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HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS: SPECIFIC BINDING OF PLATELET-ACTIVATING FACTOR AND CYTOSOLIC CALCIUM FLUX

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Abstract—An interaction of the platelet-activating factor (Paf) with endothelial cells was investigated using human umbilical vein endothelial cells. Confluent endothelial cells bound [^{3}H]Paf in the presence of 0.25% fatty acid-free serum albumin after culture in media containing either heat-inactivated foetal calf serum or serum substitute. The Scatchard analysis of the saturated specific [^{3}H]Paf binding showed a B_{max} of 2.5 fmol indicating 2800 binding sites per endothelial cell. [^{3}H]Paf binding was partially reversible at 20° and 4° and endothelial cells partially metabolized [^{3}H]Paf at 20° but not at 4°. [^{3}H]Paf binding and Paf-mediated increase of cytosolic free calcium were inhibited by specific Paf receptor antagonists which do not interfere with Paf metabolism. Immortalized umbilical vein endothelial cells bound [^{3}H]Paf specifically after culture in the presence of insulin (20 hr, 0.4 U/mL) with non-specific binding in the absence of insulin. The results show that specific Paf binding mediated calcium flux in human endothelial cells.

Key words: Paf; calcium flux; prostacyclin synthesis; human endothelial cells

Endothelial cells produce vasoactive substances that regulate cell adherence, vascular tone and vascular permeability [1, 2]. Endothelial cells synthesize Paf¶ in response to various stimuli [3, 4]. Paf is an etherphospholipid, chemically 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [4]. Paf possesses hypotensive properties, induces coronary vasoconstriction and acute oedema formation in various organs [2, 4]. Specific Paf receptor antagonists with Paf-related structures have been described [5]. Other antagonists with structures not related to Paf such as chemically defined plant extracts from *Caulis piperis futkadsura* [6], *Ginkgo biloba* or synthetic triazolothieno diazepines inhibit Paf binding and Paf-mediated effects with a close correlation and without intermediate of Paf metabolism [7–10].

Paf induces a concentration-dependent Ca^{2+} flux and synthesis of prostacyclin in cultured human endothelial cells [11–15]. Specific Paf receptor antagonists dose-dependently inhibit Paf-mediated Ca^{2+} flux in endothelial cells [11]. Endothelial cells respond more rapidly at lower Paf concentration and display longer recovery periods as compared with macrophages, suggesting differences of Paf binding sites on both cell types [14]. Using monocyte/macrophage-like cells the number of high affinity Paf receptors increases during differentiation [10].

The present work investigates specific Paf binding to intact human umbilical vein endothelial cells using radioligand binding studies, cytosolic Ca^{2+} flux in response to Paf and four specific Paf receptor antagonists. Specific Paf binding was observed on the surface of Im-Huvec only after incubation with human insulin.

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¶ Abbreviations: Paf, platelet-activating factor; [^{3}H]Paf, 1-*O*-[^{3}H]-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; Paf enantiomer, 3-*O*-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine; [^{3}H]lyso-Paf, 1-*O*-[^{3}H]-octadecyl-glycero-3-phosphocholine; AAGPC, alkyl-acyl(long-chain)-glycero-phosphocholine; Huvec, human umbilical vein endothelial cells; Im-Huvec, immortalized human umbilical vein endothelial cells; FCS, foetal calf serum; BCS, biodegradable counting scintillant; ACD, acid citrate dextrose; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; fura-2/AM, fura-2-acetoxymethyl ester; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2.

MATERIALS AND METHODS

Reagents. The materials used in this study were obtained as follows: Collagenase (type I), BSA and bovine thrombin from Sigma Chemical Co. (St. Louis, MO, U.S.A.); FCS from Hyclone (Logan, UT, U.S.A.); Ultroser (a serum substitute) from IBF (Villeneuve la Garenne, France); Heparin from Choay (Paris, France); M199 medium, RPMI-vitamins, glutamine, amino acids, folic acids, trypsin-EDTA and HBSS from Gibco (Paisley, Scotland, U.K.); specific antiserum to factor VIII antigen from Nordic Immunology (The Netherlands); HEPES and fura-2/AM from Dojin (Japan); 25, 50 and 75 cm^2 culture flasks and 35 mm culture dishes from Primaria (Falcon Labware, Oxnard, CA, U.S.A.);

