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2-arachidonyl glycerol activates platelets via conversion to arachidonic acid and not by direct activation of cannabinoid receptors

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- There are conflicting views in the literature as to whether cannabinoids have an impact on platelet activity and to what extent cannabinoid receptors are involved. This is an important issue to resolve because platelet effects of putative therapeutic cannabinoid inhibitors and stimulators will have an impact on their potential benefits and safety.

WHAT THIS PAPER ADDS

- The data presented in this manuscript clearly show that the endocannabinoid 2-arrachidonyl glycerol can activate platelet activity, but that the effects are mediated through an aspirin-sensitive pathway that is not affected by cannabinoid receptor antagonists or FAAH inhibition, but is abolished by MAGL inhibition. The findings question the role of cannabinoid receptors in platelet function and suggest that platelet function is unlikely to be directly affected by cannabinoid receptor antagonists, at least in the acute phase.

AIMS

Cannabinoid receptor-1 (CB_1) antagonists suppress appetite and induce weight loss. Direct antagonism of CB_1 receptors on platelets might be an additional benefit for CB_1 antagonists, but the role of CB_1 receptors in platelets is controversial. We tested the hypothesis that the endocannabinoid, 2-arachidonyl glycerol (2-AG), induces platelet aggregation by a COX-mediated mechanism rather than through CB_1 receptor activation, in blood obtained from healthy volunteers and patients with coronary artery disease receiving low dose aspirin.

METHODS

Aggregatory responses to the cannabinoids 2-AG and Δ^9-THC were examined in blood sampled from healthy volunteers ($n = 8$) and patients ($n = 12$) with coronary artery disease receiving aspirin using whole blood aggregometry. The effects of CB_1 (AM251) and CB_2 (AM630) antagonists, as well as fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MAGL) inhibitors and aspirin on 2-AG-induced aggregation were also assessed.

RESULTS

AM251 (100 nM–30 μM) had no effect on platelet aggregation induced by either ADP ($P = 0.90$) or thrombin ($P = 0.86$). 2-AG, but not Δ^9-THC, induced aggregation. 2-AG-induced aggregation was unaffected by AM251 and AM630 but was abolished by aspirin ($P < 0.001$) and by the MAGL inhibitor, URB602 ($P < 0.001$). Moreover, the aggregatory response to 2-AG was depressed (by >75%, $P < 0.001$) in blood from patients with coronary artery disease receiving aspirin compared with that from healthy volunteers.

CONCLUSIONS

2-AG-mediated activation of platelets is via metabolism to arachidonic acid by MAGL, and not through direct action on CB_1 or CB_2 receptors, at least in the acute phase.

Introduction

Human cannabinoid receptors, CB_1 and CB_2, bind the psychoactive compound, Δ9-tetrahydrocannabinol (Δ^9-THC), found in *Cannabis sativa* [1, 2], as well as endogenous cannabinoid entities (endocannabinoids). In the last decade, the endocannabinoid system has been recognized to be important in controlling many physiological and pathological processes and has become an area of interest for novel drug development.

Endocannabinoids are derivatives of arachidonic acid (AA) and belong to the eicosanoid family. The two main endocannabinoids, 2-arachidonoyl glycerol (2-AG) and anandamide, are synthesized in response to increasing intracellular calcium concentrations by diacylglycerol lipase [3]. Metabolism is relatively non-specific and occurs through hydrolyzing enzymes, notably fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) [4]. Hydrolysis of 2-AG produces AA, which is the main substrate for cyclooxygenase (COX) enzymes that catalyze the conversion of AA into prostaglandin-H_2 in the first step of the synthesis of prostaglandins and thromboxanes [5].

CB_1 receptors are predominantly localized in central and peripheral nerves and mediate retrograde synaptic inhibition of endogenous cannabinoids [6]. However, they are also present in other cell types and cellular fragments, including immune cells and platelets [7]. CB_2 receptors mediate inflammation and are found in many inflammatory cell types [8].

The physiological actions of endocannabinoids are diverse [9–13] but are believed to include modulation of platelet function. Platelets express CB_1 and CB_2 receptors and possess the metabolic pathways to both synthesize and degrade endocannabinoids [7]. However, data regarding the impact of CB receptor activation are contradictory: some studies have reported platelet activation by Δ^9-THC [7, 14], anandamide [15] and 2-AG [16], whilst another has demonstrated that Δ^9-THC inhibits platelet function [17]. Likewise, the mechanism by which endocannabinoids induce activation is unclear: anandamide-induced aggregation has been shown to be antagonized by a CB_1 antagonist in one study [15], but not in another [18], where platelet activation was shown to be dependent on metabolism of anandamide to arachidonic acid.

CB_1 receptor knockout mice have reduced bodyweight, reduced fat mass and reduced appetite [19]. In rat [20] and human [21, 22] studies, pharmacological CB_1 antagonism using AM251 or SR141716 reduces food intake and leads to an increase in energy expenditure. These findings led to the clinical development of the CB_1 receptor antagonist, SR141716, rimonabant, as an appetite suppressant that acts through inhibition of tonic CB_1 receptor activity [23].

