580 (inhibitor of p38MAPK) and FURA 2/AM were purchased from Merck Biosciences, Germany. SR141716 (SR1, inhibitor of type-1 cannabinoid receptor) and SR144528 (SR2, inhibitor of type-2 cannabinoid receptor) were from Sanofi-Aventis Recerche, France. URB597 (inhibitor of fatty acid amide hydrolase) and URB754 (inhibitor of monoacylglycerol lipase) were from Alexis Biochemicals. Inhibitors were diluted in saline from a stock DMSO solution immediately before each experiment. $TXB_2$ EIA kit was from Assay Designs, Inc., Ann Arbor, MI. Anti-phospho-p38MAPK, anti-phospho-$cPLA_2$, and anti-$cPLA_2$ antibodies were from Cell Signalling Technology, Inc., Danvers, MA. Anti-p38MAPK and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. ECL® system and [$^3$H] arachidonic acid were from Ge Healthcare, Little Chalfont, UK. Nitrocellulose membranes (pore size 0.45 μm) were purchased from Bio Rad Laboratories, Inc., Chapel Hill, NC.

### BLOOD COLLECTION AND PREPARATIVE PROCEDURE

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, added to $PGE_1$ (2 μM) and apyrase (2 μg/ml), was 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 in calcium-free 10 mM HEPES buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose (pH 7.4), if not otherwise indicated. The final pH of platelet suspension was 7.4. 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 ($3.0 \times 10^8$ platelets/ml) were preincubated with saline or additions for 2 min at 37°C and then stimulated with 2-AG. The amplitude of aggregation measured in the presence of the additions was compared with that measured in a control experiment carried out under the same conditions, and the percentage of inhibition was calculated. From each series of experiments an inhibition percentage curve was derived for decreasing concentrations of the tested compounds. From this curve the concentration of compound inducing 50% inhibition ($IC_{50}$) was determined. The $IC_{50}$ values reported are the averages (± SD) of those obtained from at least three determinations.

### INTRACELLULAR CALCIUM MEASUREMENT

Washed platelets ($3.0 \times 10^8$ 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 $2.0 \times 10^8$ platelets/ml in calcium-free HEPES buffer (pH 7.4), was preincubated at 37°C with saline or additions; then 2-AG was added. Fluorescence of FURA 2/AM-loaded platelets was monitored at 37°C under unstirring conditions for 3 min in a Perkin-Elmer fluorescence spectrometer model LS50B, with excitations at 340 nm 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.

### IMMUNOBLOTTING ANALYSIS

Platelet suspensions ($1.0 \times 10^9$ platelets/ml), prewarmed with saline or additions at 37°C, were stimulated with 2-AG or thrombin. Incubation was stopped by adding 2 × Laemmli-SDS

reducing sample buffer. Samples, heated for 5 min at 100°C, were separated by 5–10% SDS–PAGE, and transferred to nitrocellulose membranes. Running was performed in the presence of Colorburst™ Electrophoresis weight markers. Blots were blocked in 6% BSA 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-p38MAPK (1/200 dilution) or anti-phospho-cPLA$_2$ antibodies (1/1,000 dilution). 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 the optical density was quantified by the Bio-Rad Chemi-Doc software package. Then nitrocellulose membranes, stripped by incubation with 62.5 mM Tris/HCl (pH 6.7), 2% SDS, 100 μM β-mercaptoethanol for 30 min at 50°C, were reprobed with anti-p38MAPK or anti-cPLA$_2$ antibodies and band intensity was quantified as detailed above.

### ARACHIDONIC ACID RELEASE

Washed platelets ($3.0 \times 10^8$ platelets/ml), resuspended in Hepes buffer containing 1.0 mM EGTA and 0.2% BSA (fatty acid-free), were incubated with [$^3$H] arachidonic acid (1 mCi/ml) for 60 min at 37°C. Over this time platelets incorporated an average of 70% of the added [$^3$H] arachidonic acid. Labeled platelets were washed twice with pH 4.8 ACD and resuspended in the original volume of pH 7.4 $Ca^{2+}$-free Tyrode's-Hepes buffer containing 0.2% BSA (fatty acid-free) and 2 mM $CaCl_2$. Platelet suspensions, preincubated for 10 min at 37°C with saline or additions, were incubated with 2-AG. Incubation was stopped by adding 50 μl of cold blocking mix containing 5 mM EGTA, 5 mM theophylline and 0.2 μg/ml $PGE_1$. After a cold rapid centrifugation, suitable aliquots of supernatants were analyzed by liquid scintillation counting.

### TXB$_2$ ASSAY

Washed platelets ($3.0 \times 10^8$ platelets/ml), preincubated with saline or additions for 10 min at 37°C, were treated with 2-AG. Incubation was stopped adding 50 μM indomethacin and 2 mM EDTA. After centrifugation at 14,000*g* for 2 min at 4°C, the $TXB_2$ content of supernatant was measured by an EIA kit according to the manufacturer's protocol.

