British Journal of Pharmacology (2005) 146, 419–424
www.nature.com/bjp

Effect of clopidogrel on the expression of inflammatory markers in rabbit ischemic coronary artery

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1 Inflammation and platelet activation are critical phenomena in the setting of acute coronary syndromes. Platelets may contribute to increase ischemic injury by enhancing the inflammatory response of leukocytes and endothelial myocardial cells. Pharmacological inhibition of platelet activation prevents ischemic complications in patients with coronary diseases. Agents directed against the integrin glycoprotein IIb/IIIa (GP IIb/IIIa) receptor not only inhibit platelet aggregation but also have been demonstrated to limit the inflammatory response in acute coronary syndromes. The question then raised is if the inhibition of platelet activation by other mechanisms than the blockade of GP IIb/IIIa may also exert anti-inflammatory effects. The aim of the present study was to analyze if clopidogrel may exert anti-inflammatory effects during the acute phase of myocardial infarction.

2 A ligature was placed around the left anterior descending coronary artery of New Zealand White rabbits. After 15 min of ischemia, the myocardium was reperfused and the ischemic coronary artery was isolated 24 h after the ischemia. A group of ischemic rabbits was given a single oral dose of clopidogrel (20 mg kg^{-1}) just after the arterial occlusion and the animal was recovered. Sham-operated animals served as control.

3 P-selectin expression was significantly increased in infarcted rabbits with respect to control rabbits. Clopidogrel administration reduced P-selectin expression with respect to untreated infarcted rabbits. CD40 ligand and tissue factor expression was increased in the ischemic coronary artery and reduced after clopidogrel administration. Clopidogrel also protected endothelial nitric oxide synthase protein expression in the ischemic coronary artery, a protein that has been found downregulated under inflammatory conditions.

4 In conclusion, inhibition of platelet activation by clopidogrel exerted anti-inflammatory effects on the ischemic coronary artery.
British Journal of Pharmacology (2005) **146,** 419–424. doi:10.1038/sj.bjp.0706340;
published online 25 July 2005

Keywords: Clopidogrel; platelet activation; inflammation; coronary disease; acute myocardial infarction

Abbreviations: ACD, acid-citrate dextrose; ADP, adenosine diphosphate; AMI, acute myocardial infarction; eNOS, endothelial nitric oxide synthase; FITC, fluoresceine isothiocyanate; GP, glycoprotein; IgG, immunoglobin G; i.m., intramuscular; NO, nitric oxide; s.e.m., standard error of the mean

Introduction

Thrombosis and inflammation are associated with acute coronary syndromes. Thrombin and platelet activation have been demonstrated in the occlusive coronary in both humans and animals models of acute myocardial ischemia (Hughes *et al*., 1982; Davies & Thomas, 1984). Moreover, the inflammatory reaction seems to has detrimental consequences for the myocardial event (Liuzzo *et al*., 1994; Ridker *et al*., 2000). In this regard, inflammation has been also recognized as one of the main causes of endothelial dysfunction. Indeed, the nitric oxide (NO)-generating enzyme in the endothelium, endothelial nitric oxide synthase (eNOS), has been demonstrated downexpressed by inflammatory mediators such as cytokines and by inflammatory conditions including acute coronary syndromes (Yoshizumi *et al*., 1993; Mateos-Cáceres *et al*., 2002).

Pharmacological inhibition of platelet activation prevents ischemic complications in patients with coronary diseases (Eikelboom & Anand, 2003). In this regard, it is well known that ischemia–reperfusion induces platelet accumulation in the myocardium (Bednar *et al*., 1985), and, further to altering coronary blood flow by the mechanical obstruction of the coronary arteries, it has been postulated that platelets may contribute to the ischemic injury by enhancing the inflammatory response in both leukocytes and endothelial cells (Neumann *et al*., 1997; Gawaz *et al*., 1998). In this regard, agents directed against the integrin receptor glycoprotein IIb/IIIa (GP IIb/IIIa) not only inhibit platelet aggregation but also limit the inflammatory response (Neumann *et al*., 1999; Lincoff *et al*., 2001). Initially, it was thought that the crossreactivity of some GP IIb/IIIa inhibitors such as abciximab with other integrin receptors, rather than the reduction of platelet activity, exerted the anti-inflammatory

