ELSEVIER

European Journal of Pharmacology 302 (1996) 123–128

ejp

Biochemical and cellular effects of heparin-protamine injection in rabbits are partially inhibited by a PAF-acether receptor antagonist

Jamal Aïssa [a,*], Nathalie Nathan [b], Bernard Arnoux [a], Jacques Benveniste [a]

[a] *INSERM U 200, 32 Rue des Carnets, 92140 Clamart, France*

[b] *Département d'Anesthésiologie, CHU Dupuytren, 2 Avenue Martin Luther King, 87042 Limoges, France*

Received 28 November 1995; revised 16 January 1996; accepted 19 January 1996

Abstract

The origin of the thrombocytopenia and leucopenia induced by protamine-heparin complexes is unknown. We studied the biochemical and cellular effects of protamine (6 mg × kg^{-1}, i.v.) injected after heparin (5 mg × kg^{-1}, i.v.) in New Zealand rabbits. After protamine injection (0.5 min) increases in blood platelet-activating factor (PAF-acether, PAF) (27.6 ± 27.6 to 148.2 ± 48.9 pg × ml^{-1}, $P < 0.05$), thrombocytopenia (403 ± 64 to 166 ± 13 cells × 10^{-3} × mm^{-3}, $P < 0.05$) and leucopenia (7650 ± 930 to 4300 ± 668 cells × mm^{-3}, $P < 0.05$) were noted. Plasma thromboxane B_2 increased at 1 min (125.6 ± 24.4 to 879.7 ± 141.0 pg × ml^{-1}, $P < 0.01$). Protamine alone induced no change. Indomethacin (3 mg × kg^{-1}, i.v.) did not counteract the effects of heparin-protamine. Pretreatment with the PAF receptor antagonist BN 52021 [9*H*1,7a-(epoxymethano)-1*H*,6a*H*-cyclopenta[*c*]furo[2,3-*b*]furo-[3′,2′,3,4]cyclopenta[1,2-*d*]furan-5,9,12-(4*H*)trione,3-*tert*-butylhexahydro-4,7b,11 hydroxy-8 methyl] alone (3 mg × kg^{-1}, i.v.) delayed thrombocytopenia and reduced plasma thromboxane B_2 concentration but did not modify leucopenia. Thus thrombocytopenia and thromboxane B_2 release triggered by heparin-protamine may be potentiated by the release of PAF.

Keywords: Heparin; Protamine; Platelet; PAF (platelet-activating factor); PAF-acether; Thromboxane; BN 52021

1. Introduction

Protamine sulfate is a polycationic protein in common use for the reversal of the effects of heparin sodium after cardiopulmonary bypass. Protamine has been described to cause platelet aggregation, leucocyte sequestration (Radegran and McAslan, 1972; Wakefield et al., 1984), and sometimes adverse hemodynamic effects including significant hypotension and acute pulmonary hypertension in animals and in humans (Jaques, 1949; Marin-Neto et al., 1979). Many mediators have been implicated in the adverse hemodynamic and hematological effects of protamine such as histamine (Parsons and Mohandas, 1989) and complement (Best et al., 1983) and mediators from the cyclooxygenase pathway (Radegran and McAslan, 1972). Thromboxane A_2, which is a cyclooxygenase product of arachidonic acid and a potent vasoconstrictor and stimulus of platelet aggregation (Moncada and Vane, 1979), could be responsible for these hemodynamic responses. However, cyclooxygenase inhibitors prevent pulmonary hypertension but not thrombocytopenia after protamine infusion (Degges et al., 1987; Hobbhahn et al., 1988). PAF-acether (PAF, first described as platelet-activating factor) is a potent phospholipid mediator synthesised and released from most proinflammatory cells such as polymorphonuclear neutrophils, platelets and endothelial cells (Benveniste, 1988; Pinckard et al., 1988). Administration of PAF to animals induces thrombocytopenia and leucopenia (Robertson and Page, 1987), and pulmonary artery vasoconstriction via thromboxane A_2 generation (Heffner et al., 1983). PAF induces cardiovascular shock similar to the type III hemodynamic effects of protamine (Benveniste et al., 1983; Feuerstein and Goldstein, 1987). Recently elevated levels of PAF have been reported in human left atrium blood after protamine reversal of heparin activity during coronary bypass surgery (Nathan et al., 1992). The purpose of this study was to further investigate in a rabbit model the levels of blood PAF and plasma thromboxane B_2 (the stable degradation product of thromboxane A_2 after protamine neutralisation of heparin activity with or without pretreatment with BN 52021, a PAF receptor antagonist [9*H*1,7a-(epoxymethano)-1*H*,6a*H*-cyclopenta[c]furo[2,3-

