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The L-arginine/NO pathway in the early phases of platelet stimulation by collagen

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Abstract

Nitric oxide production, L-arginine transport and intracellular [Ca^{2+}] changes in human platelets stimulated without stirring by low doses of collagen have been evaluated. Collagen decreased in a dose-dependent manner the nitric oxide formation. A reduction of about 30% of the basal level was produced by 5 μg/mL. Aspirin did not change the collagen effect. The inhibition was reversed by EGTA. Moreover collagen reduced L-arginine uptake. The exposure of platelets to 5 μg/mL collagen diminished of about 30% L-arginine transport. The specific involvement of the system y^+ is suggested. In addition in FURA 2-loaded platelets collagen induced a dose-dependent slow sustained [Ca^{2+}] rise that was almost completely cancelled by EGTA. Finally the treatment of whole platelets with collagen affected in a dose-dependent manner the maximal nitric oxide formation, suggesting a direct effect at the level of nitric oxide synthase enzyme. The phosphorylation of specific serine/threonine residues regulated by protein kinase C could be involved. In conclusion during the early phases of platelet stimulation with collagen nitric oxide formation is diminished. This reduction can be due to a lower availability of L-arginine for cytosolic nitric oxide synthase and/or to a decreased activity related to modifications of the enzyme.

Keywords: Collagen; Human platelets; Calcium; L-Arginine; Transport; Nitric oxide; Nitric oxide synthase

1. Introduction

NO, a free radical constantly produced and released by various tissues, is implicated in many physiological processes such as neurotransmission [1], immunological reaction by macrophages [2] and regulation of vascular tone [3]. Moreover NO causes smooth muscle relaxation [4] and inhibition of platelet adhesion and aggregation [5,6]. The regulation of platelet function, crucial to prevent platelet hyperaggregation, thrombus formation and stroke, seems to be due both to endothelial and endogenous derived NO [5]. Platelets themselves synthesize NO through the action of a soluble calcium/calmodulin-dependent cNOS, that has been purified and kinetically characterised [7,8]. As shown in endothelial cells and in macrophages [9], in human platelets extracellular L-arginine modulates intracellular NO synthesis by providing the substrate for cNOS [8,10]. Thus L-arginine transport exerts a crucial role in the regulation of NO formation. Recently we have shown that in platelets L-arginine transport is mediated both by the y^+ system that accounts for the 30–40% and by y^+L system which corresponds to 60–70% of the total transport. System y^+ is characterised by low affinity for L-arginine, is unaffected by L-leucine, is sensitive to trans-stimulation and to changes of membrane potential. System y^+L has high affinity for L-arginine, is inhibited by L-leucine in the presence of Na^+ and is unaffected by changes in membrane potential [10].

Platelets play a crucial role in the processes of homeostasis and thrombosis. Following injury to blood vessels, platelets adhere to the exposed subendothelial connective tissue (collagen in particular), are activated, aggregate and release several biologically active compounds, such as ADP and Ca^{2+}.

Collagen produces an intracellular Ca^{2+} elevation that is not inhibited by a rise in platelet cAMP, whereas Ca^{2+} increase induced by thrombin has an opposite effect [11]. Moreover in activated platelets the thrombin-stimulated

Abbreviations: A23187, calcium ionophore A23187; cNOS, cytosolic nitric oxide synthase; NEM, *N*-ethylmaleimide; NO, nitric oxide; NO_x, nitrite + nitrate; Plts, platelets; WP, washed platelets

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Ca^{2+} response produces an initial and transient spike, reflecting the discharge of intracellular stores and a sustained pattern related to the Ca^{2+} transport across the plasma membrane. On the contrary, administration of a moderate dose of collagen to human platelets results in a slow and gradual Ca^{2+} increase. The majority of this increase is dependent on extracellular Ca^{2+}, via the Na^{+}/Ca^{2+} exchanger functioning in the reverse mode [12].

The objective of this study was to evaluate the efficiency of L-arginine/NO pathway in the early phases of platelet stimulation with collagen. Thus the changes in the intracellular Ca^{2+} level, the L-arginine transport, NO and cGMP formation in unstirred platelets stimulated by low doses of collagen have been measured.

2. Methods

2.1. Blood collection and preparative procedures

Human blood obtained from healthy volunteers was collected in 130 mM trisodium citrate (9:1). WP were prepared as previously described [13]. Briefly platelet-rich plasma was obtained by the centrifugation of whole blood at 100 × *g* for 25 min. Platelets, isolated from platelet-rich plasma (added to 1 μg/mL apyrase and 1 μM PGE_1) centrifugation at 1000 × *g* for 20 min, were washed once with pH 4.8 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose) and resuspended in nominally Ca^{2+}-free pH 7.4 Hepes buffer (145 mM NaCl, 5 mM KCI, 1 mM $MgSO_4$, 10 mM Glucose, 10 mM HEPES). Protein concentration, when required, was assayed according to the Lowry method [14]. All chemicals, if not otherwise indicated, were from Sigma Chemicals Co.

