



Original Contribution

Vitamin C inhibits platelet expression of CD40 ligand

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Abstract

Upon stimulation with agonists, platelets express CD40 ligand (CD40L), a transmembrane protein implicated in the initiation and progression of atherosclerotic disease. We have recently discovered that oxidative stress plays a major role in platelet CD40L expression. In this study, we sought to determine whether vitamin C, a known antioxidant, is able to influence platelet CD40L expression. In vitro experiments were done by stimulating platelets with collagen in the presence or absence of vitamin C (50–100 μ M) or vehicle as control. An in vivo study was done in 10 healthy subjects who were randomized to intravenous infusion of placebo or 1 g vitamin C for 45 min in a crossover design. At the end of infusion platelet CD40L and O_2^- were measured. The in vitro study demonstrated that vitamin C dose dependently inhibited platelet CD40L expression without affecting agonist-induced platelet aggregation. In subjects treated with placebo no changes of platelet CD40L and O_2^- were observed; conversely, vitamin C infusion caused a significant and parallel decrease of platelet O_2^- (-70% , $P < 0.001$) and CD40L (-68% , $P < 0.001$). Platelet aggregation was not modified by either treatment. This study suggests that water-soluble antioxidants, which scavenge superoxide radicals, may reduce platelet CD40L expression.

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Introduction

CD40 ligand (CD40L), a member of the tumor necrosis factor ligand family, is a transmembrane protein with proinflammatory and prothrombotic properties upon interaction with its receptor CD40 [1]. CD40L was primarily found on the immune system cells, and subsequently on macrophages, smooth muscle cells, endothelial cells, and platelets [1]. Upon agonist stimulation, platelets express CD40L that is then cleaved on the membrane surface and finally circulates in the soluble form [2]; it has been

calculated that over 95% of circulating soluble CD40L (sCD40L) originates from platelets [2]. The mechanism accounting for CD40L expression by activated platelets is still unclear. Previous studies demonstrated that platelets produce reactive oxidant species, which in turn act as intracellular signaling molecules that amplify platelet response to agonists [3].

We have recently demonstrated that platelet production of O_2^- is implicated in the expression of CD40L, as platelet incubation with SOD, which dismutates O_2^- to H_2O_2 , significantly decreased agonist-induced CD40L expression [4]. These findings were confirmed by an in vivo study performed in patients with a deficit of gp91phox, the central core of NADPH oxidase. These patients showed a CD40L down-regulation, suggesting that O_2^- generated by NADPH oxidase activation elicits an up-regulation of CD40L [4].

These data induced us to seek whether natural antioxidants play a role in modulating platelet CD40L expression.

Abbreviations: CD40L, CD40 ligand; sCD40L, soluble CD40L; PRP, platelet-rich plasma; FITC, fluorescein isothiocyanate; Mab, monoclonal antibodies; PBS, phosphate-buffered saline; BSA, bovine serum albumin; AU, arbitrary unit.

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We first performed an *in vitro* study to assess if vitamin C, a water-soluble molecule that scavenges superoxide radicals [5], was able to influence platelet CD40L expression. For the first time, we herein report that vitamin C inhibits CD40L expression *in vitro* and *in vivo*.

Methods

Platelet preparation and activation

Blood samples were taken from healthy subjects who had fasted at least 12 h and were mixed with 0.13 M Na citrate. Platelet-rich plasma (PRP) was prepared as previously described [3]. Unless otherwise reported PRP was stimulated at 37°C for 3 min with collagen [4 µg/ml) or thrombin (0.1 U/ml). Agonist-induced platelet aggregation (Born's method) was measured by light transmission (LT%) difference between PRP and platelet-poor plasma.

O₂⁻ detection

Superoxide anion (O₂⁻) production by PRP stimulated with agonists was measured by lucigenin (5 µM) [3] and expressed as stimulation index (SI = mean level of stimulated platelet luminescence/average level of luminescence in unstimulated platelets).

