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COX-2 IS NOT INVOLVED IN THROMBOXANE BIOSYNTHESIS BY ACTIVATED HUMAN PLATELETS

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The occurrence of aspirin resistance has been inferred by the assessment of platelet aggregation *ex vivo* in patients with ischemic vascular syndromes taking aspirin. Since aspirin is a weak inhibitor of the inducible isoform of prostaglandin H synthase (COX-2), it was suggested that COX-2 may play a role in aspirin resistance. However the cellular source(s) of COX-2 possibly responsible for aspirin resistance remains unknown. Recently, the expression of the inducible isoform of COX-2 in circulating human platelets was reported. To investigate the possible contribution of COX-2 expression in platelet thromboxane (TX) biosynthesis, we have compared the inhibitory effects of NS-398 and aspirin, selective inhibitors of COX-2 and COX-1, respectively, on prostanoid biosynthesis by thrombin-stimulated platelets *vs* lipopolysaccharide (LPS)-stimulated monocytes (expressing high levels of COX-2) isolated from whole blood of healthy subjects. NS-398 was 180-fold more potent in inhibiting monocyte COX-2 activity than platelet TXB_2 production. In contrast, aspirin (55 μmol/L) largely suppressed platelet TXB_2 production without affecting monocyte COX-2 activity. By using specific Western blot techniques, we failed to detect COX-2 in platelets while COX-1 was readily detectable. Our results argue against the involvement of COX-2 in TX biosynthesis by activated platelets and consequently dispute platelet COX-2 expression as an important mechanism of aspirin resistance.

Key words: *Thromboxane; platelets; COX-2; COX-1; aspirin*

INTRODUCTION

Aspirin resistance is a phenomenon initially observed in some patients with ischemic vascular syndromes by the assessment of platelet aggregation *ex vivo*. Some patients developed aspirin resistance over time and even with high doses

of aspirin (1, 2). However, the uncontrolled nature of the studies as well as the lack of measurements of platelet cyclooxygenase acetylation by aspirin makes the interpretation of these findings highly questionable. Vejar *et al.* (3) reported the occurrence of episodes of aspirin-resistant thromboxane (TX) A_2 biosynthesis *in vivo*, in a subset of patients with unstable angina.

The clinical efficacy of aspirin in the prevention of arterial thrombosis is largely accounted for by the inhibition of the synthesis of TXA_2, the main platelet agonist produced by activated platelets, through the irreversible and selective acetylation of a single serine residue of prostaglandin H synthase (PGHS) (4—6). PGHS, the first rate-limiting enzyme in the synthesis of prostanoids from arachidonic acid, exists as constitutive and inducible isoforms (designated as COX-1 and COX-2, respectively) (7). Aspirin is a weak inhibitor of human COX-2, hence COX-2 may reasonably play a role in aspirin resistance (8), but nothing is known on the cellular source(s) of COX-2 possibly responsible for this phenomenon.

Because circulating platelets lack a nucleus and consequently cannot synthesize mRNA, the involvement of constitutively expressed COX-1 in TXA_2 biosynthesis has been assumed. This is supported by cDNA cloning and immunoaffinity purification of only COX-1 from human platelets (9).

In contrast, Weber *et al.* (10) have recently reported that circulating platelets from healthy subjects express COX-2 protein and mRNA, as detected by Western blot and reverse transcription coupled polymerase chain reaction (RT-PCR), respectively.

In order to investigate the potential involvement of COX-2 expression in platelet TXA_2 biosynthesis, we have studied the inhibitory effects of NS-398, a selective COX-2 inhibitor (11), and aspirin, a selective COX-1 inhibitor (8), on constitutive and inducible prostanoid biosynthesis by platelets *vs* monocytes. Moreover, we have examined the expression of inducible COX-2 and constitutive COX-1 in circulating platelets *vs* monocytes of healthy subjects by specific Western blot techniques (11).

