De Novo Synthesis of Cyclooxygenase-1 Counteracts the Suppression of Platelet Thromboxane Biosynthesis by Aspirin

Virgilio Evangelista, Stefano Manarini, Angelo Di Santo, Marta L. Capone, Emanuela Ricciotti, Luigia Di Francesco, Stefania Tacconelli, Andrea Sacchetti, Sandra D'Angelo, Antonio Scilimati, Maria G. Sciulli, Paola Patrignani

Aspirin affords cardioprotection through the acetylation of serine[529] in human cyclooxygenase-1 (COX-1) of anucleated platelets, inducing a permanent defect in thromboxane A_2 (TXA_2)–dependent platelet function. However, heterogeneity of COX-1 suppression by aspirin has been detected in cardiovascular disease and may contribute to failure to prevent clinical events. The recent recognized capacity of platelets to make proteins de novo paves the way to identify new mechanisms involved in the variable response to aspirin. We found that in washed human platelets, the complete suppression of TXA_2 biosynthesis by aspirin, in vitro, recovered in response to thrombin and fibrinogen in a time-dependent fashion (at 0.5 and 24 hours, TXB_2 averaged 0.1±0.03 and 3±0.8 ng/mL; in the presence of arachidonic acid [10 μmol/L], it was 2±0.7 and 25±7 ng/mL, respectively), and it was blocked by translational inhibitors, by rapamycin, and by inhibitors of phosphatidylinositol 3-kinase. The results that COX-1 mRNA was readily detected in resting platelets and that [^{35}S]-methionine was incorporated into COX-1 protein after stimulation strongly support the occurrence of de novo COX-1 synthesis in platelets. This process may interfere with the complete and persistent suppression of TXA_2 biosynthesis by aspirin necessary for cardioprotection.

Aspirin affords cardioprotection inducing a complete and permanent defect in the capacity of platelets to generate thromboxane A_2 (TXA_2) through the acetylation of serine[529] of cyclooxygenase-1 (COX-1).[1,2] Because of a nonlinear relationship of inhibition of platelet TXA_2 generation with inhibition of TXA_2-mediated platelet aggregation, an excess of 95% inhibition of COX-1 activity is required to influence platelet function.[3] In fact, even tiny concentrations of TXA_2 have been shown to cause platelet activation. Thus, 10 nmol/L of the TXA_2 mimetic U46619 induces platelet adhesion and shape change,[4] and in the presence of a subthreshold concentration of collagen, U46619 (0.5 to 10 nmol/L) causes platelet aggregation.[5] Recently, Maree et al[6] showed that many patients who are treated with low-dose enteric-coated aspirin (75 mg) for secondary prevention of cardiovascular events have persistent elevated serum TXB_2 levels (>2.2 ng/mL), which translates into a more frequent occurrence of arachidonic acid (AA)–induced platelet aggregation. Reduced bioavailability of aspirin[6] and genetic variants in COX-1[7] may participate in the intersubject variable response to aspirin. The recent recognized capacity of platelets to make proteins de novo[8] paves the way to identify new mechanisms involved in aspirin failure to cause complete and persistent suppression of platelet COX-1 activity in some individuals.

Thus, in the present study, we assessed, in vitro, the hypothesis that de novo synthesis of COX-1 could account for TXA_2 biosynthesis in platelets in which the activity of preformed COX-1 was blocked by pretreatment with aspirin in vitro.

