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Mechanism of the Stimulation of Prostaglandin H Synthase and Prostacyclin Synthase by the Antithrombotic and Antimetastatic Agent, Nafazatrom

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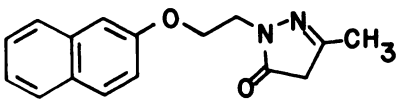
SUMMARY

Nafazatrom, an antithrombotic and antimetastatic agent containing a pyrazolone functionality, is a reducing substrate for the peroxidase activity of prostaglandin H (PGH) synthase. Nafazatrom inhibits the hydroperoxide-dependent oxidation of phenylbutazone, stimulates the reduction of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid, and is oxidized by microsomal or purified enzyme preparations from ram seminal vesicles. Consonant with the effects of other peroxidase-reducing substrates, nafazatrom stimulates the oxygenation of arachidonic acid to prostaglandin endoperoxides by the cyclooxygenase component of PGH synthase. In addition, nafazatrom causes an elevation in the levels of 6-keto-prostaglandin $F_{1\alpha}$, the non-enzymatic hydrolysis product of prostacyclin (PGI_2) biosynthesized from arachidonic acid by ram seminal vesicle microsomes. Elevation of PGI_2 biosynthetic capacity by nafazatrom occurs under conditions in which prostaglandin endoperoxide biosynthesis is maximal, suggesting that nafazatrom has a stimulatory effect on the conversion of prostaglandin endoperoxides to PGI_2. Nafazatrom has no effect on the ability of ram seminal vesicle microsomes to convert PGH_2 to PGI_2 but protects microsomal PGI_2 synthase from inactivation by 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid. Nafazatrom stimulates PGI_2 biosynthesis in ram seminal vesicle microsomes by acting as a substrate for the peroxidase-catalyzed reduction of hydroperoxy fatty acids that are irreversible inactivators of PGI_2 synthase. Several other compounds, including dipyridamole and triiodothyronine, exert similar effects. This may contribute to the reported ability of nafazatrom and related compounds to elevate the levels of bioassayable PGI_2 *in vivo* and to the antithrombotic and antimetastatic activities of nafazatrom.

INTRODUCTION

Nafazatrom, 2,4-dihydro-5-methyl-2-[2-(2-napthyloxy)ethyl]-3*H*-pyrazol-3-one (see structure below), is an



exciting new pharmacological agent that exhibits significant antithrombotic and antimetastatic activity *in vivo*, but low toxicity (1, 2). It is not a thromboxane synthase or phosphodiesterase inhibitor and it does not have a direct antiaggregatory effect on platelets (1). Its antithrombotic and antimetastatic effects may be the result of a unique mechanism of action and it may represent the prototype of a new class of pharmacologically active agents (3).

Nafazatrom elevates the levels of bioassayable PGI_2[7]

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[7] The abbreviations used are: PGI_2, prostacyclin; BW755C, 3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline; PGH, prostaglandin H; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; PB, phenylbutazone; 20:4, 5,8,11,14-eicosatetraenoic acid; RSVM, ram seminal vesicle

following administration to rats (3). Since PGI_2 is a potent inhibitor of platelet aggregation (4) and of tumor cell metastasis (5), the antithrombotic and antimetastatic activities of nafazatrom may derive from its ability to elevate PGI_2 levels *in vivo* (1–3). The mechanism by which nafazatrom raises PGI_2 levels is unknown. *A priori*, several possibilities exist. Nafazatrom could stimulate PGI_2 biosynthesis (6), elevate the levels of PGI_2 synthase, inhibit PGI_2 degradation (7), or stimulate the release of PGI_2 into plasma.

BW755C contains a pyrazoline ring structurally related to the pyrazolone present in nafazatrom, and it is a potent reducing substrate for the peroxidase component of PGH synthase (8). This peroxidase reduces PGG_2 and other hydroperoxy fatty acids to hydroxy fatty acids (9). Since hydroperoxy fatty acids, but not hydroxy fatty acids, are potent inhibitors of PGI_2 synthase (10), peroxidase-reducing substrates can lower the steady-state concentrations of hydroperoxy acids and stimulate PGI_2 synthase. Indeed, such an effect has been described for the peroxidase substrates 2-aminomethyl-4,5-butyl-6-iodophenol and propyl gallate (11, 12) and for vitamin C and tryptophan (13). We have tested nafazatrom for its effect on the peroxidase and cyclooxygenase components of PGH synthase and for its effect on PGI_2 synthase in RSVM. We report here that nafazatrom is a potent reducing substrate for the peroxidase and stimulates cyclooxygenase and PGI_2 synthase activities *in vitro*. As required of a peroxidase-reducing substrate, nafazatrom is oxidized concomitant with hydroperoxide reduction. Dipyridamole, another compound that has been reported to elevate PGI_2 levels and is antithrombotic (14), also enhances the conversion of arachidonic acid to PGI_2. The ability of these compounds to serve as peroxidase-reducing substrates and to enhance PGI_2 biosynthesis may be an important component of their pharmacological activities *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. 20:4 (99% pure) was obtained from NuChek Inc. (Elysian, Minn.). 1-[^{14}C]20:4 was from New England Nuclear Corporation (Boston, Mass.) or Amersham (Arlington Heights, Ill.). 5,6,8,9,11,12,14,15-[^{3}H]20:4, 5,8,9,11,12,14,15-[^{3}H]6-keto-$PGF_{1\alpha}$, 5,6,8,11,12,14,15-[^{3}H]PGE_2, 5,6,8,9,11,12,14,15-[^{3}H]$PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ radioimmunoassay kits were purchased from New England Nuclear Corporation. 15-HPETE was synthesized from 20:4 using soybean lipoxygenase (Sigma Chemical Company, St. Louis, Mo.) according to the method of Funk *et al.* (15). 1-[^{14}C]PGH_2 was biosynthesized from 1-[^{14}C]20:4 and purified by HPLC before use (16). Nafazatrom was a gift from Bayer AG, West Germany. Hematin was from Calbiochem-Behring Corporation (San Diego, Calif.). Other chemicals were the highest purity obtainable from Sigma Chemical Company.