METHODS

Preparation of platelets and monocytes

Blood was collected from healthy volunteers who had not taken aspirin or other aspirin-like drugs in the two-week period preceding the study. Platelets were isolated from citrated human whole blood of 7 healthy volunteers (4 male and 3 female subjects, aged 20 to 30 years), and washed as described (12). Briefly, washed platelets were prepared by centrifugating platelet-rich plasma at $1{,}100 \times g$ for 15 min after addition of 1 − μmol/L of PGE_1. The pellet was then resuspended in N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) Tyrode buffer (pH 7.4) containing 1μmol/L PGE_1 and 5 mmol/L ethylene glycol-bis (b-aminoethyl ether)-N-N-N'-N'-tetraacetic acid (EGTA) and centrifuged at $1{,}100 \times g$ for 10 min. Platelets were then resuspended in HEPES Tyrode

at a concentration of 5×10^8/mL. Mononuclear cells were separated from whole blood containing heparin (10 IU/mL) using a Ficoll-Paque gradient (11). Monocytes were isolated from the mononuclear fraction by washing off the non-adherent cells after incubation for 1 h at 37°C. The adherent cells were recovered by gently scraping with a rubber policeman and resuspended (2×10^6 cells/mL) in RPMI 1640 medium buffered with 0.05 M of HEPES, pH 7.4, supplemented with 0.5% heat-inactivated foetal calf serum (FCS) and 4 mmol/L L-glutamine; their viability (>96%) was examined by trypan blue exclusion. The cell suspension was constituted by >90% monocytes.

Effects of NS-398 on prostanoid production by platelets and monocytes

Washed human platelets (2×10^8 cells/mL) were incubated for 30 min at 37°C with increasing concentrations of NS-398 or DMSO vehicle, and then for further 4 min with thrombin (0.5 IU/mL). Isolated human monocyte (2×10^6 cells/mL) were incubated with increasing concentrations of NS-398 or DMSO vehicle in the presence of lipopolysaccharide (LPS; 10 μg/mL) for 24 h at 37°C. Supernatants of platelets and monocytes were assayed for TXB_2 and PGE_2, respectively, by previously described and validated radioimmunoassays (13, 14).

Western blot analysis of COX-1 and COX-2 in human platelets and monocytes

Platelets (5×10^8 cells/mL) and monocytes (2×10^6 cells/mL; unstimulated or incubated for 24 h with LPS, 10 μg/mL) were analyzed for the content of COX-1 and COX-2 by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques, as previously described (11). Briefly, cells were lysed and equal amounts of proteins (50 μg) were separated by 9% polyacryalmide gel electrophoresis in the presence of SDS. The resolved proteins were electroblotted onto nitrocellulose membranes and treated with blocking buffer (5% fat-free dry milk in Tris-buffered saline containing 1% Tween—20) before the incubation (at room temperature for 2 h) with specific rabbit polyclonal antisera (1:1,000 dilution) directed against COX-1 or the carboxyl-terminal portion of human COX-2 (kindly provided by Drs. W.L. Smith, Department of Biochemistry, Michigan State University, East Lansing, MI, and J. Maclouf, INSERM, Paris, France, respectively). Immune complexes were visualized by incubating the membranes with biotin-conjugated anti-rabbit IgG (1:2,000 dilution) and streptavidin-peroxidase.

Statistical analysis

The sigmoidal dose-response curves were analyzed with ALLFIT, a basic computer program for simultaneous curve-fitting based on a four parameter logistic equation (15).