2.2. Measurement of intracellular [Ca^{2+}]

Intracellular [Ca^{2+}] was measured as previously described with light modifications [15]. WP (3.0×10^8 plts/mL), resuspended in pH 7.4 Hepes buffer, were incubated with 1 μM FURA 2/AM (Calbiochem-Novabiochem Corp), for 60 min at 37°. PGE_1 (2 μM final concentration) and EGTA (1 mM final concentration) were added before centrifuging loaded platelets for 15 min at 1100 × *g*. The pellet, resuspended at 2.0×10^8 plts/mL in pH 7.4 Hepes buffer, was preincubated at 37° for 2 min with saline, EGTA (1 mM final concentration) and $CaCl_2$ (1 mM final concentration), when required, then collagen (Mascia Brunelli S.p.A.) from equine tendon or A23187 was added, when required. FURA 2-loaded platelet fluorescence was followed at 37° in unstirred conditions for 15 min in a Perkin-Elmer Fluorescence Spectrometer model LS50B with excitations at 340 and 380 nm and emission at 510 nm. The fluorescence of fully saturated FURA 2 (F_{max}) was obtained by lysing the cells with 50 μM digitonin in the presence of 2 mM Ca^{2+}, while F_{min} was determined by exposing the lysed platelets to 20 mM EGTA. The fluorescence was fully quenched with 5 mM Mn^{2+} to give the autofluorescence value. A software combined with the fluorescence spectrometer converted data into cytosolic [Ca^{2+}]. The Kd value for FURA 2 and Ca^{2+} was 135 nM.

2.3. L-Arginine uptake assay

L-Arginine uptake was measured as previously reported [8,10]. WP (2.0×10^8 plts/mL), prewarmed at 37° for 10 min with saline or 200 μM NEM, when required, were incubated in the presence of 1 μCi/mL L-[2,3,4 -^{3}H] arginine (NEN Life Science Products), 40 μM L-arginine, collagen and A23187, when required. At the indicated times aliquots of 1 mL were withdrawn, immediately filtered through a Titertek filter (Flow Laboratories) and washed twice with large volumes of cold PBS, containing 10 mM L-arginine. The radioactivity corresponding to the incorporated L-[2,3, 4 -^{3}H] arginine was directly measured by liquid scintillation counting of the filter in a Packard model TRI-CARB 1600 TR Liquid Scintillation Analyzer. Blank value, obtained by measuring the radioactivity of an ice-cold mixture of platelets, containing unlabelled and labelled L-arginine, immediately filtered, was subtracted from each experimental value.

2.4. Measurement of NO_x production

WP (1.0×10^9 plts/mL), resuspended in pH 7.4 Hepes buffer containing 1 mM $CaCl_2$ and prewarmed at 37° for 10 min with saline or additions, were incubated with 40 μM L-arginine and collagen for 15 min at 37° under mild shaking without stirring. Incubation was stopped by sonicating samples on ice. To measure the NO_x content of samples, suitable aliquots of supernatant were added to equal volumes of pH 9.7 assay buffer (15 g/L glycine–NaOH) containing cadmium beds (Fluka AG) and incubated overnight at room temperature under horizontal shaking. Cadmium beds were activated immediately before each experiment by subsequent washings with 0.2 N H_2SO_4, bidistilled water and pH 9.7 assay buffer. The NO_x formation, determined by the Griess reagent (1% sulphanilamide in 2.5% H_3PO_4, 0.1% naphtylenediamine dihydrochloride), was measured at 540 nm using a sodium nitrite calibration curve.

2.5. Measurement of cGMP formation

WP (1.0×10^9 plts/mL) resuspended in pH 7.4 Hepes buffer containing 1 mM $CaCl_2$, were prewarmed for 10 min at 37° and then incubated with 40 μM L-arginine and collagen for 15 min. The reaction was stopped by the addition of cold 2 M perchloric acid. The mixtures, sonicated for 5 s on ice, were centrifuged for 2 min at 12,000 × *g*. Supernatants, neutralised with 2 M NaOH,

were immediately analysed for cGMP content by radioimmunoassay kit (Amersham Pharmacia Biotech).

2.6. Measurements of maximal NO_x levels

WP (1.0×10^9 plts/mL), resuspended in pH 7.4 Hepes buffer containing 1 mM $CaCl_2$ and prewarmed at 37° for 10 min with saline or additions, were incubated with 40 μM L-arginine and collagen for 15 min at 37°. The reaction was stopped by sonicating samples on ice twice for 10 s in the presence of 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 100 μM dithiothreitol and centrifuged at $600 \times g$ for 20 min. Suitable aliquots of the supernatants, mixed with 100 μM dithiothreitol, 100 μM NADPH, 10 μM FAD, 10 μM FMN, 0.1 μM tetrahydrobiopterin and 1 mM $CaCl_2$, were incubated in the presence of 5 μM L-arginine. After 5 min at 37° the mixtures were added to equal volume of pH 9.7 assay buffer and treated as above detailed.

