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Paracrine up-regulation of monocyte cyclooxygenase-2 by platelets: Role of transforming growth factor-β1

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Abstract

Objective: To examine the role of platelets and platelet-derived products on cyclooxygenase-2 (Cox-2) induction in adherent monocytes and to address the signaling pathways involved.
Methods: Platelets and monocytes were obtained from peripheral blood of healthy donors. Adherent monocytes were co-cultured with autologous platelets or platelet releasates or exposed to mediators contained in platelet α-granules (either from platelet source or recombinant) for 4–24 h. Cox-2 protein and mRNA were determined by Western and RT-PCR analysis, respectively. Thromboxane B_2 (TxB_2) and prostaglandin E_2 (PGE_2) synthesis as index of Cox-2 activity, and levels of transforming growth factor-β1 (TGF-β1) in platelet releasates were measured by enzyme immunoassay (EIA).
Results: Activated platelets induce rapid and transient Cox-2 *de novo* synthesis in adherent monocytes. The effect is dependent upon the platelet number but not upon cell–cell contact. Platelet-induced Cox-2 was not affected by prevention of platelet TxA_2 synthesis or microparticle formation but was blunted by inhibition of platelet α-granule secretion. TGF-β1, either platelet-derived or recombinant (rTGF-β1), induced Cox-2 expression and activity in adherent monocytes at concentrations within the range of those detected in releasates from activated platelets; this effect was not shared by recombinant platelet-derived growth factor ($rPDGF_{BB}$). The time course of Cox-2 induction by TGF-β1 in monocytes was identical to that observed with platelet releasates. Moreover, TGF-β1 receptor blockade completely abolished platelet-induced Cox-2 expression. p38 MAPK activation represents a common transduction pathway through which activated platelets and rTGF-β1 induce Cox-2 in monocytes.
Conclusion: These findings suggest that TGF-β1 released by activated platelets has a pivotal role in Cox-2 induction in monocytes and further supports the key role of platelets in the inflammatory and reparative responses.

Keywords: Cyclooxygenase; Platelets; Growth factors; Monocytes; MAP-kinase

1. Introduction

Monocytes, once activated by inflammatory stimuli, synthesize and release eicosanoids that mediate physiological processes as cell proliferation, inflammatory and immune responses, wound healing and haemostasis. Two prostaglandin synthases catalyze the formation of eicosanoids from arachidonic acid, namely cyclooxygenase-1 (Cox-1) and cyclooxygenase-2 (Cox-2). Cox-1 is constitutively expressed in most tissues and serves in general housekeeping functions. On the other hand, Cox-2 is responsible for high-level production of prostanoids in response to proinflammatory agents, tumor promoters and growth factors [1]. Cox-2 metabolites produced by activated monocytes, mainly thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2), influence the onset and progression of the inflammatory response, including the increase of vascular permeability, leukocyte recruitment, regulation of regional blood flow, remodelling, and wound repair.

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Moreover, these monocyte-derived eicosanoids are implicated in the pathogenesis and modulation of atherosclerosis that is now viewed as an inflammatory disease [2–4].

The "inflammatory" scenario, however, requires not only the presence of monocytes at the site of inflammation but also rapid and effective mechanisms to activate them. In this respect, the role of platelets as proinflammatory cells which co-ordinate innate and adaptive responses is beginning to be highlighted [5]. Platelets represent an important link between inflammation, thrombosis and atherogenesis. Platelet-induced chronic inflammatory processes at the vascular wall may indeed contribute to the development of atherosclerotic lesions and to atherothrombosis [6]. It has been previously shown that Cox-2 is induced upon platelet–monocyte aggregate formation [7] and that microparticles shed from activated platelets induce Cox-2 expression in a monocytoid cell line [8]. Platelets participate in inflammation and its resolution through the release of a variety of mediators that occurs within minutes after activation [9]. Among them, agents stored in α-granules possess multifaceted activities [10] that are central to platelet trafficking with other cells [11]. Platelet α-granules also contain growth factors, including transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) that orchestrate cell proliferation and repair mechanisms, providing beneficial therapeutic effects observed in various surgical settings [12].

TGF-β1 is a pleiotropic inflammatory mediator characterized by multifunctional immuno-modulatory properties that differ according to cell type and activation state [13]. Several monocyte functions are either stimulated or deactivated by TGF-β1, including proliferation, chemotaxis and growth factor production [14,15]. In addition, TGF-β1 influences monocyte invasion of basement membranes and transmigration into the tissues. Both the enhancement of monocyte integrin expression and modulation of metalloprotease secretion are involved [16]. The finely tuned regulation of these stages is critical for the resolution of inflammation and wound repair [17]. At the tissue level, TGF-β1 has been identified to be responsible for the platelet-mediated cardioprotection during ischemia [18] and, more recently, as a key factor in the development of atherosclerosis, restenosis and pulmonary hypertension [19].

