'Antiflammins': Two nonapeptide fragments of uteroglobin and lipocortin I have no phospholipase A_2-inhibitory and anti-inflammatory activity

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Received 21 March 1990

The 'antiflammin' nonapeptides P1 and P2 [(1988) Nature 335, 726–730] were synthesized and tested for inhibition of phospholipase A_2 and release of prostaglandin E_2 and leukotriene C_4 in stimulated cells in vitro, and in vivo for anti-inflammatory activity in rats with carrageenan-induced paw oedema. Porcine pancreatic phospholipase A_2 was not inhibited at concentrations of 0.5–50 μM. Prostaglandin E_2 and leukotriene C_4 release by mouse macrophages stimulated with zymosan or ATP was not affected up to a concentration of 10 μM, nor was prostaglandin release by interleukin 1β-stimulated mesangial cells and angiotensin II-stimulated smooth muscle cells. Both peptides exhibited no anti-inflammatory activity in carrageenan-induced rat paw oedema after topical (250 μg/paw) or systemic administration (1 or 4 mg/kg s.c.). These results do not support the claim of potent phospholipase A_2-inhibitory and anti-imflammatory activity of the 'antiflammins' P1 and P2 [1].

Lipocortin; Uteroglobin; Nonapeptide; Phospholipase A_2; Eicosanoid; Inflammation

1. INTRODUCTION

Synthetic nonapeptides with amino acid sequences corresponding to areas of high sequence similarity of uteroglobin and lipocortin I have been reported to be potent inhibitors of porcine pancreatic phospholipase A_2 (PLA_2) in vitro and to exhibit striking anti-inflammatory effects in carrageenan-induced rat paw oedema in vivo [1]. Because of the anti-inflammatory effect, the name 'antiflammins' has been proposed for these peptides [1].

The amino acid sequence of nonapeptide P1, MQMKKVLDS, corresponds to the 9 C-terminal amino acids of α-helix 3 of uteroglobin, whereas nonapeptide P2, HDMNKVLDL, has the same sequence as the residues 246–254 of lipocortin I [1]. Uteroglobin and lipocortin I belong to a family of structurally related proteins such as renocortin, calpactin and endonexin, which have a molecular mass of about 36 kDa, are inducible by glucocorticosteroids, show calcium-dependent binding to phospholipids and inhibit PLA_2 (for review see [2]). The latter two properties appear to be intimately connected, as has been demonstrated recently [3–5].

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Abbreviations: DICD, diisopropylcarbodiimide; DMA, dimethylacetamide; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, fluorenyl-methoxy-carbonyl; HOBt, 1-hydroxy-benzotriazole; LTC_4, leukotriene C_4; MEM, modified Eagle's medium; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; TFA, trifluoroacetic acid

It has been suggested that peptide P1 corresponds to an active site of uteroglobin responsible for its PLA_2-inhibitory activity, and that an important anti-inflammatory mechanism of the peptides P1 and P2 may be the inhibition of arachidonate release from the cell membrane by PLA_2 [1]. However, van Binsbergen et al. recently synthesized the peptides P1 and P2 and could not detect any inhibitory activity on pancreatic PLA_2 in 5 different kinetics assays [6]. In view of these unexpected results and the obvious implications for the 'antiflammin' concept, we also synthesized the nonapeptides P1 and P2 and attempted to reproduce both the PLA_2-inhibitory effect in vitro and the anti-inflammatory activity in vivo, under the experimental conditions described in the original publication by Miele et al. [1].

2. MATERIALS AND METHODS

Fmoc amino acids were purchased from Novabiochem AG (CH-4448 Läufelfingen, Switzerland), porcine pancreatic PLA_2 (700 U/mg) from Boehringer (Mannheim, FRG), 1-stearoyl-2[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (58 mCi/mmol) and radioimmunoassay kits for PGE_2 and LTC_4 from Amersham (UK), λ-carrageenan and zymosan from Sigma (St. Louis, MO, USA) and angiotensin II from Calbiochem (Luzern, Switzerland). Human rec. interleukin-1β was kindly provided by the Biotechnology Department of Ciba-Geigy. Solvents and other reagents used were analytical grade.

