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The involvement of the release of nitric oxide in the pharmacological activity of the new furoxan derivative CHF 2363

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1 The mechanism of action and the pharmacological effects of the new furoxan derivative, CHF 2363 (4-ethoxy-3-phenylsulphonylfuroxan), were investigated.

2 Pre-incubation of CHF 2363 with human platelet-rich plasma produced a concentration-dependent inhibition of the platelet aggregation induced by collagen, adenosine diphosphate (ADP) and platelet activating factor (PAF). The test compound was about 5 times more potent than sodium nitroprusside. 3-Isobutyl-1-methyl-xanthine (IBMX) potentiated the antiaggregating effect of CHF 2363.

3 CHF 2363 was a potent inhibitor of rubbed endothelium rabbit aortic ring contraction induced by noradrenaline. Comparison of IC_{50} values showed that CHF 2363 was as potent as glyceryl trinitrate (GTN).

4 Increasing concentrations of CHF 2363 elevated platelet guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels. Adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels were unaffected.

5 Oxyhaemoglobin reduced all the pharmacological actions of the test compound. Moreover, CHF 2363 concentration-dependently released nitric oxide (NO) in platelet-rich plasma. The NO release was correlated to its ability to increase platelet cyclic GMP levels.

6 After exposure of rat aortic strips to supramaximal concentrations of GTN (550 μM), the vasorelaxant activity of CHF 2363 did not change, although that of GTN decreased about 55 fold.

7 It has been concluded that the new furoxan derivative CHF 2363 exerts a potent antiaggregating and vasorelaxant activity via NO release and increase of cyclic GMP levels. No *in vitro* cross tolerance between GTN and CHF 2363 was observed.

Keywords: Nitric oxide; platelet aggregation; vasodilatation; phenylsulphonylfuroxan; cyclic GMP

Introduction

Nitric oxide (NO) produced from L-arginine by the enzyme nitric oxide synthase (Bredt *et al.*, 1991; Bredt & Snyder, 1990) is an important mediator of a number of biological effects.

NO induces vasodilatation (Amezcua *et al.*, 1988) and inhibition of platelet aggregation (Radomski *et al.*, 1987) by activating soluble guanylate cyclase (Ignarro, 1989). It is also implicated in neurotransmission in the central nervous system (Garthwaite, 1991) and in regulating the cytotoxicity of the macrophages (Berdeaux, 1993).

The first proof that NO was at least one of the endothelium-derived relaxing factor(s) (EDRFs) resulted from studies by Palmer *et al.* (1987, 1988). They demonstrated that chemically detectable NO was released from cultured endothelial cells in an amount sufficient to account for all EDRF activities.

It is now generally accepted that the vasodilating and antiaggregating activity of sydnonimines, sodium nitroprusside and organic nitrates, including glyceryl trinitrate (GTN), arises as a result of their ability to release NO (Moncada *et al.*, 1991). Recently, Seth and Fung (1993) showed the presence of a membrane-bound enzyme in the bovine smooth muscle cells that appears to be primarily responsible for NO generation from organic nitrate esters.

Thus, the pharmacological and biochemical effects of EDRF, NO and nitrovasodilators are virtually identical. Consequently, nitrovasodilators may be considered potential exogenous EDRFs and therefore pharmacological substitutes in pathological situations where normal endothelium function fails. These findings have led to a renewed interest in drugs thought to act through the formation of NO, devoid of nitrate tolerance and with a duration of action longer than GTN and other organic nitrate esters.

There is increasing evidence that some compounds with a furoxan structure could initiate endogenous NO actions when applied to biological systems (Feelisch *et al.* 1992; Ferioli *et al.*, 1995). We demonstrated that 1,1-dinitro-ethyl and 4-**R**-3-(**R**-sulphonyl)furoxans possess features characteristic of NO, i.e. they induce vasodilatation and inhibition of platelet aggregation (Ghigo *et al.*, 1992; Gasco *et al.*, 1993; Civelli *et al.*, 1994).

In the present study we characterized the pharmacological properties of a novel 3-phenylsulphonylfuroxan CHF 2363 (4-ethoxy-3-phenylsulphonylfuroxan), which was found in our search for compounds with relaxant and antiaggregating effects. In particular, we investigated the effect of CHF 2363 on platelet aggregation, vascular tone and platelet guanosine 3':5' cyclic monophosphate (cyclic GMP) levels. Moreover, in order to define the mechanism of action of the compound, we studied (1) its ability to release NO in the platelet-rich plasma under the same experimental conditions used for the platelet aggregation studies, and (2) whether this NO generation was significantly correlated to the activation of soluble guanylate cyclase in the platelets produced by the test compound. In addition, we observed that the activity of CHF 2363 may be influenced by the development of tolerance.