In this study we show that platelets are potent activators of rapid Cox-2 expression in human adherent monocytes and provide the first evidence that TGF-β1 released by platelets is responsible for this effect through a receptor-mediated mechanism that involves p38 MAPK activation. This finding further supports the notion for a key role of platelets in the inflammatory reaction and wound repair.

2. Methods

2.1. Reagents

Arachidonic acid, bovine serum albumin (fatty acid-free and low endotoxin) thrombin, amiloride, EGTA (ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid), acetylsalicylic acid (ASA), methyl-β-cyclodextrin and cycloheximide were from Sigma Chemical Co. (Milano, Italy); PD98059 and platelet-derived TGF-β1 from Calbiochem (Inalco SpA, Milano, Italy); collagen from Mascia Brunelli (Milano, Italy) and SB431542 from Tocris (Biotrend Chemikalien GmbH, Kohln, Germany). SP600125, SB203580 and NS398 were from Biomol (Trimital, Milano, Italy); furegrelate, SQ-29548 and U46619 from Cayman (Spi-bio, Montigny le Bretonneux, France). Recombinant TGF-β1 (r-TGF-β1) and recombinant platelet-derived growth factor (r-$PDGF_{BB}$) were from R&D System (Space Import–Export, Milano, Italy), eptifibatide (Integrilin®) from SP Labo (SP Europe Bruxelles, Belgium). Electrophoresis reagents were from Amersham Pharmacia Biotech., Milano, Italy, and cell culture medium from BioWhittaker Italia SRL, Bergamo, Italy.

2.2. Antibodies

Monoclonal antibodies (mAbs) against cyclooxygenases (Cox-2, mAb 29 and Cox-1, mAb 10 and 11) were gift from A. Habib (American University of Beirut, Lebanon). MAbs against p38, ERK1/2 and c-*jun* (total and phosphorylated) were from Biosource Inc., (Prodotti Gianni S.p.A, Milano, Italy) and from Cell Signaling (Celbio, Milano, Italy), respectively. Peroxidase-conjugated anti-mouse IgG antibody was from Jackson ImmunoResearch Labs Inc. (Li StarFISH, Milano, Italy). Blocking and nonblocking mAbs anti-human P-selectin (CD62P) were from R&D System (Space Import–Export, Milano, Italy) and from BD Biosciences Pharmingen (Becton Dickinson Italia S.p.A. Buccinasco, Milano, Italy), respectively.

2.3. Cell isolation and platelet–monocyte co-culture

Blood from healthy donors who did not receive any medication for at least 2 weeks was collected into sodium citrate, in accordance with the principles outlined in the Declaration of Helsinki. Platelet-rich plasma was prepared (18 min, 180×*g*) and placed in separate tubes for platelet isolation (see below). Mononuclear leukocytes were separated by density centrifugation and plated (5×10^6/ml, M-199 medium supplemented with 10% human AB serum) in tissue culture dishes for 90 min at 37 °C [20]. Adherent cells were 85% to 90% monocytes, as determined by non-specific esterase staining. Platelets were isolated by centrifugation (6 min, 1400×*g*, 4 °C) from platelet-rich plasma containing EDTA (10 mM). The platelet pellet was resuspended, under sterile condition, in PBS containing 5 mM EDTA, and platelets were centrifuged (6 min, 500×*g*, RT) and resuspended (25×10^9/ml) in medium M-199 supplemented with 0.2% bovine serum albumin. Releasates were prepared by centrifugation (3 min, 10,000×*g* RT) of platelets exposed to various agonists for 10 min (37 °C, with 1000 rpm

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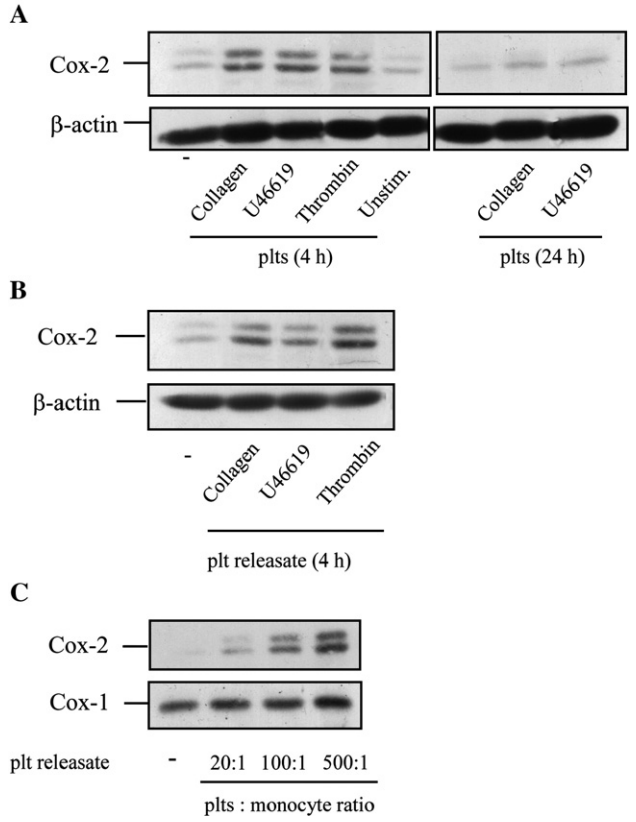