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effect of these drugs (Neumann *et al*., 1999). However, we recently demonstrated that tirofiban, another GP IIb/IIIa inhibitor that does not cross react with other integrin receptors, also induced an anti-inflammatory response in the myocardium of guinea-pigs suggesting that platelets may have a direct involvement in the myocardial inflammatory reaction (Molero *et al*., 2003).

In the last years, other antiplatelet drugs have been developed. One of these platelet drugs is clopidogrel, a thienopyridine that specifically acts on the platelet adenosine diphosphate (ADP) P2Y12 purinergic receptors (Webber *et al*., 1999). Clinical trials have consistently shown that clopidogrel prevents acute ischemic events although it only partially blocks the ADP (Mehta *et al*., 2001). Therefore, other effects of this drug apart from the simple inhibition of ADP-induced platelet aggregation may occur. The question raised is, thus, if inhibition of platelets by clopidogrel may also exert anti-inflammatory effects.

The aim of the present study was to analyze the anti-inflammatory effects of clopidogrel during the acute phase of myocardial infarction in an animal model of myocardial ischemia.

Methods

Experimental design

In all, 12 healthy male New Zealand white rabbits (3–3.5 kg body weight) were anesthetized with pentobarbital (30 mg kg^{-1} i.m.). The rabbits were intubated with an intratracheal tube and ventilated mechanically with 95% oxygen (rate, 45 breaths min^{-1}; tidal volume, 20 ml) (Harvard rodent ventilator model 683, Harvard Apparatus), as reported previously (López Farré *et al*., 1996). A polyethylene catheter was inserted into the right external jugular vein for supplementary addition of anesthesia as needed.

Under sterile conditions, a left thoracotomy was performed, the pericardium was opened, and a 3-0 silk ligature was placed around the left anterior descending coronary artery 8–10 mm from its origin. This ligature was left untied until the begining of the experimental protocol. After all the surgical procedures were completed, myocardial ischemia was induced by tightening the ligature around the left anterior descending coronary artery to occlude completely the vessel. After 15 min of ischemia, the ligature was untied and the ischemic myocardium was reperfused. Clopidogrel (20 mg kg^{-1}) was given through a nasogastric polyethylene tube to a group of ischemic animals ($n=6$). The dose of clopidogrel was chosen based on previous works that demonstrated that similar clopidogrel doses administered to rabbits reduced platelet activation (Herbert *et al*., 1998; van Gestel *et al*., 2003). Clopidogrel was administered in one dose just after the occlusion of the coronary artery. Six ischemic rabbits did not receive clopidogrel (untreated ischemic group). Sham-operated animals ($n=6$), on which all procedures were performed except tightening of the coronary artery ligature, served as controls.

After the ligature was released from the coronary artery, the animals were allowed to recover. After 24 h, the rabbits were anesthetized exanguinated and the entire ischemic coronary artery removed and quickly frozen in liquid nitrogen for molecular biology determinations. The blood was collected in both acid-citrate dextrose (ACD) for P-selectin evaluation in the platelet surface and in a glass tube for sera isolation to determine troponin I. Sera troponin I was determined using a commercially available Dade-Behring immunoassay for human cardiac troponin I, which has been shown to react with rabbit cardiac troponin I (Zaninotto *et al*., 1996; O'Brien *et al*., 1997). The experimental procedure was approved by the institutional Animal Care and Use Committee.

