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ORIGINAL ARTICLE

Nitric oxide from mononuclear cells may be involved in platelet responsiveness to aspirin

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ABSTRACT

Background Several mechanisms have been proposed to explain why some platelets have a reduced response to aspirin (ASA). Among them, it was reported an increased circulating level of vitamin-D-binding protein (DBP). In addition, nitric oxide (NO) released from mononuclear cells was involved in the antiplatelet effects of ASA. The aim was to analyse the relationship between platelet response to ASA and both NO generation and vitamin-D-binding protein content in mononuclear cells.

Materials and methods Mononuclear cells were obtained from patients with stable coronary artery disease that were divided by a platelet functionality test (PFA-100) as ASA-sensitive (n = 23) and ASA resistant (n = 27).

Results Both the release of NO (determined by nitrite + nitrate concentration) and the expression of endothelial-type NO synthase (eNOS) were higher in mononuclear cells from ASA sensitive as compared with those from ASA-resistant patients. There was a positive correlation between either the release of NO and the expression of eNOS protein in mononuclear cells with the ability of ASA to inhibit platelet activity. DBP content in mononuclear cells was higher in ASA resistant than in ASA sensitive. The level of DBP content in mononuclear cells was negatively associated with the ability of ASA to inhibit platelets. However, in vitro experiments suggested that there was no association between DBP and NO production by mononuclear cells.

Conclusions Mononuclear cells from patients with platelets with lower responsiveness to ASA showed a reduced ability to produce NO.

Keywords Aspirin, mononuclear cells, nitric oxide, platelets, vitamin-D-binding protein.

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Introduction

Clinical benefits of aspirin (ASA) have been largely attributed to its antithrombotic activity by the irreversible inhibition of cyclooxygenase-1 (COX-1) in platelets, thereby preventing thromboxane A_2 (TxA_2) synthesis [1]. However, evidences have demonstrated that clinical benefits of ASA are higher than those solely explained by the inhibition of TxA_2 production, suggesting that additional mechanisms associated with the antiplatelet effect of ASA may exist [2]. In this regard, we demonstrated the involvement of nitric oxide (NO) released from leucocytes in the antiplatelet effects of ASA [3]. Human leucocytes generate NO from L-arginine through an endothelial-type NO synthase (eNOS) similar to the endothelial one [4]. Indeed, ASA also stimulated eNOS to produce NO in endothelial cells [5].

Several meta-analyses have demonstrated that platelet from ASA-treated patients has not similar degree of platelet inhibition. Indeed, those with inadequate inhibition of their platelets showed higher risk of clinical adverse by thrombotic events [6–8]. It is important to remind that all the methods used to identify patients with platelets showing inadequate response to ASA have important limitations, although most of them have been focussed to determine alterations in the ability of ASA to inhibit platelet functions. Moreover, another important fact is that the mechanisms involved in the lack of responsiveness of platelets to ASA are not well established. In this regard, several mechanisms have been associated with the failure of ASA to inhibit platelet function. Among them, it was included noncompliance, genetic-related alterations, increased platelet turnover, particularly important for smokers patients, different pattern of protein expressed in the platelets and others [9,10]. We have also

reported that patients with ASA-resistant platelets have increased the circulating levels of vitamin-D-binding protein (DBP), the major circulating vitamin D transport protein, as compared with those with ASA-sensitive platelets [11]. Moreover, in in vitro studies DBP reduced the ability of ASA to prevent TxA_2 production by platelets, an effect that was more evident in whole blood than in platelet-rich plasma suggesting the involvement of other blood cells in such DBP-related effect [11].

Although studies have also shown that mononuclear cells contain DBP [12,13], however, in our knowledge, it was not previously analysed whether either NO released or DBP contained in mononuclear cells may be involved in a different response of platelets to ASA. Therefore, the aim was to analyse whether there is an association between the NO system and DBP content in mononuclear cells with respect to the ability of ASA to inhibit platelet activity.