* Corresponding author. Tel.: 33(1)46.32.12.07; fax: 33(1)46.31.02.77.

PII S0014-2999(96)00069-6

b]furo-[3′,2′,3,4]cyclopenta[1,2-*d*]furan-5,9,12-(4*H*)trione, 3-*tert*-butylhexahydro-4,7b,11-hydroxy-8-methyl], and indomethacin, a cyclooxygenase inhibitor.

2. Materials and methods

2.1. Animal preparation

Experiments were performed in 30 New Zealand White rabbits weighing 3–4 kg. Anesthesia was induced with 12.5 mg intramuscular levomepromazine and intravenous (i.v.) thiopental (15–20 mg × kg^{-1}). Stable anesthesia was maintained with boluses of thiopental (5 mg × kg^{-1}). A polyethylene catheter was placed into the jugular vein for drug infusion and another in the carotid artery for blood sampling. Catheters were filled with saline containing 3% sodium-EDTA (77 mM). An equilibrium period of 30 min was allowed before any experiment was started.

2.2. Experimental protocol

Animals were divided into 6 groups of 5 rabbits. Heparin (Laboratoires Choay, Paris, France), 5 mg × kg^{-1}, and protamine (Choay), 6 mg × kg^{-1}, were injected i.v. as boluses:

Group 1 received heparin followed by protamine 10 min later.

Group 2 received only protamine.

Group 3 was pretreated with indomethacin (Sigma, St.Louis, MO, USA), 3 mg × kg^{-1} i.v., 60 min before the sequence heparin-protamine.

Group 4 was pretreated with the PAF receptor antagonist BN 52021, 5 mg × kg^{-1}, i.v. (IHB, Le Plessis-Robinson, France), 2 min before heparin infusion. Protamine was injected 10 min later.

Group 5 received first BN 52021 and then indomethacin, 3 mg × kg^{-1} i.v., 2 min later. Heparin and protamine were injected 60 min later.

Group 6 received first indomethacin and 60 min later BN 52021 before heparin and protamine infusion.

2.3. Blood sampling

A plastic syringe (precooled at 0°C) was used to collect blood from the carotid artery. One milliliter of blood was immediately mixed with sodium-EDTA (77 mM) for leucocyte and platelet counts. Two milliliters of blood were added to 8 ml of absolute ethanol for PAF extraction. To measure thromboxane B_2 levels 3 ml of blood was immediately mixed with 0.1 ml (1 mg × ml^{-1}) indomethacin and 0.2 ml sodium-EDTA (123 mM). After an equilibration period of 30 min, blood samples were obtained just before (T0) and 10 min (H10) after heparin infusion. Other blood samples were collected 0.5 (P0.5), 1 (P1), 3 (P3) and 10 (P10) min after protamine infusion.

2.4. Leucocyte and platelet counts

Platelets and leucocytes were counted using an Unopette 5855 dilution system (Becton-Dickinson, Rutherford, NJ, USA).