2.7. Assessement of PKC activation by measurement of 47 kDa phosphorylation

WP (2.5×10^9 plts/mL), resuspended in pH 7.4 Hepes buffer containing 1.0 mM EGTA and 5% platelet-poor plasma, were incubated for 60 min at 37° with 250 μCi/mL [^{32}P] phosphoric acid (Amersham Pharmacia Biotech), under gentle shaking, washed once and finally resuspended to 2.0×10^8 plts/mL in the same buffer. Samples were preincubated for 10 min at 37° with saline, then collagen was added. After 15 min at 37°, activation was stopped by the addition of suitable aliquots of 2× Laemmli SDS reducing gel sample buffer. Samples were boiled for 5 min and proteins separated by 10% SDS-PAGE. Running was performed in the presence of molecular weight markers. The gels were dried and [^{32}P] phosphorylated bands were revealed using the Packard Cyclone Storage Phosphor System and quantified with the related software package.

2.8. Data analysis

Data are the mean ± S.D. of at least four independent determinations, each performed in duplicate. Reported drawings are also representative of four experiments. Statistical analysis was performed using the unpaired Student's *t*-test, considering significant the difference between control and each treatment at least at 5% level ($P < 0.05$).

3. Results

3.1. The effect of collagen on intracellular Ca^{2+} levels

Fig. 1 shows changes of intracellular Ca^{2+} levels in platelets resuspended in nominally Ca^{2+}-free medium and incubated with collagen in unstirring conditions. Collagen produces a gradual rise in the intracellular [Ca^{2+}]. The effect is dose and time dependent. In platelets stimulated with 10 μg/mL collagen for 15 min at 37° the $[Ca^{2+}]_i$ reaches the level of 206 ± 16 nM.

In platelets resuspended in EGTA-containing medium the rise in $[Ca^{2+}]_i$ evoked by collagen is significantly lower ($P < 0.0005$) than that measured in the absence of EGTA (Fig. 2A). Moreover, in the presence of 1 mM extracellular Ca^{2+}, the platelet stimulation with collagen significantly rises the Ca^{2+} levels: in the presence of 5 μg/mL collagen, after 15 min incubation, the $[Ca^{2+}]_i$ increases from 180 ± 5 nM to 430 ± 19 nM. EGTA significantly

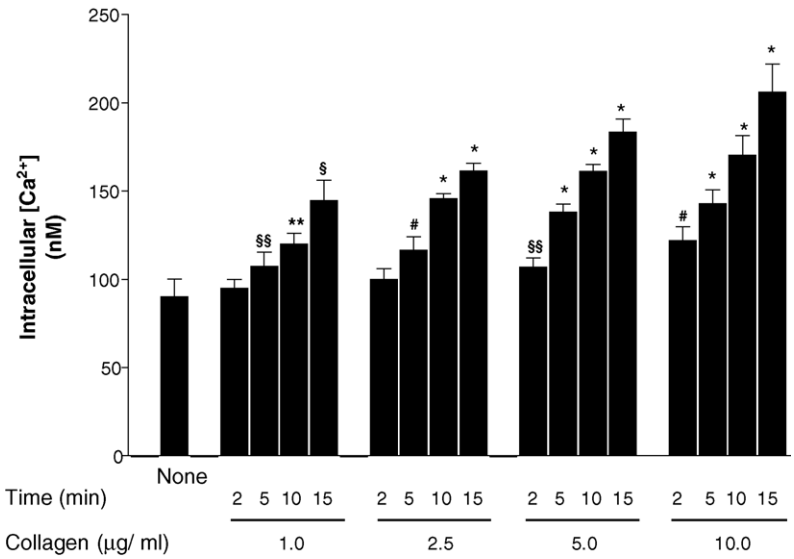

Fig. 1. Intracellular [Ca^{2+}] elevation induced by collagen. FURA 2-loaded platelets (2.0×10^8 plts/mL), prewarmed for 2 min at 37°, were stimulated with collagen as indicated. To induce platelet activation but not aggregation experiments have been carried out without stirring. Data are the mean ± S.D. of four experiments. $^{§§}P < 0.05$; $^{\#}P < 0.01$; $^{**}P < 0.005$; $^{§}P < 0.0025$; $^{*}P < 0.0005$ vs. none.