The controversy surrounding the role of CB_1 receptors in mediating platelet activation requires clarification, given that any pro- or anti-aggregating effect of drugs acting at the CB_1 receptor could have serious implications with respect to the safety or potential added benefit of CB_1 receptor antagonism in patients with cardiovascular disease. We set out to test the hypothesis that the cannabinoids Δ^9-THC and 2-AG cause platelet aggregation in whole blood in healthy volunteers and a group of coronary artery disease patients on long term, low dose aspirin. The mechanism by which 2-AG induces platelet aggregation was probed *in vitro* with the CB_1 and CB_2 receptor antagonists, the COX inhibitor, aspirin and inhibitors of FAAH and MAGL.

Methods

Ethics

This study was approved by the North of Scotland Research Ethics Committee (Ethics no. 06/S0901/46) and was conducted in accordance with the declaration of Helsinki and its amendments.

Healthy volunteers

Healthy non-smoking male volunteers aged 18–60 years, on no regular medications were recruited from local hospital staff. Volunteers were excluded if they had renal disease (creatinine >159 μM), diabetes (fasting blood glucose >6 mmol l^{-1}, $HbA1_c$ >6.5%) or had participated in a clinical trial in the previous 3 months. A venous blood sample (100 ml) was taken from the antecubital fossa using a 21G butterfly needle.

Patients with coronary artery disease

Non-smoking male patients with a clinical history suggestive of coronary artery disease and who were undergoing routine coronary angiography were screened for suitability in the study. Patients were excluded if they had renal disease (creatinine >159 μM) or had participated in a clinical trial in the previous 3 months. As part of routine coronary angiography, an arterial sheath was inserted into their right radial or femoral artery. An arterial blood sample (100 ml) was taken from this site before catheterization. Demographics were recorded from patient medical notes and coronary disease status was defined as mild (<50% occlusion) moderate (50–75% occlusion) or severe (>75% occlusion); the number of major coronary vessels affected was also recorded.

Handling of blood samples

The first 5 ml of blood was discarded to remove blood affected by haemostasis in the needle. A 100 ml sample was drawn into two 50 ml syringes and then immediately transferred into tubes containing 3.8% trisodium citrate solution. Citrate was used as an anti-coagulant because of its negligible intrinsic effect on platelets [24]. A 9 ml sample was drawn at the same time into a lithium heparin-containing tube (Sarstedt Ltd, Leicester, UK) for biochemi-

cal screening (DCA 2000+ Analyser; Bayer, Newbury, UK). Renal status (Piccolo Renal Panel Plus; Abaxis, Darmstadt, Germany) and lipid status (Piccolo Lipid Panel Plus; Abaxis, Darmstadt, Germany) were recorded. Blood cell counts, including platelet count (Beckman Coulter ACT8: Beckman Coulter, High Wycombe, UK) were also obtained. Blood was promptly taken to the research laboratory after sampling and experiments were completed within 2 h of sampling. All sampling was carried out at approximately 09.00 h, ensuring minimization of previously observed circadian platelet variation [25], following a 12 h fast.

Preparation and treatment of platelets

Citrated blood for whole blood experiments was diluted 1:1 with 0.9% saline solution, as previously described [26], before 1 ml was added to a disposable aggregometry electrode and cuvette tube (LabMedics, Manchester, United Kingdom) containing a stirrer bar (LabMedics, Manchester, United Kingdom) and pre-warmed (37°C, 5 min). Aggregometry analysis was carried out using whole blood impedance aggregometry (37°C, stirrer speed 1000 rev min^{-1}; Chrono-Log Model 700 Lumi-Aggregometer; LabMedics, Manchester, United Kingdom). Aggregation of platelets was determined by measuring the area under the curve (AUC) representing impedance change, from the baseline, over time.

Experimental protocols

Characterization of the responses of human blood to cannabinoid and non-cannabinoid platelet agonists Preliminary studies were undertaken whereby blood samples from healthy volunteers were pre-incubated (10 min, 37°C), with a range of concentrations of AM251 (0.1–30 μM) prior to activation (6 min) with different agonists; 2-AG (200 μM), Δ^9-THC (300 μM), ADP (20 μM; LabMedics, Manchester, UK) and thrombin (0.25 U ml^{-1}; LabMedics, Manchester, UK). The potential of AM251 and AM630 themselves to induce aggregation alone was therefore also determined in these experiments.

Comparison of the aggregatory responses to 2-AG in healthy volunteers and CAD patients receiving aspirin Aliquots (15 ml) of blood were transferred into seven tubes containing AM251, AM630 or DMSO (solvent control; final concentration, 0.5%). The final concentration for each drug was 30 μM, 3 μM and 300 nM. At time 10, 30, 60 and 120 min, 1 ml of blood from each tube was transferred to a cuvette within the aggregation chamber and 2-AG (100 μM) added; impedance was then recorded for the subsequent 6 min.