P-selectin expression in platelets

The degree of platelet activation was assessed by measuring platelet surface expression of the α-granule protein P-selectin as reported (de Miguel *et al*., 2000). In brief, 200 μl of anticoagulated blood in ACD were fixed with 1% paraformaldehyde and incubated overnight. The fixed cells were then washed three times, resuspended in phosphate-buffered saline and used for cytofluorometric analysis. To detect P-selectin-positive cells, the samples were incubated with a monoclonal anti-P-selectin antibody (fluorescein isothiocyanate (FITC)-anti-mouse CD62P IgG_1, Pharmingen, San Diego, CA, U.S.A.) for 30 min at room temperature. The samples were analyzed in a fluorescent-activated cell-sorter flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). A minimum of 10,000 cells for each sample was analyzed. Mean P-selectin-positive fluorescense for the entire platelet population was expressed in arbitrary fluorescense units. The percentage of the cells positive for P-selectin was generated by substracting an isotype control (mouse FITC-labeled IgG_1).

Determination of tissue factor, eNOS and CD40 ligand (CD40L) in the coronary artery

Tissue factor, CD40L and eNOS expression in the left descending coronary artery were analyzed by Western blot. In brief, the frozen coronary arteries were homogenized with an Ultra-Turrax T8 IKA-Werke homogenizer in a buffer containing 8 mol l^{-1} urea, 2% CHAPS (w v^{-1}), 40 mol l^{-1} dithiothreitol, 0.2% Bio-Lyte™ ampholyte (Bio-Rad) and 0.01% (w v^{-1}) bromophenol blue. The homogenated tissues were centrifuged at 10,000 × *g* for 30 min and the supernatant stored at −70°C until further analysis. Homogenated tissues were solubilized in Laemmli buffer containing 2-mercaptoethanol. Proteins (20 μg lane^{-1}) were separated in denaturing SDS 10% polyacrylamide gels. After the gels were blotted onto nitrocellulose, Western blot analysis were performed with a murine tissue factor monoclonal IgG_1 antibody (American Diagnostica, Greenwich, CT, U.S.A.), a goat CD40L polyclonal IgG (Santa Cruz, Santa Cruz, CA, U.S.A.), or a mouse eNOS monoclonal IgG antibody (Transduction Laboratories, Becton Dickinson, Lexington, KY, U.S.A.), respectively. Specific tissue factor, CD40L and eNOS proteins were detected by enhanced chemoluminescence (ECL, Amersham International, Buckinghamshire, U.K.) and evaluated by densitometry (Quantity One, BioRad, Hercules, CA, U.S.A.) as reported (López Farré *et al*., 2002). A parallel gel with identical samples was run and, after blotting onto nitrocellulose, Western blot was performed with a β-actin monoclonal antibody (1 : 2500) (Sigma-Aldrich, St Louis, MI, U.S.A.). Prestained protein markers (Sigma, St Louis, MI, U.S.A.) were used for molecular mass determinations.