Methods. Ram seminal vesicles were obtained from a local slaughterhouse and stored at −80°. They were homogenized, and the microsomal fraction (RSVM) was prepared as previously described (17). Microsomal protein was diluted to 10 mg/ml in 1.15% KCl containing 25 mM HEPES buffer (pH 7.8) as determined by the protein assay of Lowry *et al.* (18). Either it was used immediately or 1-ml aliquots were stored at −80° until used.

PGH synthase was purified from RSVM by ion exchange chromatography on DE53. RSVM were suspended in solubilization buffer [10 mM Tris (pH 8.0)/1% Tween 20/0.1 mM diethyldithiocarbamate/0.5 mM EDTA] for 1 hr and centrifuged at 100,000 × *g* for 60 min. The supernatant was concentrated to 20 mg of protein per milliliter (Amicon XM50 membrane) and applied to a DE53 column (30 g of gel per gram of protein) at a flow rate of 1.5 ml/min. The DE53 had been packed according to the manufacturer's recommendations and equilibrated with 10 mM Tris·HCl (pH 8.0)/0.1% Tween 20/0.3 mM diethyldithiocarbamate. The column was eluted for 5 hr at a rate of 1.5 ml/min with wash buffer followed by elution with 14 column volumes of a linear gradient of 10–80 mM Tris·HCl (pH 8.0)/0.1% Tween 20/0.3 mM diethyldithiocarbamate. Active fractions were concentrated and stored at −70°. PGH synthase purified by this procedure exhibited hematin-reconstitutable cyclooxygenase and peroxidase activities of 11 μmol of 20:4 oxidized per minute and 7 μmol of PB oxidized per minute, respectively. Quantitative purification studies indicated that it contains all of the PB peroxidase activity in RSVM.[8] The purified protein exhibited a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Inhibition of PB peroxidase. PB (150 μM) and varying concentrations of nafazatrom were incubated with RSVM (0.75 mg/ml) in 1.4 ml of 0.1 M sodium phosphate (pH 7.8). Oxidation was initiated by the addition of 15-HPETE (150 μM), and the rate and extent of O_2 uptake were measured using a Clark electrode (19).

Reduction of 15-HPETE by nafazatrom. Incubations were carried out in the dark at 22° using 0.1 M HEPES (pH 7.8). Nafazatrom (100 μM) was preincubated with RSVM (0.5 mg of protein per milliliter) or with purified PGH synthase (0.4 μM) and hematin (0.7 μM) for 1 min followed by the addition of [^{3}H]15-HPETE (25 μM). The reaction mixture was stirred for 1 min, acidified with 6 N HCl to pH 3, and extracted with hexane/ether (1:1). The organic layer was separated, and an aliquot was injected onto a Waters μ-Bondapak C_{18} column (4 × 250 mm) eluted with hexane/isopropanol/acetic acid (991:8:1) at a flow rate of 2.0 ml/min. 15-HPETE and 15-HETE were identified by comparison to authentic standards and quantitated with a Varian Varichrome UV detector set at 235 nm. Recoveries of radioactivity were determined by liquid scintillation counting.

PGH_2 biosynthesis. The conversion of 20:4 to PGH_2 by RSVM was estimated by measuring the rate of O_2 consumption (Clark electrode, Yellow Springs Instrument Company, Yellow Springs, Ohio) based on the stoichiometry of 2 mol of O_2 incorporated per mol of PGH_2 biosynthesized. The 2:1 stoichiometry was previously verified using an identical microsomal preparation (20). RSVM (0.1 ml) were added to 1.9 ml of 0.1 M HEPES buffer (pH 7.8) at 37°, to a final protein concentration of 0.25 mg/ml. Incubations with purified PGH synthase contained the protein concentrations stated in the figure legend. Nafazatrom, dissolved in ethanol or dimethyl sulfoxide, was added at concentrations of 1–200 μM. Controls and all incubations contained the same amount of ethanol or dimethyl sulfoxide. The reaction was initiated by the addition of 100 μM 20:4 dissolved in 10 μl of ethanol. The initial velocity of PGH_2 formation was determined from a tangent to the linear portion of the O_2 uptake curve.

Conversion of 1-[^{14}C]20:4 into prostaglandins. RSVM, 0.5 mg/ml, were incubated with or without nafazatrom in 0.1 M HEPES buffer (pH 7.8) at 25° for 5 min. The reaction was initiated by the addition of 1–250 μM 1-[^{14}C]20:4 (1 μCi/incubation). Nafazatrom was dissolved in ethanol, and all incubations contained the same amount of ethanol.

The reaction was terminated by the addition of 0.1 N HCl to pH 3.5 and extracted with 5 volumes of ethyl acetate. The acid residues were analyzed by thin-layer chromatography or HPLC, and the products were identified by co-chromatography with authentic standards (20, 21).

PGI_2 biosynthesis. The formation of PGI_2 from 20:4 was also measured by radioimmunoassay for 6-keto-$PGF_{1\alpha}$. RSVM (0.25 mg/ml) were added to 0.46 ml of 0.1 M HEPES buffer (pH 7.8 at 37°). Nafazatrom was added at various concentrations. All systems received the same

microsomes; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PGE_2, prostaglandin E_2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography.

[8] W. Pagels, unpublished results.

amount of ethanol, and the final volume was 0.5 ml. After a 30-sec preincubation, the reaction was initiated by the addition of 100 μM 20:4. After 5 min the reaction was terminated by the addition of acetic acid and cooled to 0°. An aliquot of each incubation mixture was diluted for the radioimmunoassay of 6-keto-$PGF_{1\alpha}$, the stable end-product of PGI_2 hydrolysis.

