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Comparison of in vitro effects of triflusal and acetysalicylic acid on nitric oxide synthesis by human neutrophils

Lourdes Sánchez de Miguel, Santos Casado, Jerónimo Farré, Margarita García-Durán, Luis A. Rico, Mercedes Montón, José Romero, Teresa Bellver, María P. Sierra, José I. Guerra, Pedro Mata, Andrés Esteban, Antonio López-Farré *

Nephrology, Hypertension and Cardiovascular Research Laboratory, Fundación Jiménez Díaz, Avda. Reyes Catolicos 2, Madrid 28040, Spain

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Abstract

Recent studies have suggested that the protective anti-ischemic effects of acetylsalicylic acid are stronger than the inhibition of platelet thromboxane A_2 synthesis. Since ischemic events still occur in acetylsalicylic acid-treated patients, the development of new drugs with more powerful protective effects is needed. We compared the effects of a new platelet antiaggregating drug, 2-acetoxy-4-trifluoromethylbenzoic acid (triflusal) and of acetylsalicylic acid on the interaction between human neutrophils and platelets, examining the capability of neutrophils to generate nitric oxide (NO). Triflusal, in the presence of neutrophils, showed a greater antiplatelet potency than acetylsalicylic acid to inhibit thrombin-induced platelet activation. Significant stimulation of NO-mediated mechanisms in the presence of acetylsalicylic acid or triflusal was demonstrated by the following findings: (1) increased metabolism of arginine to citrulline, (2) increase of cGMP in the platelet/neutrophil system and (3) the inhibitory action of the L-arginine (L-Arg) competitive analogue, N^G-nitro-L-arginine-methyl ester (L-NAME), which was reversed by L-Arg. Triflusal increased the stimulation of NO synthesis by neutrophils more than did of acetylsalicylic acid. The main metabolite of triflusal, 2-hydroxy-4-trifluoromethylbenzoic acid (HTB), alone or in combination with acetylsalicylic acid, did not modify NO production by neutrophils. Therefore, the whole molecule of triflusal is needed to stimulate NO production by neutrophils. Our results show that, in the presence of neutrophils, triflusal exerts an antiplatelet effect greater than that of acetylsalicylic acid, demonstrating a more powerful stimulation of the NO/cGMP system. The present results indicate that it is possible to develop new and more potent acetylsalicylic acid-related antiplatelet drugs for the prevention of the myocardial ischemic/reperfusion processes.

Keywords: Acetylsalicylic acid; Nitric oxide (NO); Neutrophils; Platelet

1. Introduction

During the last few years, a new concept has been emerging about thrombosis as a multicellular event in which cell-to-cell interactions amongst platelets, neutrophils and endothelium regulate the size of a growing thrombus (Marcus, 1990; Marcus and Safier, 1993). In brief, there is evidence showing that two vasodilating mediators produced by endothelial cells and neutrophils (nitric oxide (NO) and prostacyclin) have synergistic anti-aggregating effects, mediated by the cyclic GMP (cGMP)- and cyclic AMP (cAMP)-related systems, respectively (Moncada et al., 1991; Hoffmann et al., 1992).

Inhibition of platelet activation has been the focus of several pharmacologic strategies in the prevention and treatment of thrombotic events. Acetylsalicylic acid remains a keystone in these preventive and damage-limiting strategies (Coller, 1991; Willard et al., 1993). However, various compounds have been developed to improve the efficacy of acetylsalicylic acid.

Triflusal (2-acetoxy-4-trifluoromethylbenzoic acid) is a new antiplatelet drug that, despite its structural analogy to acetylsalicylic acid (Fig. 1), exhibits different pharmacologic, pharmacokinetic and biochemical properties (Francia et al., 1978; Rabasseda and García-Rafanell, 1993). Triflusal has greater antiplatelet aggregating activity than acetylsalicylic acid, mainly due to its inhibitory activity on the cAMP-phosphodiesterase system (García-Rafanell et al., 1986). The deacetylated and main metabolite of tri-

* Corresponding author. Tel.: +34-1-5504821; fax: +34-1-5494764.

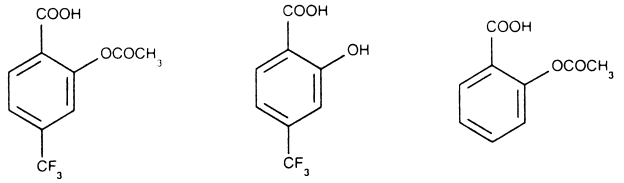

Fig. 1. Chemical structures of 2-acetoxy-4-trifluoromethylbenzoic acid, triflusal, its metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB) and acetylsalicylic acid (ASA). Note that triflusal is a fluorinated derivative of ASA.

flusal, 2-hydroxy-4-trifluoromethylbenzoic acid (HTB), retains cAMP-phosphodiesterase inhibitory activity, an effect not shown by the deacetylated metabolite of acetylsalicylic acid, salicylic acid (García-Rafanell et al., 1986).