2.5. PAF assay and characterisation

After PAF extraction, ethanolic samples were centrifuged (1500 × *g*, 20 min) and supernatants were dried under an air stream, recovered in dichloromethane-methanol (1 : 1; v/v) and submitted to normal-phase high-performance liquid chromatography (HPLC), using a microporasil column 3.9 mm ID × 300 mm length from Waters associates, Milford, MA, USA (Tencé et al., 1980). Samples were eluted with dichloromethane-methanol-water (60 : 50 : 5; v/v) as solvent at a flow rate of 1 ml × min^{-1}. One milliliter fractions were collected, dried, and suspended in 50 μl of 60% ethanol for PAF bioassay. PAF exhibited a retention time between 18 and 21 min. PAF was measured by its aggregating activity on washed rabbit platelets. Aggregation was assessed by using an aggregometer (Icare, Marseille, France) at 37°C under stirring, with aspirin-treated washed rabbit platelets in Tyrode's buffer containing creatine phosphate (0.7 mM)/creatine phosphokinase (13.9 IU × ml^{-1}), an ADP scavenger complex (Cazenave et al., 1979). PAF levels were calculated with respect to a daily assessed dose-response curve of synthetic PAF (Bachem, Bubendorf, Switzerland) and are expressed as pg PAF/ml of blood (pg × ml^{-1}). PAF was characterised on the basis of the following criteria: (1) aggregation of rabbit platelets in the presence of 0.1 mM BN 52021 and L 652-731, two specific PAF-receptor antagonists (Nunez et al., 1986); (2) aggregation after incubation of the samples with phospholipase A_2 from hog pancreas at 10 μg × ml^{-1} as well as with lipase A1 from *R. arrhizus* at 100 μg × ml^{-1} (Benveniste et al., 1977); (3) HPLC retention time, using phosphatidylcholine, lysophosphatidylcholine and synthetic PAF as standards (Tencé et al., 1980).

2.6. Thromboxane B_2 levels

Blood samples were immediately centrifuged (800 × *g*, 15 min at 4°C) and the plasma was stored at −70°C until analysis. Thromboxane B_2 was measured using an enzyme-linked immunoassay (Laboratoire des Stallergènes, Fresnes, France).

2.7. Statistical analysis

All data are presented as means ± S.E.M. A one-way analysis of variance and multiple range tests were used to determine statistical differences between groups and between times in each group. Linear correlation analyses were performed between results obtained after protamine

infusion, in order to correlate PAF, thromboxane B_2, platelet and leucocyte counts. Non-parametric variance analysis (Kruskall-Wallis) and comparison of non-parametric means (Mann-Whitney U-tests) were also used since variances were not homogeneous (Batlett's test) for PAF levels in group 2, thromboxane B_2 levels in groups 1 and 4, and platelet counts in group 2. A $P < 0.05$ and a correlation coefficient > 0.5 indicated statistical significance.

3. Results

Blood PAF levels are reported in Table 1. Plasma thromboxane B_2 levels are shown in Table 2. Platelet and leucocyte counts are summarised in Tables 3 and 4. Protamine infusion in heparin-treated animals (group 1) increased blood PAF levels ($P < 0.05$) 0.5 min after protamine administration, and levels returned to baseline thereafter. Plasma thromboxane B_2 levels dramatically increased 1 min after protamine injection ($P < 0.01$) and remained high at 3 min and 10 min. Kruskall-Wallis and Mann-Whitney U-tests showed highly significant differences for thromboxane B_2 variations as a function of time in group 1 (P = 3 E-4) and group 4 (P = 9 E-4). Acute and profound thrombocytopenia and leucocytopenia were immediately observed after protamine. The maximal decrease in platelet and leucocyte counts occurred 3 min and 10 min after protamine infusion respectively. By contrast, protamine alone (group 2) did not induce significant variations of blood PAF and plasma thromboxane B_2 levels and platelet counts, whereas leucocyte counts decreased significantly at 3 min and reached a minimum value at 10 min. In all groups, heparin alone had no effect on blood PAF, plasma thromboxane B_2 levels, and platelet and leucocyte counts.

Pretreatment with indomethacin alone (group 3) did not protect the animals against the hematological changes induced by the heparin-protamine complexes. In the BN 52021-pretreated animals (group 4), infusion of protamine was not followed by detectable changes in blood PAF levels. An increase in plasma thromboxane B_2 levels occurred 1 min after protamine but the levels reached at 3 min and 10 min were 50% of those observed in group 1 ($P < 0.05$). BN 52021 pretreatment delayed the fall in platelet counts to 3 min and in leucocyte counts to 1 min after protamine injection. Thus, as compared to group 1, platelet counts were significantly higher at 0.5 ($P < 0.01$) and 1 ($P < 0.05$) min after protamine whereas leucocyte counts were significantly lower ($P < 0.05$). There was no