Metabolism of 1-[^{14}C]PGH_2 to 1-[^{14}C]PGI_2. RSVM (0.5 mg/ml) were incubated with 0.5 or 2.0 μM 1-[^{14}C]PGH_2 in 1.8 ml of 0.1 M HEPES (pH 7.8) in the presence or absence of 186 μM nafazatrom. Incubations without nafazatrom contained the same volume of ethanol. Reactions were allowed to proceed for 3 min at 25° and were terminated by acidification to pH 3.0. Following extraction, PGI_2 was estimated by HPLC analysis for 6-keto-$PGF_{1\alpha}$ (21).

Inhibition of PGI_2 synthase by 15-HPETE. RSVM were added to 0.885 ml of 0.1 M HEPES buffer (pH 7.8) to a final protein concentration of 0.5 mg/ml. Nafazatrom (186 μM) was added in 10 μl of ethanol. Controls received 10 μl of ethanol. Varying concentrations of 15-HPETE were added and preincubated for 1 min at 25°. 1-[^{14}C]PGH_2, (4 μM) was added, and the mixture was incubated for 3 min. The reaction was terminated by the addition of 0.1 N HCl to pH 3.5 and extracted with ethyl acetate. 1-[^{14}C]6-keto-$PGF_{1\alpha}$ was isolated and quantitated by HPLC.

Spectrophotometric assay of nafazatrom oxidation. Nafazatrom (100 μM) was incubated in 3.0 ml of 0.1 M sodium phosphate (pH 7.8) in the sample cell of an Aminco DW-2a spectrophotometer operated in the dual-wavelength, single-beam mode. Varying concentrations of RSVM or hematin-reconstituted PGH synthase were added, and the suspension was preincubated at ambient temperature for 1 min. H_2O_2 was added, and metabolism was monitored by the difference in absorbance betwen 327 and 295 nm.

RESULTS

Ability of nafazatrom to serve as a reducing substrate for the peroxidase of PGH synthase. We have used two methods to determine whether nafazatrom is a reducing substrate for the peroxidase activity of PGH synthase. The first measures the ability of nafazatrom to inhibit the PGH synthase-dependent oxidation of PB. PB is oxygenated to 4-hydroxy-PB by the peroxidase of PGH synthase; other compounds that are oxidized by the peroxidase inhibit this process by acting as alternate substrates (19, 22). Nafazatrom inhibits 15-HPETE-dependent PB oxygenation by RSVM (Fig. 1). Maximal inhibition is observed at 100 μM and half-maximal inhibition at approximately 16 μM nafazatrom.

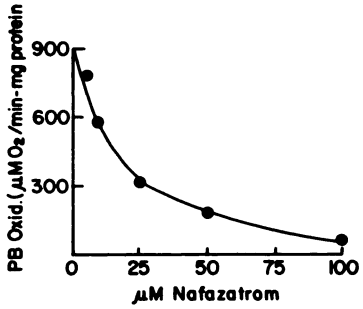

FIG. 1. *Inhibition of PGH synthase-dependent oxygenation of phenylbutazone by nafazatrom*

RSVM (0.75 mg of protein), PB (150 μM) and nafazatrom were mixed in 1.4 ml of 0.1 M sodium phosphate (pH 7.8). Oxidation was initiated by the addition of 15-HPETE (150 μM), and O_2 uptake was measured with a Clark electrode. The data points are the average of three experiments. The standard deviations are less than 10% of each value.

Direct evidence for the ability of nafazatrom to act as a peroxidase-reducing substrate is presented in Fig. 2. Nafazatrom was incubated with RSVM and 25 μM [^{3}H] 15-HPETE. The reaction was terminated after 1 min, and the acid ethyl acetate extract of the incubation mixture was analyzed by straight-phase HPLC with UV detection at 235 nm and liquid scintillation counting. In the presence of nafazatrom, all of the 15-HPETE was reduced to 15-HETE whereas in the absence of nafazatrom only 20% was reduced. The recovery of radioactivity ranged from 80% to 85% in individual experiments. Similar results were obtained with 400 nM purified PGH synthase (data not shown). Nafazatrom stimulated the reduction by purified enzyme of 80% of 70 μM 15-HPETE to 15-HETE as compared with 16% reduction in its absence.

Peroxidatic metabolism of nafazatrom by RSVM. Aqueous solutions of nafazatrom exhibited weak absorption bands at 327 and 313 nm and a strong absorption band at 230 nm. When H_2O_2 was added to solutions of nafazatrom containing RSVM or purified PGH synthase, the absorptions at 327 and 313 nm did not change appreciably. The peak at 230 nm shifted to 232 nm and increased in intensity. The absorption between 250 and 310 nm increased in intensity, and a peak appeared at 261 nm. These spectral changes were not observed when H_2O_2 was added to solutions of nafazatrom containing heat-denatured RSVM or when H_2O_2 was added to intact