CD40L and CD62P expression

CD40L and CD62P expression on platelet membrane was analyzed using specific FITC-labeled monoclonal antibodies (Mab) as previously reported [4]. An irrelevant isotype-matched antibody (anti-IgG1) was used as a negative control.

Mab (20 µl) was added to platelets (200 µl, 2 × 10⁸/ml) previously fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) (0.1% BSA) and incubated for 60 min at 4°C. The unbound Mab was removed by addition of 0.1% BSA phosphate-buffered saline and centrifugation at 500g for 3 min (twice). Fluorescence intensity was analyzed on an Epics XL-MCL Cytometer (Coulter Electronics, FL) equipped with an argon laser at 488 nm. For every histogram, 50,000 platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity has been reported in an arbitrary unit (AU) obtained by multiplying the number of positive events obtained after platelet stimulation by the mean fluorescence observed when the specific Mab was used, and correcting against the values obtained in the unstimulated sample treated with the same antibody.

Analysis of sCD40L

After 10 min of PRP stimulation with agonists, the reaction was blocked by acidification of the medium with ACD (D-sodium hydrogen citrate, D-glucose, and citric

acid), PRP was centrifuged (10 min at 360g), and the supernatant was stored at -80°C until use. sCD40L was measured with a commercial immunoassay (Quantikine CD40 Ligand, R&D Systems).

In vitro study

In the *in vitro* study, platelet suspension (3 × 10⁹ plt/ml) was treated with vitamin C (ascorbic acid, 25–100 µM) or solvent as control (30 min at 37°C) before activation for O₂⁻, CD40L, and sCD40L analysis (see Methods above).

In vivo study

We investigated whether a short-term administration of vitamin C could affect agonist-induced platelet CD40L and O₂⁻. To calculate the sample size of the study, we first measured agonist-induced platelet CD40L expression in 44 healthy subjects (24 males, 20 females; age, 32 ± 4 years); in this population, platelet CD40L expression was 32 AU ± 3.1. Assuming that vitamin C reduced platelet CD40L by 50%, at least 9 patients for each group had to be evaluated (α = 0.05, 1-β = 0.80). We planned a randomized, double-blind, placebo-controlled crossover study in 10 healthy volunteers (5 females and 5 males; mean age, 30.5 ± 1.0 years, range 24–36 years) who provided their informed consent to participate in the study. All subjects were nonsmokers, had no risk factors for atherosclerosis, and had not taken any antiplatelet agents or antioxidant drugs in the previous month; a 10-day washout was scheduled between the two phases of the study. Collagen-induced platelet CD40L, sCD40L, O₂⁻, CD62P, and aggregation were determined at baseline and immediately after 45 min of intravenous infusion of vitamin C at 24 mg/min or placebo.

Statistical analysis

Unless otherwise reported data are expressed as means ± SD. The comparison between variables in the *in vitro* study was analyzed by Student's *t* test for unpaired data; the correlation study between CD40L expression and O₂⁻ formation was evaluated using the linear correlation analysis followed by ANOVA test.

Results

Compared to unstimulated platelets, agonists such as collagen and thrombin significantly increased platelet O₂⁻ formation and CD40L expression (Fig. 1); O₂⁻ and CD40L were significantly correlated (*r* = 0.93, *P* = 0.006) with collagen (4 µg/ml, *n* = 4) and (*r* = 0.85, *P* = 0.005) with thrombin (0.1 U/ml, *n* = 4). A similar correlation was detected using several concentrations of collagen (1 to 8 µg/ml) or thrombin (0.01 to 1 U/ml) (data not shown). Vitamin C dose dependently inhibited O₂⁻ and CD40L (Figs. 1A–D).