Materials and Methods

Healthy volunteers (n=9 to 22; mean age 40±5 years) who provided blood for the study did not assume aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) within 10 days before donation. Platelet-rich plasma (PRP)[9] was incubated for 30 minutes at 37°C without or with 0.3 mmol/L of aspirin. Platelets were then isolated from plasma as described previously[9] and resuspended in RPMI 1640 supplemented with 0.5% FCS. Platelet suspension contained <1 leukocyte per 10 000 platelets. Unless otherwise indicated, 5×10^8 platelets/mL were used for each experimental point. Mononuclear cells were separated from whole blood containing heparin (10 IU/mL) by Ficoll-Paque and stimulated for 12 hours with lipopolysaccharide (LPS; 10 μg/mL).[10] Aspirin-treated or untreated platelets were incubated in the absence or in the presence of fibrinogen (0.38 mg/mL) alone or with thrombin (1 U/mL), for different times, at 37°C. The reaction was stopped by immediate centrifugation and supernatants stored at −80°C. In some experiments, exogenous AA (10 μmol/L) was added at different times, and the incubation carried on for an additional 10 minutes. In selected experiments, the following translation or signal transduction inhibitors, cycloheximide (1 mmol/L), puromicine (500 μmol/L), rapamycin (100 nmol/L), actinomycinD (1 mmol/L), staurosporine (10 μmol/L), wortmannin (100 nmol/L), LY294002 (10 μmol/L), AG490 (10 μmol/L), or vehicle (dimethylsulfoxide 0.1%), were added to platelets 10 minutes before starting incubation with fibrinogen and thrombin. The effects of a selective and reversible COX-1 inhibitor (P6),[11] aspirin, and 2 selective COX-2 inhibitors, rofecoxib and etoricoxib, were studied by their addition to platelets immediately before starting the incubation with thrombin and fibrinogen. In platelet supernatants, TXB_2 was evaluated by radioimmunoassay,[1] reflecting TXA_2 generation. All reagents were from Sigma-Aldrich.

RT-PCR of COX-1 and COX-2 and radiolabeling of platelets and immunoprecipitation of COX-1 are described in the expanded Material and Methods section in the online data supplement, available at http://circres.ahajournals.org.

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Statistical Analysis

Data, presented as means±SEM, were analyzed by repeated-measurement ANOVA. $P<0.05$ was considered statistically significant.

Results and Discussion

Aspirin (0.3 mmol/L) pretreatment of PRP of healthy individuals was associated with an almost complete suppression of TXA_2 generation in washed platelets incubated for 30 minutes with fibrinogen (0.38 mg/mL) and thrombin (1 U/mL; 162.3±30.5 versus 0.1±0.03 ng/mL; n=9). A significant increase of TXA_2 generation was detected in supernatants of aspirinated platelets cultured for 24 hours with fibrinogen and thrombin (3±0.8 ng/mL; n=22) but not in resting platelets or when they were incubated with fibrinogen alone (Figure 1A). A similar effect was detected with other platelet agonists (ADP [20 μmol/L], collagen [5 μg/mL], or thrombin receptor–activating peptide [50 μmol/L]; supplemental online Table). The generation of TXA_2 in aspirinated platelets was significantly increased in the presence of exogenous AA (10 μmol/L; at 0.5 versus 24 hours: 2±0.7 and 25±7 ng/mL [n=3]; $P=0.0004$ and $P<0.0001$ versus the same time points without exogenous AA; supplemental online Figure). These concentrations of TXA_2 are biologically active[4–6] and, in the presence of other platelet agonists, may further boost platelet responses.[4,5]

As shown in Figure 1B, TXA_2 generation was depressed in a concentration-dependent fashion by P6, a COX-1 inhibitor,[11] and by aspirin (added de novo immediately before starting incubation) but not by etoricoxib or rofecoxib, 2 COX-2 inhibitors. The potency of aspirin to inhibit the recovery of TXA_2 synthesis in aspirinated platelets at 24 hours of incubation (IC_{50}, 22 μmol/L; 95% CI, 9 to 56) was significantly lower than that found at 30 minutes (IC_{50}, 4.5 μmol/L; 95% CI, 3.8 to 5.5; data not shown). This led us to hypothesize that time-dependent de novo synthesis of COX-1 protein could account for recovered COX-1 activity. Consistently, we demonstrated that TXA_2 biosynthesis in aspirinated platelets stimulated with fibrinogen and thrombin occurred by a regulated pathway for protein translation. In fact, it was prevented by puromycin, which causes the premature release of nascent polypeptide chains, cycloheximide, which blocks the translocation reaction on ribosomes and rapamycin, a bacterially derived immunosuppressant that inhibits the translation of a specific subset of mRNAs. In contrast, actinomycin D, a transcriptional inhibitor, did not affect the biosynthesis of this prostanoid. In addition, we found that inhibitors of phosphatidylinositol 3-kinase, LY294002 and wortmannin, also blocked TXA_2 recovery, indicating that this response is phosphatidylinositol 3-kinase dependent. In contrast, AG-490, a tyrosine kinase inhibitor, and staurosporine, a protein kinase C inhibitor, did not significantly affect TXA_2 generation in this setting (Figure 1C). The apparent reduction obtained by staurosporine may be attributable to its possible interaction with phosphatidylinositol 3-kinase.

