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Platelet-activating factor (PAF) binding sites in the rabbit oviduct [1]

Sitios de unión del factor activador de plaquetas en el oviducto del conejo

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Purified membranes of the ampullar and isthmic regions of the oviduct obtained from day 3 and day 6 pregnant rabbits were prepared. The membranes from both sections of the oviduct metabolized [^{3}H]PAF. Incubation at 25°C for 120 min, even in the presence of phospholipase A_2 inhibitors, led to [^{3}H]PAF catabolism. The ampullar membranes from day 6 metabolized [^{3}H]PAF more readily than did those from day 3, and more readily than did isthmic membranes. There was no apparent effect of the stage of pregnancy on [^{3}H]PAF metabolism in the isthmic membranes. Specific binding of [^{3}H]PAF to such membranes was detected, but non-specific binding was high and the binding parameters were variable. The isthmic region on day 3 of pregnancy, had about a five-fold higher concentration of PAF binding sites compared to day 6: 2.48 ± 0.03 pmol/ml protein compared with 0.57 ± 0.21 pmol/mg protein (mean ± SEM). The isthmic membrane PAF binding sites also had a corresponding 4-5-fold lower affinity for PAF. The biological significance of such binding is still unresolved.

INTRODUCTION

Platelet-activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) is a biologically active ether lipid, especially involved in various inflammatory and pathological conditions. Recently, it has been suggested that it plays a role in various reproductive processes, ranging from ovulation, to sperm motility and fertilization, embryonic growth and implantation (1). PAF-like material has been found in various reproductive tissues, *e.g.* rabbit, human, mouse, pig and bovine spermatozoa (2-5); rat ovary (6); rat, rabbit and human uterus (7-11); and to be released from human and mouse pre-implantation embryos (12-14); and day 10 rat embryos (15). Others have been unable to detect PAF in human pre-implantation embryos (16), and even O'Neill and colleagues have admitted that the correlation between human zygotes achieving an IVF pregnancy and secretion of PAF is not absolute (17).

Nevertheless, O'Neill has argued in his various publications that embryo-derived PAF is a key ingredient in establishment of early pregnancy, since PAF antagonists can inhibit pregnancy in mice and this inhibition is reversed by administration of PAF (18). Furthermore, embryo transfer from donor to pseudopregnant recipient mice, with either group being treated with a PAF antagonist resulted in a 35% reduction of implantation when day 4 blastocysts were transferred to day 3 recipients, while treatment of only donor animals resulted in a 64% reduction when day 4 blastocysts were transferred to untreated recipients on day 4. Treatment of only recipients in this latter protocol was without effect (19). Blastocyst trophoblast outgrowth *in vitro* was also inhibited by a PAF antagonist, while exogenous PAF stimulated oxidative metabolism by 2-cell mouse embryos (20) and significantly increased the number of

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cells in expanded blastocysts and the ability of such blastocysts to implant on retransfer (21). Spinks *et al.* (19) have claimed that these results are "consistent with PAF antagonist having actions at the embryonic rather than the maternal level and that ... embryo-derived PAF acts as an essential autocrine factor for the early embryo". However, the secreted embryo-derived PAF causes a marked peripheral thrombocytopenia (13), and thus must affect the maternal environment in some as yet undefined way.

In most systems, PAF exerts its actions through membrane receptors, which have been described on a variety of cells (see (22) for references). Receptors for PAF have been described on endometrial membranes obtained from day 6 pregnant rabbits (22, 23). Thus, PAF released from developing zygotes might exert at least some of their actions on the maternal environment, through such receptors on the endosalpinx. The present study was designed to examine whether such receptors existed.