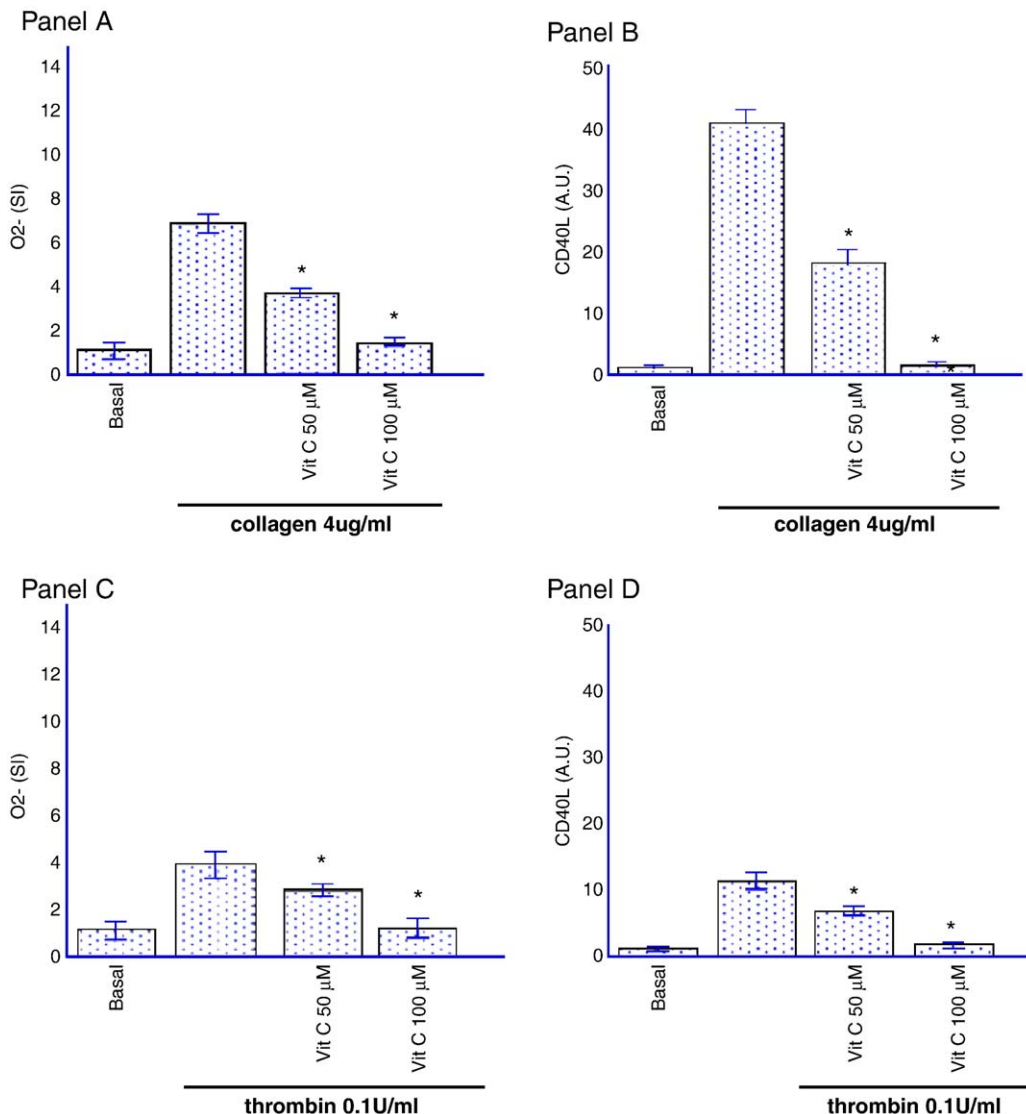


Fig. 1. O₂⁻ formation and CD40L expression in platelets treated with vitamin C. O₂⁻ and CD40L formation in platelets incubated with and without vitamin C (30 min at 37°C) and stimulated with collagen (A and B) or thrombin (C and D). Data ($n = 5$) are reported as means \pm SD. The comparison between variables in the in vitro study was analyzed using the Student t test for unpaired data (* $P < 0.01$).