2.1. *Peptides*

Peptides P1 and P2 were synthesized using a standard Fmoc solid phase approach with an automated synthesizer [7]. Side chain-protecting groups were *tert*-butyloxy-carbonyl for Lys, *tert*-butyl for Asp, and trityl for His. Couplings were performed with DICD-

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00145793/90/$3.50

Table I

Effect of the nonapeptides P1 and P2 on the activity of porcine pancreatic PLA_2

Concentration (μM)	PLA_2 activity (% control)	
	P1	P2
0.5	97.2 ± 2.6	101.8 ± 17.6
5	98.4 ± 3.4	91.4 ± 5.1
15	98.1 ± 2.7	90.7 ± 0.7
50	94.4 ± 2.7	88.5 ± 1.6

Peptides were dissolved in 10 mM Tris/HCl, pH 7.5 with 1 mg/ml fatty acid-free bovine serum albumin immediately before the assay
Values are mean ± SE, $n=3$

preformed HOBt esters for 45 min at room temperature. Fmoc was cleaved with 20% piperidine in DMA (5×2 min). Yields were determined by UV-monitoring of the Fmoc cleavage products (average of 99% per coupling). Cleavage of the peptides from the *p*-alkoxybenzylester polystyrene resin was done with a mixture of TFA/water/ethanedithiol (93/5/2, v/v/v) twice for 5 min and complete removal of protecting groups was achieved by treatment with the same reagent for 1 h. The peptides were precipitated with peroxide-free diisopropylether/petrolether (1/1, v/v).

2.1.1. Peptide 1

The product was purified to homogeneity using HPLC (Nucleosil C18, 20×250 mm, water → CH_3CN (0.1% TFA). TFA ions were exchanged to acetate ions with an AG 1-X8 anion exchange resin from Bio-Rad.

2.1.2. Peptide 2

The crude product contained approximately 60% of the Met-sulfoxide peptide according to conventional reverse-phase HPLC and FAB-MS (observed mass: 1100). The sulfoxide derivative was reduced with 5 equiv. NH_4I (1 M in 95% TFA, 30 min, room temperature) and the salts were removed by size-exclusion chromatography (10% AcOH, Biogel P2). The peptide still contained 20% of the sulfoxide derivative. There was no change in composition when both peptides were reanalyzed by HPLC after several weeks of storage in lyophilized form at −20°C. P2 is significantly more sensitive to Met oxidation than P1.

2.2. *PLA_2 assay*

PLA_2 was assayed by the method described in Table I of [1], with the following modifications: The assay was scaled up to a volume of 500 μl; the enzyme concentration used (0.15 U/ml) was selected to produce 20–30% substrate hydrolysis, within the linear range of the assay (up to 45%); liberated [^{14}C]arachidonic acid was extracted and separated as described [8]. This method avoided a possible interaction of the test peptides with arachidonic acid during TLC separation as used by Miele et al. [1].

2.3. *Cell culture*

Primary cultures of peritoneal macrophages were established from resident cells of NMRI mice by lavage with Dulbecco's modified Eagle's medium (MEM) as described [9]. The cells were plated at 4×10^4/well in 96-well plates in Dulbecco's MEM with 0.05% (w/v) lactalbumin hydrolysate.