### REACTIVE OXYGEN SPECIES (ROS) DETECTION

2′,7′-Dichlorofluorescein diacetate (DCFH-DA) is a ROS-sensitive probe that can be used to detect ROS production in living cells. DCFH oxidation yields the fluorescent adduct DCF that is trapped inside the cells. DCFH-DA was prepared from DCF according to Brandt and Keston [1965]. Washed platelets ($5.0 \times 10^7$ platelets/ml) were incubated for 15 min at 37°C with 10 μM DCFH-DA then centrifuged for 15 min at 1,100*g*. The obtained pellet was resuspended at $5.0 \times 10^7$ platelets/ml in Hepes buffer. Samples, prewarmed for 10 min at 37°C with saline or additions, were stimulated with 2-AG. Incubation, prolonged for 1 min, was stopped by sonicating samples twice for 10 s on ice. After a brief centrifugation, supernatants were appropriately diluted and the fluorescence of suitable aliquots was measured using a Perkin–Elmer fluorescence spectrometer model LS50B with 504 nm excitation and 526 nm emission. The concentration of intracellular DCF was calculated by a standard curve of the commercial compound.

### STATISTICAL ANALYSIS

Data are reported as mean ± SD of at least five independent experiments, each performed in duplicate. 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

### THE EFFECT OF 2-AG ON PLATELET AGGREGATION AND CALCIUM ELEVATION

First the effect of 2-AG on aggregation was examined. To measure platelet response to 2-AG varying concentrations of this endocannabinoid were added to washed human platelets under stirring conditions. 2-AG stimulates platelet aggregation in a dose- and time-dependent manner reaching the maximum at 10 μM. Higher doses than this (up to 50 μM) do not significantly increase platelet response any further (Fig. 1A). Next we tested the effect of 2-AG on calcium elevation. As shown in Figure 1B, 2-AG rapidly elevates intracellular calcium concentration with a spike at 10 μM. No further calcium elevation at higher concentrations of 2-AG is shown. Since both SB203580 and ETYA greatly inhibit platelet aggregation and intracellular calcium elevation (Table I), likely the p38MAPK/cPLA$_2$ pathway could be involved in 2-AG platelet activation.

### THE EFFECT OF 2-AG ON p38MAPK PHOSPHORYLATION

p38MAPK provides a key signal in the activation/aggregation of platelets stimulated by different agonists [Kramer et al., 1995; Kramer et al., 1996; Saklatvala et al., 1996; Hackeng et al., 1999; Canobbio et al., 2004]. Thus, we studied the 2-AG effect on p38MAPK phosphorylation. In Figure 2A, the dose–response relation between the phosphorylation of p38MAPK and the 2-AG concentration is shown. Ten micromolar 2-AG increases about twofold the p38MAPK basal phosphorylation level. Moreover, 2-AG increases the phosphorylation of p38MAPK in a time-dependent manner. The p38MAPK phosphorylation induced by 2-AG is very rapid, peaking after 1 min of incubation (Fig. 2B upper panel). The time-course of 2-AG effect is similar to that produced by 0.1 U/ml thrombin (Fig. 2B lower panel). The 2-AG effect on p38MAPK phosphorylation was not modified by platelet pretreatment with 50 μM indomethacin, 200 μM RGDS peptide or 1 μg/ml apyrase. In addition 20 μM URB754 or 20 μM URB597, inhibitors of monoacylglycerol lipase and fatty acid amide hydrolase, respectively, had no effect on p38MAPK phosphorylation induced by 2-AG (data not shown). To clarify whether CB1 and/or CB2 receptors could be involved in the 2-AG effect on p38MAPK phosphorylation, we pretreated platelets with SR1 or SR2, specific antagonists of CB1 and CB2, respectively. We found that only SR1 strongly impairs the 2-AG induced phosphorylation of p38MAPK, whereas SR2 was ineffective (Fig. 3A). In contrast, SR1 and SR2 do