Platelet O₂⁻ was inhibited by 50 and 93% with 50 and 100 μ M vitamin C, respectively; platelet CD40L was inhibited by 52 and 96% with 50 and 100 μ M vitamin C, respectively. Vitamin C also inhibited collagen-induced sCD40L formation in a dose-dependent fashion (50 μ M = from 6.5 ± 0.6 to 3.18 ± 0.02 , $P < 0.001$; 100 μ M = from 6.5 ± 0.6 to 0.45 ± 0.01 , $P < 0.001$); conversely vitamin C did not affect either platelet aggregation or CD62P expression (data not shown). No change in pH value was observed in the platelet sample added with vitamin C (data not shown).

As phospholipase A₂ (PLA₂) and protein kinase C (PKC) have a role in enhancing O₂⁻ production [3], we investigated the contribution of these enzymes in the production of O₂⁻ elicited by agonist. Incubation of platelets with RO318210 (30 μ M, 37°C 30 min), an inhibitor of

PKC, or with AACOCF₃ (14 μ M, 37°C 30 min), an inhibitor of PLA₂, resulted in -23% $P < 0.05$ and -93% $P < 0.001$ platelet O₂⁻ inhibition, respectively (Fig. 2).

At baseline subjects allocated to placebo or vitamin C had no significant difference in CD40L expression (25.2 ± 6.0 vs 31.4 ± 5.4 , respectively, $P > 0.05$) and O₂⁻ production (7.1 ± 1 vs 7.3 ± 1 , respectively, $P > 0.05$).

In subjects receiving placebo, no changes in platelet CD40L (25.2 ± 6.0 before and 25.6 ± 6.0 after) and O₂⁻ (7.1 ± 1 before and 7.4 ± 3 after) were observed (Fig. 2); conversely, subjects receiving vitamin C showed a reduction in platelet CD40L from 31.4 ± 5.4 to 8.9 ± 3.8 (-68% , $P < 0.001$), and in O₂⁻ from 7.2 ± 1.1 to 1.8 ± 0.5 (-70% , $P < 0.001$) (Fig. 3). A parallel decrease in platelet O₂⁻ and CD40L with a significant linear regression was observed ($r = 0.86$, $P < 0.001$); similar findings were seen using thrombin as agonist (not shown).

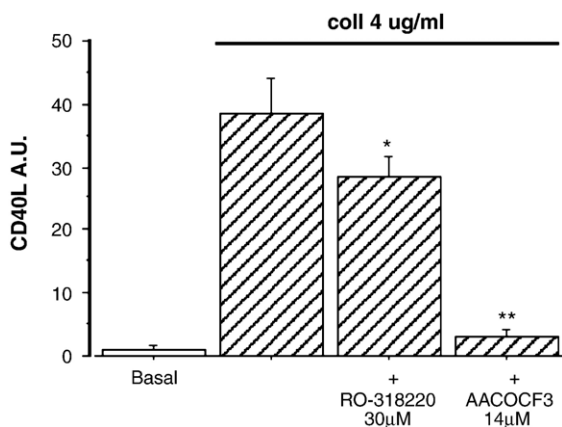


Fig. 2. Effect of PKC and PLA₂ inhibitors on CD40L expression. CD40L expression in platelets stimulated with collagen and treated with or without PKC inhibitor RO-318220 or PLA₂ inhibitor AACOCF3. Data (*n* = 5) are reported as means ± SD. (**P* < 0.05; ***P* < 0.001).

sCD40L induced by platelet stimulation with collagen was unaffected in subjects given placebo (from 6.1 ± 0.6 to 5.7 ± 0.7 , $P > 0.05$) while it was significantly inhibited in subjects given vitamin C (from 6.5 ± 0.7 to 2.2 ± 0.5 , $P < 0.001$). No changes in agonist-induced platelet aggregation or platelet CD62P expression were observed after either placebo (LT% from $80 + 4$ to $79 + 6$ and CD62P from 8.9 ± 1.3 to 8.8 ± 1.3 , $P > 0.05$) or vitamin C infusion (LT% from $82 + 3$ to $81 + 3$ and CD62P from 8.6 ± 1.3 to 8.3 ± 1.2).