Several reports have demonstrated the protective role of acetylsalicylic acid in the control of the complications of myocardial ischemia and in the prevention of coronary bypass graft thrombosis (Lewis et al., 1983; Theroux et al., 1988; Yusuf et al., 1988; Kerins and Fitzgerald, 1991; Mickelson et al., 1993). However, inhibition of cyclooxygenase activity by acetylsalicylic acid does not fully explain the antithrombotic effect of this drug (Di Gaetano et al., 1985; Gaspari et al., 1987; Metha and Metha, 1993). We have recently reported on new evidence demonstrating that the antiaggregating properties of acetylsalicylic acid do not depend solely on the described selective inhibitory effect on thromboxane A_2 production by platelets. We found that acetylsalicylic acid also inhibits platelet activation by stimulating NO generation by neutrophils (López-Farré et al., 1995). In the same line of evidence, De la Cruz et al. (1988) have demonstrated that leukocytes potentiate the antiplatelet aggregation effect of triflusal by an undescribed mechanism. The aim of the present study was to compare the effect of triflusal and acetylsalicylic acid on the interaction between human neutrophils and platelets, examining the capability of neutrophils to generate NO.

2. Materials and methods

2.1. Chemicals

Ficoll-Hypaque medium was obtained from Flow Laboratories (Madrid, Spain). Acetylsalicylic acid, L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemicals (St. Louis, MO). Triflusal and HTB were obtained from Uriach Laboratories (Barcelona, Spain). [^{3}H]L-arginine and the cyclic GMP radioinmmunoassay kit were purchased from Amersham (Buckinghamshire, UK). Thrombin was purchased from Ortho Diagnostic Systems (Raritan, NJ). All other chemicals were of the highest commercially available quality from Sigma (St. Louis, MO).

2.2. Neutrophil isolation

Human neutrophils were obtained from peripheral blood of healthy donors by Ficoll/Hypaque centrifugation as previously described (López-Farré et al., 1993; Riesco et al., 1993). Volunteer donors had not received drugs for at least 20 days before the experiments. The protocol was approved by the Institutional Ethics Committee. Neutrophils (95% pure, 98% viable by trypan blue exclusion) were resuspended in Ca^{2+}-physiological saline solution (PSS) containing (in mM:) 140 NaCl, 4.6 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 5.0 D-glucose and 10.0 HEPES, pH 7.4.

2.3. Platelet-rich plasma preparation and platelet aggregation

Platelet activation was evaluated in a lumiaggregometer (Aggrecorder, four channels) by the change in light transmission as previously reported (López-Farré et al., 1995). Platelet rich plasma was obtained from the same donor of neutrophils for each experiment. In brief, whole blood was obtained in 10% (v/v) acid-citrate–dextrose and centrifuged at 2500 rpm for 15 min. Platelet rich plasma was collected and the platelet number was counted with a Coulter counter. The platelet number was adjusted with platelet-poor plasma, obtained from the same donor, to 2.5×10^8 cells/ml plasma.

A platelet-poor sample was used as control for 100% light transmission. To correct for the possible light absorption induced by the presence of the neutrophil suspension, the platelet-poor sample contained a number of neutrophils equal to that of the platelet-rich sample.

Platelet rich plasma (500 μl) was incubated at 37°C for 3 min in the aggregometer with continuous stirring (1000 rpm) and then stimulated with submaximal concentrations of thrombin (0.025 U/ml) or ADP (10^{-6} mol/l). Before stimulation, 100 μl of the neutrophil suspension was added to plasma rich plasma to reach a final amount of 1.25×10^8 platelets and 1×10^6 neutrophils (125:1), which approximate the relative concentrations in normal blood. In other series of experiments, increasing neutrophil concentrations ranging from 10^6 to 10^7 cells/ml were added to 1.25×10^8 platelets.

To standardize the measurements, only the values of turbidimetry at 5 min were used for the calculations. This time period corresponds to the plateau of the maximum value of the first wave of platelet aggregation. This primary wave represents platelet activation rather than platelet aggregation and is partially reversible. Acetylsalicylic acid or triflusal were added to the platelet–neutrophil suspension 5 min before platelet stimulation with thrombin or ADP.

In all cases, the comparative baseline measurements were done in the presence of the solvent of the drugs, 0.8% $NaHCO_3$ (sodium bicarbonate). Triflusal and HTB, diluted in 0.8% $NaHCO_3$, were allowed to stand 30 min

before being added to the assays (as recommended by the manufacturer). A similar protocol was followed for acetylsalicylic acid dilution. No significant effects were detected in any case with these concentrations of sodium bicarbonate.

To determine the mechanisms involved in platelet aggregation inhibition, neutrophils were preincubated with the NO production antagonist, L-NAME (10^{-5} mol/l), 45 min before the experiments were performed. The L-NAME-treated neutrophils were washed before being added to the platelets. In addition, the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline 1-oxyl 3-oxide (C-PTIO, 3×10^{-5} mol/l) (Akaike et al., 1993) was added to the platelet–neutrophil suspension.

