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APOPROTEIN (A) ANTAGONISES THE GPIIB/IIIA RECEPTOR ON COLLAGEN AND ADP-STIMULATED HUMAN PLATELETS

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1. ABSTRACT

The nature of the lipoprotein (a) (Lp(a))/agonist-stimulated platelet interaction is unclear. The objective was to determine whether Lp(a) inhibits platelet aggregation by displacing fibrinogen from the platelet GPIIb/IIIa receptor. Platelets were washed in Tyrode's buffer and stimulated using 10 micromolar ADP or 2 micrograms/ml collagen. Lp(a) was isolated from plasma using lectin affinity chromatography followed by ultracentrifugation. Lp(a) inhibited aggregation of collagen- and ADP-stimulated platelets with IC-50's of about 5 mg/dl. Lp(a) inhibited ^{125}I-labeled fibrinogen binding to collagen-stimulated platelets with an IC-50 of <5 mg/dl. MAb 3B1, specific for apo(a), restored platelet aggregation to control levels, inhibited ^{125}I-labelled Lp(a) binding, and increased ^{125}I-labelled fibrinogen binding by displacing Lp(a) from the fibrinogen binding site. In conclusion, binding of Lp(a) results in displacement of fibrinogen from its receptor, leading to decreased platelet aggregation. This antagonism suggests a novel role for Lp(a) in modulating fibrinogen binding to the GPIIb/IIIa receptor on collagen- and ADP-stimulated platelets.

2. INTRODUCTION

Lipoprotein (a) (Lp(a)) is considered to be pro-atherosclerotic (1-6) in part through it's putative prothrombotic role(7-10). This prothrombotic role has been proposed based on the high degree of homology between Lp (a) and plasminogen (11,12). Such homology has been proposed to cause interference with plasminogen function (13-15), though this view is not universally held (16). Plasminogen gives rise to plasmin which digests fibrin holding the thrombus or embolus together (17-20). Thus it has been proposed that Lp (a) would reduce the opportunity for platelet disaggregation thus increasing the opportunity for clinically significant formation of platelet aggregates (21-24). However, Lp (a) has been observed to decrease collagen- and adenosine diphosphate (ADP)-stimulated platelet aggregation in whole blood, platelet-rich plasma and washed platelet suspensions (25-27). Thus there may be a moderating role for Lp(a) in terms of counteracting Lp(a)'s putative prothrombotic role. Lp(a) contains two proteins (apolipoprotein (a) (apo (a)) and apolipoprotein B (apo B))(28-31). It has been shown that apo(a) mediates the reduction of thromboxane A_2 (TxA_2) production with subsequent diminishment of platelet aggregation (25). However, the nature of the interaction between Lp(a) and agonist-stimulated platelets has never been explained. Lp (a) has been observed to bind, via apo (a) to the fibrinogen binding site on unstimulated platelets (32). However, it is not clear which aspect of Lp(a) mediates the aggregation lowering impact of that lipoprotein due to potential interaction of Lp (a) with the agonist-stimulated platelet's fibrinogen receptor. This receptor undergoes conformational change upon agonist stimulation thus exposing other binding sites to ligands (33-36). It is also unclear whether Lp (a) competes for fibrinogen binding on stimulated platelets and whether that would play a role in the observed Lp (a)-mediated aggregation decrease. The objectives of this work were to determine whether: Lp (a) competes with fibrinogen for binding at the GPIIb/IIIa receptor thus lowering platelet aggregation to collagen and adenosine diphosphate

(ADP)-stimulation and if so whether it is apo (a) or apo B mediating the antagonism of the GPIIb/IIIa receptor.

3. MATERIALS AND METHODS

3.1. Subjects and experimental materials

Six subjects participated in all experiments. The proposed study was fully explained to each subject and informed written consent was obtained. The study's protocol was approved by the Human Ethics Committee of the University of Saskatchewan where the work was performed. Lectin, KBr, polyacrylamide, fibrinogen, Lowry kit, and chemicals for washing platelets were from Sigma (St. Louis, MO). Collagen and ADP were obtained from Hormon Chemie (Munich, Germany) and Chronolog Corporation (Havertown, Pennsylvania), respectively. ^{125}I was obtained from Amersham(Chicago, IL). The aggregometer was obtained from Payton Instruments (model 800B Buffalo, NY) and the Coulter Counter and ultracentrifuge from Beckman Coulter (Miami, Florida). The Mab Trap II kit was from Pharmacia (Piscataway, NJ). Monoclonal antibodies to apo (a) and B were the generous gift of Dr. David Usher.