Table II

Effect of the nonapeptides P1 and P2 on PGE_2 and LTC_4 release by stimulated cells

Concentration (μM)	Release (% control)			
	P1		P2	
	PGE_2	LTC_4	PGE_2	LTC_4
Mouse macrophages, zymosan (100 μg/ml)[a]				
0.1	79 ± 20	114 ± 37	73 ± 19	143 ± 38
1.0	73 ± 10	142 ± 36	72 ± 18	137 ± 46
10.0	91 ± 12	128 ± 30	87 ± 24	132 ± 32
Mouse macrophages, ATP (50 μM)[b]				
10.0	131 ± 15	112 ± 6	122 ± 14	138 ± 27
Mesangial cells, interleukin-1β (1 nM)[c]				
0.05	136 ± 31		not tested	
0.5	106 ± 11		111 ± 25	
5.0	97 ± 12		89 ± 9	
Smooth muscle cells, angiotensin II (100 nM)[d]				
0.01	75 ± 27		71 ± 11	
0.1	100 ± 15		93 ± 31	
1.0	113 ± 7		71 ± 16	
10.0	125 ± 20		79 ± 10	

Peptides were dissolved in culture medium immediately before the assay.
Values are mean ± SD, $n=4$

[a] Release/10^6 cells:
PGE_2: basal 1.75 ± 0.25 ng, stimulated 31.7 ± 0.85 ng.
LTC_4: basal 0.08 ± 0.06 ng, stimulated 155 ± 29 ng.
[b] Release/10^6 cells:
PGE_2: basal 1.75 ± 0.25 ng, stimulated 4.70 ± 1.40 ng.
LTC_4: basal 0.08 ± 0.06 ng, stimulated 1.47 ± 0.60 ng.
[c] Release/10^6 cells:
PGE_2: basal 0.41 ± 0.06 ng, stimulated 1.84 ± 0.16 ng.
[d] Release/10^6 cells:
PGE_2: basal 50 ± 10 pg, stimulated 250 ± 20 pg.

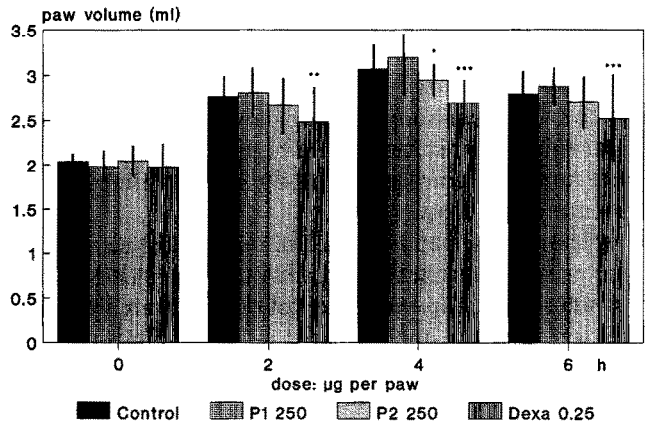

Fig. 1. Carrageenan-induced rat paw oedema, topical administration of test compounds. Vehicle control, $n = 10$; P1, 250 µg/paw $n = 8$; P2, 250 µg/paw $n = 8$; dexamethasone, 0.25 µg/paw $n = 10$. Paw volumes are expressed as means ± SD. Difference against controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

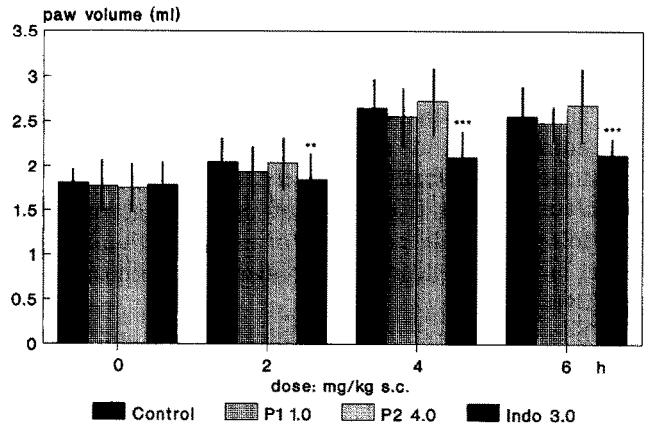

Fig. 2. Carrageenan-induced rat paw oedema, systemic (subcutaneous) administration of test compounds. Vehicle control $n = 10$; P1, 1.0 mg/kg $n = 7$; P2, 4.0 mg/kg $n = 10$; indomethacin, 3.0 mg/kg $n = 10$. Paw volumes are expressed as means ± SD. Difference against controls: ** $P < 0.01$; *** $P < 0.001$.