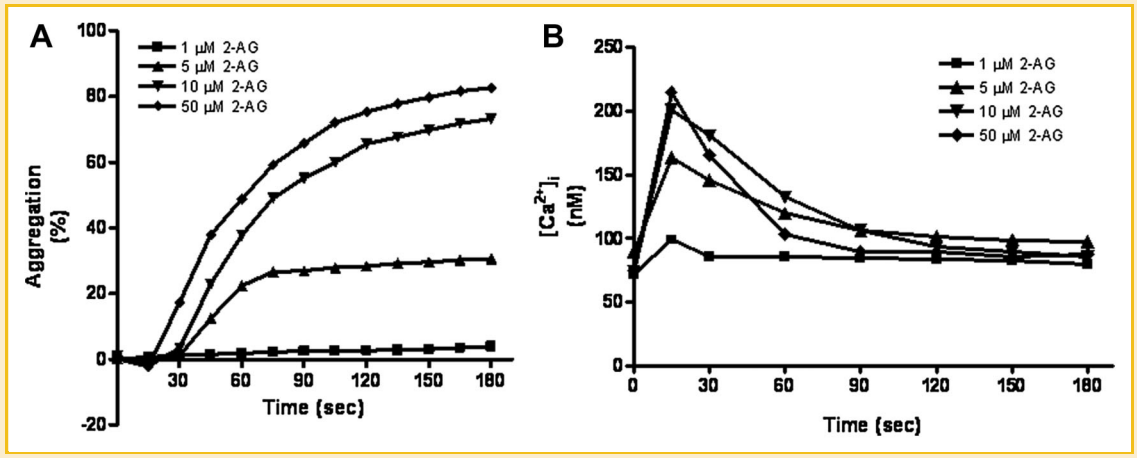


Fig. 1. Platelet aggregation and intracellular calcium elevation. Washed platelets ($3.0 \times 10^8$ platelets/ml), preincubated at 37°C in the presence of saline, were challenged with 2-AG as indicated. Panel A shows platelet aggregation induced by varying concentrations of 2-AG. Aggregation was quantified by the light transmission reached within 3 min. Panel B shows elevation of intracellular calcium quantified in FURA 2-loaded platelets ($2.0 \times 10^8$ platelets/ml), preincubated with saline at 37°C before the addition of varying 2-AG concentrations. Calcium concentration was monitored at 37°C in a fluorescence spectrophotometer, as detailed in Materials and Methods section. Tracings of A and B are representative of four independent determinations.

not affect p38MAPK phosphorylation induced by thrombin (Fig. 3B).

### PHOSPHORYLATION OF $cPLA_2$ INDUCED BY 2-AG

2-AG stimulates phosphorylation of $cPLA_2$ dose-dependently (Fig. 4A). Moreover, in Figure 4B, it is shown that the 2-AG effect was prevented by the p38MAPK inhibitor SB203580, but was not modified by the inhibitor of MEK/ERK1,2 pathway PD98059. In addition the protein kinase C inhibitor GF109203X had no effect on $cPLA_2$ phosphorylation, as previously demonstrated in thrombin or collagen stimulated platelets [Borsch-Haubold et al., 1995, 1997; Kramer et al., 1996].

### THE 2-AG EFFECT ON ARACHIDONIC ACID RELEASE

Arachidonic acid is released from membrane phospholipids and is regulated by two mechanisms: The first one involves the intracellular calcium-dependent translocation of $cPLA_2$ from cytosol to the membrane [Six and Dennis, 2003] and the second mechanism involves $cPLA_2$ phosphorylation on its ser505 residue [Kramer et al., 1996; Borsch-Haubold et al., 1997, 1998a]. Since 2-AG promotes $cPLA_2$ phosphorylation (Fig. 4) and calcium elevation (Fig. 1B), likely 2-AG can stimulate $cPLA_2$ activity. Data confirm this assumption, as 2-AG induces arachidonic acid release, being the effect dose- dependent and peaking at 10 μM (Fig. 5A). Moreover, the 2-AG effect was very rapid, peaked after 1 min of incubation and sustained for 10 min (Fig. 5B). Arachidonic acid release and $cPLA_2$ phosphorylation are in good correlation, being the parameters: $y = 0.2774x + 380.9$; $r^2 = 0.9243$; $P = 0.009$. PD98059 and RHC80267, inhibitor of diacylglycerol lipase, did not modify arachidonic acid release stimulated by 2-AG. Moreover, SR1 greatly reduced arachidonic acid released by 2-AG, whereas SR2 was completely ineffective (Fig. 5C). SB203580 inhibited arachidonic acid release, in a dose dependent-manner, peaking at 10 μM (Table II). To exclude that SB203580 and PD98059 could inhibit cyclooxygenase and thromboxane synthase [Borsch-Haubold et al., 1998b], experiments have been performed in the presence of indomethacin.

### THE 2-AG INDUCED $TXB_2$ FORMATION

2-AG stimulated formation of $TXA_2$, as measured by its stable metabolite $TXB_2$. The 2-AG effect was dose- (Fig. 6A) and time-dependent (Fig. 6B). This parameter is in good correlation with arachidonic acid release ($y = 165.7x + 1617$; $r^2 = 0.9837$;

TABLE I. Effect of SB203580 and ETYA on Platelet Aggregation and Intracellular Calcium Elevation Induced by 2-AG

| | $IC_{50}$ (μM) | |
|---|---|---|
| | Platelet aggregation | $[Ca^{2+}]$ elevation |
| SB203580 | 9.2 ± 2.4 | 10.0 ± 1.2 |
| ETYA | 44.8 ± 3.5 | 34.1 ± 4.8 |

Washed platelets ($3.0 \times 10^8$ platelets/ml) were preincubated at 37°C in the presence of saline or varying concentrations of the agents and then challenged with 10 μM 2-AG. Platelet aggregation was quantified by the light transmission reached within 3 min. The $IC_{50}$ value was calculated as detailed in Materials and Methods section.

TABLE II. Effect of SB203580 on Arachidonic Acid Release Induced by 2-AG

| | Arachidonic acid release (cpm/$3.0 \times 10^8$ platelets) |
|---|---|
| None | 1934 ± 131 |
| 2-AG | 4559 ± 186 |
| 2-AG + I | 4385 ± 197 |
| 2-AG + I + 1 μM SB203580 | 3986 ± 239 |
| 2-AG + I + 5 μM SB203580 | 2507 ± 201 |
| 2-AG + I + 10 μM SB203580 | 1986 ± 164 |

Labeled platelets ($3.0 \times 10^8$ platelets/ml) were preincubated with saline, 50 μM indomethacin and/or SB203580 as indicated for 10 min at 37°C, then treated with 10 μM 2-AG for 1 min. Results are the mean ± SD of four independent experiments carried out in triplicate.