MATERIALS AND METHODS

Materials

Tritiated platelet-activating factor (1-*O*-[^{3}H] octadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine; sp. act. 114-127 Ci/mmol; [^{3}H] PAF C 18:0) was purchased from Amersham International, Arlington Heights, IL. Unlabeled PAF C 18:0; lyso-PAF C 18:0 (1-*O*-octadecyl-2-lyso-*sn*-glycero-3-phosphorylcholine), oleoyl-PAF (3-*O*-hexadecil-2-acetyl-*sn*-glycero-1-phosphorylcholine), sphingomyelin and phosphatidylcholine were purchased from Sigma Chemical Company, St. Louis, MO. U66982 (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphoric acid-10'-trimethylammoniumdecyl ester) and U66985 (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphoric acid-6'-trimethylammoniumhexyl-ester) manufactured by Upjohn Company were gifts from Dr. Donald J. Hanahan, Department of Biochemistry, University of Texas Health Science Center at San Antonio. The ginkgolide B, BN52021 (3-t-butyl-hexahydro-4, 7, b, 11-trihydroxy-8-methyl-9H-1,7 α-epoxymethano-1H, 6α-H-cyclopenta [c] furo1[2, 3-b]furo [3', 2': 3, 4] cyclopenta [1, 2-d] furan-5,9, 12(4H) trione) was a gift from Dr. P. Braquet, Institut Henri Beaufour, Le Plessis-Robinson, France.

All reagents were of analytical quality. Dibromaoacetophenone, bacitracin, and naphthalene were purchased from Aldrich Chemical Company, Milwaukee, WI. Quinacrine dihydrochloride and BBOT (2,5-bis (5'-tert-butylbenzoxalyl-[2'])-thiophene) were purchased from Sigma Chemical Company, St. Louis, MO. BSA (fraction V) was obtained from Chemical Credential (ICN Immunobiochemicals), Lisle, IL.

Precoated thin-layer chromatographic plates (Silica gel G, 500 μm 20 x 20 cm) were purchased from Analtech, Newark, DE. Chromatographic-quality organic solvents were purchased from Fisher Scientific Company, Fair Lawn, NJ.

Animals

Mature New Zealand White-Cambridge female rabbits (body weights > 3.0 kg) were obtained from Penn Acres, Wimberley, TX, and caged individually in a controlled environment with 14L: 10D, fed 170 g rabbit pellets/day, and individually provided with water ad libitum. On the day designated Day 0 of pregnancy, rabbits were inseminated with 0.5 ml of fresh mixed sperm suspension (collected via an artificial vagina from fertile bucks). After the insemination, the rabbits were injected i.v. with 50 IU human chorionic gonadotropin (hCG, Ayerst Laboratories, New York, N.Y.).

Tissue Preparation

Immediately after euthanasia of the rabbits by an overdose of sodium pentobarbital given i.v., the oviducts were removed, placed into ice cold TEBBQ buffer (10 mM Tris-HCl, 2 mM EDTA, 100 μM bacitracin, 2 μM dibromoacetophenone, 10 μM quinacrine dihydrochloride, pH 7.4 at 25°C), trimmed of extraneous fat and divided into ampulla and isthmus. Portions of oviducts from 8-10 rabbits were pooled

prior to further processing (as described by Kudolo and Harper (22) for endometrial tissues). The oviductal tissues were washed by centrifugation at 500 x g for 10 min to remove contaminating red blood cells and then homogenized in 10 volumes of TEBBQ at polytron number 6 speed setting for 10 s, placed on ice for 2 min, and then homogenized again for 5 s. After filtration through a 350-μm mesh and 10-min centrifugation at 1000 x *g*, the membranes were sedimented at 30,000 x *g* for 30 min. The membranes were further purified on discontinuos 3-layer sucrose gradients by centrifugation at 30,000 x *g* for 30 min. A greater portion of the isthmic membrane fraction sedimented at the boundary between 1 and 2 M sucrose (prepared in TEBBQ) while that of the ampulla sedimented at the boundary of 2 and 3 M sucrose. The membranes were isolated and washed three times with 10-12 volumes of TEBBQ, and centrifuged at 30,000 x *g* for 30 min. All procedures were carried out at 0°-4°C. The protein content of the membrane preparation was estimated with a Bio-Rad Kit (Bio-Rad Laboratories, Richmond, CA) with BGG as reference protein standard. Aliquots of membrane preparations, enough for 80-100 tubes (50 μg protein/tube), were stored at -135°C until required. The length of storage never exceeded 4 wks.

