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Effect of some cyclooxygenase inhibitors on the increase in guanosine 3′:5′-cyclic monophosphate induced by NO-donors in human whole platelets

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1 The effect of the NSAIDs indomethacin, indoprofen, diclofenac and acetylsalicylic acid on the increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) induced by nitric oxide-donor agents was tested in human whole platelets and in platelet crude homogenate.

2 In whole platelets, indomethacin reduced the increase in cyclic GMP induced by the nitric oxide-donors (NO-donors) sodium nitroprusside (NaNP) and S-nitroso-N-acetylpenicillamine (SNAP) in a dose-dependent way, its IC_{50} being 13.7 μM and 15.8 μM, respectively.

3 Of the other cyclooxygenase inhibitors tested, only indoprofen reduced the increase in cyclic GMP induced by both NO-donors in a dose-dependent way ($IC_{50} = 32.7$ μM, NaNP and 25.0 μM, SNAP), while acetylsalicylic acid (up to 1000 μM) and diclofenac (up to 100 μM) were ineffective.

4 However, in platelet crude homogenate neither indomethacin nor indoprofen reduced the cyclic GMP production.

5 Indomethacin (10 μM), indoprofen (30 μM), diclofenac (100 μM) and acetylsalicylic acid (1000 μM) showed a comparable efficacy in inhibiting platelet thromboxane B_2 (TXB_2) production, suggesting that the inhibitory effect of indomethacin and indoprofen on the increase in cyclic GMP induced by both NO-donors was not mediated by inhibition of cyclooxygenase.

6 *In vitro*, the NSAIDs analysed did not interfere with nitrite production of SNAP.

7 The unhomogeneous behaviour of NSAIDs on the increase in cyclic GMP induced by NO-donors in whole platelets may contribute to the different pharmacological and toxicological characteristics of the drugs, providing new knowledge on the effect of indomethacin and indoprofen.

Keywords: Cyclic GMP; sodium nitroprusside; S-nitroso-N-acetylpenicillamine; indomethacin; indoprofen; acetylsalicylic acid; diclofenac; thromboxane B_2; 4-bromophenacyl bromide; nitrites

Introduction

Haeme-containing guanylate cyclase (HGC) is activated by several radical species, including nitric oxide (NO), nitrosothiols, superoxide anion ($\cdot O_2^-$) and hydroxyl radical (for review see Waldmann & Murad, 1987; Schmidt, 1992). Drugs known to increase the guanosine 3′:5′-cyclic monophosphate (cyclic GMP) level act primarily by releasing NO (directly or enzymatically) and are commonly termed 'NO-donors' (Nathan, 1992).

HGC is the dominant guanylate cyclase enzyme in platelets (Glass *et al*., 1977); stimulation of this enzyme by gaseous NO (Mellion *et al*., 1981), NO-donors and in particular sodium nitroprusside (NaNP) (Shafer *et al*., 1980; Mellion *et al*., 1981; Morgan & Newby, 1989; Salvemini *et al*., 1989), gaseous CO (Brüne & Ullrich, 1987; Brüne *et al*., 1990), H_2O_2 (enzymatically generated by xanthine-xanthine oxidase in the presence of superoxide dismutase, Ambrosio *et al*., 1994) increases cyclic GMP and inhibits platelet aggregation. On the other hand chemically generated $\cdot O_2^-$ by ferricytochrome c and pyrogallol enhances platelet aggregation and adhesion (Handin *et al*., 1977; Salvemini *et al*., 1989) by inhibiting HGC (Brüne *et al*., 1990).

Endogenously, these radicals derive from metabolic enzyme reactions. In particular, a relevant source of radical species is the arachidonic acid metabolism; in fact, $\cdot O_2^-$ is initially formed during arachidonic acid oxidation (Kukreja *et al*., 1986). Cyclo-oxygenase and lipoxygenase enzymes are both involved in the production of radical species and the formation of oxygen peroxide is reduced in the presence of cyclooxygenase inhibitors (Kukreja *et al*., 1986).

Moreover, micromolar concentrations of several unsaturated fatty acids including arachidonic acid and/or their metabolites can activate partially purified HGC from human platelets (Barber, 1976; Glass *et al*., 1971; Hidaka & Asano, 1977; Asano & Hidaka, 1977). According to Hidaka and Asano (1977), concentrations of arachidonic acid up to 100 μM are required for activation of HGC. The activating effect of arachidonic acid was demonstrated by using partially purified HGC from human platelets, whereas arachidonic acid does not increase the content of cyclic GMP in whole platelets (Barber, 1976). Arachidonic acid peroxides also stimulate the partially purified platelet HGC (Asano & Hidaka, 1977).