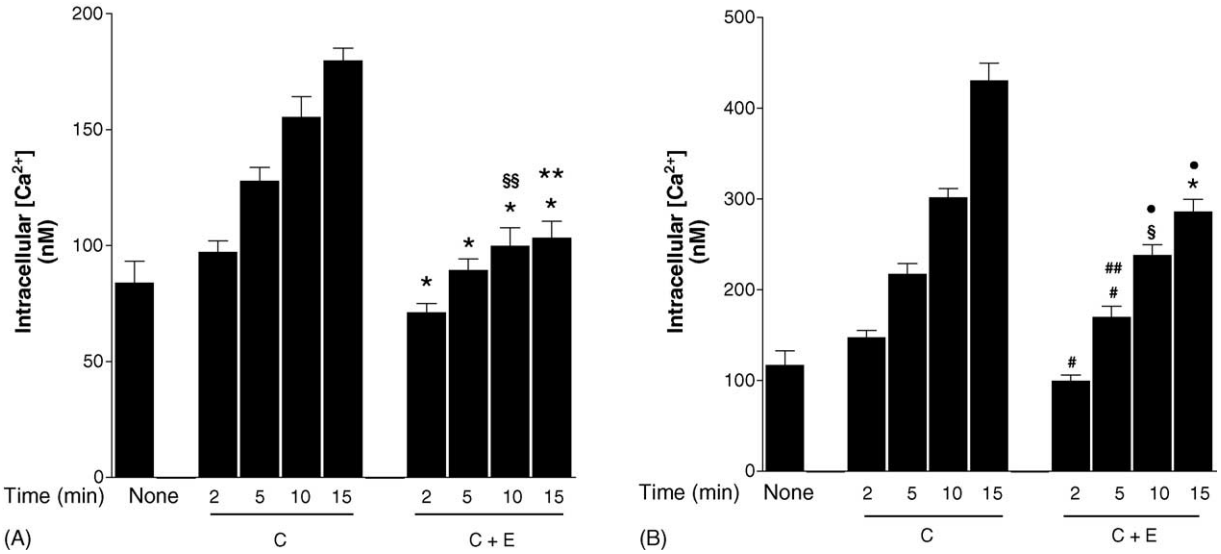

Fig. 2. Effect of EGTA on intracellular [Ca^{2+}] elevation induced by collagen. FURA 2-loaded platelets (2.0×10^8 plts/mL) were resuspended in pH 7.4 Hepes buffer in the absence (A) or in the presence (B) of 1 mM $CaCl_2$ and then challenged with 5 μg/mL collagen (C). In some experiments suitable samples were pretreated with 1 mM EGTA (E). Data are the mean ± S.D. of four experiments. $^{*}P < 0.0005$; $^{\S}P < 0.0025$; $^{\#}P < 0.01$ vs. collagen. $^{\bullet}P < 0.0005$; $^{\#\#}P < 0.0025$; $^{**}P < 0.01$; $^{\S\S}P < 0.025$ vs. none.

($P < 0.0005$) reduces the [Ca^{2+}] elevation induced by collagen (Fig. 2B).

In addition the collagen effect was compared with that produced by A23187. Results indicate that collagen and A23187 differently behave. Collagen, incubated for 2 min at 37°, produces a very small change in Ca^{2+} levels, while in platelets stimulated with 0.1 and 1.0 μM A23187 $[Ca^{2+}]_i$ peaks at 166 ± 15 and 460 ± 40 nM, respectively (Fig. 3). Moreover the $[Ca^{2+}]_i$ elevation induced by 0.1 μM A23187 maintains after 15 min, while the peak induced by 1.0 μM A23187 declines rapidly and then produces a sustained plateau. It is noteworthy that in platelets stimulated by collagen or 0.1 μM A23187 $[Ca^{2+}]_i$ reaches the same level after 10 min incubation.

3.2. Effect of collagen on L-arginine uptake

To assess whether Ca^{2+} elevation could affect L-arginine transport across plasma membrane, L-arginine uptake in platelets challenged with collagen or A23187 was measured. Two min after platelet stimulation with collagen, L-arginine uptake does not appear to be modified in respect to control, while it slows down in A23187 treated platelets. The Ca^{2+} ionophore effect is dose-dependent. At longer incubation times (15 min) of platelets exposure to collagen or 0.1 μM A23187, the L-arginine uptake is diminished of about 30%. A stronger effect is produced by 1.0 μM A23187. In the presence of EGTA, the reduction observed in response to collagen or A23187 is almost completely