Materials and methods

Patients

The study was performed in patients with clinically stable coronary artery disease taking ASA (100 mg/day). Patients were obtained from a database, and they were matched by age and gender. The patients were divided into ASA resistant (n = 27) and ASA sensitive (n = 23) using a laboratory test (PFA-100) based on a platelet functionality assay (see below). This laboratory platelet function test has been also used to predict clinical recurrences in cardiovascular patients under ASA treatment [8].

All the included patients had suffered an acute coronary event from at least 9 months before inclusion. All the patients had been taking 100 mg/day ASA as single antithrombotic therapy and/or in combination with an ADP receptor P2Y12 antagonists from the last 8 months before inclusion. However, at least during last month before inclusion the only antithrombotic drug taking by the included patients was ASA (100 mg/day). Therefore, an exclusion criterion was that patients were on other antithrombotic drugs or nonsteroid anti-inflammatory drugs than ASA within 30 days before inclusion.

Blood samples were obtained by antecubital venipuncture in the morning 2–4 h after last ASA was taken. To avoid spontaneous platelet activation, the initial 3–4 mL of blood was discarded. Blood samples were collected in citrate tubes for PFA-100 assay and in tubes containing EDTA for mononuclear cells isolation. The study was approved by the Ethical Committee of the San Carlos University Hospital, and all patients gave informed consent.

Identification of ASA resistance

PFA-100 assay (Dade Behring, W. Sacramento, CA, USA) was used to identify platelet resistance to ASA. Disposable test cartridges containing collagen-coated membrane infused with epinephrine (10 μg) were used. CT, indicative of platelet function for the whole blood sample, was defined as the time necessary for the occlusion of a small aperture (150 μm) contained in a membrane into the cartridges when whole blood was infused into the cartridge. According to the manufacturer, CT ranges between 94 and 193 s with epinephrine cartridges defined ASA-resistant platelets. ASA-sensitive platelets show prolonged CT (300 s). Therefore, only patients with extreme CT values for each of the two ASA responsiveness conditions (between 94–193 s and > 300 s) were included in the study.

To discard the effect of noncompliance in the lack of response of platelets to ASA and as previously reported [10], PFA-100 assay was performed at inclusion and 1 h after patients received an additional 100 mg ASA dose. The patients that demonstrated a similar closure time (CT) range (see below) in response to ephinephrine at inclusion and 1 h after the additional 100 mg ASA administration were included in the study.

Mononuclear cells isolation

Mononuclear cells were isolated and manipulated under sterile conditions. Mononuclear cells were isolated using Ficoll-Hypaque and resuspended in RPMI-1640 medium supplemented with 5 mM L-glutamine and 0·25% serum albumin. Mononuclear cells were then divided into aliquots containing 5×10^6 cells/mL and incubated into Eppendorf at 37 °C during 18 h. In some experiments, mononuclear cells from patients with ASA-sensitive platelets were further incubated in the presence of either DBP (15 μM) and with an L-arginine antagonist, L-N^G-nitroarginine-methyl-ester (L-NAME, 10^{-5} M). This DBP concentration was chosen based on the fact that reduced the inhibitory effect of ASA on TXA_2 production by platelets [11]. After the incubation period, mononuclear cells were centrifuged at 700 g for 10 min at 4 °C and supernatants were recovered for nitrate/nitrite determination and the pellets for Western blot determinations.