More recently, it has been demonstrated that exogenously administered NO (Rettori *et al*., 1992; Salvemini *et al*., 1995; 1996) and/or endogenously produced by induction of NO synthase (Salvemini *et al*., 1993, 1994, 1995; Inoue *et al*., 1993; Corbett *et al*., 1993) increases the final metabolic products of cyclooxygenase isoenzymes. On the other hand, NaNP and glyceryl trinitrate (GTN) did not significantly increase the activity of pure ovine

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cyclooxygenase (PGHS isoform-1), as detected spectroscopically (Tsai *et al*., 1994).

In human platelets, radioactive cyclooxygenase metabolites (measured by h.p.l.c. and radiochemical detection after administration of radioactive arachidonic acid for 5 min) are not modified by either L-arginine or NaNP. However, in the same experimental conditions, the radioactive metabolic end-products of lipoxygenase were strongly reduced (Nakatsuka & Osawa, 1994). These results suggest that NO inhibits lipoxygenase in human platelets, but does not activate cyclooxygenase.

The complex relationships between cyclooxygenase and HGC in the regulation of platelet function prompted us to study the influence of cyclooxygenase inhibitors on the activation of HGC by NO-donors in whole platelets. We used two different molecules: S-nitroso-N-acetylpenicillamine (SNAP) and NaNP. Although both molecules are frequently used as NO-donors for experimental purposes, their chemical structures as well as their NO-releasing characteristics are very different. Indeed, NaNP is a cyanoferrate and the NaNP-mediated activation of HGC is strongly increased by the addition of thiols, while SNAP is a nitrosothiol and the SNAP-mediated activation of HGC does not require addition of thiols (Ignarro *et al*., 1981).

Possible tools for investigating the role of cyclooxygenase and its metabolic products in biology are the inhibitors of cyclooxygenase isoenzymes, which have been used in medicine as drugs because of their anti-inflammatory properties. Their relative specificities prompted us to examine the effect of compounds which possess cyclooxygenase inhibitory properties having different chemical structures, kinetic and metabolic profiles as well as side-effects (Insel, 1996). The drugs that we used were indomethacin, indoprofen, acetylsalicylic acid (ASA) and diclofenac. According to the current, chemical-derived classification of cyclooxygenase inhibitors, the four compounds selected belong to different groups (Flower *et al*., 1985; Insel, 1996), whereas according to the new proposed classification (Frölisch, 1997), indomethacin and ASA are selective cyclooxygenase-1 inhibitors at low dosage; diclofenac, high dose of indomethacin and ASA are classified as non-selective cyclooxygenase inhibitors. Indoprofen is chemically classified as a propionic acid derivative, but the molecule also posesses an isoindolyl ring and therefore it was selected as another indol derivative. ASA is largely used as antiplatelet agent in the treatment of several specific thromboembolic diseases, including coronary artery disease. Indomethacin, on the other hand, induces coronary vasoconstriction in patients with coronary artery disease (Friedman *et al*., 1981). Diclofenac is a phenylacetic acid which behaves like indomethacin in inducing apoptosis (Lu *et al*., 1995).

These four molecules were selected on the basis of their different chemical, pharmacological and toxicological profiles. Moreover, recently several studies have demonstrated that individual cyclooxygenase inhibitors possess different biological effects unrelated to the block of cyclooxygenase. In particular it has been shown that sodium salicylate (1–20 mM) and ASA (1–5 mM) inhibit nuclear factor κB (NF-κB) activation in Jurkat cells (Kopp & Ghosh, 1994), whereas indomethacin (100 μM) and sodium salicylate (2–3 mM) block the ADP-ribosylation of G-protein by pertussis toxin in human neutrophils (Abramson *et al*., 1991). However, all these effects were demonstrated at high drug concentrations. Therefore, we decided to test our selected compounds at concentrations higher than those effective as cyclooxygenase inhibitors.

Methods

Platelet preparation

Blood from healthy human volunteers who had not taken drugs for at least 10 days was collected by venipuncture and immediately diluted 1/5 with citric acid: trisodium citrate: glucose (1.5% : 2.5% : 2% w/v, ACD). Platelet rich plasma (PRP) was prepared by centrifugation at 180 *g* at 20°C for 15 min.

Platelets were then washed twice in HEPES/$NaHCO_3$: ACD (4 : 1 v/v) by centrifugation and resuspended in HEPES buffer of the following composition (mM): NaCl 140, HEPES 10, $NaHCO_3$ 12, KCl 2.9, $MgCl_2$ 0.9, NaH_2PO_4 0.5, glucose 10, $CaCl_2$ 1 (HEPES/$NaHCO_3$ buffer).