Table 1
Blood PAF levels (pg × ml^{-1}) at baseline (T0), 10 min after heparin (H10), and 0.5 (P0.5), 1 (P1), 3 (P3) and 10 (P10) min after protamine administration

Group	T0	H10	P0.5	P1	P3	P10
1 = Heparin/protamine	27.6 ± 27.6	0.0 ± 0.0	148.2 ± 48.8 [b]	71.2 ± 71.2	60.2 ± 60.2	39.5 ± 39.5
2 = Protamine	63.0 ± 6.0	NA [a]	59.5 ± 21.9	66.0 ± 38.1	68.5 ± 45.4	144.2 ± 76.3
3 = Indomethacin/heparin/protamine	32.1 ± 11.3	42.5 ± 8.9	139.6 ± 18.3 [b]	57.8 ± 24.5	41.8 ± 25.5	29.3 ± 11.7
4 = BN52021/heparin/protamine	44.2 ± 44.2	36.2 ± 17.1	19.6 ± 12.7	18.8 ± 18.8	0.0 ± 0.0	0.0 ± 0.0
5 = BN52021/indomethacin/heparin/protamine	38.4 ± 39.2	83.4 ± 39.2	79.2 ± 14.1	74.2 ± 16.6	85.8 ± 28.3	80.4 ± 18.2
6 = Indomethacin/BN52021/heparin/protamine	60.9 ± 32.8	81.4 ± 54.8	75.6 ± 45.1	53.2 ± 27.9	66.4 ± 29.7	67.2 ± 38.1

[a] NA: not applicable. Statistically significant difference: [b] $P < 0.05$ in comparison with T0 value.

Table 2
Plasma thromboxane B_2 levels (pg × ml^{-1})

Group	T0	H10	P0.5	P1	P3	P10
1 = Heparin/protamine	125.6 ± 24.4	268.0 ± 51.5	362.2 ± 39.9 [b]	879.7 ± 141.0 [c]	993.8 ± 176.5	1003.2 ± 149.9 [c]
2 = Protamine	132.7 ± 6.2	NA [a]	147.0 ± 7.0	156.0 ± 12.9	160.0 ± 6.6	134.0 ± 5.9
3 = Indomethacin/heparin/protamine	130.4 ± 20.9	134.2 ± 18.6	136.0 ± 19.4	147.8 ± 21.3	197.6 ± 36.1	280.0 ± 51.2
4 = BN52021/heparin/protamine	187.5 ± 24.6	210.0 ± 23.4	290.5 ± 20.8	388.7 ± 44.3 [b]	505.0 ± 40.6 [b]	503.0 ± 49.3 [b]
5 = BN52021/indomethacin/heparin/protamine	180.0 ± 11.5	199.3 ± 5.8	233.3 ± 13.3	226.6 ± 6.6	276.6 ± 44.8	290.0 ± 4.6
6 = Indomethacin/BN52021/heparin/protamine	167.0 ± 24.9	164.0 ± 21.3	148.0 ± 17.4	165.2 ± 21.0	162.0 ± 17.1	134.2 ± 25.8

[a] NA: not applicable. Statistically significant difference: [b] $P < 0.05$, [c] $P < 0.01$ in comparison with T0 value.

Table 3
Platelet counts (number of cells × mm^{-1})

Group	T0	H10	P0.5	P1	P3	P10
1 = Heparin/protamine	403 ± 64	303 ± 54	166 ± 13 [b]	161 ± 13 [b]	103 ± 14 [c]	206 ± 55 [b]
2 = Protamine	406 ± 13	NA [a]	318 ± 21	305 ± 57	209 ± 19	257 ± 70
3 = Indomethacin/heparin/protamine	339 ± 66	297 ± 56	150 ± 68 [b]	120 ± 46 [b]	105 ± 30 [c]	198 ± 43 [c]
4 = BN52021/heparin/protamine	414 ± 60	400 ± 84	442 ± 62	340 ± 79	179 ± 39 [b]	273 ± 77
5 = BN52021/indomethacin/heparin/protamine	403 ± 33	378 ± 89	323 ± 83	320 ± 87	144 ± 31 [b]	214 ± 40 [b]
6 = Indomethacin/BN52021/heparin/protamine	389 ± 61	336 ± 35	333 ± 42	254 ± 56	144 ± 42 [b]	224 ± 66 [b]

[a] NA: not applicable. Statistically significant difference: [b] $P < 0.05$, [c] $P < 0.01$ in comparison with T0 value.