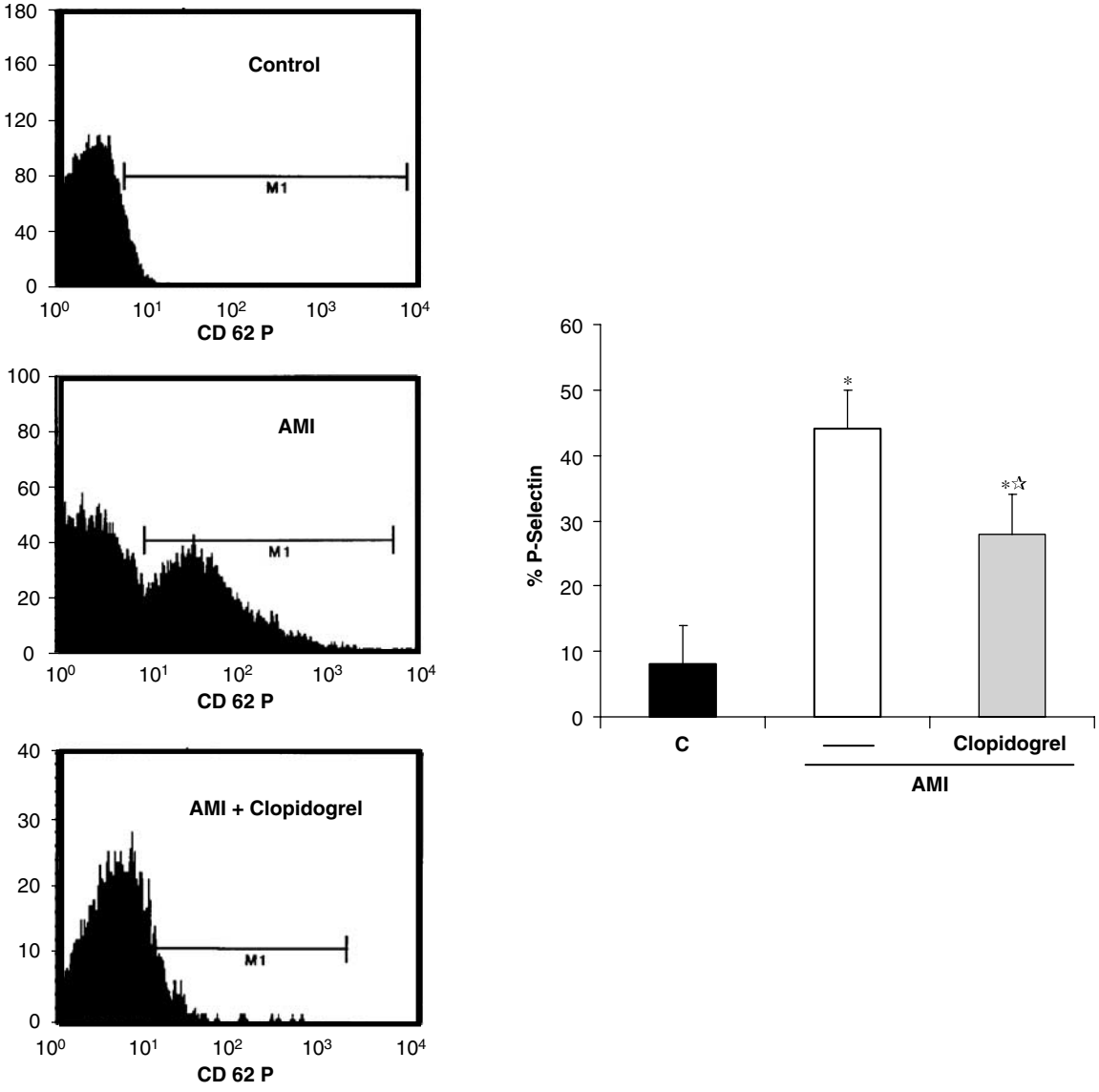

Figure 1 (Left) Plotted blots of the P-selectin expression in platelets from control, coronary ischemic (AMI) and clopidogrel-treated coronary ischemic rabbits (AMI + clopidogrel). (Right) Mean ± s.e.m. of the percentage of platelets that expressed P-selectin as arbitrary units of fluorescence. *$P<0.05$ with respect to control rabbits. $^{\star}P<0.05$ with respect to untreated ischemic rabbits.

Statistical methods

Results are expressed as mean ± s.e.m. To determine statistical significance, we performed the Mann–Whitney test. A P-value <0.05 was considered statistically significant.

Results

P-selectin expression

The percentage of platelets expressing P-selectin in their surface was used as index of *in vivo* platelet activation. P-selectin was expressed on 7.02 ± 0.2% of sham-operated control rabbits. The percentage of platelets expressing P-selectin on their surface was significantly increased in ischemic rabbits with respect to control rabbits (Figure 1). In the ischemic rabbits receiving clopidogrel, the percentage of platelet-expressing P-selectin was significantly reduced with respect to untreated ischemic rabbits (Figure 1). However, the percentage of platelets expressing P-selectin in clopidogrel-treated ischemic rabbits remained increased with respect to controls (Figure 1). The reduction in platelet activation by clopidogrel was not accompanied by a different myocardial damage since sera troponin I was similarly increased in both ischemic and clopidogrel-treated ischemic rabbits compared with controls (control: undetectable; ischemic: 2.4 ± 0.3 ng ml^{-1}; ischemic + clopidogrel: 2.8 ± 0.5 ng ml^{-1}; $P<0.05$ with respect to control).