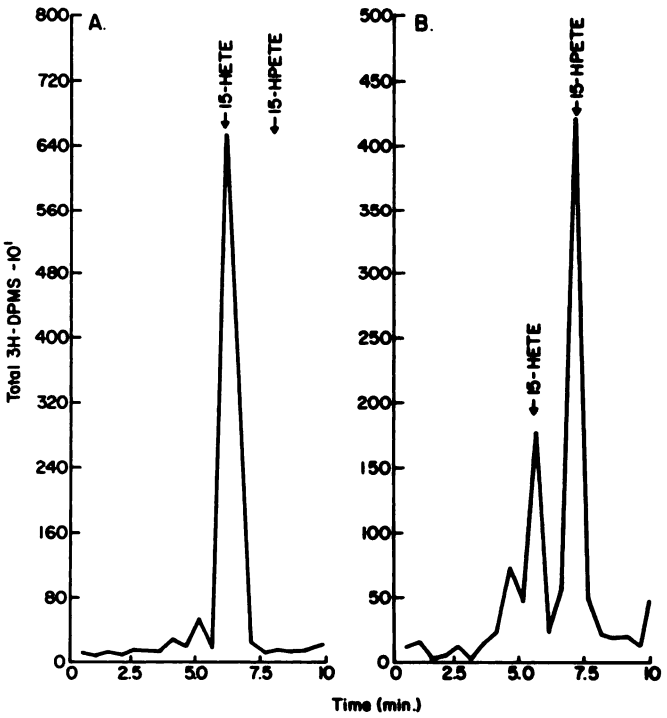

FIG. 2. *Effect of nafazatrom on the reduction of 15-HPETE by RSVM*

RSVM (0.5 mg of protein) and 15-HPETE (25 μM) were incubated for 1 min at 22° in 1.0 ml of 0.1 M HEPES (pH 7.8) with nafazatrom (100 μM) or an equal volume of solvent. Products were extracted and analyzed by HPLC as described under Experimental Procedures. Recovery of radioactivity following extraction and chromatography was 80–85%.

RSVM in the absence of nafazatrom. The increase in absorbance at 295 nm relative to 327 nm could be used to follow nafazatrom metabolism. Figure 3 displays the time course of $\Delta A_{295-327}$ following the addition of H_2O_2 to RSVM in the presence of nafazatrom. Rapid spectral changes were observed that paralleled the kinetics of oxidation of other peroxidase substrates by PGH synthase (6). The *inset* of Fig. 3 displays the linear dependence of the initial velocity of nafazatrom metabolism on the microsomal protein concentration. Similar results were obtained with purified PGH synthase. Furthermore, both microsomal and purified PGH synthase catalyzed the hydroperoxide-dependent consumption of nafazatrom as measured by HPLC.[9] These observations establish that nafazatrom is oxidized by the peroxidase component of PGH synthase in the presence of hydroperoxides. Taken with the results above, it is clear that nafazatrom fulfills all the requirements for a peroxidase-reducing substrate.

Effect of nafazatrom on prostaglandin biosynthesis. The stimulatory effect of nafazatrom on the oxygenation of 20:4 by RSVM is shown in Fig. 4; increases in the initial velocity and final extent of oxygenation are evident. Oxygen uptake in the absence or presence of nafazatrom was completely inhibited by 10 μM indomethacin, suggesting that the 20:4 oxygenation was catalyzed by cyclooxygenase. Nafazatrom also stimulated 20:4-dependent O_2 uptake by purified and hematin-reconstituted PGH synthase. Figure 5 displays the effect of varying concentrations of nafazatrom on 20:4 oxygenation by the purified enzyme.

The data presented in Fig. 6 confirm that the stimulation of oxygenation by nafazatrom reflects stimulation of prostaglandin endoperoxide biosynthesis. 1-[^{14}C]20:4 was incubated with RSVM in the presence of varying concentrations of nafazatrom. The radiolabeled products were extracted and quantitated after separation by HPLC. Nafazatrom stimulated PGE_2 biosynthesis approximately 5-fold, and 6-keto-$PGF_{1\alpha}$ (hydrolysis product of PGI_2) biosynthesis 2- to 3-fold at 20:4 concentrations of 30–100 μM. The high level of stimulation of 6-keto-$PGF_{1\alpha}$ biosynthesis by nafazatrom at 250 μM 20:4 was primarily due to the low basal level of 6-keto-$PGF_{1\alpha}$ biosynthesized in the absence of nafazatrom. The low basal level of 6-keto-$PGF_{1\alpha}$ biosynthesis at 250 μM 20:4 was due to a high rate of hydroperoxide-dependent inactivation of PGI_2 synthase (*vide infra*).

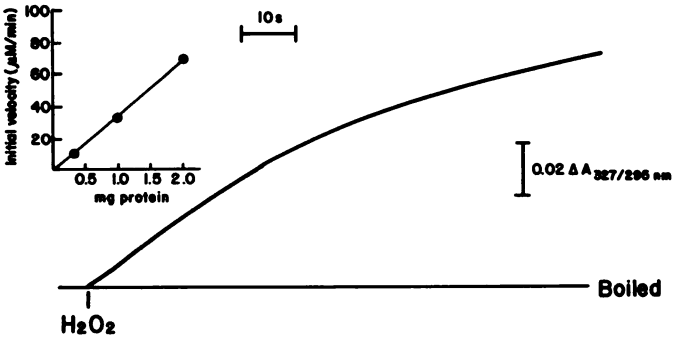

FIG. 3. *H_2O_2-dependent oxidation of nafazatrom by RSVM*
Nafazatrom (100 μM) was incubated at ambient temperature with RSVM in 3.0 ml of sodium phosphate (pH 7.8) in the sample cell of an Aminco DW-2a spectrophotometer operated in the dual wavelength mode ($A_{295}-A_{327}$). H_2O_2 (100 μM) was added and the spectral changes were monitored. *Inset*, dependence of the initial velocity of nafazatrom oxidation on the microsomal protein concentration.

[9] M. Das, unpublished results.

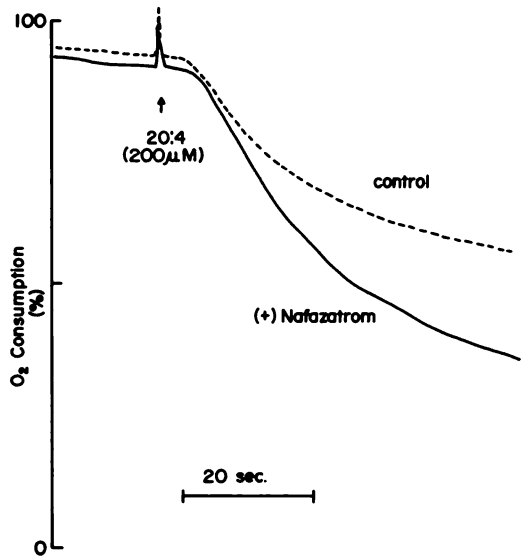

FIG. 4. *Effect of nafazatrom on arachidonate oxygenation by RSVM*
RSVM (0.25 mg of protein) were suspended in 1.0 ml of 0.1 M HEPES (pH 7.8) at 37°. Reaction was initiated by the addition of 200 μM 20:4 in ethanol. Nafazatrom (186 μM) was dissolved in ethanol. The control contained an equal volume of ethanol.