3.2. Lp(a) isolation

Lp(a) was isolated from six high level Lp(a) donors by passing human plasma over a lectin column (37,38). The eluate was ultracentrifuged in a Beckman benchtop ultracentrifuge (TLA 100.3 rotor, 85,000 rpm, 16 h) using a KBr final density of 1.215 g/ml to isolate Lp(a). The purity of the isolated Lp(a) was determined on a 2-16 % native polyacrylamide gradient gel and rocket immunoassay. Lp (a) and fibrinogen (99 % plasminogen free) were iodinated according the method of Sinn et al. (39). Protein levels were determined by the method of Lowry (40).

3.3. Platelet isolation

Blood was drawn from the antecubital vein of each subject in 15-ml vacutainers (red top) containing 2.0 ml of acid citrate dextrose (41). The washed platelets were obtained by centrifuging whole blood at 111 *g* for 15 min at 30 ^{o} C to yield platelet rich plasma (PRP). The PRP was centrifuged at 2000 g for 15 minutes at 37 ^{o}C. The derived platelet pellet was suspended in platelet suspension I (PS I) and further washed once in each of PS II and PS III based on the method of Mustard et al (42). PS I, II, and III contained Tyrodes buffer (NaCl (137 mM), KCl (2.7 mM), $NaHCO_3$ (12 mM), NaH_2PO_4 (0.4 mM), MgCl (1 mM), $CaCl_2$ (2 mM), glucose (5.5 mM) at pH 7.35, and osmolarity 200-300 mosmol. PS I, II, and III also contained 0.35 % bovine serum albumin, HEPES (2.5 mM)) and apyrase (PS I and II, 30 micrograms/ml, PS III (3 micrograms/ml). PS I also contained heparin (50 units/ml). Final platelet concentrations in PS III were adjusted to 2 x 10^8/ml with the aid of a Coulter Counter (model ZM).

3.4. Experiments performed

1. Washed platelets were incubated for 15 minutes at 37 ^{o}C with 50 mg/dl ^{125}I-labelled fibrinogen and increasing levels of unlabelled Lp(a) (Figure 1) and then stimulated for 5 min with 2 micrograms/ml collagen or 10 micromolar ADP. Platelet aggregation was measured in parallel samples by turbidometry via the method of Born (43). The aggregometer was set at 37 ^{o} C and stirring at 900 rpm. Aggregation levels were measured by taking the distance in cm from the baseline to the vertical midpoint of the aggregation plateau at 5 minutes post-agonist (equine type I collagen or ADP) introduction to the platelet. This distance was expressed as a percentage of the distance between baseline Lp (a) /platelet mix (0 % change in light transmittance) and a Tyrode's buffer blank (100 %). Non-specific binding of fibrinogen and Lp (a) was determined by mixing 50:1 mass ratio (unlabelled : labelled) of each protein or lipoprotein. ^{125}I binding was measured for all experiments in accordance with Malle *et al* (32). 2. Washed platelets were incubated with cold fibrinogen (50 mg/dl) and increasing levels of ^{125}l-labelled Lp(a) (Figure 2) and then stimulated as above with collagen or ADP. Platelet aggregation and ^{125}I binding was measured as above. 3. Monoclonal antibodies (Mabs) to apo (a) were preincubated overnight at 4^{o}C with labelled Lp(a)(0.15, 1.5 and 15 micrograms Mab)/600 micrograms Lp (a)). Labelled Lp (a) (100 mg/dl) blocked with individual Mabs to apo (a) were incubated with washed platelets (including 50 mg /dl unlabelled fibrinogen) at 37 ^{o} C for 15 minutes followed by stimulation with collagen or ADP as above. Similar experiments were also done except that labelled fibrinogen and unlabelled Lp (a) were used. Platelet aggregation and binding of ^{125}l was determined (Figure 3) as above. 4. Monoclonal antibodies (Mabs) to apo B were preincubated overnight at 4^{o}C with labelled Lp(a)(0.15, 1.5 and 15 micrograms Mab)/600 micrograms Lp (a)). Labelled Lp (a) (100 mg/dl) blocked with individual Mabs to apo B were incubated with washed platelets (including 50 mg /dl unlabelled fibrinogen) at 37 ^{o} C for 15 minutes followed by stimulation with collagen or ADP as above. and unlabelled Lp (a) were used. Platelet aggregation and binding of ^{125}l was determined (Figure 4) as above.