Effect of FAAH and MAGL inhibitors on responses to 2-AG in healthy volunteer whole blood and platelet rich plasma (PRP)

Whole blood Blood (10 ml) from healthy volunteers was incubated with the FAAH inhibitor, URB597 (300 nM), the MAGL inhibitor, URB602 (300 μM), both URB597 + URB602 (300 nM and 300 μM, respectively) or vehicle control for 15 min (37°C, with occasional gentle swirling). In each case, the total final DMSO concentration was 1% v : v). Concentrations of URB597 and URB602 were derived from literature values for maximal inhibition of respective enzymes [27]. Aliquots (500 μl) of these samples were transferred to plastic cuvettes containing 500 μl saline. A further sample was treated for 30 min with aspirin (100 μM, 37°C) following saline dilution. Each sample was then treated with 2-AG (200 μM) and aggregation assessed for 6 min by the impedance method described above.

In experiments involving PRP, citrated blood (40 ml) was centrifuged (130 *g*, 10 min, 20°C) and PRP was aspirated to a fresh tube, and kept at 37°C in a water bath, whilst platelet poor plasma was prepared from the remaining blood by centrifugation (13 000 *g*, 1 min, 20°C). PRP platelet count was normalized to 150×10^9 platelets l^{-1} by adding the appropriate volume of platelet poor plasma to PRP, which was then divided into glass cuvettes (500 μl) and treated with 2-AG (200 μM), whilst aggregation was assessed by standard turbidometric aggregometry.

Materials

Chemicals were of the purest analytical grade. 2-arachidonyl glycerol (2-AG), Δ9-tetrahydrocannbinol (Δ^9-THC), 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide (AM251) and trisodium citrate were purchased from Sigma Aldrich (Dorset, UK). Adenosine diphosphate (ADP), thrombin and collagen were purchased from LabMedics (Manchester, UK). (6-iodio-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl) (4-methoxyphenyl) methanone (AM630; Axxora, Bingham, Nottingham, UK). 2-AG and Δ^9-THC were evaporated from their stock solvent using a steady stream of nitrogen and reconstituted in DMSO. AM251 and AM630 were reconstituted in DMSO. All other chemicals were reconstituted in 0.9% saline solution. Rimonabant shares almost identical properties and structure with other CB_1 selective antagonists, including AM251, which has similar selectivity for CB_1 receptors and almost identical pharmacological actions [28, 29]. URB 597 and URB602 were obtained from Axxora (Cambridge, UK).

Statistics

The number of subjects used in each group is stated in the respective figure legends. All data on the graphs are expressed as the mean ± SEM. *P* values were obtained by the relevant statistical test as stated in the legend of each figure. $P < 0.05$ was considered to be statistically significant.

Table 1

Profiles of healthy volunteers and patients with coronary artery disease. Demographical measurements and biochemical results of two study groups

Parameter	Healthy volunteers (n = 8)	Patients (n = 12)
Age (years)	39 ± 4.3	65 ± 2.5***
BMI (kg m^{-2})	25 ± 1.6	29 ± 1
BP (mmHg)	127/79 ± 4/2	138/79 ± 4/3
Total cholesterol (mM)	4.8 ± 0.3	3.9 ± 0.2*
HDL cholesterol (mM)[a]	1.2 ± 0.1	1.4 ± 0.1
Triglycerides (mM)	1.6 ± 0.4	1.4 ± 0.2
Creatinine (μM)	93.5 ± 7.0	108 ± 6.9
Red blood cells (× 10^{12} cells l^{-1})	4.8 ± 0.1	4.3 ± 0.1*
Haemoglobin (g l^{-1})	13.0 ± 0.2	12.0 ± 0.4
Haematocrit (%)	42.7 ± 0.9	40.2 ± 1.2
Platelets (× 10^{9} cells l^{-1})	209 ± 14	202 ± 16

Abbreviations and notes: [a], calculated; BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein. Data expressed as mean ± SEM; Statistical analysis using two-tailed, unpaired t-test (*P < 0.05; ***P < 0.001).

Results

Healthy volunteer and patient characteristics

Demographic and biochemical characteristics from the two groups studied are summarized in Table 1. Table 2 characterizes the patient population profiles and medications, including the severity of coronary artery disease in each patient.

Characterization of the response of blood from healthy volunteers to cannabinoid and non-cannabinoid agonists

The CB_1/CB_2 agonist, Δ^9-THC (300 μM), had no pro-aggregatory effect in the absence or presence of either AM251 (100 nM–30 μM) or AM630 (100 nM–30 μM). 2-AG (200 μM) did induce aggregation, but this was not affected by pre-incubation with AM251 (Figure 1). Similarly, AM251 had no effect on aggregation induced by either ADP (20 μM; Figure 2A) or thrombin (0.25 U ml^{-1}; Figure 2B). AM251 itself had no aggregatory effects beyond a small, aggregatory response also induced by the vehicle control. To account for this, all subsequent responses involved subtracting individual vehicle control values for both the agonist and antagonist from the final aggregatory response to give a true value of aggregation as the area under the curve over a 6 min period.