Methods

Platelet aggregation studies

Blood was collected from normal healthy volunteers who had not taken any drugs during the previous two weeks, and

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transferred to tubes containing 0.1 volume of a 3.8% (w/v) trisodium citrate solution. Platelet-rich plasma was prepared by centrifugation at 160 g for 18 min at room temperature. Platelet-poor plasma was obtained by further centrifugation at 2000 g of the blood remaining after removal of platelet-rich plasma.

Platelet aggregation was studied photometrically (Born, 1962) with an aggregometer (Elvi Logis, Milan, Italy) connected to a linear recorder. An aliquot of platelet-rich plasma (500 μl at approximately 300,000 platelets μl^{-1}) was incubated in a cuvette at 37°C and stirred at 1000 rev min^{-1}. The test compound, dissolved in dimethyl sulphoxide (DMSO) or sodium nitroprusside, was added to platelet-rich plasma 1 min before the following aggregating agents were added: collagen (0.5–1.7 $\mu g\ ml^{-1}$), adenosine diphosphate (ADP) (1.36–2.60 μM), platelet activating factor (PAF) (0.1–1 μM).

Control samples received in parallel the same volume addition of DMSO (1 μl). This concentration of DMSO did not interfere with the platelet assay. Aggregation was recorded as % change in light transmission. The aggregation baseline was set by using platelet-rich plasma; full transmission (100%) was set by using platelet-poor plasma.

For each platelet-rich plasma preparation the concentration of aggregating agent employed was the smallest which elicited sustained maximal aggregation. The aggregation induced was irreversible with at least a 70–80% decrease in optical density in 5 min. In other experiments, oxyhaemoglobin or 3-isobutyl-1-methyl-xanthine (IBMX) was incubated for 1 min with platelet-rich plasma before the addition of CHF 2363. Experiments involving the addition of oxyhaemoglobin required a platelet-poor plasma reference blank with an equivalent amount of haemoprotein.

Preparation of rabbit aortic rings and tension recording

Male New Zealand rabbits (Charles River, Calco, Italy) weighing 2.4–2.8 kg were killed by stunning and exsanguination. The aorta was removed, cleared of adhering fat and connective tissue and cut into 4 mm wide rings. These were mounted under 2 g resting tension on stainless-steel hooks in 20 ml organ baths.

The rings were bathed in Krebs solution at 37°C containing (mM): NaCl 112.0, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 25.0, glucose 11.5, ascorbic acid 0.035 and gassed with 95% O_2 and 5% CO_2. Tension was measured isometrically by microdynamometer recorders (Ugo Basile, Milan, Italy).

Endothelial cells were removed by gently rubbing the intimal surface with a stainless-steel rod for 30–60 s. Successful removal of endothelial cells from the aortic rings was confirmed by the inability of acetylcholine to induce relaxation.

The endothelium-deprived rings were contracted by noradrenaline 0.3 μM. When a stable plateau of contraction was reached, cumulative concentrations of the test compound were added to the organ bath. Solvent addition (DMSO) in related concentrations had no appreciable effect on the contractile state of the rings. In some experiments the activity of GTN was assessed.

The relaxing effects of the compounds were referred to the sustained contraction which was considered as 100%.

When oxyhaemoglobin was used, contraction was induced with phenylephrine (0.3 μM) because the noradrenaline-induced tone was less stable in the presence of oxyhaemoglobin, as previously shown (Martin *et al.*, 1985).

When the phenylephrine-induced contraction reached a plateau, a single submaximal concentration of the test compound (0.1 μM) was added to two out of three rings obtained from the same animal. A submaximal concentration of papaverine (100 μM) was added to the third ring. When the relaxation was stable, oxyhaemoglobin 10 μM was added in order to test its ability to reverse the inhibitory activity of the compound.

Induction of in vitro *GTN tolerance*

Male albino rats (Sprague Dawley, 320–450 g, Charles River, Calco, Italy) were killed by decapitation. The thoracic aorta was removed, cleared of adhering fat and connective tissue and cut into helicoidal strips. These were mounted under 1 g resting tension on stainless-steel hooks in 20 ml organ baths.