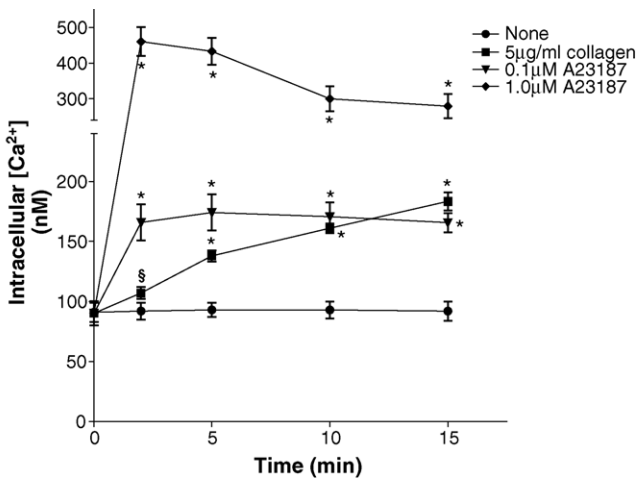

Fig. 3. Time course of intracellular [Ca^{2+}] elevation induced by collagen or A23187. FURA 2-loaded platelets (2.0×10^8 plts/mL), prewarmed for 2 min at 37°, were stimulated with collagen or A23187 as indicated. Data are the mean ± S.D. of four experiments. $^{\S}P < 0.05$; $^{*}P < 0.0005$ vs. none.

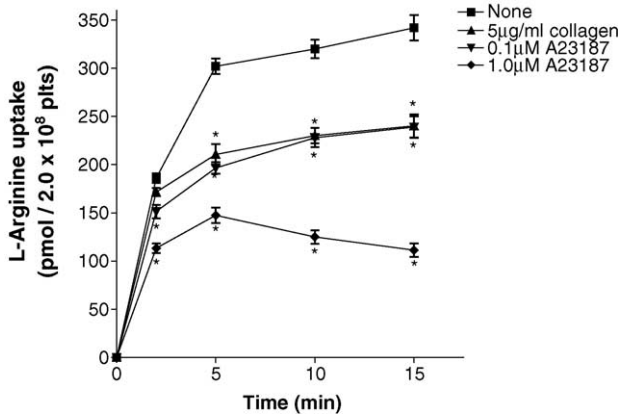

Fig. 4. Time course of L-arginine uptake in the presence of collagen or A23187. WP (2.0×10^8 plts/mL), prewarmed with saline at 37°, were incubated with 1 μCi/mL L-[2,3, 4-^{3}H] arginine, 40 μM L-arginine and collagen or A23187, when required. At the indicated times aliquots of 1 mL were withdrawn and rapidly filtered as described in Section 2. Data are the mean ± S.D. of four experiments carried out in triplicate. $^{*}P < 0.0005$ vs. none.

Table 1
Effect of collagen on L-arginine uptake in NEM-treated platelets

	L-Arginine uptake pmol/2 × 10^8 plts	Inhibition %
None	328 ± 9.5	
NEM	233 ± 6.0	29
Collagen	229 ± 8.0	30
Collagen + NEM	226 ± 5.0	31

WP (2.0 × 10^8 plts/mL), prewarmed for 10 min at 37° in the presence of saline or 200 μM NEM, were incubated for 15 min at 37° with 1 μCi/mL L-[2,3, 4 -^{3}H] arginine, 40 μM L-arginine and 5 μg/mL collagen when required. L-Arginine uptake was determined as detailed in Section 2. Each value is the mean ± S.D. of four experiments carried out in triplicate.

cancelled, suggesting that Ca^{2+} can have a role in the inhibition of L-arginine uptake (data not shown).

Recently we have shown that in human platelets L-arginine transport is taken up by the systems y^+L and y^+, which account for the 60–70% and for the 30–40% of the total, respectively [10]. These transport systems can be distinguished by platelet treatment with NEM, a selective inhibitor of the system y^+ [16]. As shown in Fig. 4, in collagen stimulated platelets L-arginine uptake is decreased at the most by 30%, suggesting the involvement of the system y^+. To clarify these findings we studied the collagen effect in platelets pretreated with NEM. Results of these experiments reported in Table 1 confirm that collagen decreases L-arginine total flux by 30% and it does not improve the NEM effect.

3.3. The collagen effect on NO_x formation and cGMP levels

Previously it was shown [8,10] that a reduced L-arginine uptake affects NO formation. To confirm these data the effect of varying amounts of collagen on NO_x formation was tested. As shown in Fig. 5A collagen dose-dependently inhibits NO_x production in whole cells, producing a significant ($P < 0.0005$) reduction at all tested concentrations. Moreover 5 μg/mL collagen and 0.1 μM A23187 produce the same effect. The addition of EGTA to platelets reverses the collagen inhibition on NO_x formation (Table 2). Moreover the pretreatment of WP with 1 mM aspirin did not change the collagen effect: in platelets challenged with 5 μg/mL collagen for 15 min at 37° the NO_x concentration was 2.0 ± 0.4 pmol or 2.1 ± 0.4 pmol/2.0 × 10^8 plts, in absence or in the presence of aspirin. In these experiments the basal NO_x levels corresponded to 2.6 ± 0.3 pmol and 2.7 ± 0.1 pmol/2.0 × 10^8 plts. Similar results have been obtained when aspirin was added to PRP. In agreement with data on L-arginine influx, collagen does not improve the NEM effect on NO_x levels (Table 3). Collagen has the same effect on L-arginine uptake (Table 1) and NO_x formation (Table 3) both in the presence and in absence of NEM ($y = 0.882128x + 4.24167$; $r^2 = 0.9996$).