Cultivation and characterization of rat renal mesangial cells was done as described [10]. The cells were grown in RPMI 1640 medium in 24-well plates.

Cultures of smooth muscle cells of rat aorta were prepared by an explant technique [11]. The cells were grown in Dulbecco's MEM in 24-well plates.

2.4. *Incubation of cells and eicosanoid analysis*

The different cell types were preincubated with the indicated concentration of P1 and P2 for 1 h. Thereafter, the macrophages were stimulated with (i) serum-treated zymosan (100 µg/ml) or (ii) ATP (50 µM) for 2 h [9]. The mesangial cells were stimulated with interleukin-1β (1 nM) for 24 h [10] and the smooth muscle cells were stimulated with angiotensin II (100 nM) for 1 h. All the incubations were performed in the continuous presence of the indicated amounts of P1 and P2. After the stimulation period the medium was withdrawn and assayed for PGE_2 and LTC_4 by radioimmunoassay [9,10].

2.5. *Carrageenan-induced rat paw oedema [12]*

Hind paw oedema was induced in male Lewis rats (LEW/TIF, SPF, 140–160 g body weight, 7–10 animals per group) by an intraplantar injection of λ-carrageenan into the right hind paw. The oedema was measured plethysmometrically before and up to 6 h after the carrageenan injection.

Topical administration of peptides [1]: intraplantar injection of 0.1 ml of 1% carrageenan in saline, followed after 30 s by peptide 1 or 2, 250 µg/paw in 0.1 ml of 10 mM Tris/HCl, pH 8. Dexamethasone 0.25 µg/paw served as a positive control.

Systemic administration of peptides: intraplantar injection of 0.05 ml of 2% carrageenan in saline. Peptide 1 (1 mg/kg) or peptide 2 (4 mg/kg) were injected subcutaneously 1 h before the irritant (5 ml/kg). They were dissolved immediately before injection in 10 mM Tris/HCl, pH 8. Indomethacin 3 mg/kg served as positive control.

3. RESULTS

The structures of the two nonapeptides used in this study were confirmed unequivocally by FAB-MS (observed masses of P1 and P2 1079 and 1084, respectively) and by Edman sequencing.

The effect of the peptides on the activity of porcine pancreatic PLA_2 in vitro is shown in Table I. No inhibition was observed over the concentration range studied, i.e. from 0.5 to 50 µM.

Effects on the release of PGE_2 and LTC_4 by stimulated cells in vitro are summarized in Table II. Both peptides had no significant activity when studied at concentrations up to 10 µM in zymosan- or ATP-stimulated mouse macrophages, in interleukin-1β-stimulated rat mesangial cells, or in angiotensin II-stimulated rat smooth muscle cells.

Finally, anti-inflammatory activity was evaluated in the carrageenan-induced rat paw oedema model. When the peptides were administered topically at 250 µg/paw or systemically at a dose of 1 mg/kg s.c. (P1) or 4 mg/kg s.c. (P2), no anti-inflammatory effects were detected, whereas the reference compounds dexamethasone and indomethacin significantly reduced oedema formation (Figs 1 and 2).

4. DISCUSSION

Two nonapeptides with amino acid sequences contained in the 36 kD proteins uteroglobin and lipocortin I were recently described by Miele et al. as potent inhibitors of PLA_2 with striking anti-inflammatory activity in vivo [1]. The authors suggested that the oligopeptides P1 and P2 are able to mimic the biological activity of uteroglobin and lipocortin because they may represent an active site, or part of an active site, of the proteins. It has been proposed that uteroglobin, lipocortin and related proteins owe their anti-inflammatory activity to a suppression of eicosanoid formation consequent to inhibition of PLA_2 [2,13–15]. Inhibition of PLA_2 – determined in vitro with the enzyme from porcine pancreas – is indeed the most frequently reported activity of lipocortin-like proteins [2–5,15–22]. However, in contrast to the earlier proposal by Hirata [23], it was recently convincingly

demonstrated that inhibition of PLA_2 by lipocortin does not result from a direct interaction with the enzyme, but rather from binding to the substrate. Inhibition of PLA_2 is prominent at low, limiting substrate concentrations and disappears gradually when the substrate concentration is increased [3–5]. It seems doubtful whether this mode of inhibition provides a physiologically relevant basis for an anti-inflammatory effect in vivo (for a critical commentary on this subject see [24]).