The strips were bathed in Krebs solution at 37°C containing (mM): NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgCl_2$ 0.54, NaH_2PO_4 1.0, $NaHCO_3$ 25.0, glucose 11.0, EDTA 0.027 and gassed with 95% O_2 and 5% CO_2. Tension was measured isometrically by microdynamometer recorders (Ugo Basile, Milan, Italy).

According to the method described by Keith *et al.* (1982), the strips were contracted by noradrenaline 0.1 μM. When a stable plateau of contraction was reached, cumulative concentrations of CHF 2363 or GTN were added to the organ bath. After washing for 60 min, the strips were incubated with GTN (550 μM) or its solvent (0.25 ml of ethanol 96%) for 90 min. Tissues were then washed every 15 min for 1 h; after this time the cumulative concentration-response curve for the compounds was repeated.

Haemodynamic activity in anaesthetized rats

Male albino rats (Sprague Dawley, 340–410 g, Charles River, Calco, Italy) were anaesthetized by sodium pentobarbitone (60 mg kg^{-1}) and maintained by i.v. infusion (6 mg h^{-1}). The trachea was cannulated to facilitate spontaneous respiration and body temperature was maintained at 37°C by Homeothermic Blanket Control System (Harvard, England). Mean arterial pressure and heart rate were continuously monitored by a pressure transducer (HP 8805B) and medium gain amplifier (HP8802A) triggered by the ECG signal (Hewlett Packard, U.S.A.) respectively. When parameters were stable (about 15 min), increasing doses of the test compound or isosorbide-5-mononitrate (ISO-5-MN) were infused through the jugular vein at 50 $\mu l\ min^{-1}$ flow rate for 10 min. Cardiovascular parameters were recorded for a further 20 min after the infusion had ceased. Infusion of the solvent (DMSO) in related amounts had no appreciable effect on cardiovascular parameters.

Each animal received only one infusion. At the end of the experiment, the animals were killed by i.v. injection of euthanasic drug (Tanax, Hoechst).

The peak hypotensive response, recorded 2 min after the start of the infusion, was expressed as % change from pre-infusion level (baseline).

Measurement of platelet cyclic GMP level

An aliquot of platelet-rich plasma (1 ml), pre-incubated (1 min at 37°C) or not in the presence of oxyhaemoglobin (30 μM), was incubated at 37°C in a cuvette of an aggregometer and stirred at 1000 rev min^{-1} with CHF 2363 at the concentrations and for the time indicated.

At the end of the incubation, 1 ml of ice-cold trisodium citrate (85 mM), dextrose (111 mM) and citric acid (71 mM) were added to platelet-rich plasma, the platelets were quickly pelleted (2000 *g* for 10 min at 4°C) and the pellets were treated with 500 μl of ice-cold 6% trichloroacetic acid.

After freeze-thawing, the cellular extracts were centrifuged at 2000 *g* for 20 min at 4°C. Trichloroacetic acid was extracted 6 times, each with 2 ml of water saturated diethyl ether. Aliquots of the aqueous phase were kept frozen (−80°C) until analysis. Concentrations of cyclic GMP were determined by radioimmunoassay following acetylation of the sample, by use of commercially available kits. Adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) levels were measured by use of similar procedures.

The addition of [^{3}H]-cyclic GMP or [^{3}H]-cyclic AMP (~600 d.p.m./sample) to platelets allowed correction of the results for recovery (85–95%).

All experiments were performed in triplicate. The results are expressed as pmol of cyclic GMP/cyclic AMP per 10^9 platelets.

Spectrophotometric determination of NO

NO release from CHF 2363 in platelet-rich plasma was determined by the method based on the rapid and stoichiometric reaction of NO with oxyhaemoglobin to yield methaemoglobin and nitrate (Feelisch & Noack, 1987). The time-dependent recording of the methaemoglobin concentration mirrors the rate of NO formation.

Briefly, each 1 ml sample was incubated with oxyhaemoglobin 5 μM at 37°C in a dual-wave length spectrophotometer and the rate of NO-induced formation of methaemoglobin was measured by continuous recording of the extinction difference between 401 and 411 nm (respectively the maximum and the isobestic point for the difference spectrum of the methaemoglobin and oxyhaemoglobin). The molar extinction coefficient (ε) was found in our experimental conditions to be 30.8 $mM^{-1}\ cm^{-1}$. This is close to the value previously reported (32 $mM^{-1}\ cm^{-1}$, Schrör *et al.*, 1991).