Binding Assays

Solubility of [^{3}H]PAF C18:0. Tritiated [^{3}H]PAF C18:0 was dried under nitrogen gas and reconstituted in 100% ethanol. Duplicate 0.01-ml aliquots (controls) were counted in 3.5 ml Aquamix (ICN Radiochemicals, Irvine, CA), or 0.1-ml aliquots were redried under nitrogen and reconstituted in 1 ml TEBBQ either alone or containing 0.25% BSA. Duplicate 0.1-ml aliquots were counted as above for determination of radioactivity.

Competitive inhibition studies. Radioinert competitive ligans (PAF C18:0, lyso-PAF C16:0, U66985, U66982 and BN 52021) were reconstituted in 0.25% BSA TEBBQ buffer and added to 50 μg of oviductal membranes (isthmic and ampullar) at 250-5,000-fold molar excess of 4 nM [^{3}H]PAF C18:0 and incubated at 25°C for 120 min.

Equilibrium saturation analyses. Both radioligand and radioinert competitors were dissolved in 0.25% BSA/TEBBQ buffer at four-fold the final concentrations required. Purified oviductal membranes were suspended in 0.25% BSA/TEBBQ buffer to produce a concentration of 0.5 mg protein/ml. The equilibrium saturation analyses were performed using 0.25-8 nM [^{3}H]PAF C18:0. The determinations were carried out in triplicate; each tube contained 0.1 ml of the membrane suspension, 0.02 ml radioligand, 0.02 ml of the competitor or buffer and were made up to 0.4 ml with 0.25% BSA/TEBBQ buffer. The incubations were carried out at 25°C for 120 min. Membrane-bound tritiated ligands were separated from the free by vacuum filtration (22), and dried filter disks with membrane-bound radioactivity were counted in 3.5 ml AquaMix scintillation cocktail.

Thin-Layer Chromatographic Analysis of [^{3}H]PAF Metabolism

A modified method of the Bligh and Dyer (24) procedure was used to extract lipids from the reaction volume, as described previously (22). Briefly, the reaction mixture was prepared in the presence or absence of 0.25% BSA. In its absence, [^{3}H]PAF was solubilized in 100% ethanol and its final concentration did not exceed 1%. After incubation, the reaction mixture was placed on ice and 1.2 ml ice-cold distilled water were added, followed by 6 ml of a mixture of chloroform and 2% acetic acid in methanol (1:2). The monophasic solution was vortexed at 15-min intervals for 1 h at room temperature, and the organic phase was separated by addition of 4 ml distilled water: chloroform mixture (1:1). The organic phase was removed and the aqueous phase was washed twice with 4 ml chloroform, each time removing the organic phase after centrifugation at 500 x *g* for 10 min. The pooled chloroform extracts were dried under nitrogen gas and reconstituted in 0.5 ml chloroform; duplicate 0.05-ml

aliquots were taken for scintillation counting and at least two of the following phospholipids added as internal standards: PAFC18:0 (2 mg/ml), 0.005 ml; lyso-PAF C16:0 (1 mg/ml), 0.04 ml; sphingomyelin (1 mg/ml), 0.01 ml; phosphatidylcholine (1 mg/ml), 0.01 ml. The extracts were dried and resuspended in 0.04 ml chloroform before loading on 20-cm x 20-cm precoated glass silica plates. The plates had been previously prepared by washing in a chloroform: methanol: water (65:35:6) solvent system and heat-activated for at least 1 h at 110°C. The plates loaded with the lipid extracts were run in chloroform: methanol: acetic acid: water (50:25:8:4). The lipids were visualized in iodine vapor and the zones coincident with the internal standards were scraped into 20-ml glass vials for radioisotopic counting in 10 ml BBOT scintillation mixture: (BBOT, 15 g; naphthalene, 300 g; toluene 2.5 liters; ethyleneglycolmonomethyl ether, 1.5 liters; distilled water, 125 ml).