CD40L, tissue factor and eNOS expression in the coronary artery

At 24 h after ischemia, the coronary artery showed an increased expression of CD40L (Figure 2). Clopidogrel administration reduced CD40L expression in the ischemic

coronary artery (Figure 2). The specificity of the changes was assessed by the fact that expression of the constitutive protein β-actin was not changed in the coronary artery of control ischemic and clopidogrel-treated ischemic rabbits (Figure 2). Tissue factor expression was also found increased in the coronary artery of ischemic rabbits (Figure 3). Oral administration of clopidogrel reduced the expression of tissue factor in the ischemic coronary artery (Figure 3).

eNOS protein expression was decreased in the ischemic coronary artery with respect to control (Figure 4). Clopidogrel administration protected eNOS protein expression in the ischemic coronary artery (Figure 4).

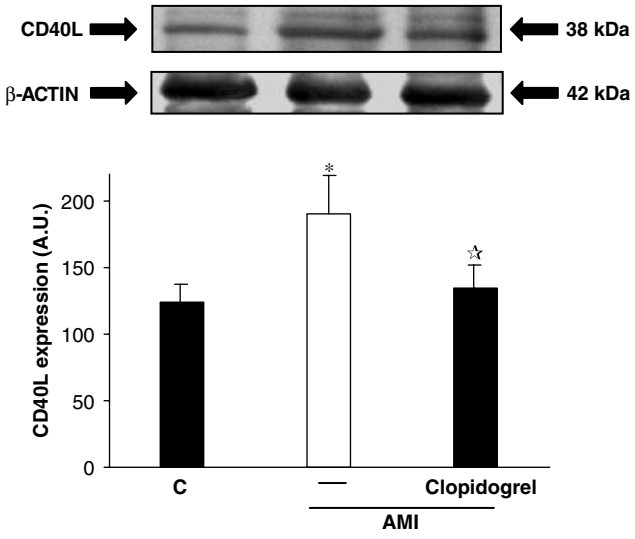

Figure 2 Representative Western blot demonstrating the expression of CD40L in coronary arteries obtained from control, ischemic and clopidogrel-treated ischemic rabbits. The expression of β-actin is also shown. The bottom shows a bar graph representing the densitometric analysis of the Western blots. Results are represented as mean $\pm$ s.e.m. $^{*}P<0.05$ with respect to control rabbits. $^{\star}P<0.05$ with respect to ischemic rabbits.

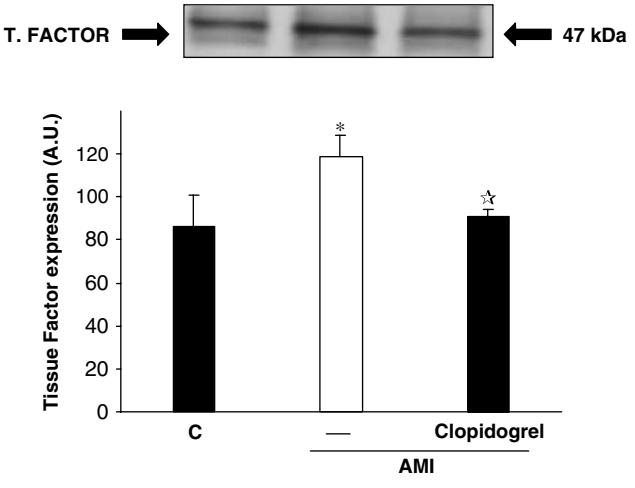

Figure 3 Representative Western blot demonstrating the expression of tissue factor in coronary arteries from control, ischemic and clopidogrel-treated ischemic rabbits. The bottom shows a bar graph representing the densitometric analysis of the Western blots. Results are represented as mean $\pm$ s.e.m. $^{*}P<0.05$ with respect to control rabbits. $^{\star}P<0.05$ with respect to ischemic rabbits.