Monoclonal antibodies to apo (a) and B were purified using the Mab Trap II kit in accordance with the manufacturer's directions.

3.5. Statistical analyses

A one-way analysis of variance (ANOVA) was used to determine if there was a statistically significant effect of differing concentrations of antibodies to Lp (a) compared to an absence of these agents on platelet aggregation or protein binding levels. In all cases, it was the mean (of duplicate measures) levels of the six platelet donors that were used in the statistical analyses. The means for each subject were then used to generate subject group means in all cases.

4. RESULTS

Increasing concentrations of unlabelled Lp (a) progressively reduced platelet binding of ^{125}I fibrinogen while decreasing platelet aggregation to collagen and ADP(Figure 1). Lp(a) inhibited aggregation of collagen-

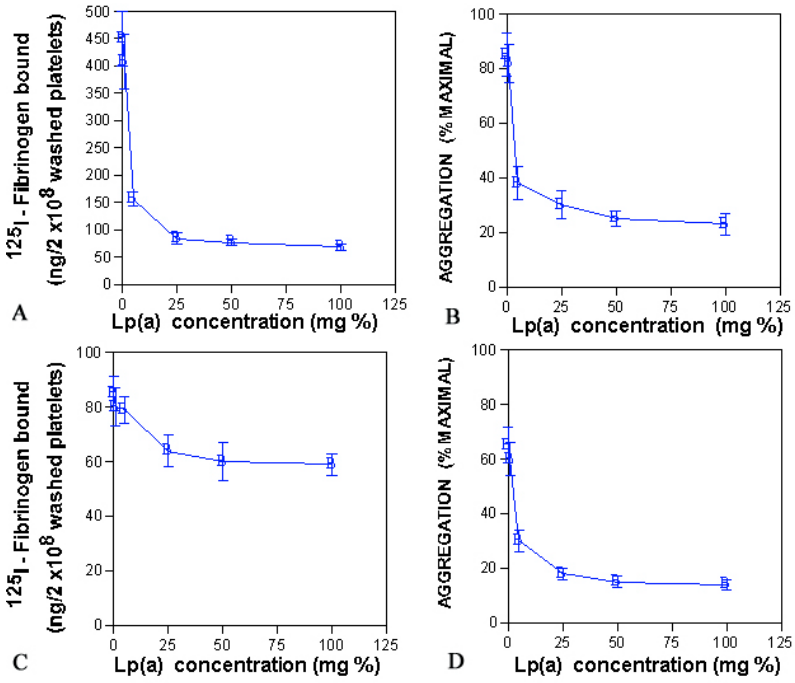

Figure 1. Washed platelets (2 x 10^8 /ml) were incubated for 15 minutes at 37 ° C with 50 mg/dl ^{125}I-labelled fibrinogen and increasing levels of unlabelled Lp(a) and then stimulated for 5 min with 2 micrograms/ml collagen (figures a and b) or 10 micromolar adenosine diphosphate (ADP) (figures c and d) Platelet aggregation was measured in parallel samples by turbidometry via the method of Born[43] and ^{125}I binding via the method of Malle *et al.*[32] The aggregometer set at 37 ° C and stirring at 900 rpm. Aggregation levels were measured by taking the distance in cm from the baseline to the vertical midpoint of the aggregation plateau at 5 minutes post-agonist (collagen or ADP) introduction to the platelet This distance was expressed as a percentage of the distance between baseline (Lp (a))/platelet mix) (0 % change in light transmittance) and a Tyrode's buffer blank (100 %) Specific activity was 355 cpm/ng protein. All values are the grand mean (± standard error of the mean) of 6 duplicate measures of 6 subjects.