Fig. 1. Activated platelets induce transient Cox-2 expression in adherent monocytes. (A) Monocytes were co-cultured (plts:monocyte ratio 100:1) for 4 and 24 h with autologous platelets (plts) either unstimulated or activated by 6 μg/ml collagen, 1 μM U46619, 1 unit/ml thrombin for 10 min (37 °C, stirring). Cox-2 was detected by Western analysis in monocytes lysates. Data shown represent 5 experiments performed with cells from different donors. β-actin was detected as a control of protein loading. (B) Releasates (plt releasate) obtained from 5×10^8 platelets activated by collagen, U46619, thrombin were incubated with adherent monocytes for 4 h (plts:monocyte ratio 100:1). Blot represents 4 experiments performed with cells from different donors. (C) Releasates from collagen-stimulated platelets (plt releasate) were incubated with adherent monocytes in various platelets/monocyte ratios, ranging from 20 to 500:1 for 4 h. Cox-2 and Cox-1 were detected by Western analysis. Data represent 3 experiments performed with cells isolated from different donors.

stirring). Centrifuge filter units (pore size 0.4 μm) were used to remove aggregates. Aliquots (100 μl) of purified platelets ($1–25\times10^8$) or platelet releasates were added to adherent monocytes (1 ml final volume) according to different platelets/monocyte ratios, ranging from 20 to 500:1. Incubations were carried out for 4–24 h. A platelet:monocyte ratio of 100:1 was used, unless specified. The endotoxin content of culture materials and reagents was measured by the *Limulus* amebocyte lysate assay (BioWhittaker) and only those containing <3 pg/ml endotoxin were used.

2.4. Western blot analysis

Cells were harvested in lysis buffer (pH 6.8), and blotting was performed as described [21]. Membranes were incubated for 1 h with antibodies directed against Cox-1 (5 μg/ml), Cox-2 (1/10,000) and p38 MAPK, phosphorylated and total (1/1000), and subsequently with peroxidase-conjugated secondary antibody (1/5000) for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech.).

2.5. Polymerase chain reaction of reverse-transcribed mRNA

Cellular RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies Italia SRL, Milano, Italy) and reverse transcribed (42 °C for 60 min). cDNA (1 μl) was applied for the amplification reaction, as described [22]. Cox-2 primers were: 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′, giving rise a 305-bp PCR product. All reactions were performed in a Bio-Rad Labs thermal cycler. PCR products were resolved on 2% agarose gel containing ethidium bromide. GAPDH mRNA was used as a control of mRNA loading.

2.6. Determination of thromboxane B_2 (TxB_2) and prostaglandin E_2 (PGE_2) release by adherent monocytes

Cox-2 activity was determined in adherent monocytes either unstimulated or exposed to releasates of collagen-stimulated platelets or to rTGF-β1 for 4 h. Cells were washed and incubated for 30 min in Hank's buffer (pH 7.4) containing 1 mg/ml bovine serum albumin and 10 μM arachidonic acid. TxB_2 and PGE_2 levels were measured by enzyme immunoassays (EIA, Cayman Chemical, Spi-bio, Montigny le Bretonneux, France).

2.7. Determination of TGF-β1 released by activated platelets

Total and active TGF-β1 were measured in releasates of collagen-stimulated platelets by EIA (Quantikine, R&D

Table 1
PGE_2 and TxB_2 production by monocytes exposed to releasates from activated platelets

	PGE_2 (ng/ml)	TxB_2 (ng/ml)
Monocytes	0.230±0.044	4.51±0.30
Platelet:monocyte ratio 20:1	0.298±0.047	11.22±2.07
Platelet:monocyte ratio 100:1	0.476±0.076*	15.35±3.88*
Platelet:monocyte ratio 500:1	0.570±0.054*	19.05±3.31*

Monocytes were incubated in medium alone or in the presence of releasates from collagen activated platelets for 4 h. Medium was then replaced with Hank's buffer containing arachidonic acid (10 μM) and bovine serum albumin (1 mg/ml). Incubation was continued for 30 min. Prostanoids were measured by EIA. Mean±S.E. of 5 separate experiments performed with cells isolated from different donors.
Statistically significant different *$p<0.05$ vs. monocytes alone.

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Systems, Space Import, Milano) (sensitivity <7 pg/ml), in accordance with the manufacturer's recommendations.

2.8. Statistical analysis

All experiments were performed with cell preparations from different donors. Data are expressed as mean±S.E. Grouped differences were compared with ANOVA (Fisher LSD and Dunnett's tests). $P \leq 0.05$ values were considered statistically different. Statistical analysis was performed by SigmaStat.