As additional evidence for the collagen effect on NO production cGMP formation was measured. Collagen dose-dependently inhibits cGMP formation (Fig. 5B). cGMP levels and NO_x formation are in strict correlation ($y = 0.003722x - 0.033352$; $r^2 = 0.996503$).

3.4. The collagen effect on maximal NO_x levels

Incubation of collagen with whole platelets decreases significantly the maximal NO_x levels. The collagen effect is dose-dependent and reaches the maximum with 10 μg/mL (Fig. 6). Moreover the collagen inhibition was potentiated by okadaic acid that is a serine-threonine phosphatase inhibitor. In the presence of okadaic acid the effect of 2.5 μg/mL collagen was potentiated of 40%. The tyrosine phosphatase inhibitor vanadate produced a poor effect (data not shown). No effect was shown when collagen was incubated directly with platelet sonicate extracts.

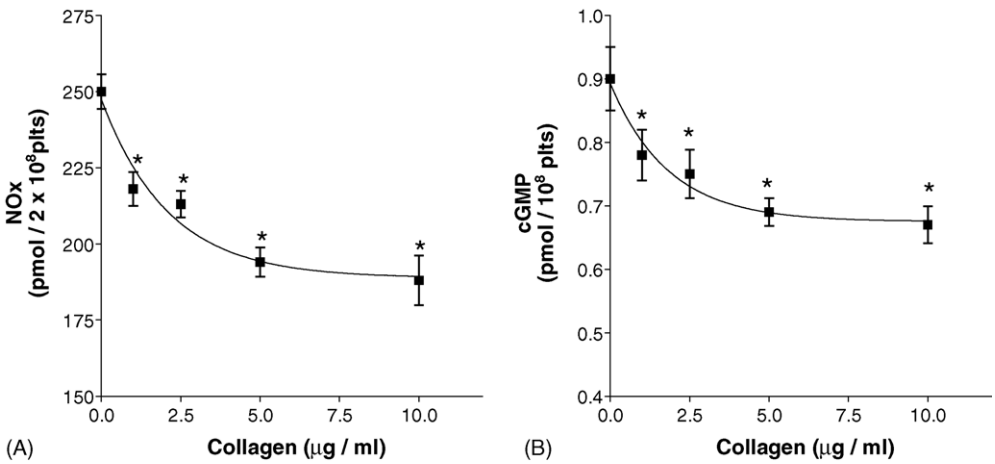

Fig. 5. Effect of collagen on NO_x formation and cGMP levels in human platelets. WP (1.0 × 10^9 plts/mL), resuspended in pH 7.4 Hepes buffer containing 1 mM $CaCl_2$, were prewarmed for 10 min at 37° with saline, and incubated with 40 μM L-arginine and collagen as indicated. After 15 min at 37°, incubation was stopped by sonicating samples on ice. NO_x formation (A) and cGMP levels (B) were measured as reported in Section 2. Each data represents the mean ± S.D. of five experiments carried out in triplicate. *$P < 0.0005$ vs. basal level.

Table 2
Effect of EGTA on NO_x formation induced by collagen

	$[NO_x]$ pmol/2 × 10^8 plts
None	265 ± 30
EGTA	248 ± 39
Collagen	209 ± 39
Collagen + EGTA	250 ± 41

WP, resuspended at 1.0 × 10^9 plts/mL in pH 7.4 Hepes buffer containing 1 mM $CaCl_2$ were prewarmed for 10 min at 37° in the presence of saline or 1 mM EGTA. Samples were then incubated for 15 min at 37° with 1 μCi/mL L-[2,3, 4 -^{3}H] arginine, 40 μM L-arginine and 5 μg/mL collagen when required. NO_x formation was quantified as detailed in Section 2. Each value is the mean ± S.D. of four experiments carried out in triplicate.

Table 3
Effect of collagen on NO_x formation in NEM-treated platelets

	$[NO_x]$ pmol/2 × 10^8 plts	Inhibition %
None	260 ± 11	
NEM	187 ± 10	28
Collagen	183 ± 13	31
Collagen + NEM	180 ± 9	31

WP, resuspended at 1.0 × 10^9 plts/mL in pH 7.4 Hepes buffer containing 1 mM $CaCl_2$, were prewarmed for 10 min at 37° in the presence of saline or 200 μM NEM. Samples were then incubated for 15 min at 37° with 40 μM L-arginine and 5 μg/mL collagen when required. NO_x formation was quantified as detailed in Section 2. Each value is the mean ± S.D. of four experiments carried out in triplicate.

3.5. The collagen effect on protein kinase C activation

Stimulation of platelets with collagen induces a receptor-related activation of phospholipase C [17,18], which leads to the phosphorylation of several proteins, among which pleckstrin (47 kDa), indicative of protein kinase C activity. Results reported in Fig. 7 show that, in conditions of mild platelet stimulation, protein kinase C is activated by collagen in a dose-dependent manner.