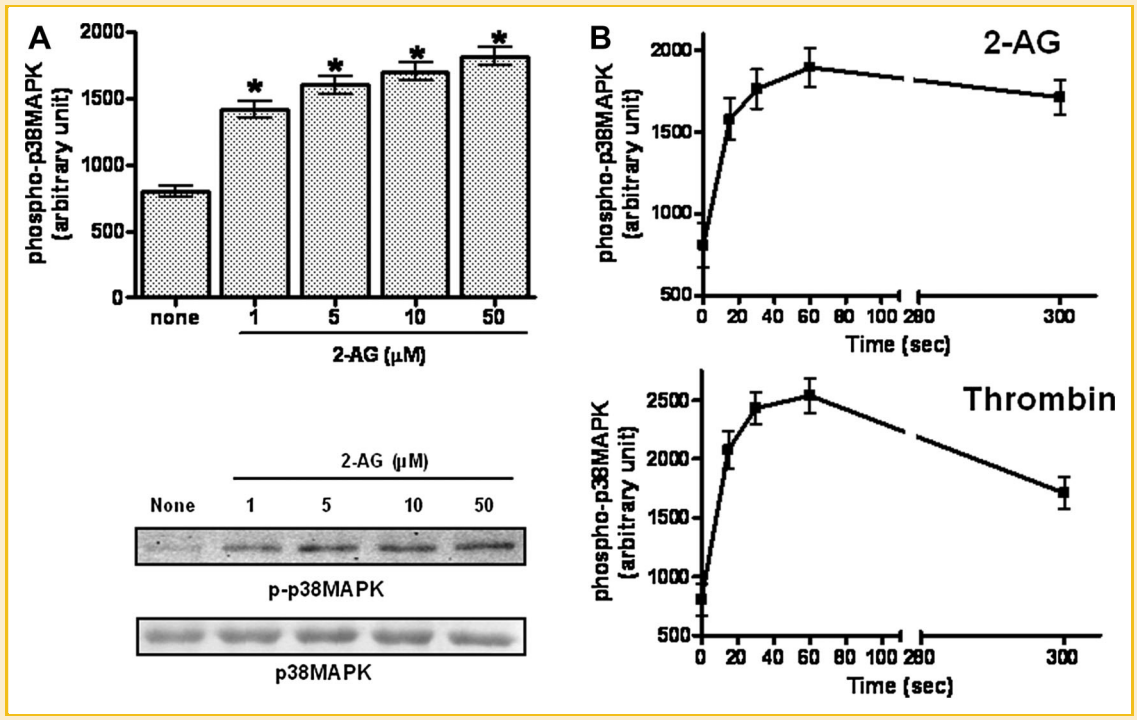


Fig. 2. Effect of 2-AG on p38MAPK phosphorylation. Washed platelets ($1.0 \times 10^9$ platelets/ml), prewarmed at 37°C, were incubated for 1 min with the indicated 2-AG concentrations (A). In B (upper panel) washed platelets were stimulated with 10 μM 2-AG for the indicated times. In B (lower panel) the time-course of 0.1 U/ml thrombin-induced p38MAPK phosphorylation is reported. At the end of incubation suitable aliquots were immunoblotted with anti-p-p38MAPK as detailed in Materials and Methods section. Blots are representative of five independent experiments. In the upper panel of A densitometric scanning ±SD of p38MAPK phosphorylation of five experiments is reported. One way ANOVA-Bonferroni's post hoc test: $^{*}P < 0.05$.