2.4. Measurement of cGMP

To reproduce the conditions of the platelet activation experiments, 1.25×10^8 platelets and 1×10^6 neutrophils were incubated in the presence of acetylsalicylic acid, triflusal or HTB. Thrombin was added 5 min thereafter and the platelet–neutrophil suspension was incubated for a further 5 min at 37°C. To stop the incubation, rgw cells were pelleted by centrifugation (2500 rpm, at 4°C, 5 min). The supernatant was aspirated and cells were extracted at 4°C with a 49:1 (v/v) mixture of ethanol/HCl 0.1 M. The extracts were evaporated, using a speed vac evaporator (Model VR-1/120/240, Heto Lab-Equipment A/S, Denmark). cGMP concentrations were measured in acetylated samples by means of a radioimmunoassay kit (Amersham) as described (Riesco et al., 1993).

2.5. Determination of [^{3}H]L-citrulline content

As detailed elsewhere (Metha and Metha, 1993; López-Farré et al., 1995), isolated neutrophils were incubated (45 min, 37°C) in PSS-Ca^{2+} containing 10^{-4} mol/l L-arginine and 1 μCi/ml [^{3}H]L-arginine. Unincorporated [^{3}H]L-arginine was washed out twice with PSS-Ca^{2+} buffer. 3×10^6 neutrophils were then incubated in the presence of triflusal or acetylsalicylic acid for 5 min at 37°C. In all cases, the comparative baseline measurements were done in the presence of 0.8% sodium bicarbonate, the solvent of triflusal and acetylsalicylic acid.

To analyze the mechanisms involved in the triflusal effects, additional experiments were performed with incubation of neutrophils with HTB, acetylsalicylic acid plus HTB, acetylsalicylic acid plus cAMP analogues (8-Br-cAMP and dibutyryl cAMP) or trifusal plus the protein kinase A inhibitor, H-89.

Neutrophils were lysed with cold ethanol and the supernatant was evaporated to dryness under N_2 at 37°C. As previously described (Riesco et al., 1993), the extracts were resuspended in 20 mmol/l HEPES/KOH, pH 5.5 and applied to columns of Dowex AG50WX8 (Na^+ form), which were subsequently eluted with water (L-citrulline fraction) and 0.5 mol/l NaOH (L-arginine fraction). The [^{3}H]L-citrulline fraction was quantified by liquid-scintillation counting. This column method separates L-arginine from L-citrulline by up to 92 ± 3%.

2.6. Statistical methods

The results are expressed as means $\pm$ S.E.M. Unless otherwise stated, each value corresponds to a minimum of eight experiments. To determine the statistical significance of our results, we used an analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons or the paired or unpaired Student *t*-test. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of triflusal and acetylsalicylic acid on platelet activation

In the absence of neutrophils, the % light transmission observed on activation of platelets with thrombin (0.025 U/ml) was 80 ± 1. As we have previously reported (López-Farré et al., 1995), no significant effect of neutrophils on thrombin-induced platelet activation was detected in the absence of acetylsalicylic acid or triflusal (Fig. 2a). In the presence of acetylsalicylic acid or triflusal, neutrophils significantly inhibited thrombin-induced platelet activation. The effects of both acetylsalicylic acid and triflusal were concentration-dependent (Fig. 2a). In the presence of neutrophils, triflusal showed a higher antiplatelet potency than acetylsalicylic acid at concentrations up to 0.93×10^{-3} mol/l (Fig. 2a). Both acetylsalicylic acid and triflusal showed their maximal inhibitory effect at 3.3×10^{-3} mol/l. Therefore, this latter concentration was used in subsequent studies.

In the absence of neutrophils, neither acetylsalicylic acid nor triflusal modified thrombin-induced platelet activation (Fig. 2a). A concentration-dependent acetylsalicylic acid and triflusal-related inhibition of platelet activation by neutrophils was also observed when ADP (10^{-6} mol/l) was used as platelet activator. A significant inhibitory effect of both acetylsalicylic acid and triflusal per se was detected on the ADP-induced platelet activation (Fig. 2b). However, in the presence of neutrophils, both acetylsalicylic acid and triflusal showed an increased antiplatelet aggregating effect (Fig. 2b).

In the presence of neutrophils, triflusal at concentrations up to 0.82×10^{-3} mol/l also was more potent as inhibitor of the ADP-stimulated platelet activation than acetylsalicylic acid (Fig. 2b).

The inhibitory effect of 0.82×10^{-3} mol/l trifusal on ADP-stimulated platelet activation was enhanced by increasing the number of neutrophils (Fig. 3a).