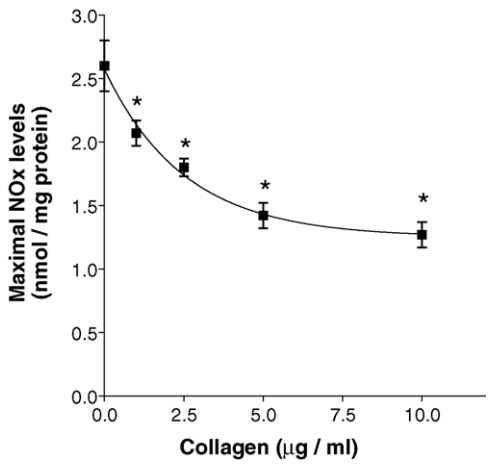

Fig. 6. Effect of collagen on maximal NO_x levels. WP (1.0 × 10^9 plts/mL), resuspended in pH 7.4 Hepes buffer containing 1 mM $CaCl_2$, prewarmed for 10 min at 37°, were incubated for 15 min at 37° with 40 μM L-arginine and collagen. The reaction was stopped by sonicating samples on ice. After a brief centrifugation, suitable aliquots of platelet sonicated extracts were incubated for 5 min at 37° in the presence of 5 μM L-arginine as detailed in Section 2. Each point is the mean ± S.D. of at least four experiments carried out in triplicate. $^*P < 0.0005$ vs. basal level.

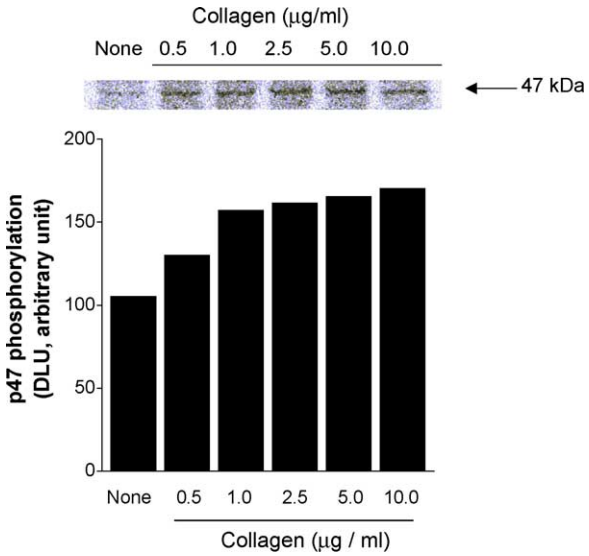

Fig. 7. Effect of collagen on pleckstrin phosphorylation. [^{32}P]-labelled WP (2.0 × 10^8 plts/mL) were preincubated with saline for 5 min at 37° then collagen was added. After 15 min at 37°, incubation was stopped by the addition of suitable aliquots of 2× Laemmli buffer. Proteins were separated by 10% SDS-PAGE and phosphorylated bands were revealed in a Phosphor Imager system. Results are representative of four similar experiments.

4. Discussion

Collagen is the most thrombogenic component of the subendothelial layer following vascular injury. Collagen supports platelet adhesion to the subendothelium and induces aggregation, secretion and procoagulant activity. The interaction between collagen and platelets is complex and mainly mediated by two receptors, the integrin $\alpha_2\beta_1$ and glycoprotein VI. $\alpha_2\beta_1$ Signal acts via phospholipase A_2 and is inhibited by aspirin, while glycoprotein VI signal activates phospholipase C and is unaffected by aspirin [19]. Data reported in the present study show that NO_x formation is unchanged by platelet treatment with aspirin ''in vitro''. Thus glycoprotein VI seems to play a central role [17] in the mechanisms leading to the formation of compounds, such as NO, very important in the regulation of platelet activation. Glycoprotein VI activation by collagen produces a cascade of signalling events including the activation of phospholipase C-γ2 [18]. Phospholipase C-γ2 leads to the formation of two second messengers, diacylglycerol, which activates protein kinase C [20] and inositol 1, 4, 5 trisphosphate, which is involved in the release of Ca^{2+} from the dense tubular system. Collagen allows the formation of inositol 1, 4, 5 trisphosphate, but unlike thrombin, it weakly mobilizes Ca^{2+} [21]. In thrombin stimulated platelets a transient initial spike and a sustained plateau are produced: the initial spike reflects the discharge of Ca^{2+} from intracellular stores and the plateau reflects Ca^{2+}

transport across the plasma membrane. Collagen exerts an activating mechanism mainly dependent on extracellular Ca^{2+} [12].