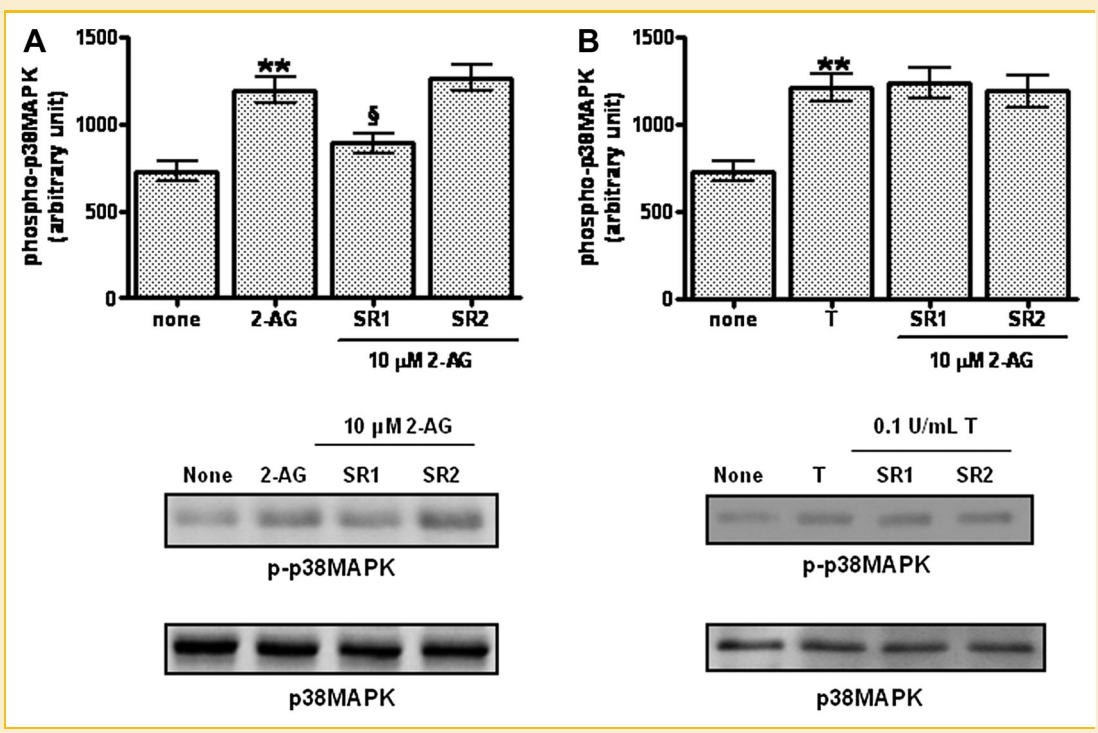


Fig. 3. Effect of SR1 and SR2 on p38MAPK phosphorylation induced by 2-AG or thrombin. Washed platelets ($1.0 \times 10^9$ platelets/ml) were preincubated with saline, 1 μM SR1 or 1 μM SR2 and then stimulated for 1 min with 10 μM 2-AG (A) or 0.1 U/ml thrombin (B). Samples were immunoblotted with anti-p-p38MAPK as detailed in Materials and Methods section. Blots are representative of five independent experiments. In the upper panels densitometric scanning ±SD of p38MAPK phosphorylation of five experiments is reported. Student's *t*-test: $^{**}P < 0.001$ vs. none; §$P < 0.01$ vs. 2-AG

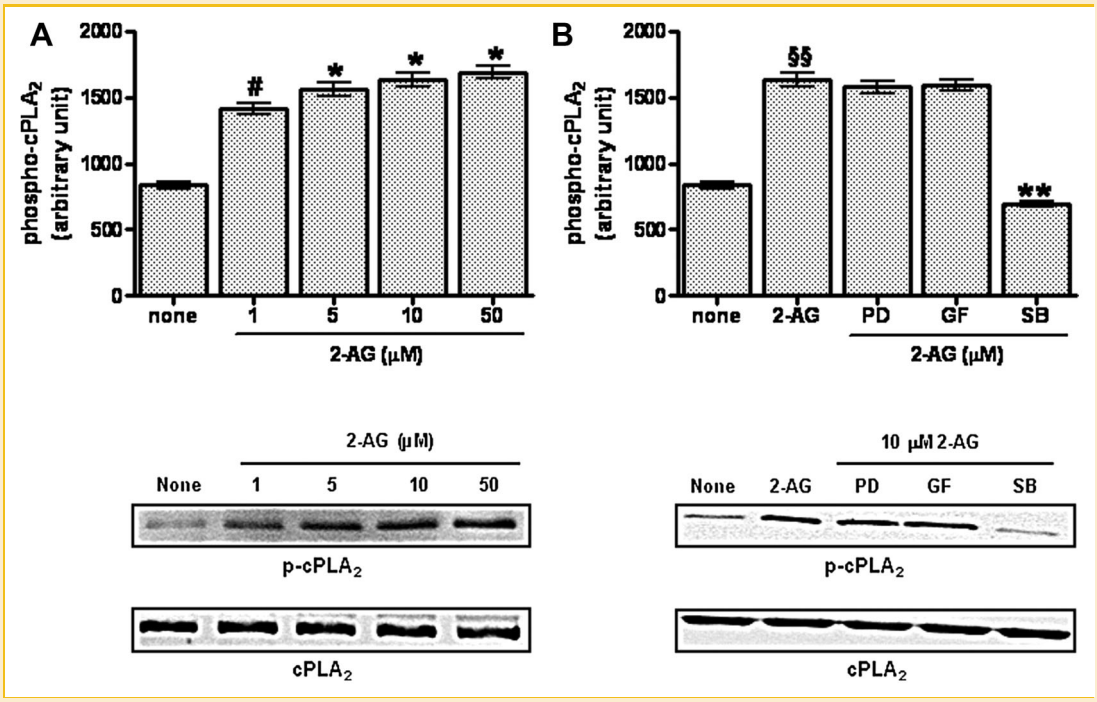


Fig. 4. Effect of 2-AG on $cPLA_2$ phosphorylation. Washed platelets ($1.0 \times 10^9$ platelets/ml), preincubated with saline, were incubated for 1 min with the indicated 2-AG concentrations (panel A). In panel B washed platelets ($1.0 \times 10^9$ platelets/ml) were preincubated with saline, 20 μM PD98059 (PD), 10 μM GF109203X (GF) or 10 μM SB203580 and then stimulated for 1 min with 10 μM 2-AG. At the end of incubation suitable aliquots were immunoblotted with anti-p-$cPLA_2$ as detailed in Materials and Methods section. Blots are representative of five independent experiments. In the upper panels densitometric scanning ±SD of $cPLA_2$ phosphorylation of five experiments is reported. One way ANOVA-Bonferroni's post hoc test: #$P < 0.01$; *$P < 0.05$; Student's *t*-test: §§$P < 0.0005$ vs. none; **$P < 0.0005$ vs. 2-AG.