The effects of AM251, AM630 and aspirin on 2-AG-induced platelet aggregation in blood from healthy volunteers and CAD patients on aspirin

Pre-incubation with AM251 and AM630 had no impact on subsequent aggregation induced by 2-AG (100 μM) at any of the incubation times tested over the 120 min incubation period in blood obtained from either normal, aspirin naïve volunteers, or from CAD patients. However, a significant difference in the aggregatory response to 2-AG (100 μM) was observed between healthy volunteers and patients (P < 0.0001) for all drug concentrations and incubations times for both AM251 and AM630 (Figure 3A,B, respectively).

Sub-analysis of the data to interrogate whether patients on clopidogrel + aspirin (n = 3) responded differently to 2-AG than those on aspirin alone (n = 9) suggested that those on combined anti-platelet therapy were more responsive to 2-AG (AUC = 23.4 ± 6.0) than those on aspirin alone (AUC = 9.5 ± 7.1). The low number of patients on clopidogrel + aspirin precluded meaningful statistics on these data.

Since all CAD patients were on aspirin therapy at the time of blood sampling, while the healthy volunteers were free of aspirin, to confirm that these observed effects were due to the presence of aspirin we assessed *in vitro* the effect of pre-incubating blood samples from the healthy volunteers with aspirin on both arachidonic acid (AA) and 2-AG-induced aggregation. Pre-incubation of blood with aspirin (100 μM) was shown to inhibit markedly aggregation induced by both 2-AG and AA (Figure 4A,B, respectively). This was statistically significant for 2-AG concentrations 100 μM (P < 0.001) and 200 μM (P < 0.05) and for AA concentrations 100 μM (P < 0.05), 300 μM (P < 0.01) and 750 μM (P < 0.001).

A final set of experiments conducted in blood from a separate cohort of healthy volunteers (n = 5) showed that a maximal concentration of the specific FAAH inhibitor, URB597, failed to have any significant effect on aggregation in response to 2-AG in either whole blood or PRP (Figure 5). However, the specific MAGL inhibitor, URB602, all but abolished aggregation (P < 0.001) in both whole blood and PRP. Similar inhibition was seen with preparations treated with both antagonists together (URB597 + URB602) and in those treated with aspirin (100 μM) as before.

Discussion

This study has demonstrated that, contrary to previous reports [16], 2-AG-induced platelet aggregation is mediated via activation of the COX pathway rather than through activation of CB_1 receptors. The effect was found to be dependent on metabolism of 2-AG by MAGL, but not by FAAH. CB_1 and CB_2 antagonism had no effect on 2-AG-induced platelet aggregation *in vitro*, in blood from healthy volunteers and CAD patients, suggesting that endocannabinoids exert no tonic effect on platelets and any potential release of endocannabinoids as a consequence of platelet activation by either ADP or thrombin does not appear to involve CB_1 or CB_2 receptors. Δ^9-THC was found to be completely inactive in platelets.

Table 2

Patient characteristics and medications

Patient	Age (years)	BMI (kg m^{-2})	BP (mmHg)	TChol : HDL ratio	Platelets (× 10^9 cells l^{-1})	CAD	Previous revascularization	T2DM	Antiplatelet therapy	Other drugs and dose (mg)
1	68	28	142/84	3	85	3 VD	CABG	Y	ASA75, Clpd75	Frus80, En2.5, Ator20, GTN
2	75	36	103/58	5.3	265	3 VD	CABG	Y	ASA75, Clpd75	Frus40, Ator80, IsoMn25, Los50, Metclp10, Omep20, Metf500, GTN
3	70	24	129/73	2.9	205	2 VD	CABG	–	ASA75	Ator80, Omep20, Ram5, Metp50
4	56	29	152/94	1.7	159	N	–	–	ASA75	GTN
5	73	30	128/74	2.4	257	2 VD	Stent	–	ASA75	Sim40, Adiz90, GTN
6	61	25	151/75	3.0	217	2 VD	–	–	ASA75	Sim40, Aten50, GTN
7	64	30	136/82	3.2	179	1 VD	–	–	ASA75	Sim40, Aten50, Per4, GTN
8	65	28	150/90	2.6	190	1 VD	–	–	ASA75	Ator10
9	65	36	135/75	3.9	188	M	–	–	ASA75, Clpd75	Ator80, Aten50, Los100, Omep15, Bfmt2.5, Aml10, GTN
10	53	28	138/84	2.4	255	N	–	–	ASA75	Sim20, Adiz120, Per8, Bfmt2.5, GTN
11	46	28	138/85	4.9	267	2 VD	–	–	ASA75	Ator40, Bis2.5, GTN
12	75	24	158/78	3.1	156	1 VD	–	Y	ASA 75	Ator20, Cand4, GTN
Mean	**65**	**29**	**138/79**	**3.2**	**202**					
SEM	**2.5**	**1**	**4/3**	**0.3**	**16**					

ASA, aspirin; Adiz, adizem; Aml, amlodipine; Aten, atenolol; Ator, atorvastatin; BMI, body mass index; BP, blood pressure; Bfmt, bendroflumethiazide; Bis, bisoprolol; CABG, coronary artery bypass graft; CAD, coronary artery disease; Cand, candesartan; Clpd, clopidogrel; En, enalapril; Frus, furosemide; GTN, glyceryl trinitrate spray; HDL, high-density lipoprotein; IsoMn, isosorbide mononitrate; LAD, left anterior descending artery; Los, losartan; M, mild; Metclp, metoclopramide; Metf, metformin; Metp, metoprolol; N, normal; Omep, omeprazole; Per, perindopril; Ram, ramipril; Sim, smivastatin; Tchol, total cholesterol; T2DM, type 2 diabetes mellitus; VD, vessel disease.