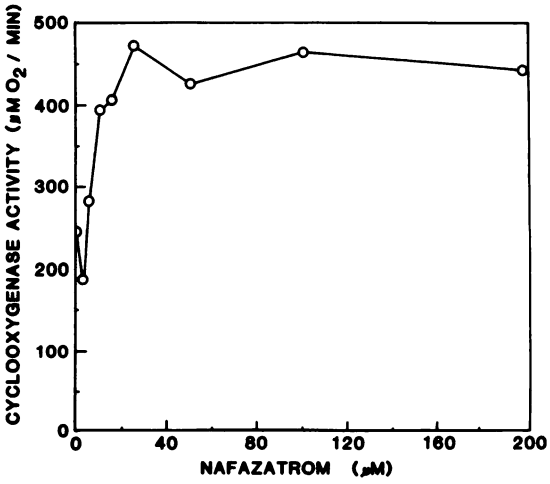

FIG. 5. *Effect of increasing nafazatrom concentrations on arachidonate oxygenation by purified and hematin-reconstituted PGH synthase*
Purified PGH synthase (0.2 μM) and hematin (0.4 μM) were stirred at 37° with varying amounts of nafazatrom in 0.1 M sodium phosphate (pH 7.8). After a 3-min preincubation, 100 μM 20:4 was added and the initial velocity of O_2 uptake determined.

Enhancement of PGI_2 biosynthesis by nafazatrom. The effect of varying concentrations of nafazatrom on 6-keto-$PGF_{1\alpha}$ biosynthesis by RSVM is illustrated in Fig. 7. These experiments were performed using 100 μM 20:4, and 6-keto-$PGF_{1\alpha}$ was quantitated by radioimmunoassay. An approximately linear increase in 6-keto-$PGF_{1\alpha}$ biosynthesis was observed from 1 to 200 μM nafazatrom (the limit of its solubility in aqueous solutions). The

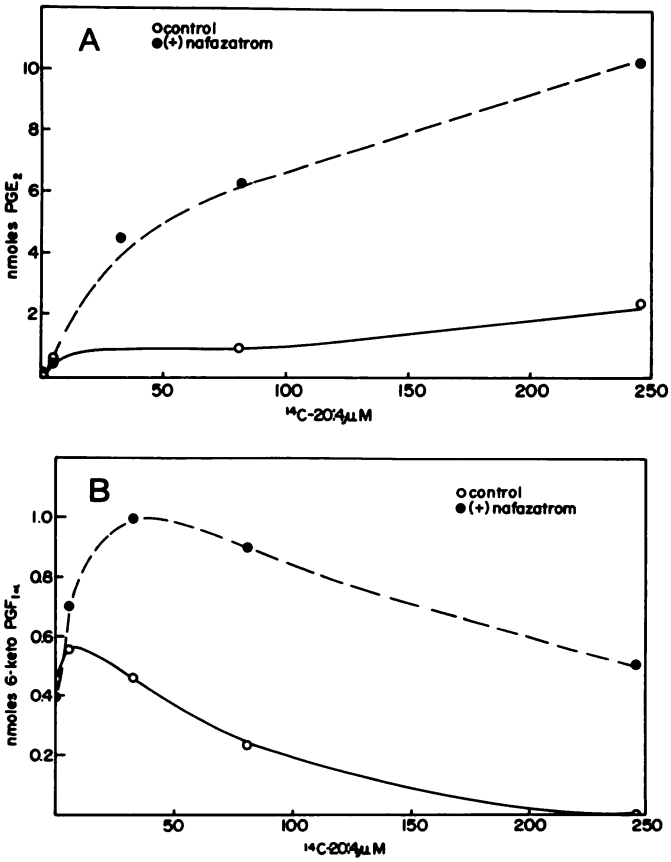

FIG. 6. *Effect of increasing arachidonic acid concentrations on PGE_2 (A) and 6-keto-$PGF_{1\alpha}$ (B) biosynthesis by RSVM in the presence and absence of nafazatrom*

RSVM (0.5 mg of protein) and nafazatrom (186 μM) or an equal volume of solvent were stirred in 0.1 M HEPES (pH 7.8) at 25°. After 3 min, 1-[^{14}C]20:4 (1–250 μM) was added and incubated for 5 min. The workup is described under Experimental Procedures. The values plotted represent the mean of two determinations.

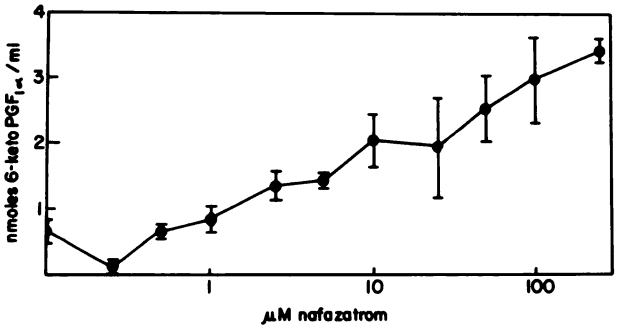

FIG. 7. *Effect of increasing nafazatrom concentrations on biosynthesis of radioimmunoassayable 6-keto-$PGF_{1\alpha}$ by RSVM*

RSVM (0.25 mg of protein/ml) was added to 0.5 ml of HEPES (pH 7.8) in the presence or absence of varying concentrations of nafazatrom. Arachidonate (100 μM) was added and the incubations were terminated after 5 min. An aliquot was removed and diluted for radioimmunoassay of 6-keto-$PGF_{1\alpha}$. Values are means ± standard deviation (n = 6).

inhibition of biosynthesis at 0.25 μM nafazatrom was slight but reproducible.