$P = 0.0009$). In agreement with data on arachidonic acid release (Fig. 5C). PD98059, RHC 80267, and SR2 had no effect on $TXB_2$ production. In contrast SB203580, indomethacin and SR1 significantly ($P < 0.0001$) reduced $TXB_2$ formation induced by 2-AG (Fig. 6C).

### THE 2-AG EFFECT ON ROS FORMATION

Resting platelets generate a continuous basal flux of ROS, including superoxide anion and hydrogen peroxide. The treatment of platelets with agonists increases the levels of these oxygen radicals [Maresca et al., 1992]. Data of Figure 7A show that 2-AG increases ROS basal level. The 2-AG effect is dose-dependent. ROS formation induced by 10 μM 2-AG was significantly reduced by SR1 and by ETYA, inhibitor of $cPLA_2$ activation and arachidonic acid metabolism. Obviously the antioxidant NAC exerted a powerful effect, as it not only abolished ROS generated by 2-AG, but also decreased ROS basal level. On the contrary RHC80267 and DPI, inhibitor of NADPH oxidase, were ineffective (Fig. 7B).

## DISCUSSION

Platelet activation represents an important contributing factor in the process of atherosclerosis and its thrombotic complications. Platelets can be stimulated "in vitro" by physiological agonists, such as thrombin, collagen, von Willebrand factor, and ADP. The literature relating to the activity of endocannabinoids in platelets is controversial. Maccarrone et al. [2001] reported that 2-AG-induced aggregation is cannabinoid receptor-dependent and metabolic breakdown-independent. Baldassarri et al. [2008] excluded the presence of CB1 and CB2 proteins in human platelets and suggested that 2-AG could act through other cannabinoid receptors such as GPR119 or GPR55. Moreover, the authors demonstrated that platelet activation is induced by 2-AG itself and not by products of its metabolism, as the 2-AG effect is not modified by inhibitors of monoacylglycerol lipase and fatty acid amide hydrolase. Recently, in whole blood or platelet-rich plasma Keown et al. [2010] have shown that 2-AG activates platelets via conversion to arachidonic acid and not by direct activation of cannabinoid receptor. In spite of these controversial results, in human platelets the presence of CB1 receptor and, to a lesser extent, of CB2 receptor has been recently demonstrated [Catani et al., 2010]. In the present study, we report that low concentrations of 2-AG activate platelet aggregation and increase intracellular calcium concentration (Fig. 1). Since both these parameters are significantly inhibited by the p38MAPK inhibitor SB203580 and ETYA (Table I) and considering that the p38MAPK/$cPLA_2$ pathway is involved in the activation and aggregation of platelets induced by numerous agonists [Kramer et al., 1995; Kramer et al., 1996; Saklatvala et al., 1996; Hackeng et al., 1999; Canobbio et al., 2004], we examined whether p38MAPK/$cPLA_2$ pathway could have a role in platelet activation by 2-AG. We found that 2-AG in time and concentration-dependent manner triggered the activation of p38MAPK as indicated by its phosphorylation. Moreover, the pretreatment of platelets with SR1