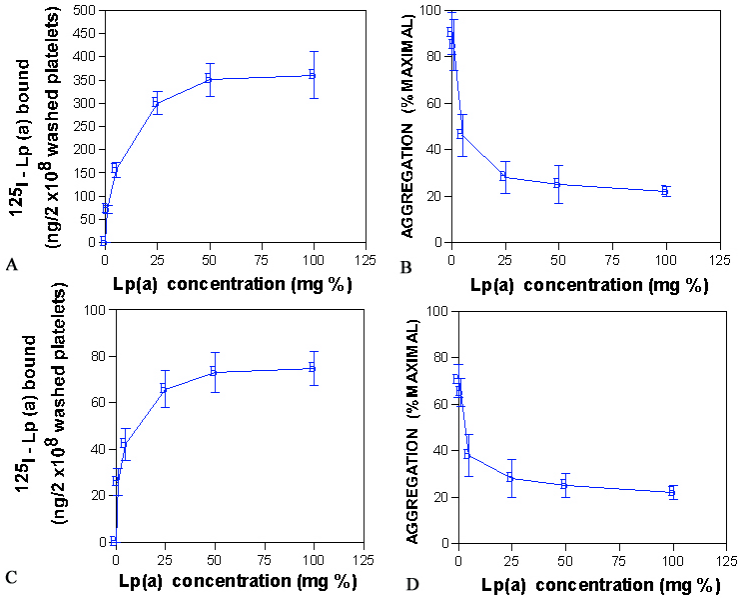

Figure 2. Washed platelets (2 x 10^8 /ml) were incubated with cold fibrinogen (50 mg/dl) and increasing levels of ^{125}I-labelled Lp(a) for 15 minutes at 37 ° C and then stimulated as above with collagen (figures a and b) or ADP (figures c and d). Platelet aggregation and ^{125}I binding were measured as above. Specific activity was 400 cpm/ng protein. All values are the grand mean (± standard error of the mean) of 6 duplicate measures of 6 subjects.

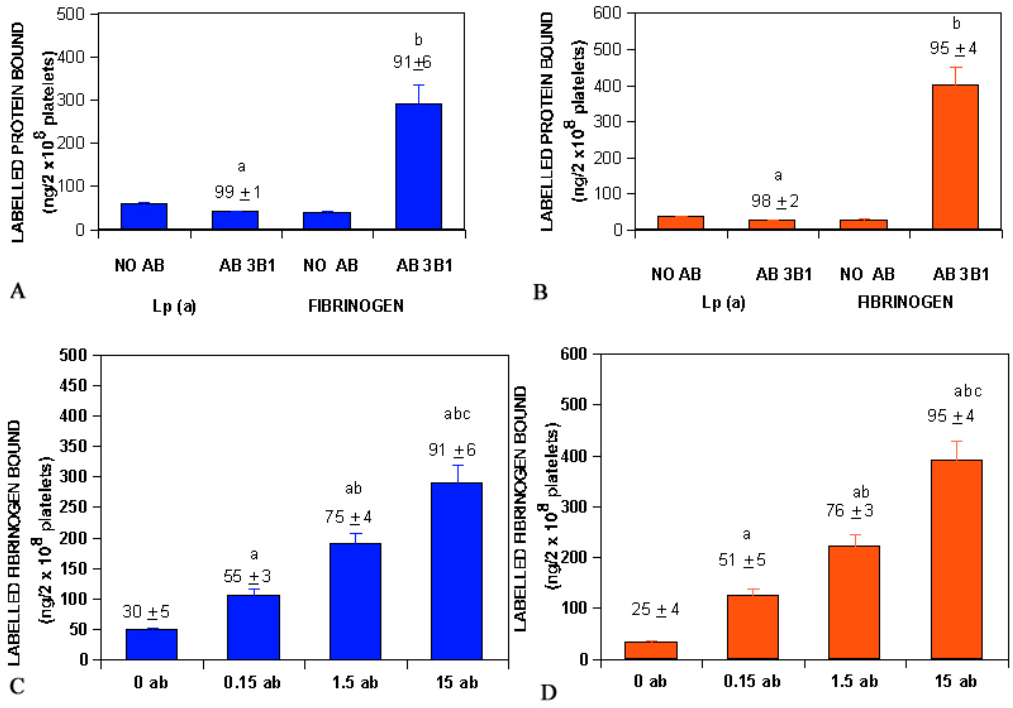

Figure 3. Monoclonal antibodies (Mabs) to apo (a) were preincubated overnight at 4°C with labelled or unlabelled 100 mg/dl Lp(a) (15 micrograms Mab/600 micrograms Lp (a) (figures a and b) or (0, 0.15, 1.5 and 15 micrograms Mab/600 micrograms Lp (a))(figures c and d). Data represents Mab 3B1. Labelled or unlabelled Lp (a) blocked with individual Mabs to apo (a) were incubated with washed platelets (2 x 10^8 /ml) in the presence of 50 mg/dl fibrinogen (unlabelled or labelled respectively) at 37 ° C for 15 minutes followed by stimulation with collagen (figures a and c) or ADP (figures b and d) as above. Platelet aggregation and ^{125}I binding were measured as above. Specific activity was 376(fibrinogen) and 427(Lp(a)) cpm/ng protein. In figures a and b the numbers above the bars represent aggregation levels while the letters above the bars represent statistically significant differences in labelled protein binding relative to controls. In figures c and d the letters above the bars represent the differences in protein binding and aggregation levels and are significantly different ($p < 0.05$) from their respective controls. Bars with different letters in Figures c and d are significantly different ($p < 0.05$) from each other. All values are the grand mean (± standard error of the mean) of 6 duplicate measures of 6 subjects.