Discussion

Several lines of evidence suggest that the CD40L/CD40 dyad is implicated in the pathogenesis of atherothrombosis. Thus, engagement of CD40L with its receptor CD40 induces several vascular responses that may potentially contribute to initiate and propagate the atherosclerotic lesion. In fact, CD40L enhances the expression of adhesive

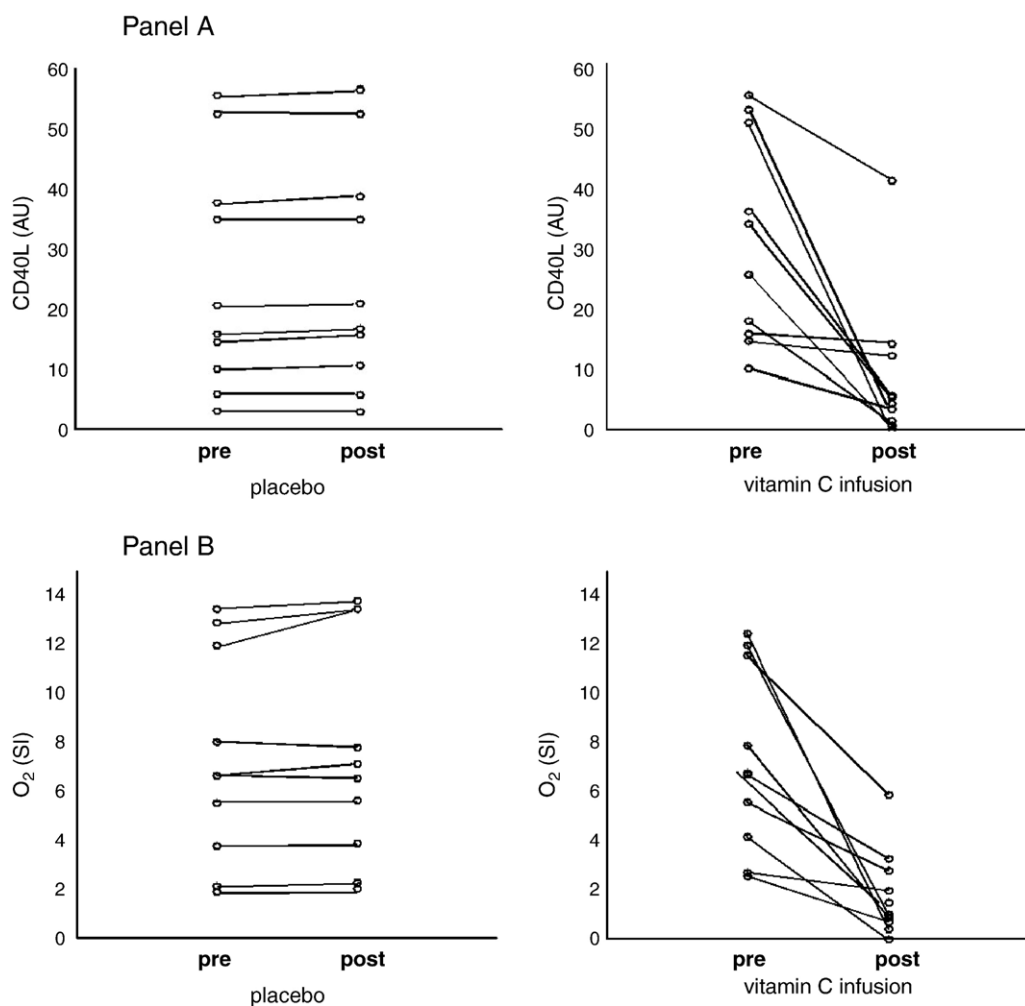


Fig. 3. Effect of placebo and vitamin C infusion on collagen-induced O₂ formation and CD40L expression in 10 healthy subjects. Collagen-induced (4 µg/ml) CD40L expression (A) and O₂ formation (B) before (pre) and after (post) treatment with either vitamin C (right side) or placebo (left side). Data are expressed as means ± SE. Student's *t* test and linear regression analysis (ANOVA) were performed. Statistical significance was accepted at $P < 0.05$.