One possibility for the increase in 6-keto-$PGF_{1\alpha}$ biosynthesis in the presence of nafazatrom is that nafazatrom stimulated PGH_2 biosynthesis by stimulating cyclooxygenase which then led to increased PGI_2 production. This must certainly occur under conditions analogous to those used for the experiments depicted in Figs. 4–6. It is also possible that nafazatrom stimulated PGI_2 synthase under conditions in which PGH_2 biosynthesis was unaffected. We designed a series of experiments using elevated enzyme concentrations so that PGH_2 biosynthesis would be maximal and could not be stimulated by nafazatrom. Any stimulation of PGI_2 synthase under these conditions would only be due to protection of PGI_2 synthase. The results are summarized in Table 1. At three different concentrations of 20:4, no stimulation of 20:4 oxygenation by nafazatrom was observed; in fact, slight inhibition was detected at 25 μM 20:4. Nevertheless, 100 μM nafazatrom approximately doubled the conversion of 20:4 to 6-keto-$PGF_{1\alpha}$, suggesting that increased conversion of 20:4 to PGI_2 can be observed under conditions in which PGH_2 biosynthesis is not increased.

In order to determine whether nafazatrom exerts a direct stimulatory effect on PGI_2 synthase, 1-[^{14}C]PGH_2 was incubated with RSVM in the absence and presence of nafazatrom. The products were extracted and the levels of 6-keto-$PGF_{1\alpha}$ quantitated following separation by HPLC. The data in Table 2 indicate that nafazatrom does not affect the conversion of PGH_2 to PGI_2 and, therefore, stimulates PGI_2 biosynthesis by lowering the steady-state concentrations of hydroperoxy fatty acids (e.g., PGG_2), which are inhibitory to PGI_2 synthase.

To test whether nafazatrom can protect PGI_2 synthase from inactivation by hydroperoxy fatty acids, we incubated RSVM with varying concentrations of 15-HPETE

TABLE 1

Effect of nafazatrom on the conversion of arachidonate to oxygenated derivatives and to PGI_2

20:4 concentration	Nafazatrom concentration	% 20:4 Conversion[a,b]	% Yield of 6-keto-$PGF_{1\alpha}$[b]
μM	μM		
25	0	98 ± 1.0	4.7 ± 1.0
25	100	83 ± 1.5	11.0 ± 1.5
50	0	98 ± 1.0	4.1 ± 0.6
50	100	96 ± 1.0	9.0 ± 0.8
100	0	98 ± 1.0	3.1 ± 0.1
100	100	96 ± 1.0	7.1 ± 0.3

[a] The concentration of RSVM was 1 mg of protein per milliliter. Conditions were as described under Experimental Procedures. % Conversion represents the total conversion of [^{14}C]arachidonic acid to oxygenated derivatives in each experiment.

[b] Values are means ± standard deviation (n = 3).

TABLE 2

Effect of nafazatrom on the conversion of PGH_2 to PGI_2 by RSVM

1-[^{14}C]PGH_2 was incubated at 22° for 5 min in the absence or presence of 186 μM nafazatrom with RSVM (0.5 mg/ml) in 0.1 M HEPES buffer (pH 7.8). The reaction was terminated, and the mixture was acidified and extracted with ethyl acetate. The residue after evaporation was analyzed by HPLC as described under Experimental Procedures. Values are averages of two determinations.

PGH_2	Control	Nafazatrom
μM	*nmol 6-keto-$PGF_{1\alpha}$*	*nmol 6-keto-$PGF_{1\alpha}$*
0.5	0.15	0.14
2.0	0.41	0.40

in the absence or presence of nafazatrom 1 min prior to the addition of 4 μM 1-[^{14}C]PGH$_2$. The production of 6-keto-PGF$_{1\alpha}$ was quantitated by the HPLC method. Figure 8 demonstrates that nafazatrom provides significant protection of PGI$_2$ synthase. As expected, the protective effect of nafazatrom was more dramatic at elevated concentrations of 15-HPETE. These data provide clear-cut evidence that nafazatrom protects PGI$_2$ synthase against hydroperoxide-dependent inactivation by acting as a reducing substrate for the peroxidase component of PGH synthase.

DISCUSSION

The present work demonstrates that nafazatrom fulfills all of the criteria that establish a compound as a peroxidase-reducing substrate. It stimulates the reduction of hydroperoxy fatty acids to alcohols by microsomal or purified preparations of PGH synthase. This protein appears to catalyze the reduction of hydroperoxides by mechanisms analogous to those of other hemeprotein peroxidases (8, 23, 24). The heme prosthetic group interacts with the hydroperoxide to generate the alcohol and an oxidized form of the heme in the first step of catalysis (25). In order to complete the catalytic cycle, the heme must be reduced by two electrons that are provided by donors such as nafazatrom. Although the peroxidase can reduce one molecule of hydroperoxide in the absence of a reducing substrate, further reaction ceases because the oxidized enzyme cannot turn over. Thus, the only way in which catalytic hydroperoxide reduction can occur is if electrons are provided by an external source. Figure 2 illustrates the reduction of a typical PGH synthase substrate, 15-HPETE, by nafazatrom in the presence of RSVM. Some reduction is detected in the absence of nafazatrom. This is due to the presence of other reducing substrates, possibly uric acid, in the microsomal preparation (26). With highly purified enzyme preparations, no reduction of 15-HPETE is detected in the absence of nafazatrom. With the microsomal or purified enzyme, nafazatrom stimulates the reduction of 15-HPETE; this can only be the result of its ability to act as a reducing substrate.