penicillin, gentamycin and streptomycin from Biopro (Strasbourg, France); [^{3}H]Paf (1-*O*-[^{3}H]-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, 80 Ci/mmol) and the BCS from Amersham (Bucks, U.K.); Paf and Paf-enantiomer from Bachem (Bubendorf, Switzerland; dissolved in 60% ethanol, final concentration 0.3%, v/v); GF/C filters from Ferrière (France); Millipore vacuum system from Molsheim (France); aspirin as lysine salt (lysine-aspirin, Aspegic®) from Amilly (France), dissolved in Tyrode's buffer; acetic acid from UCB (Leuven, Belgium). Human insulin was obtained from Hoechst (Germany). Tyrode's buffer and ACD were composed as described [7].

The specific radioimmunoassay for 6-keto-$PGF_{1\alpha}$ was performed using antibodies developed by Dr F. Dray from the Pasteur Institute (Paris, France). Im-Huvec were a gift from Patrick Vicart and Denise Paulin of the Pasteur Institute. CV 3988 was a gift of Y. Nagawa, Takeda Chemical Ind. (Osaka, Japan). The Paf receptor antagonist WEB 2086 [8] was a gift of J. Casals-Stenzel of Boehringer Ingelheim (Germany); the Paf receptor antagonist BN 52021 [7] was a gift of P. Braquet, IHB-Ipsen, Le Plessis Robinson. Kadsurenone [16] was a gift of T. Y. Shen (Merck Sharp and Dohme, Rahway, NJ, U.S.A.). CV 3988 was dissolved in water at 40°, WEB 2086 in water by adding 0.1 N HCl with ultrasonication and BN 52021 was dissolved in DMSO (Sigma). The final concentrations of vehicles used as controls were below 0.3% (v/v).

Preparation and culture of endothelial cells. Human endothelial cells were freshly isolated from umbilical cord veins according to the method of Jaffe *et al.* [17] with some modifications as described by Hirafuji *et al.* [11]. Briefly, the vein was cannulated and filled with 0.1% collagenase solution. After 10 min incubation at 37°, the detached cells were flushed out and collected by centrifugation. Huvec were resuspended in Ham's F-12 medium with 10% heat-inactivated FCS, 1% Ultroser SF and supplemented with 90 μg/mL heparin [18], 50 U/mL penicillin and 50 μg/mL streptomycin. The cells were incubated in 25 cm^2 culture flask for 1 hr. The non-adherent cells were then aspirated and the adherent cells further cultured in fresh culture medium with media changes every 2 days. When cultures reached confluence (3–5 days), the cells were harvested by brief exposure to trypsin–EDTA and plated into 35 mm culture dishes at 1:3 to 1:5 split ratios. Huvec were grown thereafter in Ham's F-12 medium with 15% FCS, 1% Ultroser and 90 μg/mL heparin with medium changes every 2–3 days. Cells reached confluence after 4–6 days and their number per dish was assessed using preparations of nine distinct cord veins with a mean of $5.2 \pm 0.15 \times 10^5$ cells per dish (±1 SEM). Huvec at first passage were used throughout this study, and were characterized as endothelial cells based on morphologic criteria [11] and by indirect immunofluorescence using a specific antiserum to human factor VIII antigen, a customary marker of endothelial cells [19]. Cell preparations contained no monocyte/macrophage cells, as defined by morphologic criteria and by indirect immunofluorescence using a monoclonal antibody to α_2-macroglobulin.

Im-Huvec were prepared by Vicart *et al.* and characterized as endothelial cells as described [20]. Im-Huvec used throughout this study were cultured in an M199 medium containing 25 mM HEPES and 2 g/L $NaHCO_3$, 10% heat-inactivated FCS, 1% amino acids, 1% folic acid, 1% RPMI vitamins, 0.1% gentamycin and 4 mM glutamine (pH 7.4). The cells were incubated in 75 cm^2 culture flask on 0.2% gelatin with media changes every 2–3 days. When cultures reached confluence (3–5 days), the cells were harvested by brief exposure to trypsin-EDTA and plated into culture dishes coated with 0.2% gelatin at 1:3 split ratio. Im-Huvec were grown thereafter 5 days with or without addition of human insulin to the medium (0.4 U/mL) 20 hr before the experiment.