Cyclic GMP assay

Cyclic GMP assay in whole platelets Cyclic GMP platelet content was measured by radioimmunoassay. After preparation of PRP, twice-washed platelets were suspended in HEPES/$NaHCO_3$ buffer containing 100 μM isobutylmethylxanthine (IBMX) at a final concentration of 10^8 platelets 100 μl^{-1}. Platelets were preincubated in the absence or presence of testing molecules at room temperature for 20 min and then at 37°C for 5 min; sodium nitroprusside (NaNP), S-nitroso-N-acetylpenicillamine (SNAP) or the buffer alone (10 μl) were added. The reaction was stopped after 5 min at 37°C by the addition of 50 μl ice-cold $HClO_4$ (20% v/v); test tubes were then placed on ice for 15 min. The reaction mixture was neutralized with 110 μl 1.08 M K_3PO_4 and after centrifugation, 2 aliquots of supernatant (100 μl) were used for the determination of cyclic GMP by use of a commercial [^{3}H]-cyclic GMP RIA Kit (Amersham International, Buckinghamshire, U.K.). The effect of indomethacin and indoprofen on the increase in cyclic GMP induced by NaNP was also tested in the presence of the irreversible phospholipase A_2 inhibitor 4-bromophenacyl bromide (4-BPB), used at a concentration near to its IC_{50} (10 μM, Duque *et al*., 1986).

Cyclic GMP assay in platelet supernatant fraction PRP was centrifuged and resuspended in 50 mM Tris-acetate buffer, pH 6 containing 20 mM 2-mercaptoethanol and 0.1 mM EGTA and then rapidly frozen at −80°C. Platelet supernatant fraction was prepared from freeze-thawed platelets, sonication of materials obtained (3 s twice) and centrifugation (105,000 *g*) for 1 h at 0°C (Glass *et al*., 1977). The guanylyl cyclase activity of platelet supernatant fraction was measured as cyclic GMP production in 50 mM Tris-HCl, pH = 7.7 containing 1 mM $CaCl_2$, 3 mM $MnCl_2$ with 0.5 mM Na guanosine 5′-triphosphate (GTP) as substrate (Asano & Hidaka, 1977). After 10 min at 37°C, the reaction was stopped by addition of 50 μl of ice-cold $HClO_4$. Cyclic GMP was measured in neutralized supernatant by radioimmunoassay. The guanylyl cyclase activity was mainly found in the supernatant fraction; the supernatant fraction was therefore used for further investigation. The guanylyl cyclase activity of platelet supernatant fraction was measured as cyclic GMP production either in control conditions or in the presence of different doses of SNAP. Either indomethacin, indoprofen or LY 83583, an inhibitor of soluble guanylyl cyclase was added to reaction mixture before incubation at 37°C for 10 min in control or in 50 μM SNAP-activated supernatant fraction.

Thromboxane B_2 assay

Thromboxane B_2 (TXB_2) platelet production was measured by radioimmunoassay. Platelets, prepared as described above, were preincubated at 37°C for 5 min in the absence or presence of (final concentrations) 10 μM 4-bromophenacyl bromide (4-BPB), 10 μM indomethacin, 30 μM indoprofen, 1000 μM ASA or 100 μM diclofenac and then either 100 μM NaNP, 50 μM SNAP or buffer alone, in an equal volume of 10 μl, was added. Reaction was stopped after 5 min by the addition of 100 μl of ice-cold nominally Ca^{2+}-free HEPES/$NaHCO_3$ buffer; test tubes were then centrifuged at 1500 *g* at 0°C for 20 min. After centrifugation, 3 aliquots of supernatant (20 μl) were diluted to 250 μl of 0.02 M phosphate buffer, pH 7.4 and used for the determination of TXB_2 according to Gentilini *et al*. (1988). The lower detection limit with this methodology is 2 pg of TXB_2 ml, i.e. 1 ng/10^8 platelets.

Spectrophotometric determination of nitrites

Nitrite production from *in vitro* spontaneous degradation of NaNP and SNAP was measured by use of the standard Griess methodology according to Nicholas and Nason (1957). Standard solutions of $NaNO_2$ at different concentrations (400 μl) or an equal volume of testing solution (either NaNP or SNAP at different concentrations in HEPES/$NaHCO_3$ buffer containing 100 μM IBMX and as indicated either 10 μM indomethacin, 30 μM indoprofen, 100 μM diclofenac or 1000 μM ASA) were incubated for 5 min at 37°C; then 150 μl of 1% (p/v) sulphanilamide in 2.5 M HCl and successively 150 μl of 0.02% (p/v) N-(1-naphthyl)ethylenediamine were added; after 15 min the absorbance of the sample was determined spectrophotometrically at 540 nm. The lower detection limit of this method was 0.2 μM of nitrites ($NaNO_2$) and the linearity ranged from 0.2 to 86 M. Calibration curves were repeated in the presence of the cyclooxygenase inhibitors tested; none of them modified the calibration curve of $NaNO_2$.

Reagents

3-Isobutyl-1-methylxanthine, indomethacin, indoprofen, diclofenac, ASA, 4-bromophenacyl bromide, sulphanilamide, N-(1-naphthyl)ethylenediamine.2HCl, 2-mercaptoethanol, guanosine 5′-triphosphate (sodium salt, Type III) were obtained from Sigma Chimica Italia (Milan), sodium nitroprusside from Merck (Darmstadt, Germany), S-nitroso-N-acetylpenicillamine from Tocris Cookson (Bristol, U.K.); LY 83583 (6-anilinoquinoline-5,8-quinone) from Calbiochem (La Jolla, CA, U.S.A.); TXB_2 standard was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.); [^{3}H]-TXB_2 (139.3 Ci mmol^{-1}) from New England Nuclear (Milan, Italy); the anti-TXB_2 antibody from Serono Biodata (Milan, Italy); all other reagents were of analytical grade.