The results are expressed as nM $min^{-1}\ mg^{-1}$ protein; the protein content was determined with the Coomassie protein assay reagent (Pierce Chemical, Rockford, U.S.A.).

Drugs

The following drugs were used: CHF 2363, 4-ethoxy-3-phenylsulphonylfuroxan, synthetized at the Dipartimento di Scienza e Tecnologia del Farmaco Torino, for Chiesi Farmaceutici S.p.A., Parma, Italy; isosorbide-5-monitrate (ISO-5-MN) synthetized by Chiesi Farmaceutici S.p.A. Parma, Italy; acetylcholine hydrochloride, L(-)noradrenaline bitartrate, L(-)phenylephrine hydrochloride, IBMX (3-isobutyl-1-methyl-xanthine), sodium nitroprusside, sodium citrate, citric acid, dextrose, papaverine hydrochloride, L-cysteine, glutathione (Sigma Chemical Co., St. Louis, USA); collagen (Mascia Brunelli, Milan, Italy); ADP (Stago, Asnieres, France); PAF C16 dissolved in saline containing 0.25% bovine serum albumin (Biomol. Plymouth Meeting, U.S.A.); sodium pentobarbitone (Grinsted Products, Copenhagen, Denmark); glyceryl trinitrate (Merck, Darmstadt, Germany). Oxyhaemoglobin was freshly prepared by reduction of human haemoglobin (Sigma) with sodium hydrosulphite (Fluka Chemika, Buchs, Switzerland) as previously described (Feelish & Noack, 1987). Kits for radioimmunoassay of cyclic GMP and cyclic AMP were supplied by Du Pont de Nemours, NEN products (Boston, MA, U.S.A.).

Data analysis

The data are expressed as mean ± s.e.mean of n experiments. Statistical analysis was performed by the two-tailed t tests for unpaired variables. P levels of <0.05 were considered significant. IC_{50} values were calculated by non-linear least square curve fitting using the Allfit computer program (De Lean *et al.*, 1978).

ED_{40} values, expressing the dose able to reduce mean arterial pressure by 40 mmHg, were calculated by least square linear equation.

Results

Inhibition of platelet aggregation by CHF 2363

Pre-incubation of CHF 2363 (0.1 – 10 μM) with human platelet-rich plasma resulted in a concentration-dependent inhibition of the platelet aggregation induced by collagen, ADP and PAF. The IC_{50} values were 0.57 ± 0.06, 0.39 ± 0.06 and 0.16 ± 0.01 μM respectively ($n=5$). The maximum anti-aggregating effect was rapid in onset (within 1 min of adding the compound to platelet-rich plasma) and did not differ significantly for a pre-incubation time up to 20 min.

The well-known nitrovasodilator sodium nitroprusside was about 5 times less potent inhibitor than CHF 2363 (IC_{50} values were: collagen = 1.95 ± 0.12 μM; PAF = 0.75 ± 0.26 μM; ADP = 1.78 ± 0.75 μM, $n=5$).

Oxyhaemoglobin has been reported to bind NO and destroy its biological activity (Gibson & Roughton, 1957; Mellion *et al.*, 1981). The addition of the haemoprotein reduced in a concentration-dependent way the effect of CHF 2363 on platelet aggregation induced by PAF without affecting aggregation *per se* (Figure 1). A similar activity of oxyhaemoglobin was also observed towards sodium nitroprusside (not shown).

The phosphodiesterase inhibitor IBMX 5 μM, a concentration which did not directly affect platelet aggregation, potentiated the antiaggregating activity of CHF 2363 (4 fold at 0.1 μM CHF 2363, $n=3$).

Vasorelaxant response of CHF 2363 on rabbit aortic rings

Increasing concentrations of CHF 2363 (0.001 – 1 μM) caused concentration-dependent relaxation of rubbed endothelium rabbit aortic rings contracted with noradrenaline ($IC_{50} = 0.025 \pm 0.002$ μM, $n=7$); the test compound was as potent as GTN ($IC_{50} = 0.027 \pm 0.002$ μM, $n=7$).

Oxyhaemoglobin 10 μM reversed completely the vasodilator effect induced by CHF 2363 0.1 μM, while it was ineffective against that induced by 100 μM papaverine (Figure 2).