RESULTS

LPS-induced expression of COX-2 in monocytes correlates with the time-dependent increase in prostanoid biosynthesis (11). As shown in the *figure* (*panel A*), NS-398 inhibited PGE_2 production by monocytes stimulated for 24 h with LPS, with an IC_{50} value of 0.0015 μmol/L. In washed platelets, NS-398 inhibited thrombin-induced TXB_2 production with an IC_{50} of 0.27 μmol/L, a 180-fold higher value than that inhibiting monocyte COX-2 activity. A concentration of NS-398 (0.01 μmol/L), largely suppressing inducible

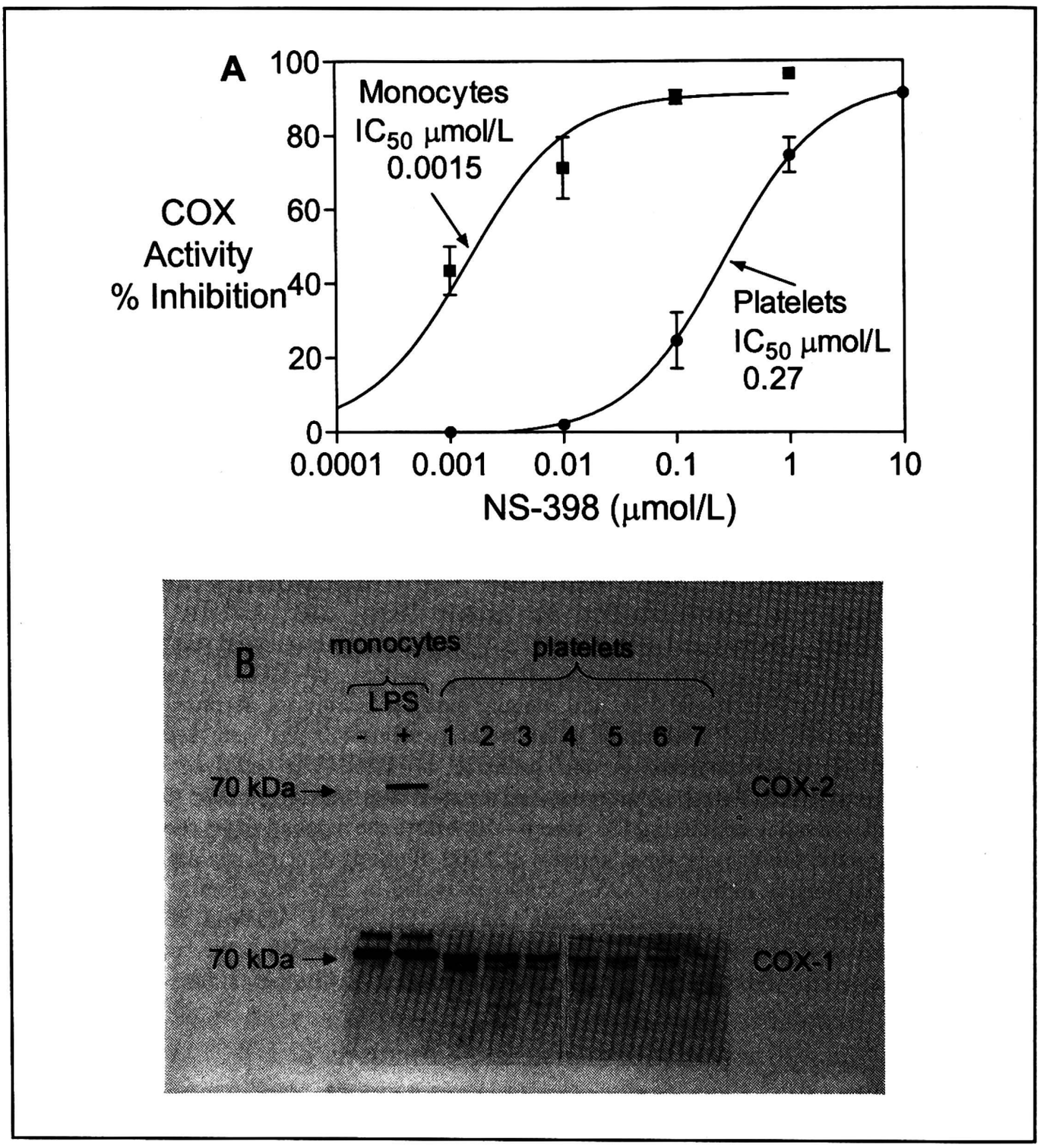

Fig. 1. (A) **Effects of NS-398, a selective COX-2 inhibitor, on prostanoid production by platelets and monocytes.** Washed human platelets (●) (2×10^8 cells/mL) were incubated for 30 min at 37°C with increasing concentrations of NS-398 or DMSO vehicle, and then for further 4 min with thrombin (0.5 IU/mL). Isolated human monocyte (■) (2×10^6 cells/mL) were incubated with increasing concentrations of NS-398 or DMSO vehicle in the presence of LPS (10 μg/mL) for 24 h at 37°C (expressing high levels of COX-2). Supernatants of platelets and monocytes were assayed for TXB_2 and PGE_2. Values are reported as mean ± SD of 3 different experiments.
(B) Western blot analysis of COX-1 and COX-2 in human platelets and monocytes. Platelets and monocytes (unstimulated or incubated for 24 h with LPS, 10 μg/mL) were analyzed for the content of COX-1 and COX-2 by SDS-polyacrylamide gel electrophoresis and immunoblotting techniques, using rabbit polyclonal antibodies against COX-1 or a carboxyl-terminal sequence present in human COX-2 but not in COX-1, respectively. The numbers (1—7) indicate the 7 healthy volunteers.

prostanoid production in monocytes, produced no statistically significant changes in platelet TXB_2 synthesis and release. In contrast, aspirin 55 μmol/L, a concentration that did not significantly affect monocyte COX-2 activity, caused an almost complete suppression (by 96%) of TXB_2 production in thrombin-stimulated platelets.

Immunoreactive COX-1 but not COX-2 was detected in circulating platelets and in unstimulated monocytes of healthy subjects. COX-2 was detected in monocytes stimulated for 24 h with LPS (*figure, panel B*).

DISCUSSION