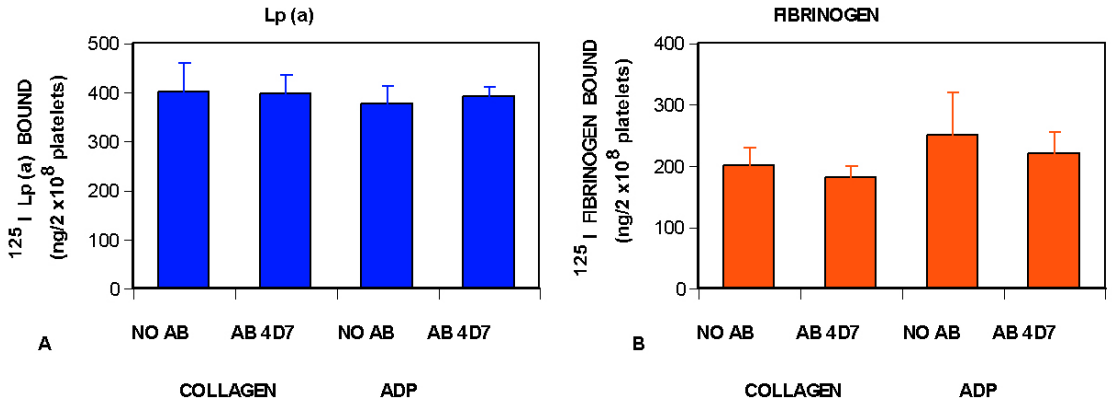

Figure 4. Monoclonal antibodies (Mabs) to apo B were preincubated overnight at 4°C with labelled 100 mg/dl Lp(a)(0, 0.15, 1.5 and 15 micrograms Mab/600 micrograms Lp (a)). Labelled Lp (a) (figure a) or unlabelled Lp (a) (figure b) blocked with individual Mabs to apo B were incubated with washed platelets (2 x 10^8 /ml) in the presence of 50 mg/dl unlabelled fibrinogen (figure a) or labelled fibrinogen (figure b) at 37 ° C for 15 minutes followed by stimulation with collagen or ADP as above. Platelet aggregation and ^{125}I binding were measured as above. The 15 micrograms Mab 4D7 /600 microgram Lp (a) data is representative of the 0, 0.15 and 1.5 micrograms Mab/600 micrograms Lp (a)) data and all other tested Mabs against apo B and the latter two points are not shown. Platelet aggregation levels were statistically identical whether the Mab was present. Specific activity was 359(fibrinogen) and 405(Lp(a)) cpm/ng protein. All values are the grand mean (± standard error of the mean) of 6 duplicate measures of 6 subjects.

and ADP-stimulated platelets with IC-50's of about 5 mg/dl. Lp(a) inhibited ^{125}I-labelled fibrinogen binding to collagen-stimulated platelets with an IC-50 of <5 mg/dl (ADP with an IC-50 of ~25 mg/dl). Increasing concentrations of labelled Lp (a) progressively increased platelet binding of ^{125}I Lp(a) while decreasing platelet aggregation to collagen and ADP (Figure 2). In all cases, non-specific binding was less than 10 % of the value observed when only a given concentration of radiolabelled protein or lipoprotein was incubated with platelets.

The binding of a Mab (6B1) to apo (a) in intact Lp (a) progressively (to increasing 6B1 antibody levels) inhibited platelet binding of ^{125}I labelled Lp(a) and increased platelet binding of ^{125}I fibrinogen to collagen- and ADP- stimulated platelets while restoring platelet aggregation to control levels (Figure 3).

The binding of progressively increasing concentrations of a series of different Mabs to apo B in intact Lp (a) failed to inhibit platelet binding of ^{125}I labelled Lp(a) and failed to increase platelet binding of ^{125}I fibrinogen to collagen- and ADP-stimulated platelets (data represented by Mab 4D7 is representative of all apo B Mabs tested and is shown in Figure 4). Platelet aggregation levels remained at low levels (statistically equivalent to the absence of all Mabs tested to apo B) and were not restored to control levels.