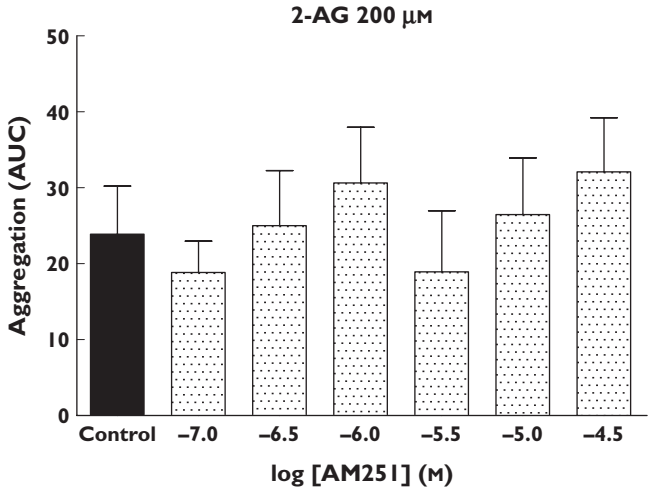

Figure 1

The aggregatory effect of 2-AG (200 μM) on its own (control) and after pre-incubation with AM251 (100 nM–30 μM), in whole blood samples from healthy human volunteers ($n = 6$). It is important to note that data were standardized by subtracting vehicle-induced aggregation in each case and are expressed as the mean ± SEM; statistical analysis was performed using a one-way ANOVA and no significance was found ($P = 0.78$)

The literature relating to the activity of endocannabinoids in platelets is controversial. Maccarrone *et al*. showed that high concentrations (1.3 mM) of anandamide caused platelet activation in human PRP [15]. The effect was unaffected by COX1 and FAAH inhibition, suggesting a cannabinoid receptor-dependent mechanism. Similar results were obtained in a later paper [16] with 2-AG, leading the authors to suggest that 2-AG-mediated aggregation is via activation of an as yet uncharacterized CB receptor. In contrast, Braud *et al*. showed similar anandamide-induced platelet aggregation, though at lower concentrations (3–10 μM), in washed platelets from rabbits, but activation was insensitive to CB_1 antagonism and completely inhibited by COX1 and FAAH inhibition [18].

Our findings are in keeping with a previous report in human PRP [16], in that 2-AG induced platelet activation in whole blood *in vitro*. However, in stark contrast to the previous study, we found that the actions of 2-AG were not mediated by either CB_1 or CB_2 activation, but were abolished by the COX inhibitor, aspirin. The inference from these results is that 2-AG is metabolized in whole blood to generate arachidonic acid, the substrate for COX-mediated generation of the platelet activator, TXA_2. The inability of AM251 or AM630 to inhibit 2-AG-mediated platelet aggregation throughout a 2 h incubation period infers that neither the antagonists themselves, nor any blood-derived metabolites that might infer inhibitory activity, were capable of inhibiting 2-AG-mediated aggregation. Whilst it is feasible that AM251 was ineffectual as a CB_1 antagonist, we have evidence from *in vivo* studies in rats that the same batch of AM251 completely abolished the depressor responses to the CB_1 agonist ACEA (unpublished observations).

In the present studies, we found that the CB_1/CB_2 agonist, Δ^9-THC, at a concentration relevant to reported peak plasma concentrations observed post-marijuana