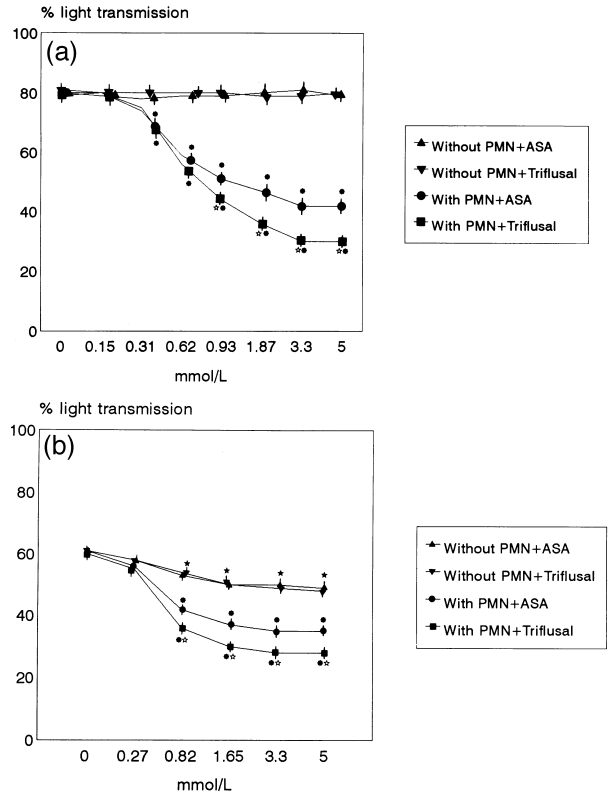

Fig. 2. Plots showing the effect of increasing concentrations of acetylsalicylic acid (ASA) and triflusal on: (A) thrombin (0.025 U/ml)-induced platelet activation in the presence ($n=6$) or absence ($n=6$) of neutrophils (PMN) and (B) ADP (10^{-6} mol/l)-stimulated platelet activation. Platelet activation was plotted as % light transmission 5 min after the addition of thrombin or ADP. Results are presented as means ± S.E.M. ★$P<0.05$ with respect to % light transmission in the absence of ASA or triflusal. *$P<0.05$ with respect to % light transmission in the absence of neutrophils. ☆$P<0.05$ with respect to the experiments performed with ASA + neutrophils.

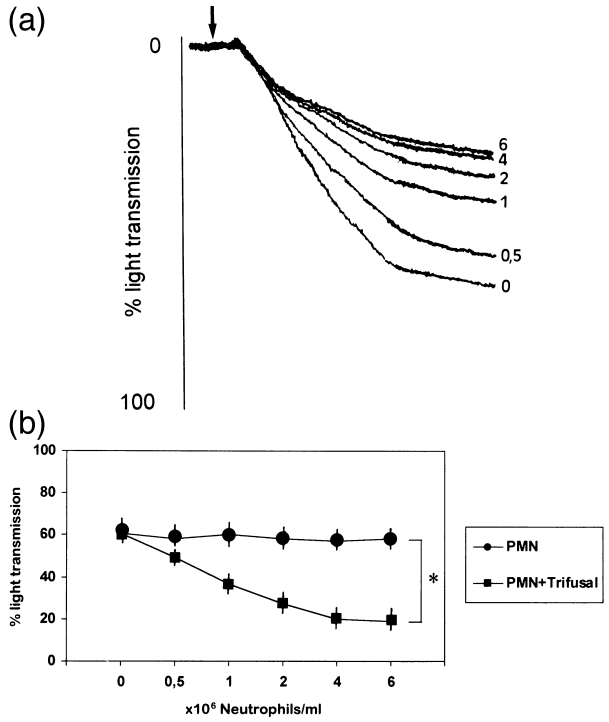

Fig. 3. (A) Representative graph showing an actual trace of ADP (10^{-6} mol/l)-induced platelet activation in the presence of increasing amounts of neutrophils and triflusal (0.82×10^{-3} mol/l). The number of incubated neutrophils are given as $\times10^6$ cells. (B) Plot showing the effect of increasing amounts of neutrophils on ADP-stimulated platelet activation in the presence or in the absence of triflusal (0.82×10^{-3} mol/l). Platelet activation was plotted as % light transmission. Results are presented as means ± S.E.M. of five different experiments. *$P<0.05$ with respect to neutrophils alone.

In the absence of trifusal, none of the neutrophil concentrations tested modified platelet activation (Fig. 3b).

Spontaneous platelet activation (% light transmission < 5%) was not changed by the incubation of platelet rich plasma with neutrophils and acetylsalicylic acid or triflusal.

3.2. Role of NO in the acetylsalicylic acid and triflusal-dependent antiplatelet aggregating response in the presence of neutrophils

To examine the implication of NO in the aforementioned effects, neutrophils were preincubated with the L-arginine antagonist, L-NAME (10^{-5} mol/l). Incubation of neutrophils with L-NAME blocked both acetylsalicylic acid and triflusal-related inhibition of platelet activation by neutrophils (Fig. 4).

In order to rule out the possibility that washing of L-NAME from neutrophils could account for the lack of effect of these cells on platelets, further experiments were performed with unwashed neutrophils. Under these conditions, L-NAME-treated neutrophils also prevented both acetylsalicylic acid and trifusal-related inhibition of platelet activation by neutrophils (% platelet inhibition: 75 ± 4; $n=4$).