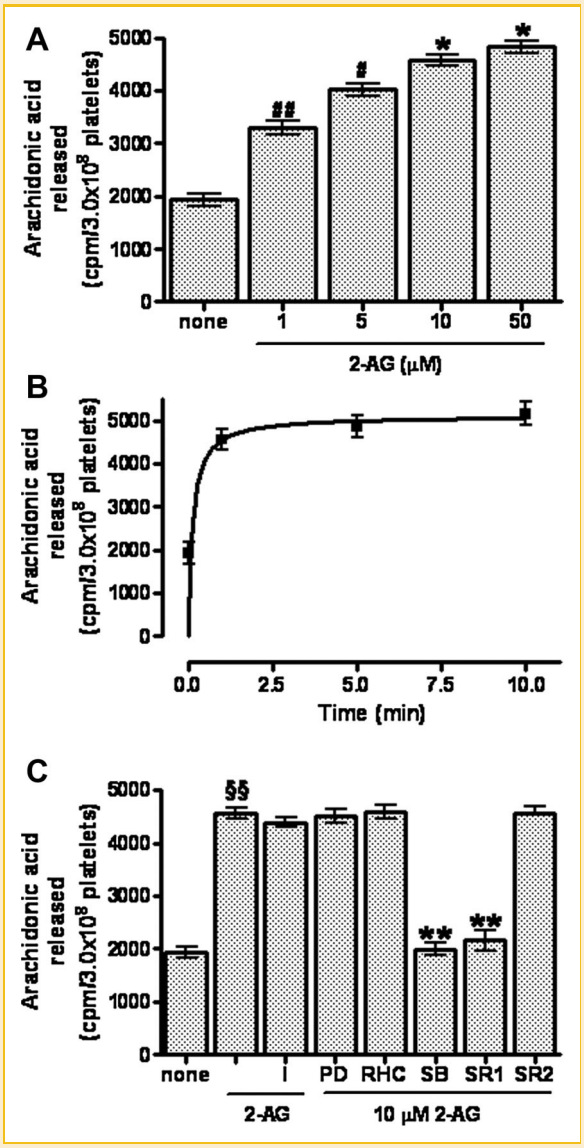


Fig. 5. Effect of 2-AG on arachidonic acid release. In (A) labeled platelets ($3.0 \times 10^8$ platelets/ml) were preincubated with saline and then stimulated for 1 min with 2-AG. In (B) the time-dependence of the arachidonic acid release was determinate. In (C) labeled platelets were preincubated with saline, 50 μM indomethacin (I), 20 μM PD98059 (PD), 1 μM RHC80267 (RHC), 10 μM SB203580 (SB), 1 μM SR1 or 1 μM SR2 and then stimulated with 10 μM 2-AG for 1 min in the presence of 50 μM indomethacin, as indicated. Values are the mean ± SD of three experiments carried out in triplicate. One way ANOVA-Bonferroni's post hoc test: $\#\#P < 0.001$, $\#P < 0.01$, $^{*}P < 0.05$; Student's *t*-test: §§$P < 0.0001$ vs. none; $^{**}P < 0.0001$ vs. 2-AG.

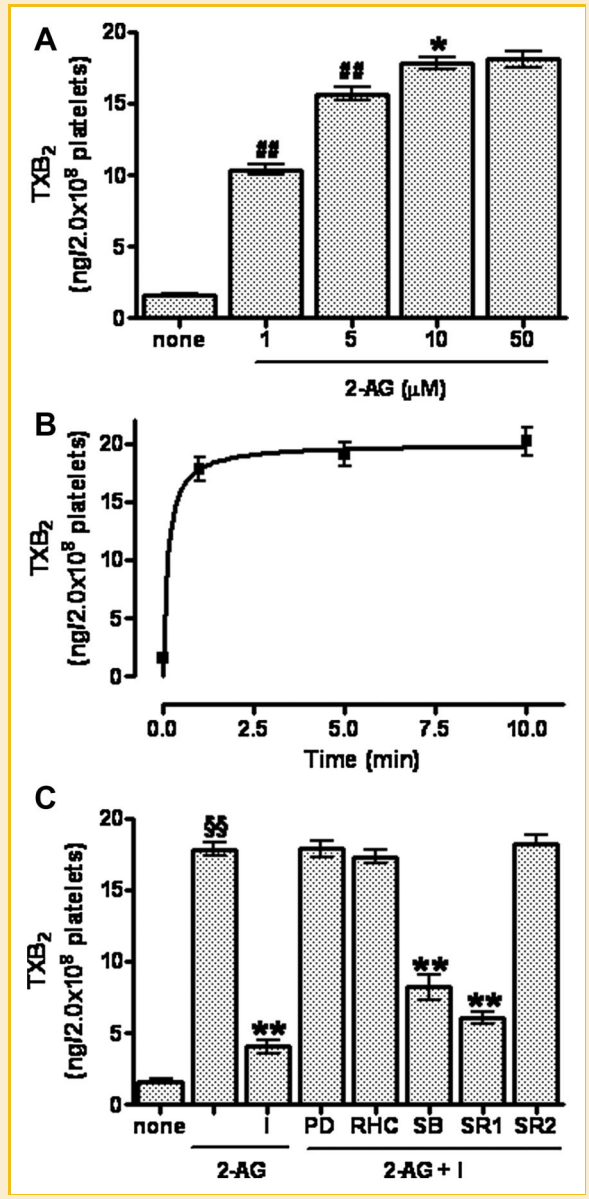


Fig. 6. Effect of 2-AG on $TXA_2$ formation. In (A) washed platelets ($3.0 \times 10^8$ platelets/ml) were preincubated with saline and then stimulated for 1 min with 2-AG. In (B) the time-dependence of the txA2 formation was determined. In (C) washed platelets were preincubated with saline, 50 μM indomethacin (I), 20 μM PD98059 (PD), 1 μM RHC80267 (RHC), 10 μM SB203580 (SB), 1 μM SR1 or 1 μM SR2 and then stimulated with 10 μM 2-AG for 1 min in the presence of 50 μM indomethacin, as indicated. Values are the mean ± SD of three experiments carried out in triplicate. One way ANOVA-Bonferroni's post hoc test: $\#\#P < 0.001$, $^{*}P < 0.05$; Student's *t*-test: §§$P < 0.0001$ vs. none; $^{**}P < 0.0001$ vs. 2-AG.

abolished the 2-AG rapid activation of p38MAPK, suggesting that the response induced by 2-AG is mediated through the CB1 receptor. Moreover, 2-AG enhanced $cPLA_2$ phosphorylation (Fig. 4) and its activity measured as arachidonic acid release (Fig. 5A) and $TXB_2$ formation (Fig. 6A). Both $cPLA_2$ phosphorylation (Fig. 4) and $cPLA_2$ activity (Figs. 5 and 6A) have a similar sensitivity for SB203580, indicating that p38MAPK mediates $cPLA_2$ phosphorylation. Since both $cPLA_2$ phosphorylation and arachidonic acid release induced by 2-AG are abolished by SB203580, likely the phosphorylation may have a great importance in the regulation of $cPLA_2$ activation by 2-AG. However, since 2-AG elevates intracellular calcium concentration, calcium may participate to $cPLA_2$ activation, as it occurs in other biological systems [Six and Dennis, 2003]. The phosphorylation of $cPLA_2$ by 2-AG was unaffected by the specific