3. Results

3.1. Activated platelets induce rapid and transient Cox-2 expression in adherent monocytes

Human adherent monocytes were left untreated or incubated with resting or activated platelets (platelets/monocyte ratio 100:1, unless otherwise specified). After 4–24 h, platelets were removed and Cox-2 expression was determined in monocyte lysates. Monocytes did not express appreciable Cox-2 levels under resting condition or after incubation with unstimulated platelets (Fig. 1, A). In contrast, we found that mixing monocytes with platelets activated by various agents (collagen, thrombin, and the endoperoxide PGH_2 analogue U46619) resulted in Cox-2 expression (Fig. 1, A). Cox-2 induction was transient. Protein bands were detected in samples of monocytes incubated with activated platelets for 4 h, while they were almost undetectable when incubation was prolonged until 24 h (Fig. 1, A). Platelets obtained from different donors behaved similarly in the capacity to induce Cox-2 in monocytes.

Cox-2 induction was observed also when monocytes were exposed to cell-free releasates from platelets stimulated with various agents (Fig. 1, B). The extent of Cox-2 expression was dependent on the concentration of platelets used to prepare the releasates (Fig. 1, C). A platelet–monocyte ratio of 500:1 (platelets/monocyte) yielded maximal Cox-2 expression (Fig. 1, C). In contrast, Cox-1, which is expressed constitutively in monocytes, remained constant (Fig. 1, C). Platelet-induced Cox-2 was enzymatically active: TxB_2 and PGE_2 levels increased in the medium of monocytes exposed to platelet releasates after the addition of exogenous arachidonate. The amount of Cox-2 metabolites was dependent upon the concentration of platelets used to prepare the releasates (Table 1).

Cox-2 protein levels and TxB_2 synthesis induced by releasates were abolished by preincubation of monocytes with cycloheximide (Fig. 2, A), indicating that the enzyme is synthesized "*de novo*". TxB_2 production by monocytes exposed to releasates was completely prevented by NS398, a selective inhibitor of Cox-2 activity, identifying Cox-2 as the Cox isoform induced by platelets (Fig. 2, A). The occurrence of Cox-2 was consequent to the appearance of mRNA levels, which were undetectable in resting adherent monocytes (Fig. 2, B).

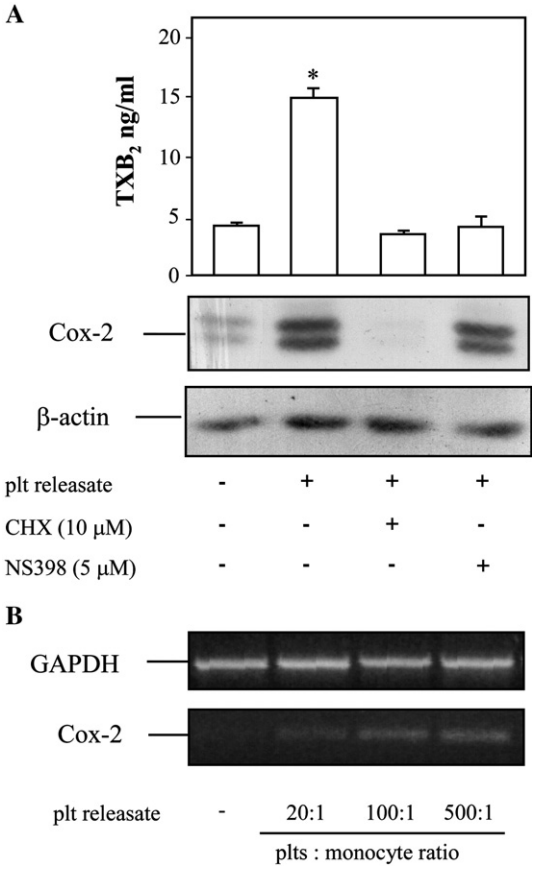

Fig. 2. Releasate from activated platelets induces *de novo* Cox-2 synthesis in adherent monocytes. (A) Monocytes were incubated with releasate of collagen-stimulated platelets (plt releasate), ratio 100:1, for 4 h. Cyclohexamide (CHX) and NS398 were added to monocytes 1 h before the addition of the releasate. Cox-2 protein was detected by Western analysis. Data represent 5 experiments performed with cells from different donors. β-actin was detected as a control of protein loading. TxB_2 levels were measured by EIA. Data are the mean±S.E. of 5 experiments performed with cells from different donors. *$p<0.0001$ vs. monocytes either untreated or exposed to CHX or NS398. (B) Monocytes were incubated with releasates from collagen-stimulated platelets (platelets/monocyte ratios from 20 to 500:1) for 2 h. Cox-2 mRNA was detected by RT-PCR. GAPDH mRNA is shown as a control for RNA loading. Data shown represent 3 experiments.