Indomethacin and indoprofen were dissolved in hydro-alcoholic buffer solution. Ethanol did not interfere with enzyme activities at the highest concentration used.

Statistical analysis

All values are means ± s.e. mean of the indicated number of experiments. For statistical comparisons, Student's *t* test for paired or unpaired data was used as indicated and a *P* value <0.05 was taken as significant. Dose-response curves were analysed by use of ANOVA and correlations were tested by linear regression analysis (Least Square's Method). Two-way ANOVA was used to compare dose-response curves obtained with different treatments; $P<0.05$ was considered to be statistically significant. The median effective concentration (EC_{50}) and the median inhibitory concentration (IC_{50}) with 95% confidence limits were calculated by linear regression analysis from at least 3 separate inhibitory dose-response curves.

Results

Effect of indomethacin, indoprofen, ASA and diclofenac on the increase in cyclic GMP induced by NO-donors in human platelets

Effect of NaNP and SNAP The platelet cyclic GMP content in basal conditions was 0.23 ± 0.028 pmol per 10^8 platelets ($n=29$). NaNP in the range 1 to 3000 μM increased cyclic GMP in a concentration-dependent way (Figure 1, $r^2=0.80$, ANOVA: $F=191$, $P<0.001$, $n=7$), the EC_{50} being 100 μM (81 – 126 μM). With 100 μM NaNP, the increase in cyclic GMP was 13.3 fold the unstimulated level. The higher NaNP dose, 3000 μM, represented the upper limit of NaNP solubility in our experimental conditions.

In parallel experiments, SNAP was used as an NO-donor. As shown in Figure 1 (closed triangles), SNAP induced a bell-shaped increase in cyclic GMP, the maximal effective dose being between 10 and 50 μM ($n=$ at least 3). The increase in cyclic GMP induced by 50 μM SNAP was 31 fold the unstimulated level and significantly different from those obtained with 100 and 250 μM SNAP.

Effect of indomethacin Preincubation with 10 and 100 μM indomethacin did not modify the basal cyclic GMP (0.23 ± 0.047 and 0.18 ± 0.020 pmol per 10^8 platelets, $n=14$ and $n=8$, respectively). In the presence of 10 μM indomethacin, NaNP still increased the cyclic GMP in a dose-dependent way (Figure 1, $r^2=0.83$, ANOVA: $F=227$, $P<0.001$, $n=$ at least 3), but this increase was significantly less than that obtained in the control ($F=24$, $P<0.01$ two-way ANOVA, Figure 1), the effect of indomethacin being particularly evident at the higher doses of NaNP. Indomethacin (10 μM) also reduced the increase in cyclic GMP induced by 50 μM SNAP ($F=11$, $P<0.01$ two-way ANOVA, $n=$ at least 3, Figure 1).

In light of the results obtained, dose-response curves for indomethacin were performed with 100 μM NaNP and 50 μM

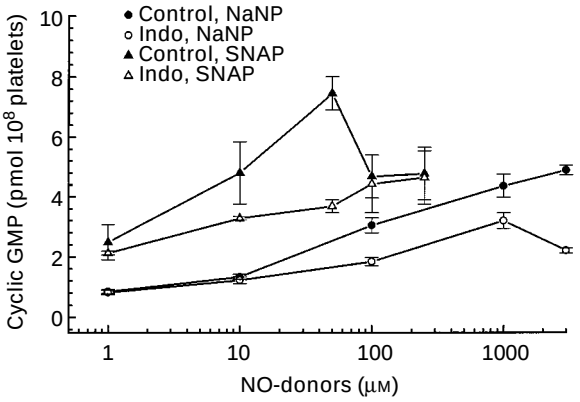

Figure 1 Effect of 10 μM indomethacin (Indo) on the dose-dependent increase in platelet cyclic GMP content (pmol/10^8 platelets) induced by sodium nitroprusside (NaNP) and S-nitroso-N-acetylpenicillamine (SNAP). Values are the mean of at least 7 experiments (controls) and 3 experiments (indomethacin) performed in duplicate; vertical lines show s.e.mean.