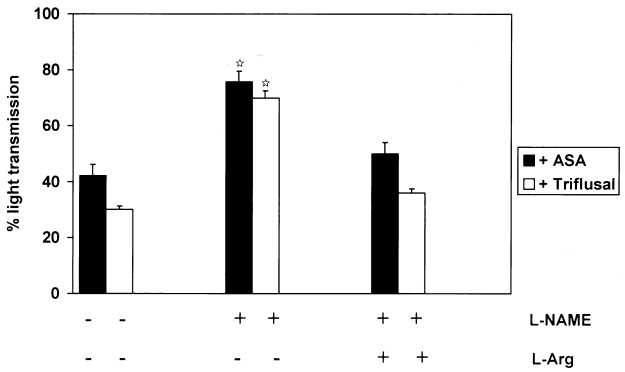

Fig. 4. Bar graph showing inhibition of thrombin-stimulated platelet activation by neutrophils in the presence of acetylsalicylic acid (ASA, 3.3×10^{-3} mol/l) or triflusal (3.3×10^{-3} mol/l). Experiments using the L-arginine antagonist L-NAME (10^{-5} mol/l), alone or in combination with L-arginine (10^{-4} mol/l), are also shown. Results are presented as means ± S.E.M. of six different experiments. ☆$P<0.01$ with respect to platelet activation in the presence of acetylsalicylic acid or triflusal.

A role of NO was further confirmed by the incubation of neutrophils with L-arginine (10^{-4} mol/l), which restored the acetylsalicylic acid and triflusal-sensitive inhibitory effect of neutrophils in the presence of L-NAME (10^{-5} mol/l) (Fig. 4). Furthermore, the NO scavenger C-PTIO (3×10^{-5} mol/l) prevented the inhibitory effect of neutrophils and trifusal (0.82×10^{-3} mol/l) on thrombin-induced platelet aggregation (% light transmission: platelets + trifusal: 80 ± 1, platelets + neutrophils + trifusal: 35 ± 4[a], platelets + neutrophils + triflusal + C-PTIO: 76 ± 3; $n = 4$; [a]$P < 0.05$ with respect to platelets + trifusal). In the absence of neutrophils, C-PTIO did not modify thrombin-induced platelet activation (% light transmission: platelets + triflusal + C-PTIO: 81 ± 1; $n = 4$, P = NS).

Although with lower capacity than neutrophils, platelets also could generate NO (for review see Moncada et al., 1991). Therefore, we determined the putative role of this platelet-dependent NO in the aforementioned results. In the absence of neutrophils, L-NAME (10^{-5} mol/l) did not modify thrombin-induced platelet activation in either the presence or in the absence of acetylsalicylic acid or triflusal (data not shown). In further experiments, we preincubated platelets with L-NAME for 30 min and, after washing, neutrophils were added. Therefore, only NO released from platelets was blocked. When we added the neutrophils, both triflusal and acetylsalicylic acid inhibited the activation of thrombin-stimulated L-NAME-treated platelets to the same extent as with L-NAME-untreated platelets (Table 1). These results suggest that the NO released from neutrophils and not from platelets is the only NO involved in the antiplatelet effects of acetylsalicylic acid and triflusal.

3.3. Effect of acetylsalicylic acid, triflusal and HTB on cGMP levels

The inhibition of thrombin-stimulated platelet activation mediated by acetylsalicylic acid, triflusal and neutrophils was accompanied by a potentiated increase in cGMP levels (Fig. 5). Since the cGMP generation induced by triflusal was greater than that induced by acetylsalicylic acid (Fig. 5), we analyzed the effect of the deacetylated metabolite of triflusal, HTB. Alone HTB increased cGMP levels slightly though statistically significantly (basal: 18 ± 1, HTB: 23 ± 1 pmol, $P < 0.05$). On the other hand, although HTB significantly increased acetylsalicylic acid-induced cGMP generation, the level obtained was lower than that obtained with triflusal (Fig. 5).

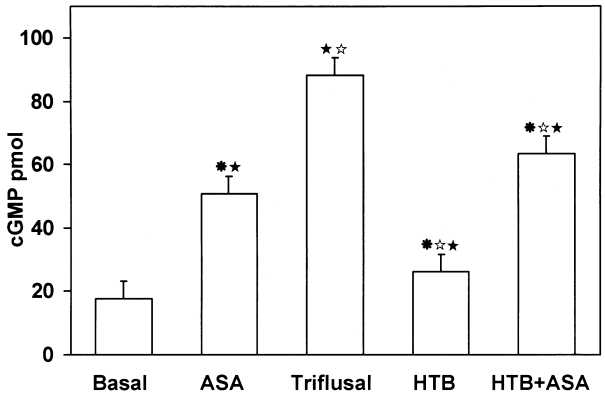

Fig. 5. Bar graph showing cGMP production by platelets and neutrophils in the presence or absence of 3.3×10^{-3} mol/l acetylsalicylic acid (ASA, $n = 6$) or 3.3×10^{-3} mol/l triflusal ($n = 6$). Additional experiments were performed with the main metabolite of triflusal, HTB (3.3×10^{-3} mol/l, $n = 6$), or the combination of HTB + ASA ($n = 6$). Results are represented as means ± S.E.M. $^{*}P < 0.05$ with respect to triflusal-incubated neutrophils. ☆ $P < 0.05$ with respect to acetylsalicylic acid (ASA)-incubated neutrophils. ★ $P < 0.05$ with respect to basal.