Our results using pharmacological and immunological tools argue against the involvement of COX-2 in TX biosynthesis by activated platelets. This is consistent with the results of over 50 clinical trials showing saturation of the antithrombotic efficacy of aspirin at low-doses (50—100 mg) (16). In fact, in a recently completed randomized comparison of different aspirin doses (81, 325, 650 and 1,300 mg/d) in patients undergoing carotid endarterectomy (17), the rate of major vascular events was significantly reduced in patients receiving lower *vs* higher doses. Moreover, we could not detect COX-2 in washed platelets. The reasons for the discrepancy between our results and those reported by Weber *et al.* (10) are unclear. In our study platelet suspensions contained less than 0.5 leukocytes per 1,000 platelets and particular attention was paid to using platelets immediately after isolation in order to avoid any *ex vivo* expression of COX-2 by contaminating leukocytes. Our findings are consistent with those of Takahashi *et al.* (9) who have identified only COX-1 in platelets.

The occurrence of episodes of aspirin-resistant thromboxane (TX) A_2 biosynthesis in unstable angina patients has been reported previously by Vejar *et al.* (3). Thus, despite >90% suppression of platelet COX activity by low-dose aspirin, as monitored *ex vivo*, incomplete suppression of TXA_2 biosynthesis *in vivo*, as reflected by the urinary excretion of its major enzymatic metabolites 11-dehydro-TXB_2 and 2,3-dinor-TXB_2, was demonstrated in a subset of patients (3).

Indirect evidence supports a role for extraplatelet sources of TXA_2, possibly expressing COX-2, in episodes of enhanced TXA_2 biosynthesis *in vivo* in patients with unstable angina receiving low-dose aspirin. In fact, in patients with unstable angina treated with indobufen, an antiplatelet drug that largely suppresses both platelet COX-1 and monocyte COX-2 at therapeutic plasma concentrations, the rate of thromboxane biosynthesis was approximately 50% lower than in patients treated with aspirin (8). COX-2 expression in nucleated cells (eg monocytes/macrophages) in response to a local inflammatory milieu (18) may be involved in this phenomenon.

In conclusion, our results argue against the involvement of COX-2 in TX biosynthesis by activated platelets and consequently dispute platelet COX-2 expression as an important mechanism of aspirin resistance.

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