Binding studies with [^{3}H]*Paf.* [^{3}H]Paf binding studies were performed as described [8] using adherent human umbilical vein endothelial cells at first passage. Cells were washed three times with Tyrode's buffer (pH 6.4) containing 0.25% BSA without addition of Ca^{2+} to remove culture medium. Next, [^{3}H]Paf binding experiments were performed (pH 7.4; 1.3 mM Ca^{2+}) using Paf or indicated Paf receptor antagonists as unlabelled ligands at 20° for an incubation period of 30 min or at 4° for 20 hr.

In a first set of experiments Huvec were cultured in a medium containing 10% (v/v) heat-inactivated calf serum. Washed Huvec were incubated at 20° with [^{3}H]Paf (100 μL, 65 pM, final concentration) in the absence and presence of unlabelled Paf (500 nM) in Tyrode's buffer (900 μL) for various time periods (3, 5, 15, 30 min). Next, different concentrations of [^{3}H]Paf (32–650 pM, final concentrations) were incubated with Huvec in the absence or presence of unlabelled Paf (500 nM) at 20° for 30 min as described above. The Paf analogue CV 3988 (3, 10 and 30 μM, final concentrations), the Paf receptor antagonists WEB 2086 (1, 10 and 1000 nM) or BN 52021 (6, 10 and 60 μM) were added together with [^{3}H]PAF (650 pM) for 30 min at 20° as compared with vehicles. Huvec were also incubated with [^{3}H]-Paf (650 pM) in the presence and absence of the Paf-enantiomer or 0.1 mM lysine-aspirin.

In a second set of experiments Huvec were kept for 48 hr in a medium containing 15% (v/v) serum substitute instead of heat-inactivated FCS to minimize a putative interference of plasma (lipo)proteins. In this context, [^{3}H]Paf binding studies were performed at 4° (20 hr) in order to avoid Paf metabolism. Huvec were preincubated with the Paf analogue CV 3988 (10 μM) or vehicle for 15 min and then incubated at 4° for 20 hr in the presence of different [^{3}H]Paf concentrations (160–650 pM).

Next, [^{3}H]Paf binding to Im-Huvec was investigated (0.25% BSA, 30 min at 20°). Im-Huvec were cultured 20 hr in a medium containing human insulin ($0.4\ IU \times mL^{-1}$) or without addition of insulin. Im-Huvec were washed as described above and incubated with [^{3}H]Paf (690 pM) with or without unlabelled Paf (50 nM, 30 min at 20°).

After the binding procedure, free radioactivity was washed twice from the confluent monolayer with Tyrode's buffer (pH 6.4) containing BSA and then once with cold isotonic NaCl–EDTA (5 mM)

solution. Cells were detached by incubation in the latter medium with the dishes kept on ice for at least 30 min. Detached cells were separated from the medium by vacuum filtration onto GF/C filters in a Millipore vacuum system. Incubation and washing buffers as well as control buffers were filtered to recover cells detached during the washing procedures. Filters were washed twice with 10 mL cold Tyrode's buffer and the radioactivity of the filter-bound endothelial cells was counted under standard conditions in scintillation fluid (BCS). Filter-bound radioactivity without endothelial cells was subtracted from filter-bound radioactivity in the presence of endothelial cells. [^{3}H]Paf was calculated in fmol bound to an indicated number of adherent endothelial cells.

Cytosolic Ca^{2+} *flux.* Cytosolic Ca^{2+} flux in adherent Huvec was measured as described [11]. Huvec were seeded onto coverglasses (9 × 20 mm), grown to confluence and washed thrice with HBSS (pH 7.4; 37°) containing 10 mM HEPES and 0.1% BSA. Huvec were incubated for 30 min with 5 μM fura-2/AM with excess fura-2/AM removed by washing after loading. The cover glass containing cells was put in a quartz cuvette (2 mM in length) placed diagonally in the cuvette holder with the cell monolayer facing the excitation beam, while the fluorescence emission was detected at the right angle to the excitation beam. Huvec were continuously superfused with HBSS (pH 7.4, 37°, 0.1 BSA) and test agents at the rate of 1 mL per min by a peristaltic pump through the tubings attached to the cuvette. Huvec were superfused with or without increasing concentrations of CV 3988, BN 52021, WEB 2086 or Kadsurenone for 3 min before and during stimulation with 100 nM Paf.