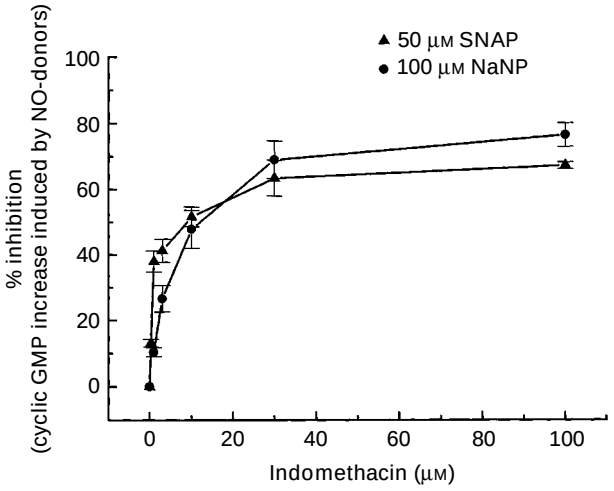

Figure 2 Dose-dependent inhibitory effect of indomethacin on the increase in platelet cyclic GMP content (pmol/10^8 platelets) induced by 100 μM sodium nitroprusside (NaNP; $r^2=0.75$, ANOVA: $F=83$, $P<0.001$) and 50 μM S-nitroso-N-acetylpenicillamine (SNAP; $r^2=0.70$, ANOVA: $F=88$, $P<0.001$). Abscissa scale: indomethacin concentration (μM); ordinate scale: inhibition of the increase in cyclic GMP induced by NaNP and SNAP as percentage of control value. Values are the mean of at least 6 experiments performed in duplicate; vertical lines show s.e.mean.

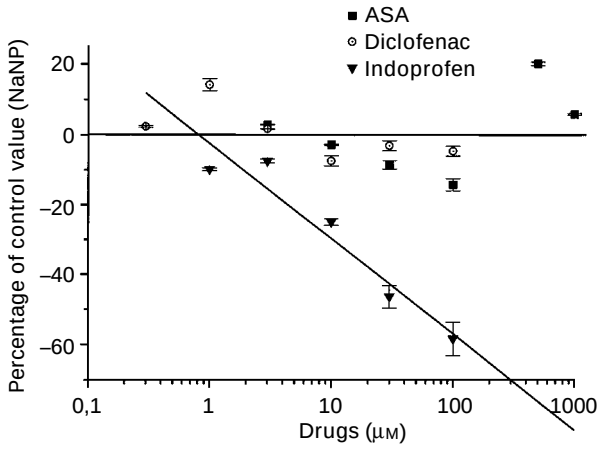

Figure 3 Effect of acetylsalicylic acid (ASA), diclofenac and indoprofen on the increase in cyclic GMP induced by 100 μM sodium nitroprusside (NaNP). Abscissa scale: drug concentration (μM); ordinate scale: cyclic GMP content expressed as percentage of control value. Values are the mean and s.e.mean of at least 3 experiments performed in duplicate.

SNAP (Figure 2). Indomethacin dose-dependently reduced the increase in cyclic GMP induced by both NO-donors ($r^2=0.75$, one-way ANOVA $F=83$, $P<0.001$, $n=6$ and $r^2=0.70$, ANOVA $F=88$, $P<0.001$, $n=$at least 6, respectively).

Effect of ASA Acetylsalicylic acid (100 μM) did not affect platelet basal cyclic GMP (0.30 ± 0.119 pmol per 10^8 platelets, $n=9$). In the presence of 100 μM ASA, NaNP still increased cyclic GMP, ($r^2=0.81$, ANOVA: $F=186$, $P<0.001$, $n=4$, not shown); the extent of this dose-dependent increase was superimposable on the control.

Effect of some other cyclooxygenase inhibitors As shown in Figure 3, of the cyclooxygenase inhibitors tested, only indoprofen dose-dependently prevented the increase in cyclic GMP induced by NaNP, whereas ASA (3–1000 μM) and diclofenac (0.3–100 μM) were totally ineffective at the highest concentrations tested (Figure 3 and Table 1). Similar results were obtained when SNAP was used as a NO-donor. However, the potency of indoprofen was lower than that of indomethacin; the IC_{50}s of indomethacin, indoprofen, ASA and diclofenac are shown in Table 1.

Effect of the phospholipase A_2 inhibitor 4-BPB The irreversible phospholipase A_2 inhibitor 4-BPB (10 μM) did not modify basal cyclic GMP platelet content. In the presence of 10 μM 4-BPB, the inhibitory effect of indomethacin on the increase in platelet cyclic GMP induced by 100 μM NaNP was not modified (Tables 1 and 2). Similarly, the inhibitory effect of indoprofen was not modified by 4-BPB (Table 1).

Table 1 Median inhibitory concentrations (IC_{50}s) of cyclooxygenase inhibitors on the increase in platelet cyclic GMP content induced by 100 μM sodium nitroprusside (NaNP) or 50 μM S-nitroso-N-acetyl penicillamine (SNAP)

Indomethacin	(NaNP)	13.71 μM	(10.20–20.88)
Indomethacin + 4-BPB§	(NaNP)	16.36 μM	(14.45–20.54)
Indoprofen	(NaNP)	32.68 μM	(30.12–35.72)
Indoprofen + 4-BPB§	(NaNP)	32.58 μM	(30.52–34.93)
Acetylsalicylic acid	(NaNP)	> > 1000 μM	(no effect)
Diclofenac	(NaNP)	> > 100 μM	(no effect)
Indomethacin	(SNAP)	15.82 μM	(11.43–25.69)
Indoprofen	(SNAP)	25.02 μM	(19.58–34.63)
Acetylsalicylic acid	(SNAP)	> > 100 μM	(no effect)
Diclofenac	(SNAP)	> > 100 μM	(no effect)

Values are the IC_{50}s with the confidence limits in parentheses. §4-Bromophenacylbromide (10 μM).