Determination of eNOS and inducible NOS-type expression and DBP content in mononuclear cells

Protein expression of eNOS, inducible NOS (iNOS), and DBP were analysed in the mononuclear cells by dot blot. As reported [14], 10 μg of total mononuclear cells protein, determined by bicinchoninic acid reagent (Thermo scientific, Rockford, IL, USA), was loaded onto nitrocellulose membranes. Nitrocellulose membranes were blocked with 5% (w/v) bovine serum albumin and incubated with monoclonal antibodies against eNOS (sc-653; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA, dilution 1 : 500), iNOS (sc-8310; Santa Cruz Biotechnology, Inc, dilution 1 : 1500) and DBP antibody (sc-18705; Santa Cruz Biotechnology, Inc, dilution 1 : 2000). Nitrocellulose membranes were then revealed with a peroxidase-conjugated

anti-rabbit IgG (1 : 2500) and developed using enhanced chemiluminescence reagents (ECL; GE Healthcare, Little Chalfont Buckinghamshire, UK). The expression of a constitutive protein, β-actin (A-5441; Sigma-Aldrich, St.Louis, MO, USA, dilution 1 : 7500), was also determined as protein load control.

Western blot analysis was further performed in seven samples per experimental group. For this purpose, 40 μg of total mononuclear cells proteins was developed in 10% SDS/PAGE. The gels were then blotted onto nitrocellulose membranes, incubated with the anti-eNOS, anti-iNOS, anti-DBP and anti-β-actin antibodies and developed, as above mentioned for dot blot assays, using ECL.

Determination of total nitrate/nitrite and interleukin-6 (IL-6) production

Total nitrate/nitrite released from the mononuclear cells was measured as indirect estimation of the NO released by mononuclear cells. Nitrate + nitrite measurements were performed in the supernatant of the in vitro incubated mononuclear cells using a colorimetric assay kit (780001; Cayman Chemical Company, Ann Arbor, MI, USA) following manufacturer's instructions. The sensitivity of the assay was 2·5 μM. The intra- and interassay variation coefficients were 2·7% and 3·4%, respectively.

Interleukin-6 (IL-6) was also determined in the supernatants using a commercial kit (D6050; R and D systems, Abingdon, UK) following manufacturer's instructions. The sensitivity of the assay was 0·7 pg/mL. The intra- and interassay variation coefficients were 2·0% and 3·8%, respectively.

Statistical analysis

Results are expressed as mean ± SEM. To compare levels of DBP, iNOS, eNOS, IL-6 and nitrite + nitrate between ASA-sensitive and resistant patients, different generalized linear models ANOVA for independent samples were used. To adjust the results by treatment with angiontensin I-converting enzyme inhibitors (ACEI), this covariate was introduced in the model. Because of the asymmetry of the variables DBP, eNOS and nitrate + nitrite, we conducted a logarithmic transformation to approximate normal distribution. Correlations analyses were performed using Spearman's ρ statistic. In the experiments in which L-NAME and DBP were added to the incubation medium, Wilcoxon's test was used for the statistical analysis. A P-value < 0·05 was considered statistically significant.

Reporting of the study conforms to STROBE along with references to STROBE and the broader EQUATOR guidelines [15].

Results

Clinical features of the included patients are shown in Table 1. All the included patients were of Caucasian origin and showed similar concomitant risk factors and drug treatments (Table 1). At the inclusion time, none of the included patients were active smokers, but a number of them had smoking history (Table 1). A slight smaller number of ASA-resistant patients treated with ACEI were observed as compared with patients with ASA-sensitive platelets, although it did not reach statistical significance (Table 1).

Table 1 Clinical features of ASA-sensitive and ASA-resistant patients at inclusion

	ASA-sensitive platelets (n = 23)	ASA-resistant platelets (n = 27)
Age (years)	69.3 ± 1.6	69.8 ± 1.4
Male/female	21/2	26/1
Risk factors (%)		
Previous smoking history	19 (82.6)	21 (77.7)
Hypertension	15 (65.2)	11 (40.7)
Hyperlipemia	13 (56.5)	18 (66.7)
Diabetes mellitus	7 (30.4)	11 (40.7)
Medication at inclusion (%)		
ASA	100	100
P2Y12 ADP receptor antagonists	0	0
β-Blockers	69.6	63.0
Calcium antagonist	30.4	40.7
ACE inhibitors	56.5	22.2
Nitrates	30.4	22.2
Statins	100	100
CT at inclusion (s)	> 300	111.1 ± 7.0
CT at 1 h after ASA administration (s)	> 300	130.8 ± 12.8

ASA, aspirin; ACE, angiotensin-converting enzyme; CT, closure time. CT is the time necessary for the occlusion of the aperture in the PFA-100 cartridge, and it is indicative of platelet function.
The upper limit of the test is 300 s. Age is represented as mean ± SEM.