The spectrofluorophotometer (Hitachi model F-4000) was programmed to shuttle between the two excitation wavelengths of 340 and 380 nm every 5 sec while maintaining an emission wavelength of 505 nm. The addition of Paf produced reciprocal changes in the fluorescence recorded at two wavelengths, from which $[Ca^{2+}]_i$ was calculated as described [11].

Prostacyclin release. The stable prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ was measured in the supernatants of Huvec cultured in a medium containing FCS [15]. Huvec (3×10^4) were incubated 30 min at 37° with Paf (1, 5, 10 nM, final concentrations) or 0.1 mM lysine-aspirin. Cells were incubated with thrombin (0.5 U) as a positive control.

At the end of the incubation period, the supernatant medium was removed and stored at −20° until tested for 6 keto-$PGF_{1\alpha}$, using a specific radioimmunoassay. The cross-reactivity of the assay was 2.8% with $PGF_{2\alpha}$, 4% with PGE_2, 0.5% with PGD_2 and sensitivity was 3 pg/100 μL.

Metabolism of [^{3}H]*Paf.* Huvec were washed and incubated with different concentrations of [^{3}H]Paf (650 pM) either at 20° or at 4° as described above for binding studies. In these experiments endothelial cells were cultured for 48 hr either in a medium containing heat-inactivated FCS or with the serum substitute ultraser (15%, v/v). Filter-bound endothelial cells and their collected supernatants were then extracted separately with dichloromethane/methanol (1:2, v/v). Water containing 2% acetic acid and dichloromethane (1:1, v/v) were added after 24 hr extraction at 4°. The water phases were then washed twice with one volume dichloromethane. Next, HPLC was performed as described [8]. Using appropriate standards, HPLC retention times of 10–11 min, 18–21 min and 28–33 min corresponded to compounds comigrating with AAGPC, Paf and lyso-Paf, respectively. Labelled compounds were calculated as a percentage of the total added radioactivity (650 pM) and then expressed in fmol per ml buffer in one dish. The radioactivity in HPLC void volume (8.0 ± 2.0 pM, mean ± 1 SD, N = 3) was retained for calculations.

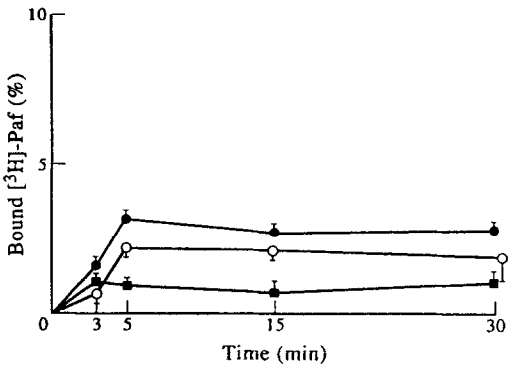

Fig. 1. Kinetics of [^{3}H]Paf binding (65 pM) to confluent endothelial cells at 20°. Huvec were incubated with [^{3}H]-Paf for indicated time periods in the absence (●) or presence (○) of unlabelled Paf (500 nM) at 20° before adherent endothelial cells were washed, detached and filtered as described [8]. Specific [^{3}H]Paf binding (■) is the difference between total binding of [^{3}H]Paf alone and non-specific [^{3}H]Paf binding in the presence of 500 nM unlabelled Paf. Values were calculated in percent of added radioactivity and represent means ± 1 SD of three experiments.

RESULTS

Binding of [^{3}H]*Paf to Huvec*

Confluent Huvec bound [^{3}H]Paf time-dependently (Fig. 1). Incubation of adherent endothelial cells with [^{3}H]Paf (65 pM) together with unlabelled Paf (500 nM, 0.25% BSA) resulted in the reduction of total [^{3}H]Paf binding by 33.0 ± 6.5% after 3 min incubation at 20° (mean ± 1 SD, N = 3).

Huvec bound [^{3}H]Paf (32–650 pM) in a concentration-dependent manner (Fig. 2). The binding was partially reversible when [^{3}H]Paf was added together with unlabelled Paf (500 nM, 30 min). Scatchard analysis showed a B_{max} of 2.5 fmol indicating 2800 binding sites per endothelial cell. The specific [^{3}H]Paf binding reached saturation, indicating a constant number of specific Paf binding sites. The Paf-enantiomer did not interfere with total [^{3}H]Paf binding (650 pM, 10.8 fmol versus 10.3 fmol, one experiment representative of three).