When we studied the 'antiflammins' P1 and P2 for possible inhibition of pancreatic PLA_2 in vitro under the assay conditions of Miele et al. [1], we found them to be completely inactive, even at a 1000-fold higher concentration than that used by Miele et al. (Table I). Our results thus are in line with those of van Binsbergen et al. [6]. These authors also tested the two nonapeptides on porcine pancreatic PLA_2 and did not find inhibition. Under a variety of assay conditions, e.g. varying the type, the concentration and the physical structure of the substrate, they could not obtain any evidence for an interaction of the peptides with either the enzyme or the substrate.

We have also studied the peptides P1 and P2 in vitro using intact cells in culture, whose endogenous PLA_2 had been stimulated by different receptor-mediated ligands, i.e. zymosan, ATP, interleukin 1 and angiotensin II. However, also under these more physiological assay conditions no inhibition of eicosanoid release – a process dependent on PLA_2 activity – was observed (Table II). While we are not aware of published data on effects of the 'antiflammins' P1 and P2 on PLA_2 activity or eicosanoid release in intact cells, controversial results in cellular assays have been reported to lipocortins. Errasfa et al. observed inhibition of arachidonic acid release after A 23187 stimulation in mouse thymocytes [25] and guinea pig alveolar macrophages [26] by purified lipocortin from mice lung and thymocytes, respectively, whereas Northup et al. found human placental lipocortin I (calpactin II) to increase – rather than inhibit – arachidonic acid release in zymosan-stimulated mouse macrophages [20].

The striking anti-inflammatory activity of the nonapeptides P1 and P2 in the carrageenan oedema model in vivo reported by Miele et al. [1] is not supported by our results (Figs 1 and 2). Topical administration of the peptides at a dose comparable to the highest dose used by Miele et al. and systemic administration by the subcutaneous route were totally inactive during the early and late phase of the carrageenan oedema. On the other hand, two reference compounds – the glucocorticosteroid dexamethasone and the cyclooxygenase inhibitor indomethacin – produced the expected significant oedema inhibition in this standard model for the evaluation of anti-inflammatory compounds. Ialenti et al. [27] recently also synthesized and tested two nonapeptides for anti-inflammatory activity. One of them, designated as Antiflammin 1, which has the same amino acid sequence as peptide P2 [1], caused a moderate inhibition of 30–35% only during the late phase of carrageenan-induced paw oedema after subcutaneous administration of a high dose, 1 mg/kg. We were unable to confirm this result with a 4 times higher subcutaneous dose.

Although the peptides used in our study had the correct primary structure as confirmed by FAB-MS and Edman sequencing, we could not demonstrate PLA_2 inhibition in vitro, nor mediator release inhibition from stimulated cells and anti-inflammatory activity in vivo. Therefore, the reasons for the discrepancy between the results of Miele et al. [1] and our findings together with those of van Binsbergen et al. [6] are not clear at present and need further clarification. Whether 'antiflammins' is an appropriate name for these nonapeptides seems to be questionable at this time.

Acknowledgements: We thank F. Raschdorf for performing the FAB-MS, R. Knecht for the amino acid sequencing and C. Brühl, U. Rindisbacher and B. Thüring for technical assistance.