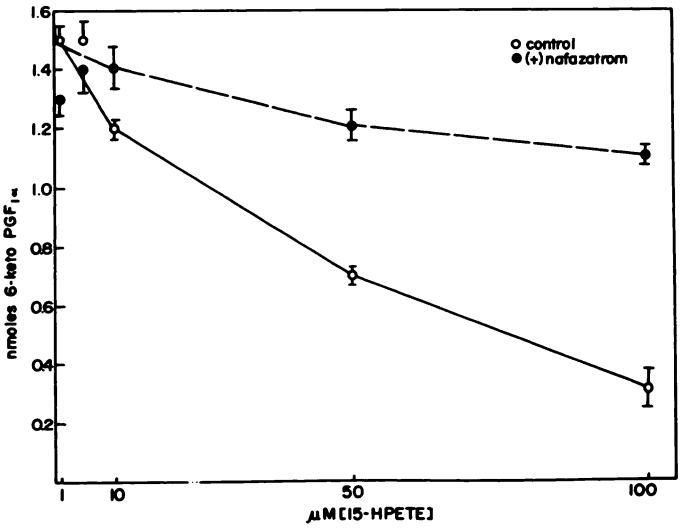

FIG. 8. *Protection of PGI$_2$ synthase from hydroperoxide-dependent inactivation by nafazatrom*

RSVM (0.5 mg/ml) were incubated with 4.0 μM 1-[^{14}C]PGH$_2$ in 1.8 ml of 0.1 M HEPES (pH 7.8) in the presence of 186 μM nafazatrom or an equal volume of ethanol. Reactions proceeded for 3 min at 25° and were terminated by acidification to pH 3.0. PGI$_2$ was estimated by HPLC analysis for 6-keto-PGF$_{1\alpha}$ (19).

Nafazatrom inhibits the oxidation of other peroxidase-reducing substrates because it acts as an alternate electron donor. The I_{50} for inhibition of PB oxidation by PGH synthase is 16 μM, identical with the I_{50} for inhibition of PB peroxidation by BW755C (8). The latter compound is an excellent reducing substrate for PGH synthase and the best inhibitor of PB oxidation that we have tested to date. It contains a pyrazoline ring that serves as the electron donor for the peroxidase. Nafazatrom contains a pyrazolidone group that is the likely source of electrons. This may account for the similarity of the biochemical actions of BW755C and nafazatrom.

Nafazatrom is oxidized by the peroxidase of PGH synthase in a hydroperoxide-dependent reaction. Incubation of nafazatrom with PGH synthase and H_2O_2 results in the appearance of a new absorption band at 261 nm. Omission of any of the components or heat denaturation of the enzyme abolishes the shift in the UV spectrum. Similar spectral changes are seen with RSVM or purified PGH synthase, indicating that they are not the result of the oxidation of an impurity in the microsomal preparation. Extraction of the spent reaction mixture and analysis by HPLC indicates that the nafazatrom has been consumed. The ease of oxidation of nafazatrom has been confirmed in a recent study of its reaction with oxidizing free radicals (27). The rate coefficient for reaction with Br_2^- is 1.4×10^9 M^{-1} sec^{-1}, a value approaching the diffusion-controlled limit. This rate coefficient indicates that nafazatrom is more reactive than phenoxide ions, NADH, or ascorbate to one-electron oxidizing agents. The oxidation product of nafazatrom has not yet been identified. The structural similarity between the enol tautomer of nafazatrom and phenols suggests that the oxidation product(s) may be analogous to those of phenols which are derived from phenoxyl radicals. The latter tend to give complex mixtures of radical coupling and disproportionation products (28).

At concentrations that reduce hydroperoxy fatty acids to alcohols in the presence of RSVM, nafazatrom enhances the conversion of 20:4 to PGI$_2$. Fischer *et al.* (29) recently reported that nafazatrom stimulates PGI$_2$ biosynthesis by rabbit kidney cortex microsomes. Wong *et al.* (7) have reported that nafazatrom is an inhibitor of the NAD-dependent oxidation of PGI$_2$ by 15-hydroxyprostaglandin dehydrogenase of bovine lung and mesenteric artery cytosols. Since oxidation of the 15-hydroxyl group to a ketone is an important step in the inactivation of prostaglandins, including PGI$_2$, inhibition of this reaction by nafazatrom could elevate the steady-state level of PGI$_2$. The enhancement of the conversion of 20:4 to PGI$_2$ in RSVM by nafazatrom is not due to inhibition of 15-hydroxyprostaglandin dehydrogenase. There is no detectable dehydrogenase activity in RSVM under the conditions of our assays. We have never detected the oxidation of prostaglandins to 15-keto derivatives in exper-

iments in which 20:4 is added to RSVM. This is not surprising, since in all tissues examined the dehydrogenase has been detected in the 105,000 × *g* supernatant, not in the microsomal fraction (30). In addition, we do not add oxidized pyridine nucleotides, the cofactors for the dehydrogenase, to the incubation mixtures. Furthermore, nafazatrom has absolutely no stimulatory effect on the conversion of PGH_2 to PGI_2 by RSVM, which would be expected if it either directly stimulated PGI_2 synthase or inhibited the dehydrogenase-catalyzed oxidation of PGI_2 to its 15-keto derivative. Therefore, the enhancement of the conversion of 20:4 to PGI_2 exhibited in the present experiments must occur by a mechanism different from that reported by Wong *et al.* (7).