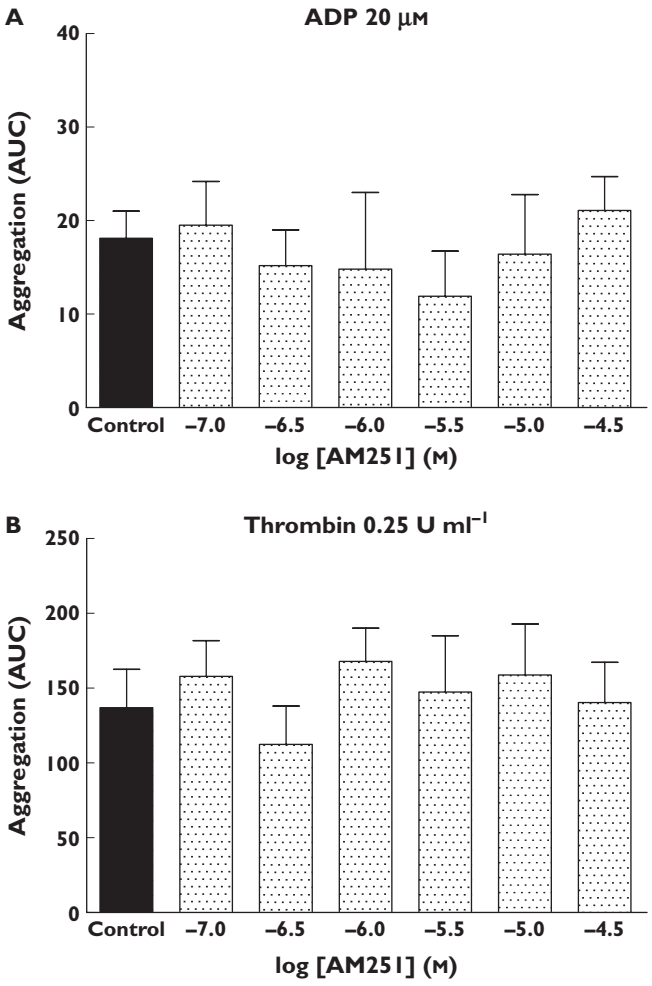

Figure 2

The aggregatory effect of (A) ADP (20 μM) and (B) thrombin (0.25 U ml^{-1}) on their own (control) and after pre-incubation with AM251 (100 nM–30 μM), in whole blood samples from healthy human volunteers ($n = 6$). It is important to note that data were standardized by subtracting vehicle-induced aggregation in each case and are expressed as the mean ± SEM; statistical analysis was performed using a one-way ANOVA and no significance was found [(A) $P = 0.90$; (B) $P = 0.86$]

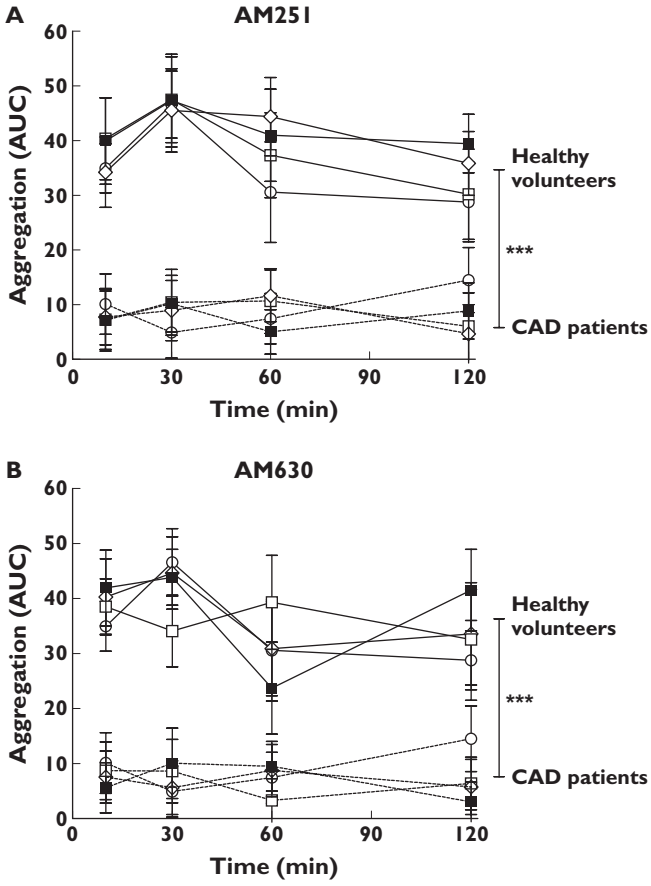

Figure 3

The aggregatory effect of 2-AG on its own (control) and after incubation with (A) AM251 (0.3, 3, 30 μM) and (B) AM630 (0.3, 3, 30 μM) at different time points (10–120 min), in whole blood samples from healthy human volunteers ($n = 8$; solid lines) and patients ($n = 12$; dotted lines). Data were standardized by subtracting vehicle-induced aggregation in each case and are expressed as the mean ± SEM; statistical analysis was performed using a two-way ANOVA. Statistical significance was observed (***$P < 0.001$) in the difference between the average 2-AG-induced aggregation of healthy volunteers compared with patients for both drugs at all three concentrations of AM251 or AM630, as well as with the control. (A) Control (—○—); AM251 30 μM (—□—); AM251 3 μM (—■—); AM251 300 nM (—◇—); (B) Control (—○—); AM630 30 μM (—◇—); AM630 3 μM (—□—); AM630 300 nM (—■—)

inhalation [30], had no impact on platelet aggregation in blood from healthy volunteers (Figure 4). This is inconsistent with previous reports of Δ^9-THC-mediated induction of GPIIb/IIIa exposure [7] and in conflict with anti-aggregatory effects of Δ^9-THC reported elsewhere [17]. It is possible that by activating both CB_1 and CB_2 receptors, Δ^9-THC could induce opposing responses mediated by the two receptors. However, the observation Δ^9-THC failed to induce aggregation in the presence of either CB_1 or CB_2 receptor blockade does not support this notion and lends weight to the argument that CB_1 (or indeed CB_2) receptors play little or no part in platelet activation.