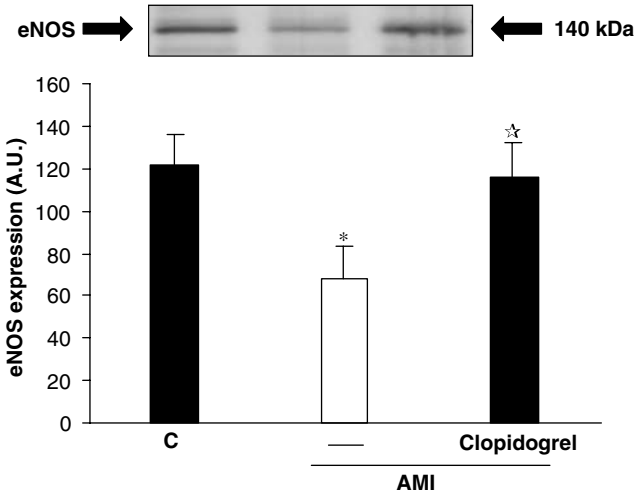

Figure 4 Representative Western blot demonstrating the expression of endothelial nitric oxide synthase (eNOS) in the descending coronary artery from control, ischemic (AMI) and clopidogrel-treated ischemic rabbits (AMI + clopidogrel). The bottom shows a bar graph representing the densitometric analysis of the Western blots. Results are represented as mean $\pm$ s.e.m. $^{*}P<0.05$ with respect to control rabbits. $^{\star}P<0.05$ with respect to ischemic rabbits.

Discussion

We have shown here that inhibition of platelets by clopidogrel reduced the expression of two proinflammatory proteins, CD40L and tissue factor in ischemic coronary arteries of rabbits. Moreover, eNOS protein expression in the ischemic coronary artery was also protected by clopidogrel treatment.

There is evidence that the acute inflammatory response to ischemia and the presence of inflammatory mediators after the ischemic event influence the functional recovery of the tissue and the clinical outcome of the ischemic patients (Morrow *et al*., 1998; Biasucci *et al*., 1999; Ridker *et al*., 2000). CD40L is a member of the tumor necrosis factor-α family of proteins. CD40L has been identified as proinflammatory mediator and risk factor for cardiovascular events (Schönbeck *et al*., 2001). CD40L was originally identified in T-lymphocytes, although now it has also been identified in others cells, including endothelial and smooth muscle cells and leukocytes (Hakkinen *et al*., 2000). We have observed that in the ischemic coronary artery, the expression of CD40L was enhanced, whereas it was reduced in the ischemic coronary artery of clopidogrel-treated rabbits. Similar results were obtained when we analyzed the expression of tissue factor. Tissue factor is the primary activator of the extrinsic coagulation cascade and its expression is rapidly induced in response to inflammatory stimuli such as cytokines (Mackman, 1997; Molero *et al*., 2003). In the ischemic coronary artery, the expression of tissue factor was increased, suggesting the presence of an inflammatory reaction. Clopidogrel treatment also reduced the expression of tissue factor in the ischemic coronary artery. Taken together, these data support an anti-inflammatory effect of clopidogrel. It is noteworthy that our experimental model was designed to resemble a situation of secondary prevention with clopidogrel during the acute phase of a coronary syndrome. In this regard, the effect of clopidogrel was rapid since 24 h after clopidogrel administration platelet activation was diminished. This was suggested by the fact that the number of platelets expressing

P-selectin on their surface was significantly reduced, which was also accompanied by anti-inflammatory effects in the ischemic coronary artery. In this regard, Herault *et al*. (1999) have also observed a rapid effect of clopidogrel in rats since they demonstrated that thrombin generation in rats was reduced 2 h after oral clopidogrel administration.