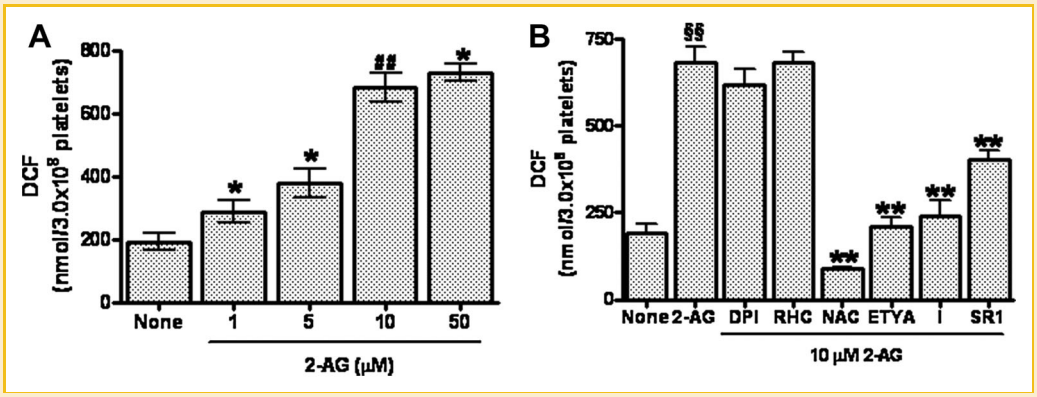


Fig. 7. Effect of 2-AG on ROS formation. In (A) washed platelets ($5.0 \times 10^7$ platelets/ml), loaded with 10 μM DCFH-DA, were preincubated with saline and then stimulated for 1 min with 2-AG. In (B), washed platelets ($5.0 \times 10^7$ platelets/ml), loaded with 10 μM DCFH-DA and preincubated with saline, 50 μM DPI, 1 μM RHC80267 (RHC), 5 mM *N*-acetyl-L-cysteine (NAC), 50 μM ETYA, 1 μM SR1 were stimulated for 1 min with 10 μM 2-AG. Results represent the mean ± SD of four experiments carried out in triplicate. One way ANOVA-Bonferroni's post hoc test: $##P < 0.001$, $^{*}P < 0.05$; Student's *t*-test: §§$P < 0.0001$ vs. none; $^{**}P < 0.0001$ vs. 2-AG.

inhibitor of protein kinase C, GF109203X, as shown in platelets stimulated by thrombin or collagen [Borsch-Haubold et al., 1995]. Moreover, both $cPLA_2$ phosphorylation and arachidonic acid release were not modified by platelet pretreatment with PD98059, inhibitor of MEK/ERK1, 2 system, excluding the involvement of these enzymes in the phosphorylation/activation of $cPLA_2$. However, in HL-60 cells the activation of ERK 1,2 by 2-AG takes place in a cannabinoid CB2 receptor-dependent manner [Kobayashi et al., 2001]. In addition $cPLA_2$ activity, tested as arachidonic acid release and $TXB_2$ formation was not impaired in platelets treated with the diacylglycerol lipase inhibitor RHC80267, indicating that arachidonic acid is released through $cPLA_2$, whereas cPLA2 activation appears to be mediated by CB1 receptor, as SR1 but not SR2 significantly reduced or cancelled the 2-AG effect. The pretreatment of platelets with the fatty acid amide hydrolase or monoacylglycerol lipase inhibitors URB597 or URB754, respectively, did not modify p38MAPK, $cPLA_2$ phosphorylation, arachidonic acid release, and $TXA_2$ formation, ruling out that 2-AG acts through the release of its arachidonic acid metabolite. Finally in this study we have demonstrated that 2-AG stimulated ROS formation (Fig. 7A). These oxygen species were significantly inhibited by indomethacin and ETYA, inhibitor of $cPLA_2$ activation and arachidonic acid metabolism, but lightly reduced by the NADPH oxidase inhibitor DPI, suggesting that ROS are intermediate metabolites mainly produced from arachidonic acid metabolism through cyclooxygenase and lipoxygenase reactions. Since ROS participate in cell signaling process and their regulation, 2-AG could potentiate its platelet aggregating power increasing ROS formation. Recently it has been demonstrated that antagonism of CB1 receptor depresses platelet activation in a mouse model of type 2 diabetes mellitus and reduces cardiovascular risk by lessening proinflammatory and proatherosclerotic cascades [Schäfer et al., 2008]. Treatment with rimonabant reduced fibrinogen binding, reduced thrombin-induced platelet aggregation and decreasing ADP-stimulated expression of p-selectin, a platelet activation marker. Thus, our finding that 2-AG activates platelets through a CB1 receptor-dependent pathway suggests that CB1 antagonism might produce some benefits by lowering the thrombotic potential and the consequent cardiovascular risk.

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