Endothelial-type NO synthase and iNOS expression and NO and IL-6 release in mononuclear cells

As Fig. 1 shows, total nitrate + nitrite production was significantly higher in supernatants of mononuclear cells from patients with ASA-sensitive platelets with respect to those from ASA-resistant platelets. Dot blot analysis demonstrated that eNOS protein expression was also higher in mononuclear cells

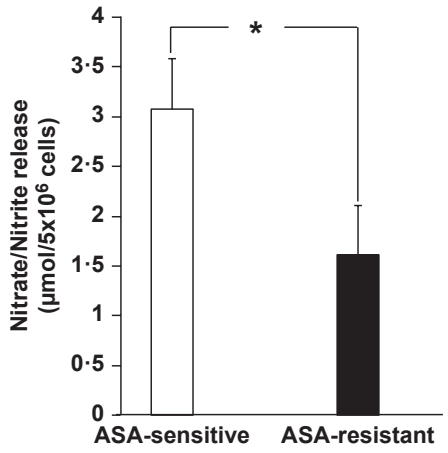

Figure 1 Bar graph shows nitrite + nitrate released from mononuclear cells obtained from patients with aspirin (ASA)-resistant and ASA-sensitive platelets. Results are represented as mean ± SEM. *$P < 0{\cdot}05$ with respect to ASA-sensitive patients.

from ASA-sensitive than those from ASA-resistant patients (Fig. 2). In dot blots determinations, the iNOS protein signal was almost undetectable, and it was not different between mononuclear cells from ASA-sensitive and ASA-resistant patients (Fig. 2). Western blot analysis assessed that eNOS and iNOS monoclonal antibodies used in dot blot experiments recognized in the mononuclear cells a single-band protein with apparent molecular weights of 140 and 130 KDa, respectively, similar to those reported for eNOS and iNOS [5] (Fig. 2).

It was further analysed whether the different number of ACEI-treated patients in the ASA-resistant group as compared with ASA-sensitive may influence on either the release of NO and the expression of eNOS protein in the mononuclear cells. Statistical analysis adjusted by ACEI treatment also demonstrated that both eNOS protein expression and nitrite + nitrate release were significantly lower in mononuclear cells from ASA-resistant patients as compared with those from ASA-sensitive patients (eNOS: $P = 0{\cdot}006$; nitrite + nitrate: $P = 0{\cdot}0001$).

Spearman's analysis showed a positive correlation between total nitrite + nitrate released from mononuclear cells and CT values in the PFA-100 assays (Table 2). The level of eNOS expression in mononuclear cells was also positively associated with CT values in the PFA-100 assays (Table 2).

IL-6 released from mononuclear cells was also determined, and it was very similar between ASA-sensitive and ASA-resistant patients (IL-6 in pg/5 × 10^6 mononuclear cells: ASA resistant: 42·07; ASA sensitive: 57·7; P NS).

Vitamin-D-binding protein content in mononuclear cells

Dot blot analysis showed that mononuclear cells from ASA-resistant patients contained higher content of DBP than those from ASA-sensitive platelets (Fig. 2). Western blot analysis demonstrated that the monoclonal antibody used in the dot blot analysis to recognize DBP, identified in the mononuclear cells a single band of 55 KDa that correspond to the reported apparent molecular weight for DBP [16].