The Paf receptor antagonist WEB 2086 and the Paf analogue CV 3988 (30 μM) decreased the total [^{3}H]Paf binding at 20° with a dose-dependent

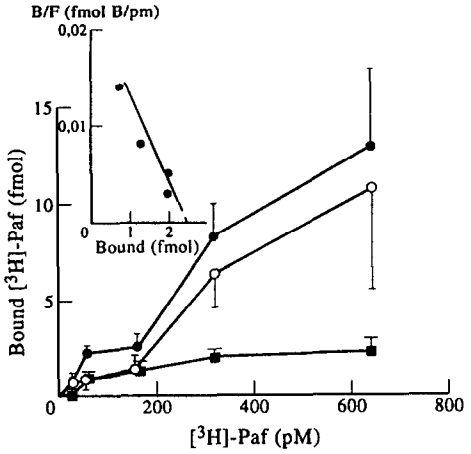

Fig. 2. Concentration-dependent [^{3}H]Paf binding to confluent endothelial cells at 20° (30 min). Total [^{3}H]Paf binding (●) and non-specific [^{3}H]Paf binding (○) were determined in the absence or presence of 500 nM unlabelled Paf to yield saturated specific [^{3}H]Paf binding (■). Values were calculated in fmol × mL^{-1} buffer with [^{3}H]Paf bound to one dish of confluent Huvec. Values represent means ± 1 SD of three experiments. Inset: Scatchard plot analysis of specific [^{3}H]Paf binding.

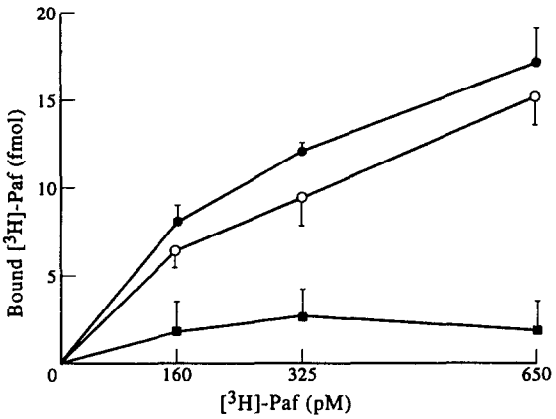

Fig. 3. [^{3}H]Paf binding to confluent Huvec at 4°. Huvec were cultured 48 hr before experiments in a medium containing serum substitute. [^{3}H]Paf binding was performed in the absence (●) and presence (○) of the antagonist with an inhibitory effect (■) of the Paf analogue CV 3988 (10 μM, 20 hr). Values were calculated in fmol/mL buffer with [^{3}H]Paf bound to one dish of confluent Huvec. Data represent means ± 1 SD of three experiments.

Table 1 (A). Specific [^{3}H]Paf binding to confluent Huvec determined with specific Paf receptor antagonists

WEB 2086		BN 52021		CV 3988	
1 nM	0.7 ± 0.2	6 μM	1.6 ± 1.4	3 μM	2.2 ± 1.4
10 nM	1.5 ± 1.1	10 μM	4.8 ± 3.5	10 μM	2.7 ± 1.9
1 μM	3.0 ± 0.7	60 μM	3.9 ± 3.1	30 μM	4.4 ± 1.4

Specific [^{3}H]Paf binding was determined as described [8] using the indicated differences of [^{3}H]Paf binding with or without specific Paf receptor antagonists. [^{3}H]Paf binding was calculated in fmol [^{3}H]Paf bound to 5.2 × 10^5 confluent cells. Values are means ± 1 SD of three experiments.

Table 1 (B). Concentrations of specific Paf receptor antagonists inhibiting 50% (IC_{50} values) of the Paf-mediated $[Ca^{2+}]_i$ flux in Huvec

WEB 2086	BN 52021	CV 3988
23.1 ± 2.9 nM	7.5 ± 0.4 μM	8.2 ± 1.5 μM

For cytosolic Ca^{2+} flux confluent adherent endothelial cells were superfused with a buffer containing various concentrations of specific Paf receptor antagonists for 3 min before and during stimulation by Paf (100 nM) as described [11]. Values are means ± 1 SD of three experiments.

increase of specific [^{3}H]Paf binding (Table 1A). The Paf receptor antagonist WEB 2086 reached inhibitory effects at lower concentrations as compared with BN 52021 or the Paf analogue CV 3988 (Table 1A).