Thus, aspirin-treated platelets recovered their ability to produce TXA_2 via a signal-dependent de novo protein synthesis. We hypothesized that this protein could be COX-1 itself. First, we explored whether platelets contained mRNA for COX isozymes. As shown in Figure 2A, COX-1, but not COX-2, mRNA can be detected by RT-PCR in resting platelets (lanes 1 and 3) as well as in platelets incubated for

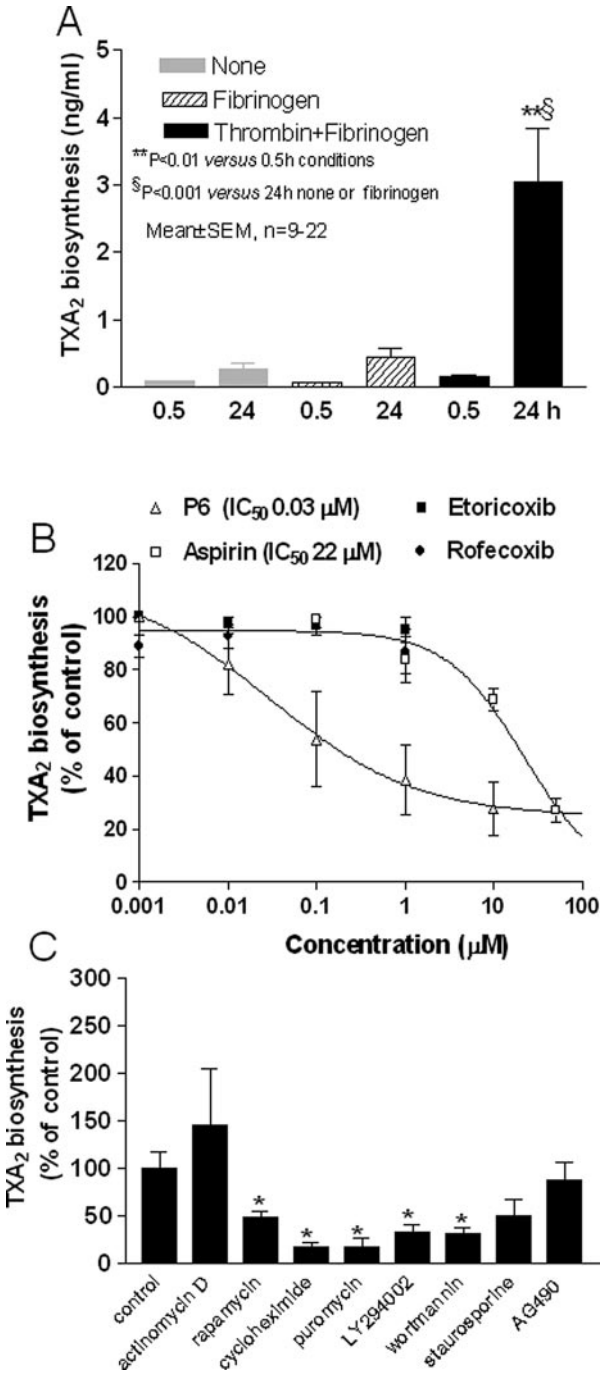

Figure 1. Aspirin-treated platelets recover COX-1–dependent capacity to synthesize TXA_2: Role of translational and signal transduction pathways. A, PRP from healthy donors was treated for 30 minutes at 37°C with aspirin (0.3 mmol/L), and washed platelets were incubated for 0.5 or 24 hours without or with fibrinogen (0.38 mg/mL) alone or with thrombin (1 U/mL), and TXA_2 biosynthesis was assessed.[1] B, Aspirin-treated platelets, 5×10^8/mL, activated by thrombin and fibrinogen were incubated for 24 hours without or with aspirin, P6 (a reversible and selective COX-1 inhibitor),[11] and 2 selective COX-2 inhibitors, etoricoxib and rofecoxib, and with inhibitors of translation and signal transduction (C).