The data in Fig. 8 clearly demonstrate that nafazatrom protects PGI_2 synthase from inactivation by hydroperoxy fatty acids. When PGH_2 is incubated with RSVM that have been preincubated with 15-HPETE in the presence or absence of nafazatrom, significantly higher yields of 6-keto-$PGF_{1\alpha}$ are obtained at all levels of 15-HPETE when the preincubations are performed in the presence of nafazatrom. Nafazatrom even provides 75% protection against the inactivating effects of 100 μM 15-HPETE, a concentration that is much higher than could be expected under normal physiological conditions. Deckmyn *et al.* (31) have recently developed a method for measuring local TxB_2 and 6-keto-$PGF_{1\alpha}$ production by platelets and vessel walls of rabbits stimulated by insertion of a nylon thread into the external jugular vein. TxB_2 levels increased dramatically after stimulation and remained high for 5 hr. 6-Keto-$PGF_{1\alpha}$ levels also increased dramatically but declined to basal levels within 5 hr. Deckmyn *et al.* (31) attributed the decrease of 6-keto-$PGF_{1\alpha}$ levels to "exhaustion" of the ability of vascular endothelial cells to synthesize prostacyclin resulting from prolonged stimulation. One mechanism for "exhaustion" of the PGI_2 biosynthetic capacity of endothelial cells is irreversible inactivation of PGH synthase and/or PGI_2 synthase by hydroperoxy fatty acids (32). When animals were pretreated with nafazatrom 1 hr before stimulation, the initial increase in 6-keto-$PGF_{1\alpha}$ levels was of the same magnitude as in controls, but the decrease with time was very substantially prevented. These observations suggest that nafazatrom prevents exhaustion of the PGI_2 biosynthetic capability of endothelial cells that results from prolonged stimulation. This is consistent with our finding that nafazatrom protects PGI_2 synthase by acting as a peroxidase-reducing substrate and with previous observations that nafazatrom inhibits hydroperoxy fatty acid synthesis via lipoxygenases (33). Kent *et al.* (32) have recently shown that arachidonate oxygenation by vascular endothelium leads to more extensive inactivation of PGH synthase than PGI_2 synthase. They attribute this to a proximity effect based on the presence of a peroxidase activity as a component of PGH synthase. Thus protection by nafazatrom of PGH synthase from inactivation (Figs. 4 and 5) may be as or more important than protection of PGI_2 synthase. Regardless of the target of inactivation by peroxides, the effects of nafazatrom are exerted locally and "on demand." No major effect of nafazatrom on basal, systemic PGI_2 biosynthesis can be seen. In this regard, the apparent selective localization of orally administered nafazatrom in vasculature takes on added importance.[10]

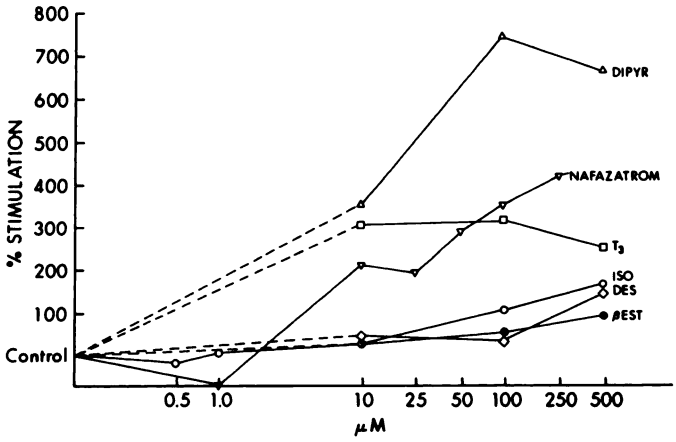

FIG. 9. *Comparison of effects of several phenols and amines on PGI_2 biosynthesis by RSVM*

RSVM (0.25 mg of protein per milliliter) was added to 0.5 M HEPES (pH 7.8) in the presence or absence of varying concentrations of each agent. Arachidonate (100 μM) was added and the incubations were terminated after 5 min. An aliquot was removed and diluted for radioimmunoassay. *DIPYR*, dipyridamole; T_3, triiodothyronine; *ISO*, isoproterenol; *DES*, diethylstilbestrol; *βEST*, 17β-estradiol.

We have tested dipyridamole and triiodothyronine as well as isoproterenol, diethylstilbestrol, and 17β-estradiol for their ability to enhance PGI_2 biosynthesis in RSVM (by protecting PGI_2 synthase from inactivation) (Fig. 9). Although all of the agents enhance biosynthesis to some degree, dipyridamole and triiodothyronine are clearly the best, particularly at low concentrations (10–100 μM).[11] The degree of enhancement is comparable to that by nafazatrom. It is important to note that dipyridamole is not an inhibitor of 15-hydroxyprostaglandin dehydrogenase, yet is a potent enhancer of PGI_2 biosynthesis (14). Like nafazatrom and BW755C, dipyridamole is easily oxidized, which should render it an excellent peroxidase reducing substrate. Nitrogen heterocycles may be more efficacious than phenols as enhancers of PGI_2 biosynthesis at neutral pH.

In summary, we have shown that nafazatrom is an electron donor for the reduction of hydroperoxy fatty acids to hydroxy fatty acids by the peroxidase activity of PGH synthase. Because hydroperoxy fatty acids but not hydroxy fatty acids are irreversible inactivators of PGH synthase and PGI_2 synthase, nafazatrom protects both enzymes from inactivation and enhances the conversion of 20:4 by PGI_2 by RSVM. Other peroxidase-reducing substrates, especially nitrogen heterocycles, enhance PGI_2 biosynthesis as well. The ability to serve as peroxidase-reducing substrates may be an important determinant of the pharmacological activities of electron-rich compounds.

[10] W. Ritter, personal communication.

[11] Blass *et al.* (14) have reported that dipyridamole stimulates PGI_2 biosynthesis by rat stomach fundus homogenates. However, Bult *et al.* (34) have been unable to demonstrate the stimulation of PGI_2 biosynthesis by dipyridamole using RSVM. We cannot explain the difference between our results and those of Bult *et al.*

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