Cross-tolerance to the vasorelaxant effects of GTN and CHF 2363

This study was carried out on rat aortic strips, because the rabbit aortic preparations are somewhat resistant to GTN tolerance development and require peculiar conditions, i.e. alkaline medium (Keith *et al.*, 1982). The investigation was restricted to cross-tolerance between CHF 2363 and GTN, because preliminary experiments showed that after incubation of the aortic strips with supramaximal concentrations of CHF 2363 for extended periods of time, there was an incomplete recovery of noradrenergic tone after washing with fresh Krebs buffer. On the other hand, normal recovery was observed in the concentration range used to obtain the concentration-response curve (0.001 – 1 μM).

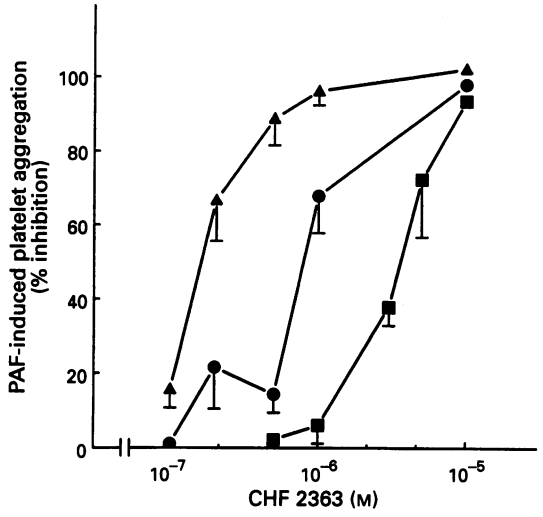

Figure 1 Influence of increasing concentrations of oxyhaemoglobin on CHF 2363-elicited inhibition of platelet-activating factor (PAF)-induced platelet aggregation. (▲) CHF 2363 in the absence of oxyhaemoglobin; (●) CHF 2363 + oxyhaemoglobin 10 μM; (■) CHF 2363 + oxyhaemoglobin 30 μM. Oxyhaemoglobin was pre-incubated for 1 min with platelet-rich plasma before the addition of the test compound and then 1 min later PAF was added. Each value represents the mean ± s.e.mean of 3 – 5 experiments.

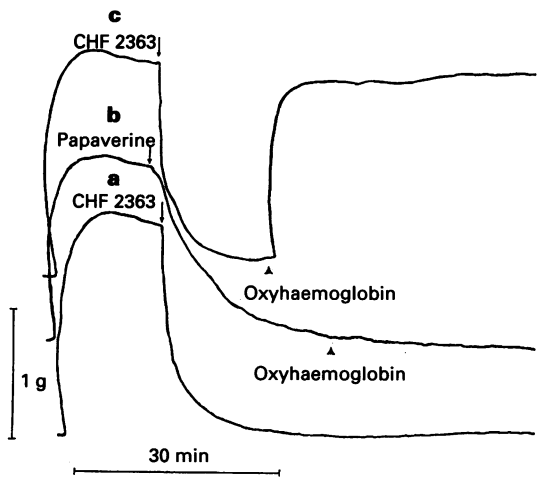

Figure 2 Effect of oxyhaemoglobin 10 μM on CHF 2363 and papaverine-elicited relaxation of phenylephrine contracted rabbit aortic rings. The relaxation induced by CHF 2363 in the absence of oxyhaemoglobin (a) was stable, while in the presence of oxyhaemoglobin (c) it was almost completely reversed in a few minutes. The relaxation induced by papaverine was not changed by oxyhaemoglobin (b). Typical recording representative of 5 different experiments.

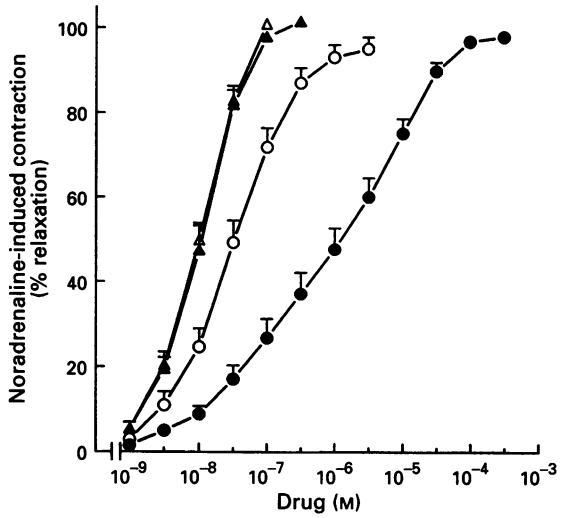

Figure 3 *In vitro* GTN tolerance and CHF 2363 cross-tolerance. Concentration-response curves for relaxation of noradrenaline-contracted rat aortic strips by GTN (○) and CHF 2363 (Δ) before (open symbols) and after (solid symbols) 90 min exposure to 550 μM GTN. Each point represents the mean ± s.e.mean of 12 experiments.