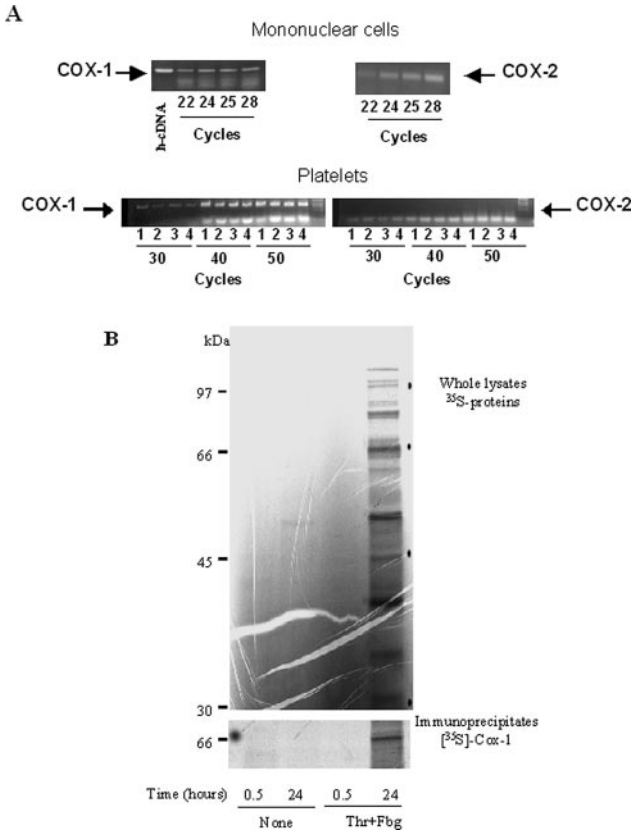

Figure 2. Platelets synthesize de novo COX-1 from pre-existing mRNA. A, Detection of COX-1 but not COX-2 mRNA in resting or activated platelets. Total RNA was extracted from $2–3\times10^6$ mononuclear cells, stimulated for 12 hours with LPS (10 μg/mL) or from 8×10^9 platelets, from 2 donors (lanes 1 and 2, donor 1; lanes 3 and 4, donor 2), before (lanes 1 and 3) or after 3 hours of incubation with thrombin and fibrinogen (lanes 2 and 4). One microgram of total RNA served as template for RT-PCR. This figure is representative of 3 different experiments. B, Radiolabeled incorporation of [^{35}S]-methionine into COX-1 in platelets activated with thrombin and fibrinogen. Platelets (1×10^9/mL) were incubated with 50 μCi [^{35}S]-methionine in the absence or in the presence of both fibrinogen and thrombin for 0.5 or 24 hours. Total cell lysates and immunoprecipitates, with a goat anti-human COX-1 antibody, were subjected to SDS-PAGE.[10] The figure is representative of 5 different experiments.

3 hours in the presence of both fibrinogen and thrombin (lanes 2 and 4). As positive control, COX-2 mRNA was detected in purified human mononuclear cells activated with LPS. To explore platelet ability to translate mRNA in new proteins, they were loaded with [^{35}S]-methionine and then cultured in the absence or presence of both fibrinogen and thrombin. As shown in Figure 2B, several [^{35}S]-methionine–labeled proteins were synthesized in activated platelets. Immunoprecipitation with an anti–COX-1–specific antibody yielded a [^{35}S]-methionine–labeled protein corresponding to the exact COX-1 size.

In conclusion, signal-dependent de novo synthesis of COX-1 occurring in aspirin-treated platelets after activation may represent a mechanism involved in the interference of complete and persistent suppression of TXA_2 biosynthesis by aspirin necessary for cardioprotection. The occurrence of this phenomenon in vivo in patients with cardiovascular disease treated with low-dose aspirin remains to be verified. Our results may contribute to shed some light on the rather nebulous concept of aspirin resistance.