Table 4
Leucocyte counts (number of cells × mm^{-1})

Group	T0	H10	P0.5	P1	P3	P10
1 = Heparin/protamine	7650 ± 931	6300 ± 39754	4300 ± 668 [b]	3575 ± 390 [b]	2900 ± 615 [b]	2250 ± 512 [b]
2 = Protamine	8650 ± 1587	NA [a]	7125 ± 813	4775 ± 802	3500 ± 580	2975 ± 460
3 = Indomethacin/heparin/protamine	5760 ± 1215	4560 ± 1134	2160 ± 595 [b]	2420 ± 922 [b]	620 ± 170 [c]	1980 ± 994 [b]
4 = BN52021/heparin/protamine	5860 ± 103	6420 ± 648	3860 ± 342	2340 ± 566 [b]	840 ± 338 [b]	2500 ± 775 [b]
5 = BN52021/indomethacin/heparin/protamine	5880 ± 844	3120 ± 667	2200 ± 635 [b]	2040 ± 734 [b]	840 ± 401 [b]	1700 ± 202 [b]
6 = Indomethacin/BN52021/heparin/protamine	6480 ± 374	5020 ± 813	2780 ± 751 [b]	1920 ± 489 [b]	1560 ± 621	2660 ± 531 [b]

[a] NA: not applicable. Statistically significant difference: [b] $P < 0.05$, [c] $P < 0.001$ in comparison with T0 value.

change in blood PAF in all groups treated with BN 52021. Plasma thromboxane B_2 levels did not vary after protamine injection in animals pretreated with indomethacin alone. It did not vary either in rabbits receiving BN 52021 and indomethacin (groups 5, 6). In the latter groups the decrease in platelet counts was similar to that observed in the group receiving only BN 52021 (group 4). In groups 5 and 6 leucopenia began at 0.5 min after protamine and remained significantly lower at 3 min ($P < 0.05$) as compared to that of group 1. PAF and thromboxane B_2 levels were not correlated with one another within each group. In group 1 PAF levels were not correlated with other experimental data. Thromboxane B_2 levels were correlated with leucocyte counts in group 1 ($r = -0.60$, $P < 0.02$) and in group 4 ($r = -0.69$, $P < 0.003$) and with platelet counts in group 4 ($r = -0.68$, $P < 0.003$).

4. Discussion

In this rabbit model, reversal of heparin activity by protamine produced an immediate and transitory release of PAF, thrombocytopenia, leucopenia and a sustained release of thromboxane B_2. After heparin-protamine administration, PAF production in human and animal blood varies according to differences in animal species, dose and rate of infusion, and the delay between blood sampling and protamine infusion. By contrast with our results, Habazettl et al. (1990) and Montalescot et al. (1992) did not observe an increase in PAF levels during the heparin-protamine reaction. In Habazettl's study, the PAF assay did not include PAF extraction and purification and was therefore most probably less sensitive than ours. In Montalescot's study, blood samples were collected 1 min after protamine whereas we detected increased blood PAF levels at 0.5 min after protamine injection. Such rapid kinetics are consistent with the short half-life of PAF in blood, resulting either from its rapid metabolism, its binding to specific receptor (Schlondorff and Neuwirth, 1986), or its inclusion into plasma albumin or lipoprotein (Benveniste et al., 1988).

No significant increase in PAF levels was seen in the animals receiving the PAF receptor antagonist. At present we have no plausible explanation for these results. On the one hand the anti-receptor compound should impair PAF binding to its receptor but should not theoretically influence PAF production and/or catabolism. On the other hand our failure to detect any PAF increase in this group cannot be attributed to in vivo interference between the BN compound and PAF or to their co-elution or HPLC

since the PAF levels recorded at time 0 before any heparin/protamine injection were similar in all groups.