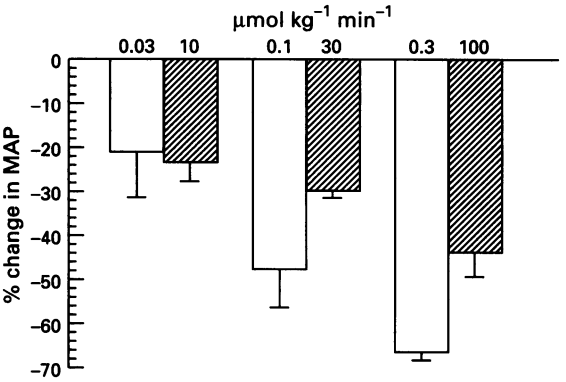

Figure 4 Peak effect induced on mean arterial pressure (MAP) by intravenous infusion of CHF 2363 (open columns) and IS-5-MN (cross-hatched columns) in the anaesthetized rat. Each value represents the mean ± s.e.mean of 4–9 experiments.

Because of the presence of ethanol (1.25%) in the GTN solution used for tolerance development (see Methods), in preliminary experiments the activity of both drugs was evaluated before and after a 90 min incubation with the same concentration of ethanol. This did not interfere with the vasorelaxant activity of either drug. The IC_{50} values for CHF 2363, respectively before and after ethanol incubation, were: 0.013 ± 0.0007 and 0.005 ± 0.0002 μM ($n = 11$) and for GTN 0.032 ± 0.002 and 0.016 ± 0.001 μM ($n = 12$). In our experimental conditions, a 90 min incubation in 550 μM GTN produced an approximate 55 fold shift to the right of the GTN concentration-response curve ($IC_{50} = 0.029 \pm 0.003$ μM, $n = 12$ and 1.57 ± 0.33 μM, $n = 12$, respectively before and after tolerance induction) (Figure 3).

In contrast, previous treatment of rat aortic strips with GTN did not reduce the vasorelaxant activity of CHF 2363 ($IC_{50} = 0.011 \pm 0.001$, $n = 12$ and 0.011 ± 0.0009 μM, $n = 12$, respectively before and after tolerance induction) (Figure 3).

Haemodynamic activity of CHF 2363 in anaesthetized rats

The continuous infusion of CHF 2363 in anaesthetized rats caused a marked and dose-dependent fall in mean arterial pressure ($ED_{40} = 0.053$ μmol kg^{-1} min^{-1}, 95% confidence interval 0.021–0.086, $n = 5$). The reference compound IS-5-MN was markedly less potent and effective than CHF 2363 ($ED_{40} = 33.11$ μmol kg^{-1} min^{-1}, 95% confidence interval 19.48–59.97, $n = 8$) (Figure 4). No significant change was induced by either drug on heart rate.

Effect of CHF 2363 on cyclic GMP levels in human platelets

When preincubated with human platelet-rich plasma, CHF 2363 produced a rise in platelet cyclic GMP content (Figure 5). The maximum accumulation obtained with 10 μM CHF 2363 was observed after 1 min and corresponded to a 500% increase in cyclic GMP levels over the baseline (basal level was 1.84 ± 0.08 pmol per 10^9 platelets). The test compound was approximately 10 times more potent than sodium nitroprusside; neither compound had any significant effect on platelet cyclic AMP levels (not shown).

Oxyhaemoglobin 30 μM reduced the activity of CHF 2363 on cyclic GMP levels (Figure 5). The inhibitory activity of oxyhaemoglobin towards the rise in cyclic GMP levels elicited by sodium nitroprusside (about 92% inhibition at 100 μM sodium nitroprusside, $n = 5$) was similar to that previously reported (Salvemini *et al.*, 1990).

NO formation in platelet-rich plasma from CHF 2363

We studied the correlation between NO liberation and CHF 2363 concentration in the platelet-rich plasma, under the same experimental conditions used for the platelet aggregation studies, by uses of a spectrophotometric assay specific for the detection of NO (Feelisch & Noack, 1987). CHF 2363 concentration-dependently released NO in platelet-rich plasma in the concentration range 0.5 and 10 μM (Figure 6).