Table 2 Effect of 10 μM 4-bromophenacylbromide (4-BPB) on the indomethacin dose-dependent inhibition of the increase in platelet cyclic GMP content (pmol × 10^8 platelets) induced by 100 μM sodium nitroprusside (NaNP)

	Indomethacin (μM)				
	0	*1*	*3*	*10*	*30*
Control	3.4 ± 0.33	3.0 ± 0.13	2.5 ± 0.15	1.8 ± 0.06	1.1 ± 0.09
4-BPB	3.6 ± 0.16	2.9 ± 0.20	2.7 ± 0.26	2.0 ± 0.18	1.3 ± 0.04

Values are the mean ± s.e.mean of at least 6 experiments performed in duplicate.

Effect of indomethacin and indoprofen on the increase in cyclic GMP induced by NO-donors in the platelet supernatant fraction

The platelet supernatant fraction produced a linearly, protein-content related increase in cyclic GMP in the range 0.1–10 μg ml^{-1} of total protein ($r^2=0.994$, $P<0.001$, Table 3). The cyclic GMP production of platelet supernatant fraction was still dose-dependent in the presence of 10 μM and 50 μM SNAP ($r^2=0.939$, $P<0.01$ and $r^2=0.833$, $P<0.05$, respectively, Table 3), both dose-dependent curves being significantly increased compared to the control curve (Table 3). The cyclic GMP production of 1 μg ml^{-1} platelet homogenate was inhibited by 1 μM LY 83583; this inhibition was about 70% in the basal condition and 45% in the presence of 50 μM SNAP (Table 4). The effect of indomethacin and indoprofen was therefore tested with platelet homogenate containing 1 μg ml^{-1} protein in basal conditions and in the presence of 50 μM SNAP. However, neither indomethacin nor indoprofen were able to decrease cyclic GMP production in the basal condition or in the presence of 50 μM SNAP (Table 4).

Table 3 Cyclic GMP production of platelet supernatant fraction

	Cyclic GMP production (fmol/100 μl) *Total protein of platelet supernatant fraction* (μg ml^{-1})				
	0.1	*0.5*	*1*	*5*	*10*
SNAP (μM)					
0	0.3±0.07	6.9±1.22	14.4±2.43	55.0±6.33	137.2±8.33
10	1.9±0.36	16.7±2.67	27.0±3.80	116.7±12.31	151.3±0.67§
50	6.7±0.89	20.3±2.19	32.4±3.26	145.7±13.97	151.0±1.00*

Values are the mean±s.e.mean of 3 experiments performed in duplicate. §$F=22$, $P<0.05$ two-way ANOVA vs basal curve. *$F=10$, $P<0.05$ two-way ANOVA vs basal curve.

Table 4 Effect of indomethacin, indoprofen and LY83583 on cyclic GMP production of platelet supernatant fraction (1 μg ml^{-1})

	Cyclic GMP production (% of control value)	
	Basal conditions	*SNAP* 50 μM
Indomethacin 10 μM	111.8±6.66	104.7±1.85
Indoprofen 30 μM	117.9±6.25	102.2±0.74
LY83583 1 μM	33.3±2.08*	55.5±5.02*

Values are the mean±s.e.mean of 3 experiments performed in duplicate. *$P<0.05$, Bonferroni test.

Table 5 Effect of sodium nitroprusside (NaNP), S-nitroso-N-acetylpenicillamine (SNAP) and collagen on thromboxane B_2 production (ng × 10^8 platelets) of human washed platelets

	Control	*4-BPB* 10 μM
No drugs	12.4±2.37	10.5±2.25
Collagen 5 μg ml^{-1}	71.0±10.71***	33.7±3.12°°
NaNP 100 μM	23.9±2.7*	12.9±3.24°
SNAP 50 μM	30.9±4.37**	12.0±4.42°

§4-Bromophenacylbromide. Data are the mean±s.e.mean of at least 3 experiments performed in triplicate. *$P<0.05$ vs no drugs (paired data). **$P<0.01$ vs no drugs (paired data). ***$P<0.001$ vs no drugs (paired data). °$P<0.05$ vs NaNP and SNAP respectively (unpaired data). °°$P<0.01$ vs collagen (paired data).