3.4. Generation of NO by neutrophils in the presence of acetylsalicylic acid or triflusal

We determined NO production by neutrophils by measuring the accumulation of [^{3}H]L-citrulline in [^{3}H]L-arginine-loaded neutrophils. In the platelet aggregation experiments we observed that the NO-mediated platelet

Table 1
Effect of platelet treatment with the NO donor antagonist, L-NAME, on thrombin-induced platelet activation in the presence of neutrophils

	% Light transmission			
	L-NAME-untreated PLT + PMN		L-NAME-treated PLT + PMN	
Concentrations (mmol/l)	ASA	Triflusal	ASA	Triflusal
0	80 ± 3	80 ± 3	80 ± 3	80 ± 3
0.62	62 ± 4	61 ± 5	64 ± 3	60 ± 3
0.93	55 ± 3	45 ± 2[a]	56 ± 4	44 ± 3[a]
1.87	50 ± 1	39 ± 3[a]	52 ± 2	41 ± 4[a]
3.3	44 ± 2	36 ± 2[a]	46 ± 2	36 ± 3[a]
5	45 ± 4	35 ± 3[a]	45 ± 2	34 ± 3[a]

Effect of increasing concentrations of acetylsalicylic acid (ASA) and triflusal on thrombin-induced platelet activation. Platelets (PLT) were preincubated with the NO donor antagonists, L-NAME (10^{-5} mol/l), for 30 min. After washing, thrombin and neutrophils (PMN) were added. Triflusal and acetylsalicylic acid inhibited platelet activation to a similar extent in L-NAME treated or in untreated platelets. Platelet activation was plotted as % light transmission 5 min after the addition of thrombin. Results are presented as means ± S.E.M.
[a]$P < 0.05$ with respect to the experiments performed with ASA + neutrophils.

inhibition by acetylsalicylic acid and trifusal was mainly dependent on neutrophils, therefore, these experiments were performed in the absence of platelets. Furthermore, we have previously demonstrated that the presence of activated platelets does not alter NO production by neutrophils (López-Farré et al., 1995).

In the presence of acetylsalicylic acid, the generation of [^{3}H]L-citrulline by neutrophils increased significantly (Fig. 6a). As shown in Fig. 6a, triflusal stimulated [^{3}H]L-citrulline formation to a significantly higher degree than acetylsalicylic acid. Thrombin per se did not change NO production by neutrophils in either the presence or the absence of acetylsalicylic acid or trifusal (Table 2).

An additional set of experiments was performed to examine whether the HTB-related moiety of triflusal mediated the above described findings. HTB alone did not change [^{3}H]L-citrulline formation by neutrophils (Fig. 6a). In addition, HTB did not significantly modify the acetylsalicylic acid-stimulated [^{3}H]L-citrulline generation by neutrophils (Fig. 6b).