There was a highly significant correlation between NO release from CHF 2363 in the platelet-rich plasma and its ability to stimulate platelet cyclic GMP levels ($n = 0.988$, $P < 0.01$, inset to Figure 6).

The compound released NO not only upon incubation with platelet-rich plasma, but also following reaction with reduced thiols such as 5 mM L-cysteine and glutathione. In these conditions, 1 μM CHF 2363 in 1 ml of 50 mM phosphate buffer,

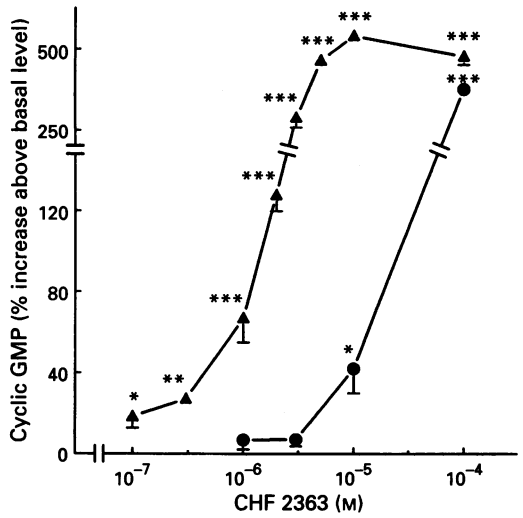

Figure 5 Effect of CHF 2363 on cyclic GMP levels in human platelets. When preincubated with human platelet-rich plasma, CHF 2363 (▲) produced a concentration-dependent elevation in platelet cyclic GMP content. The maximum accumulation (10 μM CHF 2363) corresponded to a 500% increase in cyclic GMP levels over the baseline (basal level was 1.89 ± 0.08 pmol per 10^9 platelets). Oxyhaemoglobin 30 μM (●) inhibited the activity of CHF 2363 on cyclic GMP levels. Each point is the mean ± s.e.mean of 3 experiments; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significant difference when values for CHF 2363 were compared to basal levels (Student's t test).

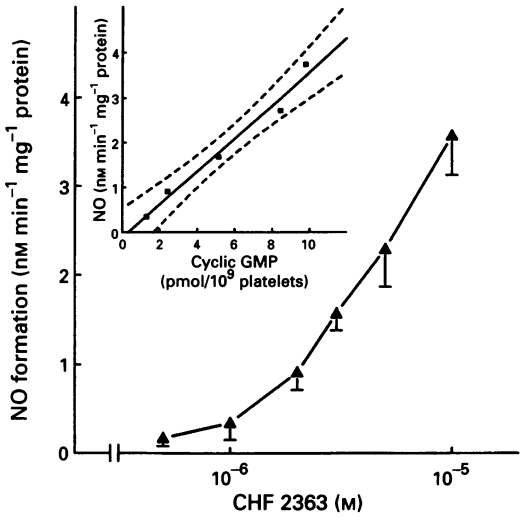

Figure 6 NO formation in platelet-rich plasma from CHF 2363. Each value is the mean ± s.e.mean of 6 separate experiments. The inset represents the correlation between NO formation and platelet cyclic GMP levels. Dashed lines indicate the 95% confidence intervals.

pH 7.4 at 37°C, released 28 ± 6 and 32 ± 5 nM min^{-1} of NO. No NO generation from CHF 2363 was detected in the buffer in the absence of thiols.

Discussion

The data shown in this study indicate that the new 3-phenylsulphonylfuroxan derivative CHF 2363 exerts its pharmacological activity via release of NO. In fact, its potent relaxant effect in rabbit isolated aortic rings was independent of endothelium integrity as already noted for NO and NO-donating vasodilators, such as GTN and sodium nitroprusside (Furchgott, 1984; Ignarro & Kadowitz, 1985).