After adjustment for ACEI treatment, the level of DBP contained in mononuclear cells remained significantly higher in ASA-resistant than in ASA-sensitive patients ($P = 0{\cdot}004$). Fur-

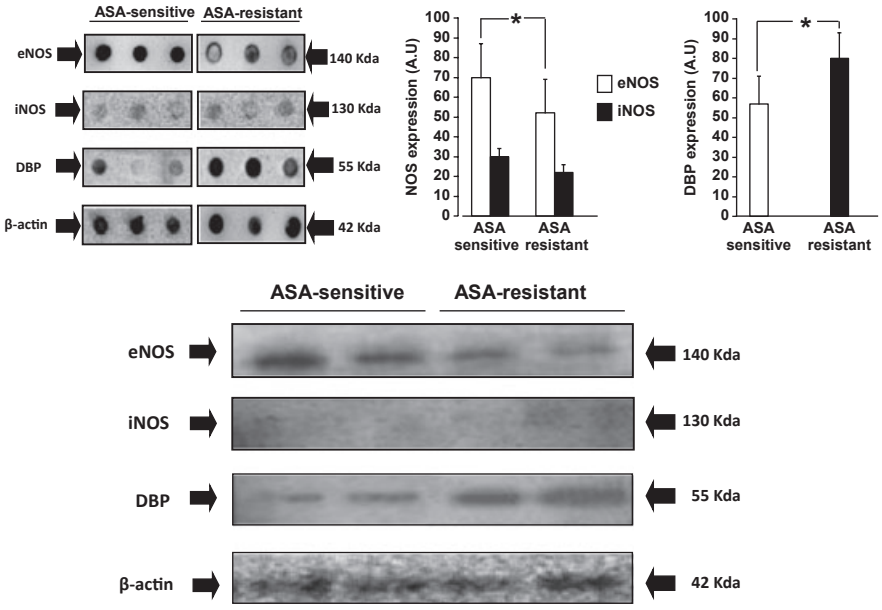

Figure 2 On the top left, it is shown a representative dot blot determining the protein expression level of endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS) and vitamin-D-binding protein (DBP) in mononuclear cells from aspirin (ASA)-sensitive and ASA-resistant patients. The protein expression level of β-actin is also shown and it was used as control for protein loading. On the top right, bar graphs show the densitometric analysis, in arbitrary units (AU), considering all dot blots. On the bottom, it is shown a representative Western blot to demonstrate the specificity of the monoclonal antibodies used in the dot blot experiments for eNOS, iNOS, DBP and β-actin. Results are represented as mean ± SEM *$P < 0{\cdot}05$ with respect to ASA-sensitive patients.

Table 2 Spearman's analysis to determine the association between CT values in PFA-100 assays and nitrate + nitrite release, eNOS expression and DBP content in mononuclear cells

	CT values	
	Spearman's ρ coefficient	*P*-value
Nitrate + nitrite	0·770	0·001*
eNOS protein	0·438	0·020*
DBP	−0·349	0·030*

CT, closure time; DBP, vitamin-D-binding protein; eNOS, endothelial-type nitric oxide synthase.
*$P < 0.05$.

thermore, Spearman's analysis showed a negative association between the level of DBP content in mononuclear cells and CT values in the PFA-100 assays (Table 2).

Relationship between the level of DBP contained in mononuclear cells and eNOS expression

It was analysed whether DBP may be involved in the lower expression of eNOS protein observed in mononuclear cells from patients with ASA-resistant platelets. For this purpose, a subgroup of mononuclear cells from ASA-sensitive patients ($n = 7$) were incubated in the presence and in the absence of 15 μM DBP for 18 h at 37 °C. Addition of DBP to mononuclear cells obtained from ASA-sensitive patients failed to modify neither the level of expression of eNOS protein nor the amount of nitrite + nitrate released from mononuclear cells as compared with mononuclear cells from ASA-sensitive patients incubated in the absence of DBP (Fig. 3). Moreover, when mononuclear cells from patients with ASA-sensitive platelets were incubated in the presence of the L-arginine antagonist, L-NAME (10^{-5} M; $n = 7$), the level of DBP contained in the mononuclear cells was similar to that found in mononuclear cells from ASA-sensitive patients incubated in the absence of 10^{-5} M L-NAME ($n = 7$, Fig. 3). In addition, considering together all the sensitive and resistant patients, Spearman's analysis did not demonstrated correlation between the content of DBP in mononuclear cells and either the level of eNOS expression and the amount of nitrite + nitrate released from them (Table 2).