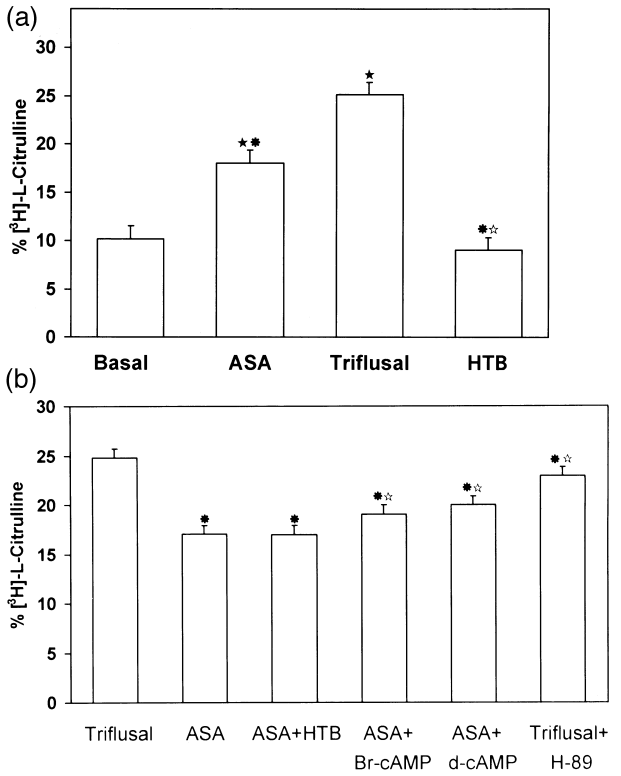

Fig. 6. (A) Nitric oxide synthesis by neutrophils was evaluated by [^{3}H]L-citrulline formation from [^{3}H]L-arginine as described in Section 2. Neutrophils were incubated in the absence (baseline) or in the presence of acetylsalicylic acid (ASA, 3.3×10^{-3} mol/l), triflusal (3.3×10^{-3} mol/l), HTB (3.3×10^{-3} mol/l). (B) Determination of nitric oxide synthesis by human neutrophils, analyzing the effect of HTB or two different cAMP analogues, 8-Br-cAMP (10^{-4} mol/l) and dibutyryl cAMP (d-cAMP,10^{-4} mol/l) on acetylsalicylic acid (ASA)-stimulated [^{3}H]L-citrulline generation. The effect of a protein kinase A antagonist, H-89 (10^{-6} mol/l), on triflusal-dependent NO synthesis was also tested. Results are presented as means ± S.E.M. of five different experiments. ★$P < 0.05$ with respect to basal. $^*P < 0.05$ with respect to triflusal-incubated neutrophils. ☆$P < 0.05$ with respect to ASA-incubated neutrophils.

Table 2
Measurement of nitric oxide production by neutrophils determined as the generation of [^{3}H]L-citrulline from [^{3}H]L-arginine

	% [^{3}H]L-citrulline content	
	+ thrombin	− thrombin
PMN	10.2 ± 0.8	11.4 ± 0.6
PMN + ASA	16.4 ± 0.6[a]	17.4 ± 0.5[a]
PMN + Triflusal	24.4 ± 0.8[a]	25.3 ± 0.4[a]

Nitric oxide production from neutrophils (PMN) was evaluated from the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline as described in Section 2. Thrombin was used at 0.025 U/ml. Results are presented as means ± S.E.M. of five different experiments.
[a] $P < 0.05$ with respect to PMN alone.

One of the effects that differs between acetylsalicylic acid and triflusal is that triflusal is an inhibitor of cAMP-phosphodiesterase (Rabasseda and García-Rafanell, 1993). Therefore, we further analyzed whether cAMP could be implicated in the triflusal-sensitive NO formation by human neutrophils. The analogues of cAMP, 8-Br-cAMP (10^{-4} mol/l) or dibutyryl cAMP (10^{-4} mol/l), significantly enhanced acetylsalicylic acid-stimulated [^{3}H]L-citrulline generation by neutrophils (Fig. 6b). However, triflusal-incubated neutrophils still generated higher amounts of NO than did those incubated with acetylsalicylic acid plus the cAMP analogues (Fig. 6b). A protein kinase A inhibitor, H-89 (10^{-6} mol/l), slightly reduced trifusal-stimulated NO generation by neutrophils (Fig. 6b).

4. Discussion

This study showed that triflusal, a new acetylsalicylic acid-related drug, stimulates NO generation by human neutrophils to a greater extent than acetylsalicylic acid. The functional implication of these findings is that, in the presence of neutrophils, triflusal showed a greater capacity to inhibit platelet activation than did acetylsalicylic acid. We have previously reported that acetylsalicylic acid acts as an antiplatelet-activating agent not only through the traditionally described mechanisms, but also by favouring the platelet-inactivating effects of neutrophils through a NO/cGMP-related pathway (López-Farré et al., 1995).

De la Cruz et al. (1988) evaluated the platelet antiaggregating potency of triflusal, using platelet rich plasma or platelet poor plasma plus leukocytes, and found a greater platelet inactivating effect of triflusal in the presence of leukocytes, mediated by an undescribed mechanism. This phenomenon was not observed with the main metabolite of triflusal, HTB (De la Cruz et al., 1988). Therefore, we tested the effect of triflusal and its metabolite, HTB, on the NO/cGMP system.

Our first observation was that, in the presence of neutrophils, triflusal and acetylsalicylic acid showed a greater

capacity to inhibit both ADP and thrombin-induced platelet activation, suggesting that platelet activation was blocked at a postreceptor level. In the presence of neutrophils, concentrations up to 0.93×10^{-3} mol/l triflusal showed a higher potency than acetylsalicylic acid to inhibit thrombin-induced platelet activation. The therapeutic dose of triflusal used in humans is between 300 to 900 mg once daily. These triflusal concentrations are close to but do not reach 0.93×10^{-3} mol/l in vitro. Therefore, one possibility suggested by the present results is that, to obtain antiplatelet effects more potent than those of acetylsalicylic acid, the in vivo doses of triflusal should be increased.

The potential functional involvement of NO in the triflusal-sensitive platelet inactivation by neutrophils was first suggested by their inhibition with the L-arginine competitive analogue, L-NAME. The specificity of this inhibition was established by reversing the L-NAME effect with excess L-arginine. Furthermore, in the presence of neutrophils, the NO scavenger, C-PTIO, reversed the antiaggregating platelet effects of triflusal.