Table 6 Effect of indomethacin, indoprofen, diclofenac and acetylsalicylic acid (ASA) on thromboxane B_2 production (ng × 10^8 platelets) of human washed platelets

Control	*Indomethacin* (10 μM)	*Indoprofen* (30 μM)	*Diclofenac* (100 μM)	*ASA* (1000 μM)
100±3.2	ND	5.7±1.81*	1.1±1.13*	9.9±1.17*

Values are the mean±s.e.mean of at least 3 experiments performed in triplicate and are presented as a percentage of basal TXB_2 production. Non-parametric tests were used to calculate the s.e.mean and the statistical significance. ND = not detectable (< 1 ng × 10^8 platelets). *$P<0.05$ vs control Bonferroni test.

Effect of NaNP, SNAP, indomethacin, ASA, indoprofen, diclofenac on platelet TXB_2 production

In parallel sets of experiments, platelet TXB_2 production (an index of platelet cyclooxygenase activity) was measured. Platelet TXB_2 production after treatment with 100 μM NaNP and 50 μM SNAP was significantly increased (Table 5). Indomethacin (10 μM) totally inhibited TXB_2 production in the presence of both NO-donors. The phospholipase A_2 inhibitor 4-BPB did not reduce TXB_2 production in basal conditions, but it antagonized the increase in TXB_2 induced by 100 μM NaNP and 50 μM SNAP or 5 μg ml^{-1} collagen (Table 5). Indomethacin, indoprofen, ASA and diclofenac inhibited to a similar extent basal TXB_2 platelet production. Indomethacin (10 μM) and diclofenac (100 μM) totally abolished TXB_2 production (< 1 ng × 10^8 platelets). Indoprofen (30 μM) and ASA (1000 μM) reduced it by 90% (Table 6). Similar results were obtained after collagen stimulation (not shown).

In vitro *nitrite production*

The possibility that indomethacin and the other cyclooxygenase inhibitors could non-specifically interfere with NO generated by NO-donors *in vitro* was tested by measuring the dose-dependent nitrite productions of NaNP and SNAP. A dose-dependent increase in nitrites was detected with SNAP in the range 10–100 μM. Nitrite production was detectable only when the NaNP concentration was higher than 1000 μM. Nitrite production dependent on the *in vitro* degradation of SNAP was not modified by either 10 μM indomethacin, 30 μM indoprofen, 100 μM diclofenac or 1000 μM ASA (Figure 4).

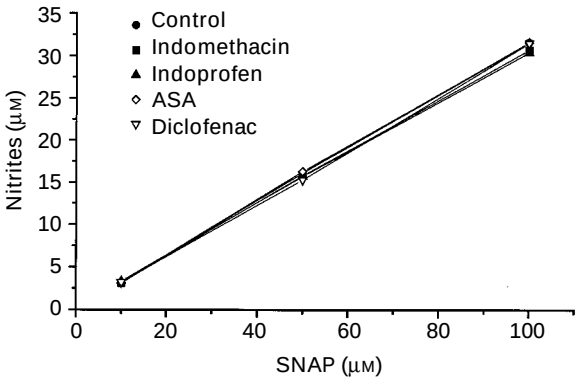

Figure 4 Nitrite production by S-nitroso-N-acetylpenicillamine in control and in the presence of 10 μM indomethacin, 30 μM indoprofen, 100 μM diclofenac and 1000 μM acetylsalicylic acid (ASA).

Discussion

Our data demonstrated that in human whole platelets, indomethacin and indoprofen antagonize the increase in cyclic GMP induced by NaNP and SNAP (Figure 2 and Table 1). The antagonism of cyclic GMP production by NO-donors was observed only when whole platelets were used. In fact, the cyclic GMP production of the platelet supernatant fraction was not influenced by either indomethacin or indoprofen (Table 4). The inhibitory effect of indomethacin and indoprofen in whole platelets cannot be ascribed to the inhibition of the cyclooxygenase metabolic pathway. While indomethacin, indoprofen, diclofenac and ASA inhibited

platelet TXB_2 production to a similar extent, 100 μM diclofenac and 1000 μM ASA were ineffective at inhibiting the increase in cyclic GMP induced by both NO-donors in whole platelets. Therefore, at least in whole platelets, arachidonic acid itself, its lipid metabolites or oxygen radicals produced by the arachidonic acid metabolism do not appear to interfere with HGC activity in either control conditions or during NO-donor activation. These results do not agree with those of other authors with regard to the activation of partially purified platelet HGC by arachidonic acid or their lipid metabolites (Barber, 1976; Glass *et al*., 1977; Hikada & Asano 1977; Asano & Hikada, 1977). However, it is clear that they used very different experimental conditions. In fact, they added, to partially purified HGC, arachidonic acid or arachidonic acid peroxide at very high concentrations (1–10 μM or even 100 μM), concentrations commonly used to induced platelet aggregation. In addition, Barber (1976), who described an increase in the activity of HGC in the soluble fraction of platelet cytosol, demonstrated a decrease in the activity of the enzyme in isolated whole platelets. In our experiments, we used whole platelets and arachidonic acid, its lipid metabolites and oxygen radicals were self-generated by platelet metabolism. The experiments performed in the presence of the PLA_2 inhibitor 4-BPB are also in line with our interpretation. In the presence of 4-BPB, indomethacin and indoprofen were still able to inhibit the increase in cyclic GMP induced by NaNP. At the concentration tested, 4-BPB did not statistically inhibit the basal production of TXB_2, but inhibited the increase in TXB_2 induced by both NO-donors and by collagen (Table 5). This suggests either that a small amount of arachidonic acid was still present before the addition of 4-BPB or that 4-BPB only inhibited a particular isoform of PLA_2 activated by NO-donors or by collagen. Moreover, small amounts of arachidonic acid were also formed by other phospholipase isoenzymes.