3.2. Platelet-derived thromboxane A_2 (TxA_2) is not involved in monocyte Cox-2 induction

TxA_2 is released in large amount by activated platelets and the α isoform of the thromboxane receptor (TPα) has been characterized in human peripheral blood monocytes [23]. Moreover, it has been reported that platelet-derived TxA_2 can act in paracrine manner to up-regulate Cox-2 in endothelial cells [24]. Prior incubation of platelets with ASA, that inhibits Cox-1, or with the thromboxane synthase inhibitor furegrelate, at concentration that reduces TxB_2 synthesis by 95.67±0.96% and 95.65±0.35%, respectively

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(n=3), did not influence Cox-2 induction (Fig. 3, A). In addition, Cox-2 was not affected in monocytes exposed to the TP antagonist SQ29548 prior to the addition of releasates (Fig. 3, A). This *in vitro* finding was reinforced by results from *ex vivo* experiments carried out in cells isolated from healthy volunteers who ingested 300 mg aspirin 16 h before blood sampling. Under these conditions, Cox-2 induced by activated platelets was still detectable (Fig. 3, B).

3.3. Role of platelet microparticles and glycoprotein IIb–IIIa complex in the modulation of Cox-2 induction by adherent monocytes

Platelet releasates were prepared by centrifugation of activated platelets in centrifuge filter units with a pore size of 0.4 μm. Therefore, a role of platelet microparticles that are rather heterogeneous in size, from 0.05 to 1 μm [25], cannot be ruled out. Microparticle formation is dependent on calcium and lipid raft integrity. Preincubation of platelets with EGTA that blocks the increase in cytosolic calcium and, as consequence, the release of microparticles [26], or with methyl-β-cyclodextrin, a non-invasive cholesterol acceptor that disrupt lipid rafts [25], did not alter Cox-2 induction by releasates (Fig. 3, C).

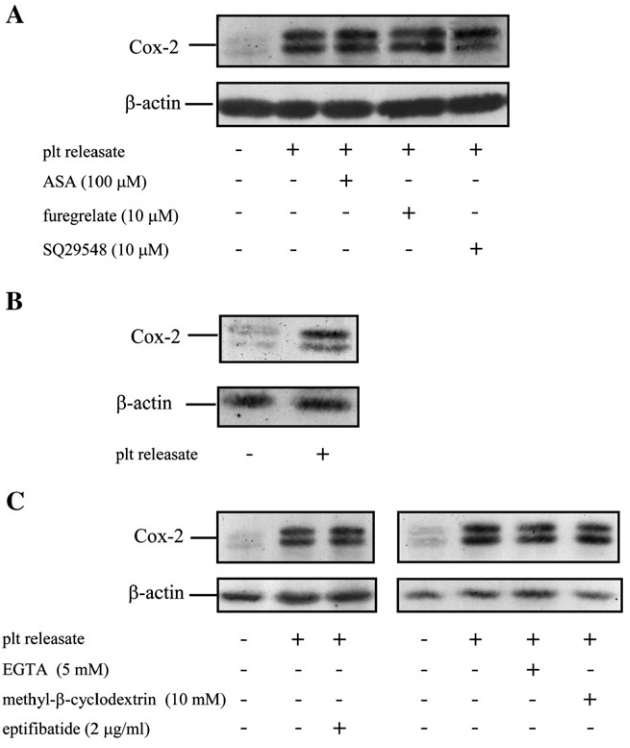

Fig. 3. Platelet-derived thromboxane A_2 (TxA_2) and platelet microparticles are not involved in monocyte Cox-2 induction. (A) Platelets were incubated with ASA or with the thromboxane synthase inhibitor furegrelate for 30 min. Platelet releasate was then added to adherent monocytes (plts:monocyte ratio 100:1) for 4 h. The thromboxane receptor antagonist SQ29548 was incubated with monocytes for 30 min before the addition of releasates. Cox-2 was detected in monocyte lysates by Western analysis. Data shown represent 5 experiments performed with cells from different donors. (B) Monocytes and platelets were isolated from healthy volunteers who ingested 300 mg aspirin 16 h before blood sampling. Monocytes were incubated with platelet releasates (plts:monocyte ratio 100:1) for 4 h. Cox-2 was detected by Western analysis. Data shown represent 5 experiments performed with cells from different donors. (C) Platelets were incubated with EGTA or eptifibatide (5 min) or with methyl-β-cyclodextrin (30 min). Releasates were then added to adherent monocytes (plts:monocyte ratio 100:1) for 4 h. Cox-2 was detected in monocyte lysates by Western analysis. Data shown represent 4 experiments performed with cells isolated from different donors.