Previous studies have demonstrated that triflusal and its deacetylated derivative, HTB, induce cAMP accumulation in platelets by inhibiting cAMP-phosphodiesterase activity (García-Rafanell et al., 1986). The more potent antiplatelet effect of triflusal as compared to that of acetylsalicylic acid could result from the inhibition of cGMP-phosphodiesterase activity in platelets, thus aiding the action of NO. In the platelet/neutrophil system, HTB alone produced a slight but significant accumulation of cGMP and also increased acetylsalicylic acid-stimulated cGMP levels which could be explained by the inhibition of cGMP-phosphodiesterase activity favouring the action of NO. However, the experiments on [^{3}H]L-citrulline production by isolated neutrophils revealed that triflusal stimulated NO generation by neutrophils directly with higher potency than acetylsalicylic acid.

Several recent findings have indicated that acetylsalicylic acid might contribute to increase NO synthesis or NO effects. In this regard, an antagonistic effect of acetylsalicylic acid has been observed on the vasoconstrictor properties of the L-arginine competitive analogue, L-NMMA (Rosemblum et al., 1992). In addition, acetylsalicylic acid might alter the contractile effects of neutrophils on the pulmonary artery (Patterson et al., 1992). More recently, we have shown that acetylsalicylic acid increases NO production by neutrophils (López-Farré et al., 1995), an effect that is enhanced after myocardial ischemia (López-Farré et al., 1996).

In a second set of experiments, we studied the mechanism by which triflusal caused a higher activation of NO production by neutrophils. We could not exclude that the aforementioned effects of triflusal on NO generation by neutrophils were exerted not only by triflusal itself but also by the combination of triflusal and the HTB generated in the solution. Therefore, we evaluated the involvement of the deacetylated moiety, HTB. HTB alone modified neither basal NO generation by neutrophils nor that induced by acetylsalicylic acid, indicating that the whole molecule of triflusal causes the activation of NO generation by neutrophils.

In a further set of experiments, we analyzed the involvement of cAMP in the mechanism that favors triflusal-stimulated NO production by neutrophils. Phosphorylation of enzymes represents a fast regulation mechanism of enzymatic activity. Several authors have demonstrated phosphorylation of NO synthase by cAMP-dependent protein kinase A (Brüne and Lapetina, 1991; Bredt et al., 1992), but to date the various findings regarding the effects of protein kinase A-dependent phosphorylation on the catalytic activity of NO synthase present a less than homogeneous picture (Brüne and Lapetina, 1991; Bredt et al., 1992; Tamaoki et al., 1995). Although the cAMP analogues enhanced acetylsalicylic acid-induced NO generation by neutrophils, the levels obtained were lower than those found in the triflusal experiments. Furthermore, the protein kinase A antagonist, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) slightly affected triflusal-dependent NO generation by neutrophils. These results suggested that the triflusal-sensitive stimulation of NO formation by neutrophils was unrelated to the activity of the cAMP/protein kinase A system. The present data do not allow us to identify the putative mechanism(s) involved in the greater increase in NO production induced by triflusal than by acetylsalicylic acid. However, the finding that the combination of acetylsalicylic acid plus HTB did not produce the levels of NO generation seen with triflusal-incubated neutrophils might indicate that to obtain these effects the action of the whole molecule of triflusal must be necessary.

The main difference between the molecular structures of acetylsalicylic acid and triflusal lies in the fluorinated moiety (Fig. 1) of the latter drug. The introduction of fluorine atoms changes the chemical and biological properties of drugs. A known example in cardiology is flecainide, a fluorinated derivative of procainamide (Hudak et al., 1984). There are several reports of differences between acetylsalicylic acid and triflusal with respect to inhibition of both cyclooxygenase (De la Cruz et al., 1986; De la Cruz et al., 1992) and cAMP-phosphodiesterase activities (Rabasseda and García-Rafanell, 1993). The mechanism by which acetylsalicylic acid activates NO generation by neutrophils remains unknown. Although preliminary data from our own laboratory have suggested a potential role for arachidonic acid and its non-cyclooxygenase derivatives (López-Farré et al., 1994), we could not exclude a direct action of acetylsalicylic acid on NO synthase, i.e. acetylation (Roth and Siok, 1978). If this latter possibility applies triflusal, by means of its fluorinated moiety, could activate NO synthesis by human neutrophils more efficiently than acetylsalicylic acid. Further studies, beyond the scope of the present research, are needed to elucidate this hypothesis.

Myocardial ischemia/reperfusion results in a diminished NO release from endothelial cells (Xin-Liang et al., 1993). Attempts have been made to supplement the reduced NO in order to attenuate myocardial injury. In animal models, the infusion of either organic NO donors, NO itself or the substrate for NO synthesis, L-arginine, provides significant cardioprotective effects against ischemia (Johnson et al., 1991; Weyrich et al., 1992; Lefer et al., 1993). Although acetylsalicylic acid is used in patients with ischemic heart disease because of its antithrombotic properties, additional protective effects could be obtained by enhancing the NO synthesis in neutrophils as found in our in vitro experiments with triflusal. Our results suggest that it is possible to develop new and more effective acetylsalicylic acid-related antithrombotic drugs whose therapeutic potential should be tested in clinical trials.

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