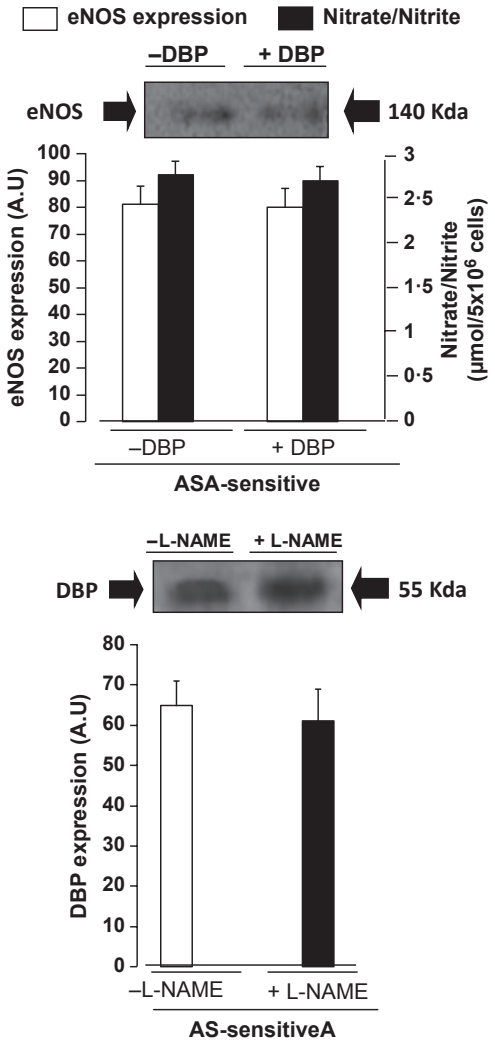

Figure 3 Representatives Western blots to analyse the effect of addition of both vitamin-D-binding protein (DBP) (15 μM), on the level of endothelial-type nitric oxide synthase protein expression, and the L-arginine antagonist, L-N^G-Nitroarginine-Methyl-Ester (10^{-5} M) on the level of DBP content in mononuclear cells obtained from seven different aspirin-sensitive patients. Bar graphs show both the densitometric analysis of the Western blots expressed in arbitrary units (A.U) and the nitrite + nitrate released from the mononuclear cells incubated in the presence and in the absence of DBP. Results are represented as mean ± SEM of seven different experiments.

Discussion

The present work demonstrated a relationship between the ability of mononuclear cells to generate NO and the platelet responsiveness to ASA for the first time. Mononuclear cells from patients with ASA-sensitive platelets showed higher expression of eNOS protein and released more NO than those with ASA-resistant platelets. Moreover, a positive association was found between the level of eNOS expression and the NO released from mononuclear cells with the ability of ASA to inhibit platelet activity.

Different mechanisms have been raised to explain why some patients have altered the response of their platelets to ASA. In a previous work, we demonstrated the association between a

reduced platelet response to ASA with the circulating levels of DBP [11].

In the present study, although mononuclear cells from patients with ASA-resistant platelets contained higher levels of DBP than those from ASA-sensitive patients, however, there was no association with the ability of mononuclear cells to produce NO because (i) the presence of L-NAME, an inhibitor of NO production, did not modify the content of DBP in mononuclear cells from ASA-sensitive patients; (ii) in mononuclear cells from ASA-sensitive patients, addition of DBP did not modify either eNOS protein expression and the ability to release nitrite + nitrate; (iii) Spearman's analysis did not demonstrated association between the content of DBP and either the level of eNOS expression and nitrite + nitrate released from mononuclear cells.