Estimation of Radioactivity

Radioactivity was counted in a Pharmacia Wallac System 1400 liquid scintillation counter (Wallac Oy, Turku, Finland). Filter disks were counted at 50% efficiency and the silica gels at 38%. Specific binding was defined as the difference in bound radioactivity between total binding (in the absence of competitors) and nonspecific binding (in the presence of a 2500-fold molar excess of PAF C18:0, lyso-PAF C16:0, U66985 or U66982). Thus, ligands whose nonspecific binding tubes have radioactivity greater or equal to total binding are ineffective in displacing [^{3}H]PAF from its specific binding sites. Tubes containing the various buffers in the absence of membranes were run to estimate residual radioactivity on the filter disks.

Values of the major metabolites of [^{3}H]PAF C18:0 are expressed as percentages of the total radioactivity recovered from the scraped silica gels. Values for the radioactivity at the origin and the solvent fronts are not shown but represent < 0.5 and 1% respectively in control tubes ([^{3}H]PAF C18:0 without oviductal membranes) and < 0.5 and 5% respectively in experimental tubes (with membranes). Recovery of the radioactivity added, defined as the total radioactivity in lipid extracts/radioactivity added x 100, was always greater than 85%.

Statistical Evaluation of Results

All values quoted are expressed as the mean ± SEM for at least three independent experiments using membrane preparations from 8-10 pregnant rabbits. Each set of data from the equilibrium saturation binding studies were analyzed using the Lundon computer program (Lundon Software, Inc., Chagrin Falls, OH).

RESULTS AND DISCUSSION

As was the case with the endometrial membranes, purified membranes from both ampulla and isthmus metabolized [^{3}H]PAF. Under optimal conditions for the binding assays (120 min at 25°C) and in the presence of phospholipase A_2 inhibitors, but in the absence of BSA, only 79.5 ± 4.6% of [^{3}H]PAF remained intact after incubation with day 3 ampullar membranes. On day 6 of pregnancy, however, ampullar membrane [^{3}H]PAF metabolism was enhanced leaving only 67.4 ± 1.1% [^{3}H]PAF C18:0 unchanged. The amount of radioactivity found in the fractions corresponding to [^{3}H]lyso-PAF and [^{3}H]alkylacyl-GPC for day 3 were 6.9 ± 1.33% and 7.3 ± 2.1% and for day 6, 14.9 ± 1.2% and 10.0 ± 0.2%, respectively. There was no apparent effect of the stage of pregnancy on the [^{3}H]PAF metabolizing-abilities of the isthmic membranes. Thus, 78.0 ± 32% and 73.2 ± 3.8% of native [^{3}H]PAF C18:0 remained after incubation with isthmic membranes of days 3 and 6, respectively. The amount of [^{3}H]lyso-PAF and [^{3}H]alkylacyl-GPC formed were also parallel: 13.1 ± 1.2% and 3.0 ± 2.0% for day 3 and 16.7 ± 1.6 and 4.8 ± 0.5% for day 6. This indicates that the acetylhydrolase and acyltransferase activities were both relatively more active in the

ampullar (and more active on day 6 than on day 3) than in the isthmic membranes. In the presence of 0.25% BSA, 94.1 ± 1.1% and 82.0 ± 4.6% of [^{3}H]PAF C18:0 remained intact after incubation with ampullar membranes on days 3 and 6, respectively. After incubation with isthmic membranes, 85.5 ± 5.2% and 92.5 ± 1.0% of intact [^{3}H]PAF C18:0 remained for days 3 and 6, respectively.

Evidence of specific PAF binding was provided by 1) the ability of radioinert PAF C18:0 (C16:0 was equally effective), lyso-PAF and PAF antagonist U66985 but not U66982, BN52021 or the inactive PAF enantiomer, oleoyl-PAF to displace bound [^{3}H]PAF and 2) apparent saturation of the binding sites. However, nonspecific binding was very high (up to 70%) and at concentrations greater than 6-7.5 nM [^{3}H]PAF, no apparent specific binding was observable. The interassay coefficients of variation were also very high.