REFERENCES

[1] Miele, L., Cordella-Miele, E., Facchiano, A. and Mukherjee, A.B. (1988) Nature 335, 726–730.
[2] Flower, R.J. (1988) Br. J. Pharmacol. 94, 987–1015.
[3] Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J.R. (1987) J. Biol. Chem. 262, 1698–1705.
[4] Haigler, H.T., Schlaepfer, D.D. and Burgess, W.H. (1987) J. Biol. Chem. 262, 6921–6930.
[5] Aarsman, A.J., Mynbeek, G., Van den Bosch, H., Rothhut, B., Prieur, B., Comera, G., Jordan, L. and Russo-Marie, F. (1987) FEBS Lett. 219, 176–180.
[6] Van Binsbergen, J., Slotboom, A.J., Aarsman, A.J. and De Haas, G.H. (1989) FEBS Lett. 247, 293–297.
[7] Atherton, E., Logan, C.J. and Sheppard, R.C. (1981) J. Chem. Soc., Perkin Trans. I. 538–543.
[8] Märki, F. and Franson, R. (1986) Biochim. Biophys. Acta 879, 149–156.
[9] Pfeilschifter, J., Thüring, B. and Festa, F. (1989) Eur. J. Biochem. 186, 509–513.
[10] Pfeilschifter, J., Pignat, W., Vosbeck, K. and Märki, F. (1989) Biochem. Biophys. Res. Commun. 159, 385–394.
[11] Pfeilschifter, J. and Rüegg, U.T. (1987) Biochem. J. 248, 883–887.
[12] Winter, C.A., Risley, E.A. and Nuss, G.W. (1962) Proc. Soc. Exp. Biol. Med. 111, 544–547.
[13] Parente, L., Di Rosa, M., Flower, R.J., Ghiara, P., Meli, R., Persico, P., Salmon, J.A. and Wood, J.N. (1984) Eur. J. Pharmacol. 99, 233–239.
[14] Cirino, G., Peers, S.H., Flower, R.J., Browning, J.L. and Pepinsky, R.B. (1989) Proc. Natl. Acad. Sci. USA 86, 3428–3432.
[15] Fradin, A., Rothhut, B., Poincelot-Canton, B., Errasfa, M. and Russo-Marie, F. (1988) Biochim. Biophys. Acta 963, 248–257.
[16] Levin, S.W., Butler, J.D., Schumacher, U.K., Wightman, P.D. and Mukherjee, A.B. (1986) Life Sci. 38, 1813–1819.
[17] Haigler, H.T., Schlaepfer, D.D. and Burgess, W.H. (1987) J. Biol. Chem. 262, 6921–6930.
[18] Rothhut, B., Comera, C., Prieur, C., Errasfa, M., Minassian, G. and Russo-Marie, F. (1987) FEBS Lett. 219, 169–175.
[19] Fauvel, J., Salles, J.P., Roques, V., Chap, H., Rochat, H. and Douste-Blazy, L. (1987) FEBS Lett. 216, 45–50.

[20] Northup, J.K., Valentine-Braun, K.A., Johnson, L.K., Severson, D.L. and Hollenberg, M.D. (1988) J. Clin. Invest. 82, 1347–1352.
[21] Cartwright, P.H., Ilderton, E., Sowden, J.M. and Yardley, H.J. (1989) Br. J. Dermatol. 121, 155–160.
[22] Machoczek, K., Fischer, M. and Söling, H.D. (1989) FEBS Lett. 251, 207–212.
[23] Hirata, F. (1981) J. Biol. Chem. 256, 7730–7733.
[24] Davidson, F.F. and Dennis, E.A. (1989) Biochem. Pharmacol. 38, 3645–3651.
[25] Errasfa, M., Rothhut, B. and Russo-Marie, F. (1989) Biochem. Biophys. Res. Commun. 159, 53–60.
[26] Errasfa, M., Bachelet, M. and Russo-Marie, F. (1988) Biochem. Biophys. Res. Commun. 153, 1267–1270.
[27] Ialenti, A., Doyle, P.M., Hardy, G.N., Simpkin, D.S.E. and Di Rosa, M. (1990) Agents Actions 29, 48–49.