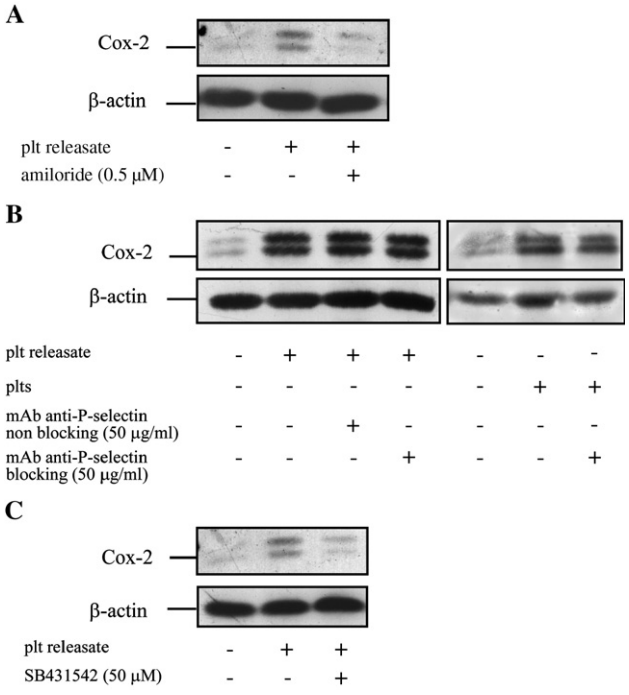

Fig. 4. Role of platelet α-granule in monocyte Cox-2 induction. (A) Platelets were incubated with amiloride (10 min) or (B) with monoclonal antibodies (mAb) against P-selectin (5 min) and then stimulated with 6 μg/ml collagen. Releasates (plt releasate) or whole platelets (plts) were incubated with monocytes (plts:monocyte ratio 100:1) for 4 h. (C) Adherent monocytes were incubated with the TGF-β1-receptor antagonist SB431542 for 30 min and subsequently exposed to platelet releasate for 4 h. Cox-2 protein was detected in monocyte lysates by Western analysis. Data represent 5 experiments performed with cells isolated from different donors.

Platelet microparticle formation is also linked to the glycoprotein IIb–IIIa complex activation. Indeed, RGD-containing peptides impair the release of microparticles [27]. Platelet incubation with eptifibatide (Integrilin®), that prevents the binding of fibrinogen to IIb–IIIa complex [28], did not affect Cox-2 levels induced by releasates (Fig. 3, C). In this condition, platelet aggregation was inhibited by more than 80% (data not shown).

3.4. Role of platelet α-granule secreted components in monocyte Cox-2 induction

The release of platelet α-granules is central to heterotypic interactions. It occurs more readily than dense granule secretion in response to weak agonists and is not prevented by cyclooxygenase and glycoprotein IIb–IIIa inhibitors

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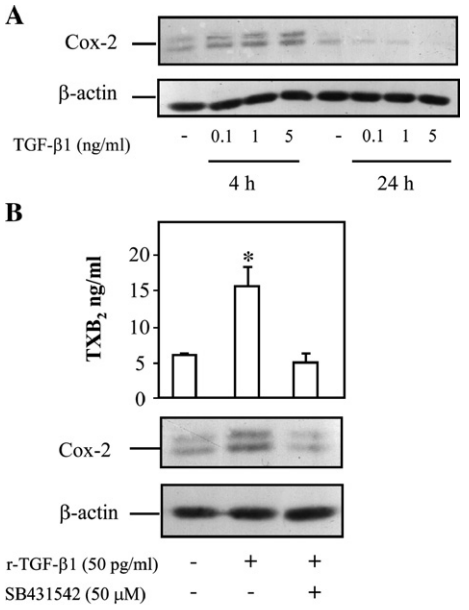

Fig. 5. TGF-β1 induces Cox-2 protein and activity in human adherent monocytes. (A) Monocytes were exposed to TGF-β1 from platelet source for 4–24 h. Cox-2 protein was detected by Western analysis. Data shown represent 4 experiments performed with cells isolated from different donors. (B) Monocytes were incubated for 30 min with the TGF-β1 receptor antagonist SB431542 before the addition of rTGF-β1 for 4 h. Cox-2 protein was detected by Western analysis. Data shown represent 4 experiments performed with cells isolated from different donors. TxB_2 levels were measured by EIA in the medium containing arachidonic acid. Data are the mean±S.E. of 4 experiments performed with cells from different donors. *$p<0.01$ vs. monocytes either untreated or exposed to SB431542.

[29,30]. In contrast, the decrease in intracellular pH has a negative effect on α-granule release [29]. Cox-2 induction was prevented by amiloride that reduces intracellular pH through inhibition of the Na^+/H^+ transporter, indicating that the release of α-granules is essential for Cox-2 induction by platelets (Fig. 4, A).

P-selectin and growth factors are stored in relevant amounts in α-granules. Following platelet activation P-selectin translocates from α-granules to the outer surface where it allows the interaction with other cells. The contribution of individual mediators released from granules or exposed on platelet surface was assessed by the use of inhibitors that target specific signaling pathways. Whole platelets or releasates were incubated with antibodies to P-selectin for 5 min and subsequently added to monocytes. Nonblocking (negative control) or blocking mAb both did not affect Cox-2 levels (Fig. 4, B). In contrast, incubation of monocytes with SB431542 that antagonizes the TGF-β1 receptor [31], completely abrogated Cox-2 induction (Fig. 4, C). Similar results were obtained with whole platelets (data not shown).