Acknowledgments

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KEY WORDS: aspirin ■ thromboxane ■ platelets ■ protein synthesis

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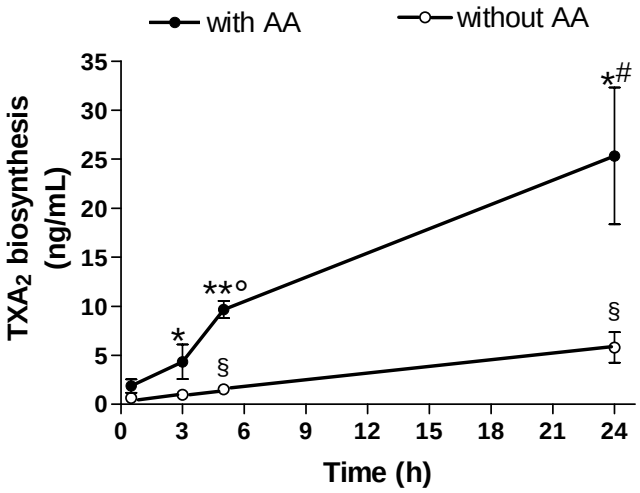
SUPPLEMENTARY INFORMATION

Evangelista *et al*
De novo synthesis of cyclooxygenase-1 counteracts the suppression of platelet thromboxane biosynthesis by aspirin

Expanded Material and Methods

RT(Reverse Transcription)-PCR
Total RNA was isolated from washed platelets ($8x10^9$/mL) and from human mononuclear cells($2-3x10^6$/mL). One µg of total RNA served as a template for RT-PCR of COX-1, COX-2 and GAPDH as previously described.[1] PCR was performed for 30, 40 or 50 cycles using a cycling program of 1 minute at 94°C, 1 minute at 64°C and 1 minute at 72°C for COX-1 and 1 minute at 94 °C, 1 minute at 60°C and 1 minute at 72°C for COX-2.
Radiolabeling of platelets and immunoprecipitation of COX-1. Platelets($1x10^9$/mL) resuspended in methionine-free M199 medium were incubated with 50 µCi [^{35}S]-methionine(Amersham) in the absence or in the presence of fibrinogen alone or with thrombin for 24h. The reaction was stopped by addition of one volume lysis buffer. Total cell lysates and immunoprecipitates, with a goat anti-human COX-1 polyclonal antibody(Santa Cruz, Biotechnology), were boiled for 10minutes and then subjected to SDS-PAGE.[2]

Supplementary Figure



Time-dependent recovery of COX-1 activity in aspirin-treated platelets. PRP from healthy donors was treated for 30 minutes at 37°C with aspirin(0.3mmol/L) and washed platelets[3] ($5x10^8$/mL) were incubated for 0.5-24h with thrombin(1U/mL). At 0.5, 3, 5 and 24 h, AA (10 μmol/L) was added and the incubation carried on for further 10 minutes at 37°C. The reaction was stopped by immediate centrifugation and supernatants stored at -80°C until assayed for TXB_2 levels by radioimmunoassay[4], as a reflection of TXA_2 biosynthesis. Values are reported as mean±SEM, n=3. *P<0.05, **P<0.01 AA versus the same time without AA; §P<0.05 versus time 0.5h without AA; #P<0.05, °P<0.01 versus time 0.5h with AA.

Supplementary Table

Aspirin-treated platelets recover COX-1-dependent capacity to synthesize TXA_2 in response to platelet agonists

Time (h)	None	Fibrinogen	Collagen fibrinogen	ADP fibrinogen	TRAP fibrinogen
	TXA_2 biosynthesis (ng/mL)				
0.5	0.08±0.01	0.06±0.01	0.19±0.03	0.08±0.02	0.07±0.02
24	0.3±0.1	0.4±0.1	4.99±1.40*§	2.51±0.09**§	2.0±0.19**§

PRP from healthy donors was treated for 30 minutes at 37°C with aspirin (0.3 mmol/L) and washed platelets[3] ($5x10^8$/mL) were incubated for 0.5 and 24 h without (none) or with fibrinogen (0.38 mg/mL) alone or fibrinogen and collagen (5 μg/mL), ADP (20 μmol/L) or thrombin receptor–activating peptide (TRAP, 50 μmol/L). The reaction was stopped by immediate centrifugation and supernatants stored at -80°C until assayed for TXB_2 levels by radioimmunoassay[4], as a reflection of

TXA_2 biosynthesis. *P<0.05, **P<0.001 *versus* time 0.5 h; §P<0.001 *versus* none 0.5, 24 h and *versus* fibrinogen 0.5, 24 h. Values are reported as mean±SEM, n=3.

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