Moreover, in human platelet-rich plasma CHF 2363 both reduced the platelet aggregation induced by PAF, collagen or ADP and produced a concentration-dependent elevation in the platelet cyclic GMP levels. The time-course of the anti-aggregating activity and that of the changes in platelet cyclic GMP levels were similar (the maximal antiplatelet effect and the peak of cyclic GMP were observed within 1 min of adding the compound to platelet-rich plasma), indicating that these two phenomena were associated. In line with this evidence, the phosphodiesterase inhibitor IBMX at 5 μM, a concentration which did not directly affect platelet aggregation, potentiated the antiaggregating action of the test compound. Finally, oxyhaemoglobin, which binds NO and destroys its biological activity, reduced the inhibition of platelet aggregation, the relaxation of vascular smooth muscle and the elevation of the platelet cyclic GMP levels elicited by CHF 2363, suggesting that the pharmacological effects of the test compound are related to the stimulation of guanylate cyclase by means of NO liberation. This mechanism was confirmed by the ability of CHF 2363 to release NO in the platelet-rich plasma and by the close correlation between the rate of NO formation and the rise in platelet cyclic GMP levels.

In accordance with data previously reported by Feelisch *et al.* (1992) on the role of sulphydryl groups of low molecular weight thiols and proteins in the mechanism underlying the NO-released from some 3,4 furoxandicarboxamide derivatives, CHF 2363 also released NO when platelet-rich plasma was replaced by a buffer containing reduced thiols such as L-cysteine and glutathione, which serve as a source of thiols equivalent to the mercapto groups present in plasma.

It is worthwhile noting that low concentrations of CHF 2363 (0.1 – 0.3 μM) caused a significant increase in platelet cyclic GMP levels (Figure 5) without releasing a measurable amount of NO after incubation with platelet-rich plasma (Figure 6). This apparent discrepancy might be explained by the fact that intraplatelet NO levels produced by CHF 2363 0.1 – 0.3 μM were sufficient to stimulate the soluble guanylate cyclase, although NO levels in the plasma were below the detection limit of the oxyhaemoglobin technique (< 0.15 nM min^{-1} mg^{-1} prot.). On the other hand, even at concentrations as low as 0.1 – 0.3 μM, the pharmacological activity of CHF 2363 can be ascribed to the release of NO, as indicated by the inhibitory effect of oxyhaemoglobin on (1) the reduction of platelet aggregation, (2) the relaxation of vascular smooth muscle and (3) the elevation of the platelet cyclic GMP levels.

After exposure to a very high concentration of GTN (550 μM) for 90 min, a significant development of tolerance was observed for GTN in rat aortic strips but there was no cross-tolerance between CHF 2363 and GTN. This finding is consistent with similar observations reported for a 3,4-furoxan-dicarboxamide derivative (ipramidil) (Feelisch *et al.*, 1992) and suggests that nitrates and furoxans release NO through different mechanisms. Although the mechanism(s) leading to tolerance development of nitrates is still a matter of debate, recent observations point to impairment of the process of their bio- transformation to NO (Forster *et al.*, 1991; Zhang *et al.*, 1994). In this respect, the hypothesis of nitrate tolerance due to depletion of critical intracellular sulphydryl groups (Needleman & Johnson, 1973; Ignarro *et al.*, 1981) may provide a good explanation for the different action of GTN and CHF 2363 in rat aortic strips made tolerant to GTN. In fact, it has been demonstrated that the metabolism of organic nitrates, including GTN, to NO requires sulphydryl groups as co-factors, present mainly in a few thiols (e.g. cysteine or N-acetylcysteine) (Feelisch, 1991). In contrast, NO release from CHF 2363 does not appear to depend on the presence of specific thiol compounds, but it seems that all available thiols are able to react with the test compound. However, some authors have recently suggested that nitrate tolerance is not necessarily associated with reduced thiol levels. Other possible mechanisms have been proposed, such as desensitization of guanylate cyclase (Waldman *et al.*, 1986), an involvement of the enzymatic bioactivation pathway (Kojda *et*

al., 1993) and a biochemical damage by the reactive free radicals NO and superoxide radical ions formed during bio-transformation (Yeates & Schmid, 1992).

In conclusion, we have shown that the new 3-phenylsulphonylfuroxan derivative CHF 2363 exerts a potent anti-aggregating and vasorelaxant activity via NO release and increase of cyclic GMP levels; it may therefore be classified as a nitrovasodilator. An agent that relaxes vascular smooth muscle and simultaneously inhibits platelet aggregation could be a potent therapeutic tool in pathological situations where the endothelium is deficient, such as atherosclerosis, its ischaemic and thrombotic complications, and other vasospastic disorders. Further investigations with CHF 2363 in animal models will explore its haemodynamic profile and reversibility of effects, and will assess whether tolerance occurs after long-term treatment with the compound.

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