Next, Paf binding studies were performed with Huvec at 4° after 48 hr culture in a medium with serum substitute instead of heat-inactivated FCS (Fig. 3). The Paf analogue CV 3988 inhibited [^{3}H]-Paf binding at 4°. CV 3988 was added 15 min prior to [^{3}H]Paf, reaching a maximal inhibitory effect at 325 pM [^{3}H]Paf (Fig. 3). Paf degradation was excluded here and before at 4° [7, 21].

Another set of control experiments was performed. Addition of lysine-aspirin did not inhibit the binding of [^{3}H]Paf (325 pM, 7.5 ± 1.5 fmol) as compared with vehicle (5.1 ± 1.7 fmol, mean ± 1 SD, N = 6). When [^{3}H]Paf was added to endothelial cells prior to unlabelled ligands, unlabelled Paf (500 nM) was not inhibitory (4.2 fmol versus 4.7 fmol, one experiment of three). When endothelial cells were detached with trypsin instead of cold NaCl-EDTA [^{3}H]Paf binding was not inhibited by unlabelled Paf (5.2 fmol versus 5.2 fmol, one experiment representative of three).

[^{3}H]*Paf binding to Im-Huvec*

We first investigated the binding of [^{3}H]Paf (690 pM) to Im-Huvec with or without insulin (20 hr, 0.4 U/mL). Insulin mediated specific [^{3}H]Paf binding to Im-Huvec as compared with non-specific [^{3}H]Paf binding to non-treated cells. The total [^{3}H]Paf binding to insulin-treated cells was 13.7 ± 1.2 fmol and unlabelled Paf decreased 2.6 ± 0.2 fmol of the bound [^{3}H]Paf. In contrast, unlabelled Paf did not inhibit [^{3}H]Paf binding to non-treated Im-Huvec (4.2 ± 1.2 fmol versus 6.5 ± 1.0 fmol). The values represent means ± 1 SD of three experiments.

Cytosolic Ca^{2+} *flux*

The Paf receptor antagonists WEB 2086, BN

Table 2. [^{3}H]Paf metabolism in Huvec

	Retention time (min)		
	10–11	18–21	28–33
	AAGPC (fmol/mL)	[^{3}H]Paf (fmol/mL)	[^{3}H]lyso-Paf (fmol/mL)
A			
Cells	0.62	11.0	—
	0.63	10.1	—
Supernatants	—	625	6.9
	—	622	6.3
B			
Cells	0.52	6.3	—
Supernatant	—	631	5

Endothelial cells (5.2×10^5) and their supernatants were extracted separately after 30 min incubation of confluent cells at 20° with [^{3}H]Paf (650 pM) and HPLC was performed as described in Ref. 8. Labelled compounds comigrating with AAGPC were found in endothelial cells and labelled lyso-Paf in their supernatants. Endothelial cells were cultured 48 hr before experiments either in a medium containing heat-inactivated foetal calf serum (A, 2 experiments) or serum substitute (B). No metabolites were found at 4° or without cells [7–10, 21, 22].

52021 and CV 3988 inhibited the Paf-mediated increase of cytosolic calcium in Huvec. The 50% inhibitory concentrations (IC_{50} values) are shown in Table 1B. WEB 2086 inhibited Ca^{2+} flux in response to Paf (100 nM) at lower concentrations as compared with BN 52021 or CV 3988. The Paf antagonist kadsurenone also inhibited the increase of cytosolic calcium, exhibiting an inhibitory IC_{50} value of 7.1 ± 2.6 μM (mean ± 1 SD, N = 3). The dose–response curves of cytosolic calcium flux in the presence of Paf have been published previously [11].

Prostacyclin release

Following incubation of Huvec with Paf (1, 5, 10 nM) the amount of the stable prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ in the supernatants increased to 121 ± 24 and 137 ± 6 and 177 ± 28 pg/mL as compared with 96 ± 10 pg/mL without Paf and 218 ± 12 pg/mL after addition of thrombin (0.5 U). The values represent means ± 1 SD of three experiments.