Several studies have shown that cannabinoids, notably 2-AG, can have a co-agonistic effect with normal physiological agonists such as ADP and thrombin [16] and it is recognized that, at least in the central nervous system, cannabinoid receptors mediate a tonic activity that is reversed by antagonists in the absence of exogenous cannabinoid agonists [23]. Our observations that AM251 alone did not have any effect on platelet activation induced by the physiological agonists, ADP or thrombin, suggests that there is no tonic activity associated with CB_1 receptors in platelets.

The above studies in blood from healthy volunteers suggest that 2-AG-mediated activation is CB-receptor independent, but that the same is not necessarily true in patients with coronary artery disease, where changes in enzyme and receptor expression might alter the activation pathway(s) involved. Furthermore, all 12 CAD patients

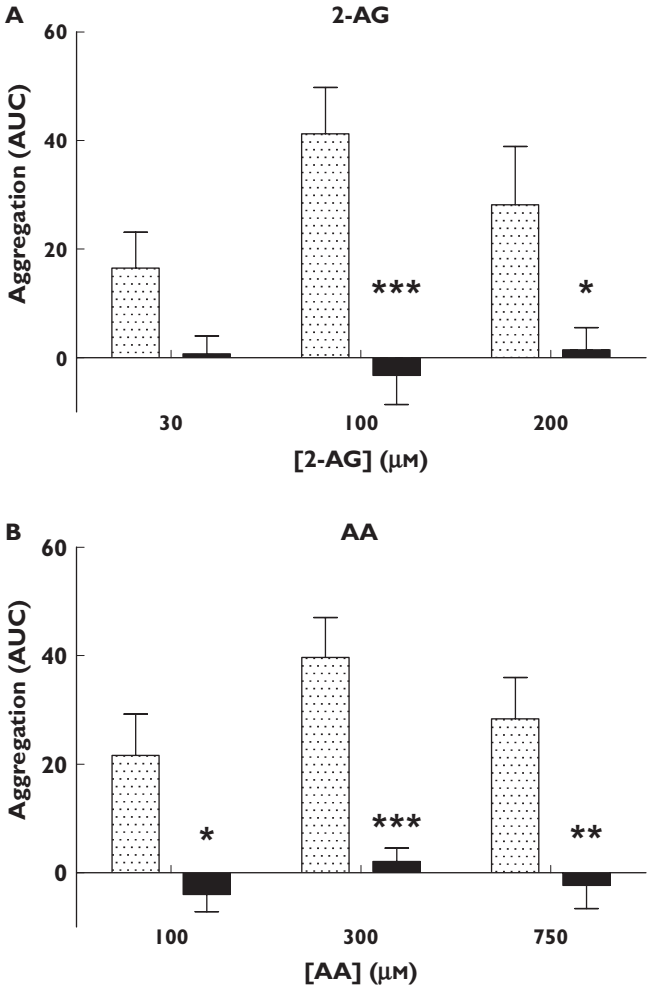

Figure 4

The inhibitory effect of aspirin (100 μM) on (A) 2-AG (30, 100 and 200 μM) and (B) AA (100, 300 and 750 μM) induced aggregation ($n = 8$). Data were standardized by subtracting vehicle-induced aggregation in each case and are expressed as the mean ± SEM; statistical analysis was performed using a two-way ANOVA. 2-AG-induced aggregation was significantly inhibited by aspirin at concentrations of 100 μM (***$P < 0.001$) and 200 μM (*$P < 0.05$). AA-induced aggregation was significantly inhibited at all three concentrations, 100 μM (*$P < 0.05$), 300 μM (***$P < 0.001$) and 750 μM (**$P < 0.01$). Control (□); + aspirin 100 μM (■)

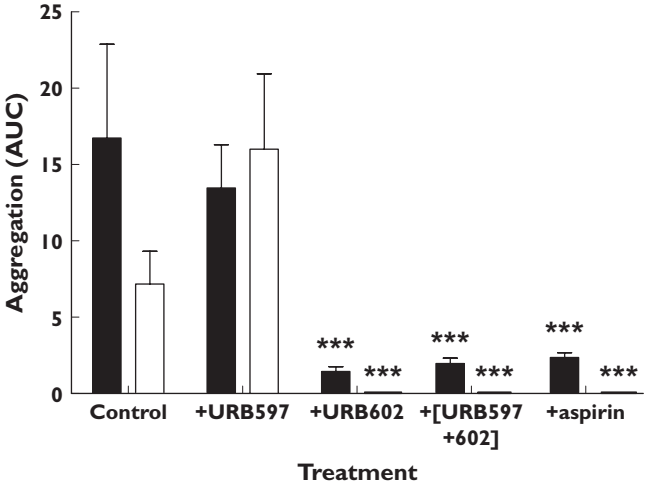

Figure 5

The inhibitory effect of URB597 (300 nM), URB602 (300 μM) and aspirin (100 μM) on 2-AG (200 μM)-induced aggregation in whole blood and platelet rich plasma. 2-AG-induced aggregation was inhibited by URB602, [URB597 + URB602] and aspirin in both whole blood and platelet rich plasma. ***$P < 0.001$ (two-way ANOVA with Bonferoni post-test; $n = 5$). whole blood (■); PRP (□)