Table 2
TGF-β1 levels in releasates from activated platelets

TGF-β1 (ng/ml)		
Platelets/ml	Active	Total
5×10^7	0.355±0.07	3.289±0.23
25×10^7	1.544±0.81	23.670±4.49

Levels of total and active TGF-β1 were measured by EIA in releasates from platelets stimulated with collagen (6 μg/ml) for 10 min (37 °C, stirring). Results are the mean±S.E. of 3 separate experiments performed with platelets isolated from different donors.

3.5. Effect of rTGF-β1 and $rPDGF_{BB}$ on Cox-2 induction in monocytes

TGF-β1 derived from human platelets increased monocyte Cox-2 levels within 4 h (Fig. 5, A). The effect was transient, similarly to what was observed with platelets (Fig. 5, A). Cox-2 levels also increased in monocytes exposed to rTGF-β1 (Fig. 5, B). TxB_2 levels produced were fully comparable to those measured when monocytes were incubated with platelet releasates (Fig. 5, B). Blocking TGF-β1 receptor with SB431542 reduced Cox-2 levels and TxB_2 synthesis (Fig. 5, B). The effect of TGF-β1 was exclusive since it was not shared by $rPDGF_{BB}$ at concentration of 10–20 ng/ml (data not shown).

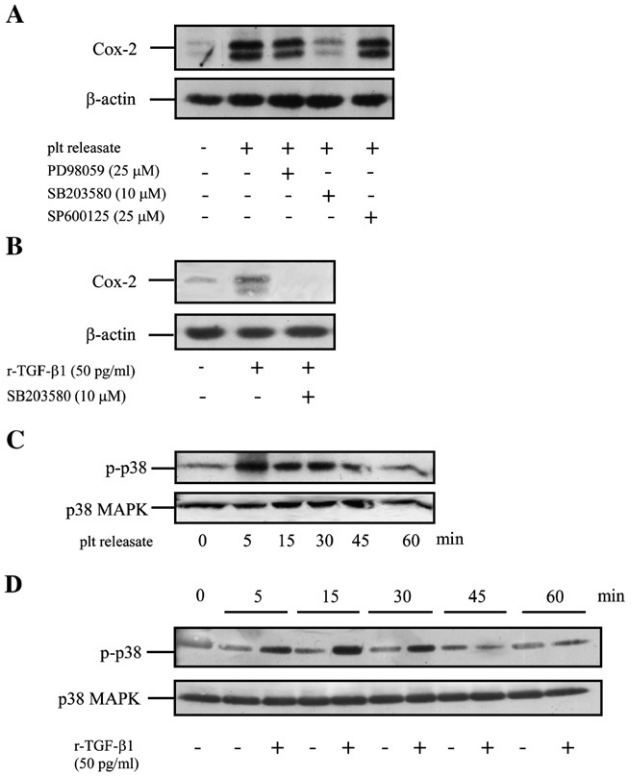

Fig. 6. p38 MAPK is involved in Cox-2 induction by platelet releasates and by rTGF-β1. (A, B) Monocytes were incubated with the MAPK inhibitors for 30 min before the addition of platelet releasate (plts:monocyte ratio 100:1) or rTGF-β1 for 4 h. Cox-2 protein was detected by Western analysis. Data shown represent 4 experiments performed with cells isolated from different donors. β-actin was detected as a control of protein loading. (C, D) Time-dependent p38 MAPK phosphorylation by platelet releasates or rTGF-β1. Monocyte lysates were analyzed by Western analysis for phosphorylated and total p38 MAPK. The blots shown are from 4 to 5 experiments performed with cells isolated from different donors.

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3.6. Determination of TGF-β1 levels in platelet releasates

In response to activation, platelets release latent TGF-β1 that can be activated through a variety of mechanisms [32]. Levels of TGF-β1 (total and active) measured in releasates of collagen-stimulated platelets were dependent upon the concentration of platelets used to prepare the releasates. Of note, the amount of active TGF-β1 measured in releasates from 5×10^7 platelets (ratio 100:1) was consistent with the concentration of exogenous TGF-β1 capable of inducing Cox-2 in monocytes (Table 2).

3.7. Selective activation of p38 MAPK by platelets and TGF-β1

To elucidate the role of MAP-kinase activation in Cox-2 induction by both releasates and rTGF-β1, monocytes were preincubated for 30 min with PD98059 or SB203580 or SP600125 that inhibit ERK1/2, p38 and c-*jun* N-terminal kinase, respectively. They were subsequently exposed to releasates from activated platelets for further 4 h. SB203580 completely abrogated Cox-2 induction whereas PD98059 or SP600125 had no effect (Fig. 6, A). Similarly to what was observed with releasates, SB203580 completely prevented Cox-2 induction by rTGF-β1 (Fig. 6, B). Both releasates and rTGF-β1 caused rapid phosphorylation of p38 MAPK, detectable at 5 min incubation and declining after 30 min (Fig. 6, C and D). Of note, the kinetic fashion of p38 MAPK phosphorylation induced by releasates or by rTGF-β1 was fully comparable. No difference was detected in control points at different incubation times (see panel D).