Two methods of data analysis were employed to estimate the binding parameters, K_d and B_{max}: only the Eadie-Hofstee plots (25, 26) revealed an apparent significant difference in the binding parameters in the isthmic, but not in the ampullar region, on day 3 of pregnancy (Table 1). The values obtained using the Scatchard-Rosenthal algorithm (27, 28) are of a much lower affinity (about a 100-fold lower than the values generated by the Eadie-Hofstee) than those obtained for day 6 endometrial membranes (K_d = 26.6 ± 4.0 nM by Scatchard-Rosenthal or 0.42 ± 0.03 nM by Lundon 2-site method (23)). The Eadie-Hofstee method revealed that the isthmic portion of the oviduct on day 3 might possess about 5 times more binding sites than on day 6. The value for the day 3 isthmic membranes was similar to that for the endometrial membranes. The Scatchard-Rosenthal method shows a corresponding higher PAF binding capacity for day 6 oviductal membranes than for day 6 endometrial membranes. But this may simply be a reflection of its lower estimate of the affinity of the binding sites. Unlike the endometrial membranes, where two PAF binding entities were detected (22, 23), in the oviductal membranes only a one-site model fitted the data best. Use of the Lundon 2-site method for the endometrial data resulted in much lower binding capacities (Type 1 site: 0.11 ± 0.01 and Type 2 site: 1.59 ± 0.22 (mean ± SD) (23). The large standard deviations observed in the oviductal membranes are partly due to high levels of nonspecific binding and also partly to inclusion of myosalpinx in the membrane preparations. In the uterus most of the [^{3}H]PAF binding was to endometrial, rather than to myometrial, membranes

TABLE 1

Summary of equilibrium binding studies of oviductal membranes on day 3 and day 6 of pregnancy

		Ampulla Method of analysis		Isthmus Method of analysis	
Binding parameters	Day of pregnancy	Eadie-Hofstee	Scatchard-Rosenthal	Eadie-Hofstee	Scatchard-Rosenthal
K_d (nM)	D3	14.33 ± 5.59	0.12 ± 0.08(x10^3)	22.00 ± 3.31[a]	32.53 ± 3.27(x10^3)
	D6	4.78 ± 1.99	0.31 ± 0.25(x10^3)	6.44 ± 4.01a	0.33 ± 0.18(x10^3)
B_{max}	D3	1.32 ± 0.46	11.87 ± 7.84	2.48 ± 0.03[b]	4.11 ± 0.88
(pmol/mg protein)	D6	1.13 ± 0.24	26.3 ± 15.10	0.57 ± 0.21[b]	34.53 ± 25.62

* Values represent the means ± SEM of three independent experiments.

** Values with the same superscripts are significantly different by the Student's t test: a, $p < 0.05$; b, p. < 0.001.

(22). The endometrial membranes sedimented at the boundary of 2 and 3 M sucrose, as did the ampullar fraction of the oviduct. The purified isthmic membrane fraction however, sedimented at the boundary of 1 and 2 M sucrose. This may be related to the small amount of endosalpinx, particularly in the isthmus. The differential abundance of myosalpinx in the two preparations, may introduce large changes in the binding parameters. Owing to the small amount of endosalpinx, particularly in the isthmus, making preparations of pure endosalpinx was not practical. Inclusion of large amounts of myosalpingeal membranes with little or no specific [^{3}H]PAF binding in the final preparation would tend to obscure even quite large changes in binding parameters of the small amount of membranes derived from the endosalpinx.

Thus, the tentative conclusion at this time can be only that there are specific receptors for PAF in rabbit oviductal tissue. To have biological significance differences would be expected between tissue derived from times when zygotes are in the oviduct compared to those when they are not. Such differences were observed for membranes derived from the isthmus, the region where the zygotes would spend most time. Nevertheless, given the variability of the binding data, the question must remain open as to whether embryo-derived PAF exerts actions via PAF receptors located on the endosalpinx.

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