molecules, chemokines, metalloproteinases, and tissue factor [2]. An important support to the putative proatherosclerotic activity of CD40L was provided by an experimental study showing that antibodies against CD40L reduce the atherosclerotic lesion [6].

Observational, cross-sectional and prospective studies in humans suggested a role for CD40L in the pathogenesis of cardiovascular events that complicate the atherosclerotic disease. Thus, high levels of sCD40L have been described in patients at risk of cardiovascular events or in patients with acute coronary syndrome [1,8–10]; also, in patients with acute coronary syndrome, high levels of sCD40L were predictive of future cardiovascular events [10].

The mechanisms accounting for CD40L up-regulation are still unclear. Recent studies were focused on the possible role of oxidative stress in enhancing CD40L expression. Schonbeck and co-workers [7] demonstrated that incubation of human vascular endothelial cells and smooth muscle cells with oxidized LDL enhanced CD40 and CD40L expressions.

Another study conducted in patients with hereditary deficiency of gp91 phox, the central core of NADPH oxidase, reinforced the hypothesis that oxidative stress plays a role in CD40L expression [4]. These patients were characterized by an almost complete suppression of platelet O_2^- production and by a significant reduction of platelet CD40L expression compared to healthy subjects. Even if the exact mechanism through which oxidative stress enhances CD40L expression requires further investigation, these data suggested that antioxidants could be a useful tool to reduce it. To explore this hypothesis, we performed in vitro and in vivo studies to assess whether a natural antioxidant may influence platelet oxidative stress and CD40L. In vitro incubation of platelets with vitamin C inhibited CD40L expression in a dose-dependent manner. As vitamin C was able to inhibit platelet CD40L at concentrations that may be achieved in vivo following supplementation [11], we conducted an interventional study in healthy volunteers to evaluate whether this effect could be reproduced in vivo. This study demonstrated that the infusion of 1 g of vitamin C inhibited both platelet O_2^- formation and CD40L expression, a phenomenon that was independent of agonist-induced platelet aggregation.

Together these data suggest that vitamin C inhibits the expression of platelet CD40L via reduction of platelet O_2^- , the oxygen species that plays a crucial role in enhancing platelet CD40L [4] but does not provide definite data concerning the exact mechanism through which vitamin C exerts this antioxidant effect. Vitamin C could inhibit platelet production of O_2^- and in turn CD40L through an enzymatic and/or a nonenzymatic mechanism. As vitamin C is able to scavenge superoxide radicals [5], inhibition of CD40L could depend on its capacity to quench O_2^- as far as the enzymatic pathway is concerned. It seems unlikely, however, that vitamin C interferes with PKC as this enzyme seems to have a minor role in enhancing platelet

CD40L. Conversely, the rate of platelet CD40L inhibition by vitamin C is quite similar to that observed with the inhibitor of PLA2; this could suggest that vitamin C interferes with PLA2 activity but this hypothesis deserves further investigation.

In conclusion our data provide the first evidence that vitamin C inhibits platelet CD40L expression, probably by virtue of its antioxidant activity as suggested by a parallel decrease in platelet O_2^- and CD40L following vitamin C infusion. While the mechanism eliciting platelet O_2^- formation and CD40L expression deserves further investigation, these findings indicate that scavenging O_2^- with vitamin C or other antioxidants having similar properties may represent a new therapeutic approach to reduce the proatherogenic properties of CD40L. We also suggest that platelet CD40L is a marker that may prove useful for monitoring the biological and clinical efficacy of antioxidants in the treatment of atherosclerotic disease.

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