Platelet releasates failed to induce ERK1/2 and c-*jun* phosphorylation, thus excluding a role of these pathways in Cox-2 induction and supporting the results obtained with pharmacological agents (data not shown).

4. Discussion

In addition to their crucial activity in haemostasis, platelets play a complex role in inflammatory and immune responses [5], and in wound healing [12]. This occurs through a variety of mechanisms, including a number of mediators, either preformed or newly synthesized, surface molecules, and the recently recognized synthetic capacity [33]. All these tools equip the platelet for signaling inflammatory cells.

In this study, we report on the discovery of a novel mechanism through which platelets participate to an immediate inflammatory/reparative response. As we were able to show, activated platelets induce rapid and transient Cox-2 expression in adherent monocytes. This effect is fully mimicked by platelet releasates and by TGF-β1, either recombinant or from platelet source. The time course of Cox-2 induction is peculiar for its transience. Cox-2 levels were observed after 4 h incubation and declined when incubation time was prolonged to 24 h, suggesting that the newly induced enzyme equips monocytes for an immediate response that is self-limiting in time. Of interest, this temporal profile is similar to that reported in vascular smooth muscle cells for the expression of thrombin receptors in response to platelet-derived products [34].

A role for Cox-2 expressed by monocyte/macrophages in promoting the inflammatory process of atherogenesis and plaque destabilization has been highlighted [35,36,3]. Indeed, Cox-2-mediated PGE_2 overproduction by monocytes is proposed as a marker of sub clinical atherosclerosis [2]. By contrast, monocytes/macrophage derived PGE_2 has been implicated in down-regulating expression of inflammatory cytokines [4] and in the self-limitation of the inflammatory response [37].

TGF-β1 and PDGF are present in large amounts in α-granules that are the richest source of TGF-β1 in humans [38]. TGF-β1 is stored in a latent, biologically inactive form and released upon platelet stimulation. The hypothesis that TGF-β1 is the physiologically relevant mediator of Cox-2 induction by aggregating platelets is supported by the finding that TGF-β1 induces Cox-2 expression at concentrations that are measured in releasates of stimulated platelets. The dose-dependent effect of TGF-β1 is, however, less apparent than that of platelet releasates, suggesting that other mechanisms may contribute to increase Cox-2 levels in monocytes exposed to releasates.

The ability of TGF-β1 to induce Cox-2 has been reported in smooth muscle cells from human airway [39,40] and, more recently, in glomerular mesangial cells [41]. Cox-2 is crucial for monocyte differentiation into macrophages [22]. In addition, it protects monocytes against apoptosis [42] that represents a crucial event for their survival during the inflammatory reaction and tumorigenesis. Moreover, Cox-2 is recognized as key regulator of bone biology, being critically involved in fracture repair [43]. Within this context, TGF-β1 induces osteoblastic cell proliferation through the local release of PGE_2 [44].

Cell to cell contact and the involvement of P-selectin have been shown as essential for chemokine synthesis and Cox-2 expression in platelet–monocyte aggregates [45,46]. In our condition, rapid Cox-2 induction was observed also in monocytes exposed to releasates of activated platelets, excluding the requirement of cell to cell contact. The condition of monocyte culture (i.e. suspended or adherent), of platelet–monocyte co-incubation and stimulation, and the engagement of monocyte P-selectin receptor by adhesion to immobilized P-selectin, may be critical for the different results obtained. Of interest, a positive paracrine regulation of Cox-2, not requiring cell–cell contact, has been observed also in adherent monocytes co-cultured with T lymphocytes [47].

Among signal transduction pathways leading to Cox-2 induction in monocytes, activation of p38 MAPK plays a prominent role independently to the type of stimulus [48,49]. The activation of p38 MAPK by TGF-β1 and its involvement in TGF-β1-induced transcriptional activation

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has been described [50]. In addition, the presence of a TGF-β1-response element on Cox-2 promoter [51] may account for the up-regulation of Cox-2 observed in our condition. It seems likely, however, that the transient Cox-2 induction by rTGF-β1 or by platelet releasates is attributable to a mechanism involving mRNA stabilization, as occurs in other cell types [52,53]. Of interest, p38 MAPK activation is essential for stabilizing Cox-2 mRNA in lipopolysaccharide-treated human monocytes [48].

In conclusion, the present study shows that aggregating human platelets stimulate the expression of Cox-2 in adherent monocytes. The effect occurs through the release of TGF-β1 that acts as a positive paracrine regulator of Cox-2 induction. Our finding fits well with the newly described role of TGF-β1 as a "recovery" signal incorporated in both repair and regulation [54]. More in general, it supports the view that the interaction of monocytes with platelet products at vascular interfaces may establish a typical gene expression profile, enabling the cells to afford a co-ordinate response to injury in terms of inflammatory reaction and tissue repair. In this scenario, the observation that Cox-2 inhibitors may increase the risk of cardiovascular events [55] strengthens the beneficial role of Cox-2 in the repair process of injured endothelium.

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