involved in the study were taking aspirin and three patients were also prescribed clopidogrel (Table 2). We found that the pro-aggregatory response was blunted in patients compared with healthy volunteers, most likely as a result of aspirin-mediated inhibition of the COX pathway, adding weight to the hypothesis that 2-AG-induced aggregation is via metabolism to arachidonic acid. No further inhibition was seen with either the CB_1 or CB_2 receptor antagonist, suggesting that cannabinoid receptors are not involved in the modest aggregation seen in response to 2-AG in patients. The observation that blood from the patients prescribed aspirin + clopidogrel appeared to be more responsive to 2-AG than that from patients on aspirin alone requires further exploration in a study specifically designed to test this hypothesis. However, the finding that blood from patients with coronary artery disease receiving aspirin showed reduced aggregatory responses led us to investigate whether or not this was due to COX inhibition. We therefore confirmed this in blood from healthy volunteers by studying the effects of COX inhibition *in vitro* with aspirin. We then went on to confirm that responses to 2-AG were abolished by the MAGL inhibitor, URB602, but not the FAAH inhibitor, URB597, adding conclusive evidence that metabolic conversion of 2-AG to AA by MAGL is a prerequisite for 2-AG-mediated platelet activation.

Our findings challenge the previous literature that suggests 2-AG-induced aggregation is cannabinoid receptor-dependent and metabolic breakdown-independent [16]. The conclusive impact of URB602 on 2-AG-induced aggregation (Figure 5) in both whole blood and PRP, combined with the inhibitory effect of aspirin, strongly suggests that the mechanism of action of 2-AG is not through CB_1 or CB_2 receptors, but via MAGL-mediated conversion of 2-AG to AA prior to activation of COX-1. This finding goes some way to supporting the findings of Braud *et al*. [18] in that an endocannabinoid is shown to require metabolic conversion for activity. It also might explain the lack of effect of Δ^9-THC on platelets. The specificity of MAGL for 2-AG clearly does not extend to Δ^9-THC, rendering the latter ineffectual in this setting despite the possibility of CB_1 receptors on platelets. The differences between our data and that of a previous report [16] are not due to the use of whole blood aggregometry in our study as opposed to platelet rich plasma previously: inhibition of MAGL in both preparations had the same inhibitory effect.

Our finding that 2-AG mediates a pro-aggregatory effect via a CB_1 receptor-independent pathway implies that CB_1 receptor antagonism might not have any benefit with respect to reducing thrombotic potential in patients, at least in the acute phase. However, a recent study, measuring platelet-bound fibrinogen in unstimulated whole blood, suggests that CB_1 receptor antagonism depresses platelet activation in a mouse model of type 2 diabetes mellitus [31]. Treatment with rimonabant reduced fibrinogen binding (reflecting reduced glycoprotein IIb/IIIa exposure), reduced thrombin-induced platelet aggregation and reduced ADP-stimulated expression of P-selectin, a platelet activation marker. These findings suggest that chronic treatment with a CB_1 receptor antagonist may cause changes in platelet function, although it is as yet unclear whether this is a species-dependent phenomenon or one that is due to subtle effects exerted during protracted therapy.

Limitations of study

The patient group and healthy volunteers used in this study were not matched. The aim was not to compare directly the effects of AM251 in platelets in the two groups but to establish whether the same principles of activation applied in CAD patients, given that their pathophysiology might differ from a healthy group. The major differences between the healthy and patient groups include increased age and the polypharmacy related to high-risk patients undergoing interventional therapies for cardiac disease. Drug therapies in these patient groups include medications directly targeting platelet function, such as aspirin and clopidogrel, and other medications, such as statins and anti-hypertensives, which may cause unexpected changes in platelet function. These differences must be taken into consideration when directly comparing the results from the two groups in this study. In addition, platelet aggregometry, whether in whole blood or PRP is nevertheless some distance from *in vivo* measures of platelet function. Whilst aggregometry is an accepted measure for this kind of pharmacological study, direct correlations to *in vivo* findings should be viewed with caution [32].

Conclusion

In conclusion, 2-AG induces aggregation in human platelets. The mechanism of platelet activation is via metabolism by the enzyme MAGL, to arachidonic acid rather than through direct activation of CB_1 or CB_2 receptors. Whilst the clinical potential of endocannabinoid receptor antagonists as therapies to modulate cardiovascular risk is potentially exciting, the current study demonstrates that there is likely to be minimal acute effects of these compounds in terms of platelet function. There is some evidence in animal models, however, to suggest that long-term therapy may inhibit platelet function, and this warrants further study.

Competing interests

There are no competing interests to declare.

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