On the other hand, the fact that the inhibitory effect of indomethacin and indoprofen was observed only in whole platelets suggests that either during the platelet homogenate preparation an important regulatory mechanism is lost or several HGC characteristics in intact whole platelets are different from those found in platelet homogenate. The IC_{50} of indomethacin at inhibiting the increase in cyclic GMP induced by both NO-donors was lower than that at which indomethacin inhibits the lipoxygenase pathway in platelets (Siegel *et al*., 1980), and, according to Nakatsuka and Osawa (1994) the lipoxygenase pathway is inhibited by NaNP. Therefore, it seems unlikely that the indomethacin inhibitory effect on the increase in cyclic GMP induced by NO-donors could depend on lipoxygenase inhibition. Further research is needed to clarify this point. It was also interesting to note that indomethacin prevented the increase in cyclic GMP only at doses of NO-donors fully active, with either low concentrations of NaNP or low or high concentrations of SNAP the effect of indomethacin was less pronounced or absent. This unusual behaviour might indicate an interference by indomethacin of the transport mechanism of NO in platelets. In fact, according to McDonald *et al*. (1993), the mechanisms of transport and delivery of NO into platelets is mediated at least in part by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme; S-nitrosylation of the enzyme by NO inhibits its enzymatic activity (Molina *et al*., 1992). An interference by indomethacin of yeast GAPDH has been described (Brownlee & Polya, 1981): indomethacin, as do adenosine, colchicine, alprenolol, propranolol and other indole derivatives, displaces cyclic AMP from yeast GAPDH. In addition, the increase in cyclic GMP induced by SNAP is a bell-shaped dose-response curve. The fact that high concentrations of NO inhibit its own transport mechanism may explain the bell-shaped form of the curve representing the increase in cyclic GMP induced by SNAP. At the higher SNAP concentration used, 10 μM indomethacin did not reduce the increase in cyclic GMP induced by SNAP, supporting the hypothesis that indomethacin might interfere with the mechanisms of transport and delivery of NO. A similar behaviour (i.e. bell-shaped dose-response curve) was not observed with NaNP since it produces less NO, as indicated by the lower increase in cyclic GMP and the small amount of nitrite produced.

In addition, we observed a significant increase in TXB_2 production after administration of both NO-donors, their potency being related to their effectiveness as NO-donors, evaluated as cyclic GMP increase and nitrite production (Figures 1 and 4). This result does not agree with the ineffectiveness of NaNP seen in platelets by Nakatsuka and Osawa (1994). In their experimental conditions (i.e. 5 min preincubation with radioactive arachidonic acid and quantification of labelled arachidonic acid metabolites by radiochemical h.p.l.c. technique), presumably only the activity of cyclooxygenase played a role, whereas in our experiments, TXB_2 production was the metabolic product of both phospholipase A_2 and cyclooxygenase metabolism. An increase in the activity of the calcium-independent phospholipase A_2 has been demonstrated by Gross *et al*. (1995) in macrophage-like cell line RAW 2647 after treatment with NaNP. Therefore it seems that in our experiments the activation not only of cyclooxygenase but also of phospholipase A_2 can account for the increase in TXB_2. In agreement with this explanation is the results obtained in the presence of 10 μM 4-BPB: the phospholipase A_2 inhibitor totally inhibited the increase in TXB_2 induced by both NO-donors.

Moreover, at least *in vitro* the four cyclooxygenase inhibitors tested do not interfere with the nitrite production of SNAP, an index of NO release from the molecule. Therefore, it seems unlikely that our results were influenced by other chemicals. In conclusion, our data show that the cyclooxygenase inhibitors we tested vary in their activity on the increase in cyclic GMP induced by NO-donors in whole human platelets. This unhomogeneous effect of the cyclooxygenase inhibitors may contribute to the different pharmacological and toxicological characteristics of these drugs.

Although the IC_{50} of indomethacin at inhibiting the increase in cyclic GMP induced by both NO-donors was 10 fold higher than the plasma level found in patients (indomethacin plasma concentration has been shown to be near to 0.5 μg ml^{-1} = i.e. 1.4 μM; Insel, 1996), our results contribute to the possible reasons for the different pharmacological characteristics of indomethacin and other cyclooxygenase inhibitors.

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