In vitro studies have suggested the involvement of platelets in the inflammatory reaction. As example, Gawaz *et al*. (1998) have demonstrated that activated platelets stimulated the expression and release of inflammatory-related proteins in cultured endothelium. Moreover, *in vivo* studies have also suggested that platelets may participate in the inflammatory reaction through the activation of leukocytes (Neumann *et al*., 1999). However, most of these *in vivo* findings were obtained by blocking the GP IIb/IIIa receptors with abciximab. Since it is well known that abciximab not only recognizes GP IIb/IIIa receptors but also other proteins such us Mac-1 and vitronectin, the anti-inflammatory effect of abciximab may be unrelated to platelet inhibition. In this regard, we have also recently demonstrated that a more specific GP IIb/IIIa blocker, tirofiban, also produced anti-inflammatory effects in the myocardium and in leukocytes (Molero *et al*., 2003). Therefore, the here reported observation that clopidogrel treatment prevented the inflammatory response in the ischemic coronary artery supports the hypothesis that platelets are not only involved in blood flow reduction in the coronary artery but also they have a direct involvement in the inflammatory response of the ischemic vessel. In accordance with our observation, it has been demonstrated that clopidogrel inhibits the platelet–leukocyte interaction after ADP and thrombin stimulation and in patients with atherosclerotic vascular disease (Klinkhardt *et al*., 2002; 2003). Moreover, Quinn *et al*. (2004) have recently demonstrated that clopidogrel pretreatment of patients undergoing percutaneous coronary intervention reduced platelet CD40L levels and serum interleukin-6 levels, which may have important clinical consequences and support the anti-inflammatory effect of clopidogrel.

To our knowledge, our work demonstrates for the first time an anti-inflammatory effect of clopidogrel on the coronary ischemic artery. In this regard, we have also observed that the expression of eNOS protein in the ischemic coronary artery, the enzyme that produces nitric oxide in the endothelium, was protected by clopidogrel administration. The expression of eNOS protein is downregulated under inflammatory conditions, which has been associated with endothelial dysfunction (Yoshizumi *et al*., 1993). Therefore, the finding that clopidogrel protected eNOS expression in the ischemic coronary artery may suggest that platelets are also involved in the endothelial dysfunction associated with ischemia. In the same line of evidence, the differences in inflammatory markers including their expression in the coronary artery from ischemic untreated and clopidogrel-treated rabbits were not related to different infarcts due to the occlusion processes since serum troponin I levels, a cardiac necrosis marker, were very similar.

Our experimental design did not allow us to determine whether the protection by clopidogrel of eNOS expression in the ischemic coronary artery was related to an anti-inflammatory effect of this drug due to the inhibition of platelets and/or through a direct effect of platelets on the regulation of eNOS protein expression. In this regard, Zeiher *et al*. (1991) have demonstrated that intracoronary thrombi cause vasoconstriction in epicardial arteries of patients with coronary artery disease, which is was completely reversed by intracoronary injection of the NO-donor nitroglycerin suggesting a direct effect of platelets on vascular function. Moreover, it is also important to remark that 24 h after ischemia, clopidogrel treatment only partially reduced platelet activation, as determined by P-selectin expression on the platelet surface, but almost reduced the inflammatory response in the ischemic coronary artery to the level observed in sham-operated animals. This observation may support the critical importance of platelets in the inflammatory response of the vascular wall after ischemic injury. However, further *in vitro* and *in vivo* studies are needed to study in depth this observation.

In summary, inhibition of platelet activation by clopidogrel exerted anti-inflammatory effects on the ischemic coronary artery and protected eNOS protein expression. Therefore, the here reported reduction of inflammation and protection of eNOS protein by clopidogrel in the ischemic coronary artery suggests that inhibition of platelet activation by clopidogrel has further biological effects than only inhibiting thrombus formation and probably may contribute to explain some of the clinical beneficial effects of clopidogrel observed in the clinical trials.

This work has been supported by grants from Brystol Myers Squibb and Fondo de Investigaciones de la Seguridad Social (Red HERACLES G03/045 and PI041205). We thank Begoña Larrea for secretarial assistance.

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(Received December 21, 2004
Revised May 4, 2005
Accepted June 16, 2005
Published online 25 July 2005)