5. DISCUSSION

The Lp (a) displacement of fibrinogen is found in Figure 1. Increasing concentrations of unlabelled Lp (a) progressively reduced platelet binding of ^{125}I fibrinogen and platelet aggregation to collagen- and ADP-stimulation. It is clear that the minor fibrinogen displacement by Lp (a) is of far greater impact on ADP- compared to collagen-induced aggregation (Figure 1). This is consistent with the literature (44) indicating the far greater importance of fibrinogen to the success of ADP- compared to collagen-stimulated platelets. The difference in fibrinogen binding between collagen and ADP stimulation is the result of fewer fibrinogen binding sites exposed during 10 micromolar ADP stimulation (45) making it easier for the Lp(a) to antagonise its binding and again is consistent with the greater importance of fibrinogen to aggregation of ADP- compared to collagen- stimulated platelets.

Increasing concentrations of labelled Lp (a) (Figure 2) in the presence of 50 mg/dl fibrinogen progressively enhanced platelet binding of ^{125}I Lp(a) while decreasing platelet aggregation to collagen and ADP. The data in Figures 1 and 2 further support the notion that there is actual competition between Lp (a) and fibrinogen for the GPIIb/IIIa binding site on agonist-stimulated platelets. GPIIb/IIIa is the sole receptor site for fibrinogen in agonist-stimulated platelets (33,34,46).

The binding of progressively increasing concentrations of a Mab (6B1) to apo (a) in intact Lp (a) inhibited platelet binding of ^{125}I labelled Lp (a) and increased platelet binding of ^{125}I fibrinogen to collagen- and ADP-stimulated platelets while restoring platelet aggregation to control levels (Figure 3). This data suggests that it is apo (a) mediating the competition of Lp (a) and fibrinogen for the GPIIb/IIIa binding site on agonist-stimulated platelets. The lack of cross-reactivity between 6B1 and plasminogen suggests that any Lp (a) interference with the role of plasminogen function may be by a region of Lp(a) other than that mediating the antagonism of the fibrinogen receptor.

The binding of progressively increasing concentrations of a series of Mabs to apo B in intact Lp (a) failed to inhibit platelet binding of ^{125}I labelled Lp(a) and failed to increase platelet binding of ^{125}I fibrinogen to collagen- and ADP-stimulated platelets. Platelet aggregation levels remained at low levels and were not restored to control levels (Figure 4). Thus apo B cannot be mediating the Lp(a)'s antagonism of the GPIIb/IIIa receptor.

In summary, the evidence that apo (a)'s antagonism of the GPIIb/IIIa receptor is responsible for Lp (a)'s –mediated reduction of collagen- and ADP-stimulated aggregation is:

1. decreasing collagen- and ADP-stimulated aggregation in the face of: inhibition of platelet fibrinogen binding by increasing concentrations of Lp (a), progressive platelet binding of Lp(a) in the presence of fibrinogen and increasing concentrations of Lp (a), and blockage of apo B in Lp (a) via a series of Mabs (representative example 4D7) failing to result in Lp(a)-induced inhibition of platelet fibrinogen binding and aggregation.

2. progressive blockage of apo (a) via a Mab leading to an progressive increase in platelet fibrinogen binding and restoration of aggregation to control levels.

It is concluded that apo (a) mediates binding of Lp(a) to the GPIIb/IIIa receptor site on collagen- and ADP-stimulated human platelets and that this binding is at least partially responsible for the Lp (a)-mediated decreases in platelet aggregation levels. This paper is believed to be the first indication of an endogenous regulator of fibrinogen binding to its agonist-stimulated platelet receptor. The epitope (s) of apo (a) and GPIIb/IIIa receptor mediating this binding is (are) currently under investigation. That the Lp(a) anti-aggregatory role is physiologically relevant has been repeatedly demonstrated (25-27).

On a strictly speculative note, the thrombogenic properties ascribed to Lp(a)(7-10) are perhaps, at least partially, attenuated by Lp (a)'s antagonism of the fibrinogen receptor in collagen- and ADP-stimulated human platelets. Interestingly LDL, not containing apo(a), increases platelet aggregation(47).

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Key Words: Human, Platelet, Lipoprotein(a), Apolipoprotein(a), GPIIb/IIIa antagonism

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