Metabolism of [^{3}H]*Paf*

Endothelial cells metabolized added [^{3}H]Paf at 20° with formation of cell-associated compounds co-migrating with labelled AAGPC. Labelled lyso-Paf was found in the supernatants (Table 2).

A similar metabolism of [^{3}H]Paf occurred at 20° when endothelial cells were precultured for 48 hr in a medium containing serum substitute as compared with culture in a medium containing heat-inactivated FCS. Our data indicate that putative contamination with plasma (lipo)proteins did not mediate Paf metabolism in endothelial cells. [^{3}H]Paf degradation was excluded at 4° here and before [7–10, 21].

DISCUSSION

Our study investigated Paf binding to freshly prepared endothelial cells. Specific Paf binding functioned as a receptor because Paf mediated cytosolic Ca^{2+} flux and various specific Paf receptor antagonists inhibited [^{3}H]Paf binding as well as Paf-mediated cytosolic Ca^{2+} flux with similar inhibitory concentrations. Insulin mediated specific [^{3}H]Paf binding to immortalized Huvec as compared with non-specific [^{3}H]Paf binding in the absence of insulin.

Specific [^{3}H]Paf binding to Huvec was functionally coupled to endothelial calcium homeostasis. Three specific Paf receptor antagonists inhibited Paf binding and cytosolic calcium flux with similar inhibitory concentrations suggesting an interaction with the same Paf receptor. A greater added concentration of Paf as compared with the receptor-bound [^{3}H]Paf was required to induce cytosolic calcium flux, indicating that only a high and rapid rate of Paf receptor occupancy mediated intracellular transducing events in endothelial cells. This has been demonstrated before with various cellular effects using human platelets and neutrophils [7, 21, 22].

Endothelial cells released lyso-Paf and formed cell-associated compounds from added [^{3}H]Paf comigrating with AAGPC during the binding studies. However, following observations showed here that Paf metabolism did not mediate calcium flux in endothelial cells. The specific Paf receptor antagonists WEB 2086, BN 52021 or kadsurenone inhibited Paf-mediated calcium flux and did not interfere with Paf metabolism [6–10, 22]. The Paf analogue CV 3988 [23] competed with labelled Paf for binding at 4° but Paf metabolism was excluded at 4° here and before [21, 22]. It has been established that Paf receptors mediate cellular signals without intermediate of Paf metabolism using intact human blood platelets [7–9]. Paf binds to receptors in neutrophil membranes that trigger function or, alternatively, the ligand is converted to an acetylated product [21] which may be an important source of metabolizable arachidonic acid interfering with prostacyclin synthesis.

Intact Paf might enter the endothelial membranes to interact with specific sites endowed with functional activity, perhaps explaining why preincubated [^{3}H]-

Paf could not be displaced here. Preincubation with low concentrations of Paf decreases the functional responses of venous and arterial endothelial cells upon a second exposure to the mediator; this functional desensitization has been used as an argument in favour of Paf receptors in endothelial cells [11–15]. Our previous study suggested very high affinity Paf binding sites in endothelial cells with a K_D value of 0.043 nM as compared with high affinity Paf receptors on platelets having a K_D value of 0.54 nM [9]. High-affinity Paf receptors have been cloned using guinea pig lungs and the human heart [24, 25].

The use of Im-Huvec excluded contamination with fibroblasts or smooth muscle cells. Here, insulin mediated specific Paf binding to Im-Huvec as compared with non-specific binding to non-treated Im-Huvec. For example, insulin might interfere with the transport of lipoprotein-associated Paf [26] from inside the vessels out via expression of endothelial Paf receptors. We are presently completing the characterization of endothelial Paf receptor(s) using Im-Huvec to perform further studies with a lucid account of compounds affecting endothelial Paf binding [27]. In summary, we showed that specific Paf binding to endothelial cells functioned as receptor using the inhibitory effects of specific Paf receptor antagonists on endothelial calcium flux. Clinically, specific Paf receptor antagonists might prevent vascular diseases and allergic inflammation. The expression of Paf binding sites on the surface of endothelial cells in the presence of insulin could interfere with the disturbance of the endothelial barrier during hyperinsulinaemia.

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