The present experimental design did not allow us to assess the origin of the higher content of DBP in mononuclear cells from ASA-resistant patients. Some authors have identified DBP into mononuclear cells granules [12,13], postulating that most of the DBP content in mononuclear cells is acquired from plasma [17]. In this regard, higher DBP circulating plasma levels were demonstrated in ASA-resistant than in ASA-sensitive patients [11].

The higher DBP content in mononuclear cells from ASA-resistant patients may be reflexing greater inflammatory activity. In this regard, association between pro-inflammatory biomarkers and residual platelet reactivity was demonstrated in patients with acute coronary syndrome [18]. However, although mononuclear cells are a main source of pro-inflammatory mediators such as IL-6 [19] in the present study, mononuclear cells released similar amount of IL-6 independently of the platelet response to ASA. Moreover, the level of expression of iNOS protein, an enzyme upregulated under inflammatory conditions [5, 20], was almost undetectable and not differently expressed between mononuclear cells from patients with ASA-resistant and ASA-sensitive platelets. Accordingly with these findings, Ziegler *et al.* [21] did not find relationship between inflammation and the ability of ASA to inhibit platelet activity.

Vitamin-D-binding protein is mainly involved in mononuclear cells chemotaxis. Chemotaxis may affect the interaction between platelets and mononuclear cells, effect that may also modify both platelet aggregation and TXA_2 production by platelets [22,23]. Therefore, as speculation, DBP could be involved in platelet–mononuclear cell interaction, and it may be associated with the platelet resistance to ASA.

The main finding of the present work was that mononuclear cells from patients with ASA-resistant platelets showed lower ability to produce NO and have reduced eNOS expression. Both NO production and eNOS expression were positively associated with the responsiveness of platelet to ASA.

The mechanism by which eNOS expression was lower in mononuclear cells from ASA-resistant patients remains to be established. As mentioned, the in vitro experiments incubating mononuclear cells with DBP discard the involvement of DBP in the regulation of eNOS expression. Although different inflammatory mediators have been associated with downexpression of eNOS protein [24], however, as mentioned, mononuclear cells from ASA-resistant patients did not show higher inflammatory state than those from ASA-sensitive patients. It could be also plausible that genetic reason, such as the existence of eNOS polymorphisms associated with reduction in eNOS expression, could be more frequently present in ASA-resistant patients [25]. Further experiments are needed to dilucidate this hypothesis.

The main contribution of the present findings is a new possible mechanism that involves other cells than platelets in the ability degree of ASA to inhibit platelets. In this regard, the better knowledge of the mechanisms involved in the lack of platelet responsiveness to ASA may favour new therapeutic strategies. Indeed, if the lack in the ability of mononuclear cells to produce NO may be involved in platelet resistance to ASA, it is possible that combination of ASA with NO donors may contribute to enhance the responsiveness of platelets to ASA.

Study limitations and comments

As we mentioned in previous publications [10,11], a possible study limitation is the lack of a standardized definition and validated method to identify ASA-resistant patients. In the present study, it was used the PFA-100 test to identify the responsiveness of platelets to ASA. Different meta-analyses have shown that ASA nonresponders detected by PFA-100 had significantly higher risk of cardiovascular events [7,8]. However, whether the here reported results could be extrapolated to other methods than PFA-100 is unknown, and it is out of the scope of the present study.

The main conclusion of the present work is that mononuclear cells from patients showing platelets with lack of responsiveness to ASA have a reduced ability to produce NO.

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Author contributions

Antonio Lopez Farre performed the experimental design, analysed research data, wrote and reviewed the manuscript. Javier Modrego researched data and analysed research data. Luis

Azcona provided blood samples from patients and contributed to discussion. Reddy Guerra provided blood samples from patients. Antonio Segura performed statistical analysis. Pablo Rodriguez and Jose J. Zamorano-Leon researched data and analysed research data. Vicente Lahera performed the experimental design and reviewed the manuscript. Carlos Macaya participated in revising the intellectual content and reviewed the manuscript.

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