The sustained increase in thromboxane B_2 levels after protamine, as compared to PAF, is consistent with other studies (Wakefield et al., 1984; Nguyenduy et al., 1986). The partial inhibitory effect of BN 52021 on thromboxane B_2 production suggests that thromboxane B_2 release is amplified by the early PAF production. Nevertheless because PAF receptor blockade was unable to totally inhibit thromboxane B_2 production, other mechanisms might trigger thromboxane A_2 synthesis. Platelets and leucocytes have not been implicated as the source of thromboxane B_2 during heparin-protamine reactions (Jastrzebski et al., 1975; Montalescot et al., 1989). However neutrophils and platelets could represent possible sources of PAF-induced effects because PAF triggers thromboxane A_2 release from platelets in some experimental models (Shaw et al., 1981).

As suggested by the leucopenia observed after protamine infusion alone, a direct cytotoxic effect of protamine cannot be excluded in this model. Involvement of other mediators such as C5a and C3a complement fractions (Camussi et al., 1981; Best et al., 1983) and histamine (Parsons and Mohandas, 1989) could be evoked. Indeed, C5a has been reported to trigger PAF release in rabbit plasma (Camussi et al., 1981). Of interest is that the clinical effects of complement fractions do not seem to be mediated by PAF (Smallbone et al., 1987). PAF infusion induces thrombocytopenia and leucopenia by marginating these cells in the microvasculature (Björk et al., 1983). Heparin-protamine complexes may trigger thrombocytopenia and leucopenia by the same mechanism (Wakefield et al., 1984). In our study PAF levels did not correlate with hematological changes but this is probably due to the short period (between 0 and 1 min) in which PAF levels were significantly elevated. However it is striking that the drop in platelet counts in the first minute occurred only in groups 1 and 3, which exhibited high PAF levels. That the effect on platelets was relatively long-lasting as compared to the length of the period with high PAF levels is hardly surprising: once aggregated, platelets will remain trapped in the vasculature before desaggregation takes place.

Thromboxane A_2 is a potent aggregating and thrombocytopenic agent which has been implicated in the hematological effects of protamine. In the heparin-protamine group, and in the BN 52021-pretreated animals, the correlation between thromboxane B_2 levels and the drop in leucocyte counts could have suggested a relationship between thromboxane A_2 and leucopenia. However the intense leucopenia with the lack of a thromboxane B_2 increase in group 3 (indomethacin-treated) rules out this possibility. Other authors have observed that cyclooxygenase inhibitors prevent protamine-induced hemodynamic adverse effects but not thrombocytopenia (Radegran and McAslan, 1972; Conzen et al., 1989; Morel et al., 1988). In our study pretreatment with both the PAF receptor antagonist and the cyclooxygenase inhibitor only suppressed the early drop in platelet counts. Recently, eicosanoids have been implicated in the protamine-induced rat lung edema (Chen et al., 1994).

Hemodynamic effects as well as hematological changes may be observed after heparin-protamine injection, yet the link between them is not well-defined since thrombocytopenia is a constant adverse effect and hemodynamic changes fortunately occur more rarely. The role of cyclooxygenase metabolites in acute pulmonary hypertension and the protective effect of cyclooxygenase inhibitors are well-documented (Nguyenduy et al., 1986; Degges et al., 1987; Morel et al., 1988; Hobbhahn et al., 1988; Conzen et al., 1989). We did not examine the hemodynamic effects of reversal of heparin activity by protamine.

In summary, it appears that the causes of the biological effects of the heparin-protamine treatment are multiple and presently not clearly defined. The main result of the present study is the release of PAF after protamine neutralisation of heparin activity in the rabbit and its possible (at least partial) implication in thromboxane A_2 generation. An increase in blood PAF concentrations was reported after protamine/heparin administration in humans (Nathan et al., 1992). However, the effect of PAF receptor antagonists on the adverse hemodynamic effects of protamine has not been documented in humans. This should be investigated in order to identify the role of PAF in heparin-protamine adverse reactions.

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