Data of this study show that collagen in a dose- and time-dependent manner significantly increases $[Ca^{2+}]_i$ Since EGTA blocks the Ca^{2+} elevation (Fig. 2), Ca^{2+} influx seems to be involved in the early collagen action. Adhesion of platelets to the vessel is prevented by NO produced both by endothelial cells and platelets [5]. Malinsky et al. [22] have shown that NO formation is enhanced during the platelet aggregation induced by collagen, but not by thrombin, suggesting that platelet-derived NO could be involved in the regulation of platelet function and recruitment [23]. Nevertheless other authors have shown that NO formation is reduced during collagen-induced platelet aggregation [24]. All previous data reported in [22–24] have been obtained in stirred platelet suspensions, in which a full aggregation was produced. Thus to evaluate the efficiency of L-arginine/NO pathway and the consequent NO formation during the early phases of platelet activation, experiments have been carried out in unstirred platelets, stimulated with low doses of collagen. Results of this study show that collagen significantly decreases NO production. In agreement with previous reports [22,25], the stimulation of platelets with 0.1 U/mL thrombin did not change NO release, whereas lower doses of thrombin (0.025–0.05 U/ml) significantly diminished ($P < 0.0005$) NO basal level. The same effect in platelets stimulated with 0.1 μM A23187 was observed (data not shown). Thus the mild rise in cytosolic Ca^{2+} induced by low concentrations of collagen, thrombin or A23187 could modulate NO formation through a reduced availability of L-arginine for cNOS. However during the early phases of platelet activation other events involved in the regulation of L-arginine/NO pathway can occur. These include alterations in NO signalling and decreased availability of NOS cofactors, such as tetrahydrobiopterin. Nevertheless one of the most important mechanisms of impaired NO bioactivity is the increased reactive oxygen species formation. Platelets produce a constant flux of oxygen free radicals. These species significantly increase in platelets activated by agonists [26,27]. One of the oxygen radicals, superoxide anion, rapidly interacts with NO to form peroxynitrite. Peroxynitrite not only decreases NO bioavailability, but also induces lipid peroxidation and oxidation of protein thiols and/or tyrosine residues.

Recently it has been shown that extracellular L-arginine plays a crucial role in modulating NO intracellular levels [8,10] and L-arginine transport is the most determinant of L-arginine availability for cNOS. In platelets stimulated with collagen or A23187 L-arginine influx is significantly reduced (Fig. 4), while EGTA almost completely normalizes L-arginine transport. These data suggest that the Ca^{2+} rise could play a role in the inhibitory mechanism. The system y^+ [10] seems to be specifically involved, as indicated by data obtained in NEM-treated platelets (Table 1). System y^+ has a restricted substrate specificity and is influenced by membrane potential [28]. Thus system y^+ can be regulated by changes in the extracellular or intracellular medium composition and should make a very important contribution to intracellular NO formation in human platelets. If biosynthesis of NO is compromised, agonist induced platelet activation may be enhanced. In platelets from type 2 diabetic patients reduced NO production was shown [29,30]. The reduced NO formation, related to the reduced L-arginine uptake [31] could contribute to generate the platelet hyperactivity described in those patients. Moreover a strict relationship between reduced vascular NO formation and increased platelet activation ''in vivo'' in young spontaneously hypertensive rats was shown [32]. It is known that the inhibition of NO biosynthesis promotes P-selectin expression in platelets [33]. Enhanced expression of adhesive molecules on the platelet surface leads to platelet activation and contributes to the development of atherosclerosis. In the present report we have shown that in platelets activated by collagen NO formation is diminished. The reduced substrate availability for cNOS can be one of the causes of the decreased NO levels. However other events such a direct effect at the level of the cNOS enzyme could be involved. Modifications of the protein, such as the phosphorylation of specific amino acid residues can be produced by the collagen treatment. Recent studies have shown that serine/threonine phosphorylation of cNOS controls cNOS activity [34,35]. Michell et al. [35] have reported that cNOS is primarily phosphorylated on serine and threonine and to a lesser extent on tyrosine residues. Data of the present study indicate that okadaic acid, a serine/threonine protein phosphatase inhibitor, potentiates the collagen effect, while vanadate, a tyrosine phosphatase inhibitor, has a poor effect. Recently it has been shown that protein kinase C phosphorylates cNOS specifically at thr497 in the calmodulin-binding domain and negatively regulates cNOS activity [36]. Thus the phosphorylation of serine/threonine residues on cNOS, probably controlled by protein kinase C, reduces NO formation [24]. Likely diminished NO further stimulates protein kinase C [33]. Finally protein kinase C could regulate NOS by direct phosphorylation, resulting in further decrease in NO production [36,37].

In conclusion this study shows that in the early phases of platelet stimulation with collagen intracellular Ca^{2+} levels are increased, while NO formation and L-arginine uptake are decreased. The protein kinase C, activated during the signalling cascade induced by collagen, could control the cNOS activity through the phosphorylation of specific residues.

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