PGF_{2α}, a Prostanoid Released by Endothelial Cells Activated by Hypoxia, Is a Chemoattractant Candidate for Neutrophil Recruitment

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Despite increasing evidence supporting the involvement of neutrophils in ischemic and postischemic damages, the mechanisms underlying the early recruitment of these cells are not completely understood. In this report, the effects of conditioned media from hypoxic endothelial cells on neutrophil chemotaxis were investigated by biochemical and morphological studies. We showed that conditioned media collected from several endothelial cell origins submitted to hypoxia as well as ischemic rat liver perfusion liquids have a chemotactic activity for neutrophils. The role of various chemoattractant molecules like HETEs, platelet-activating factor, and cytokines such as interleukin-8 and interleukin-1 was examined in the same model. Chemotactic peptide contribution was ruled out as boiled conditioned media still trigger chemotaxis. However, cell treatment with cyclooxygenase inhibitors, neutralization of PGF_{2α} biological activity with polyclonal antibodies, and the neutrophil preincubation with a specific PGF_{2α} antagonist, all dramatically inhibited neutrophil chemotaxis. A strong chemoattractant effect of pure exogenous PGF_{2α} or of a synthetic analog was also observed. The major effect of PGF_{2α} on neutrophil chemotaxis was confirmed *ex vivo* in a rat liver perfusion ischemic model. These results suggest that PGF_{2α}, a prostanoid abundantly released by the endothelium of hypoxic or ischemic tissues, is a chemoattractant molecule that might be involved in the early recruitment of neutrophils in ischemic organs. (*Am J Pathol* 2001, 159:345–357)

A rapid leukocyte accumulation is a common consequence of an ischemic event observed in several organs or tissues and polymorphonuclear neutrophils (PMNs) have been identified as responsible for many of the early tissue damages associated with the ischemic and/or the postischemic period.^{1–4} Neutrophil recruitment in an

ischemic and/or a reperfused tissue is mainly dependent on chemotactic factors released at the site of injury.⁵ Various endogenous substances known to be chemoattractants for neutrophils have been shown to be released from ischemic organs such as complement (C5a)⁶; platelet-activating factor (PAF)^{7,8}; several lipoxygenase products such as leukotriene B₄ or HETES, mainly produced by already trapped inflammatory cells⁹; some cyclooxygenase products¹⁰; and chemokines such as interleukin (IL)-8.¹¹ The conserved mechanisms of directional sensing and the signal transduction events involved in gradient detection by eukaryotic cells have recently been reviewed.¹²

Neutrophil chemoattraction and activation in ischemic and reperfused tissues are probably the result of an amplification loop involving several mediators. However, the early event and the cellular origin of this inflammatory process remain poorly understood. Extravasation and activation of leukocytes are complex processes that involve multiple and sequential steps: rolling mediated by selectins followed by a firmer integrin-dependent adhesion between leukocytes and endothelium eventually leading to the transmigration and the infiltration.^{13,14} Endothelial cells play an active role in these processes: they are not only capable of expressing various adhesion molecules in response to numerous mediators¹⁵ but they are also able to release chemoattractants. These molecules induce the transient appearance of binding sites for several pleckstrin homology domain-containing proteins on the inner face of the membrane.¹² Soluble chemokine gradients generated by endothelial cells, eg, for IL-8 and/or membrane-associated PAF expression, well illustrate this notion.^{16,17}

Changes in oxygen tension during ischemia are able to

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modulate the interactions between endothelial cells and neutrophils. Endothelial cells exposed to hypoxic conditions have been found to be activated and they synthesize large amount of prostaglandins and PAF.¹⁸ Their adhesiveness for neutrophils also increases,^{19–21} which is at least in part dependent on the hypoxia-induced increase in PAF synthesis.²¹ In addition, neutrophils adherent to hypoxic endothelial cells become activated and release LTB₄ and superoxide anions.²² However, mediators involved in the early neutrophil recruitment remain to be discovered. In this study, we investigated the release of chemotactic factors for neutrophils by endothelial cells exposed to hypoxia and we analyzed the possible contribution of several chemoattractant molecules. Using several approaches, we showed that PGF_{2α}, a prostanoid released in large amounts by hypoxic endothelial cells from various sources and detected in ischemic rat liver perfusion liquids, is able to trigger neutrophil migration *in vitro*.

Materials and Methods

Chemicals and Reagents

Indomethacin, cycloheximide, salicylic acid, nordihydroguaric acid (NDGA) and the different pure prostaglandins (PGF_{2α} and PGI₂) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human interleukin-1β (rhIL-1β), the anti-human IL-8 neutralizing antibody, and the recombinant human IL-8 (rhIL-8) were purchased from R&D Systems Europe (Abingdon, UK). PAF, fluprostenol (a synthetic analog of PGF_{2α}), and PGF_{2α} dimethyl amide (a PGF_{2α} antagonist), were purchased from Cayman Chemical (Ann Arbor, MI). PAF receptor antagonist WEB 2086 was generously provided by Boehringer (Ingelheim, Germany). The rabbit polyclonal antibodies anti-prostaglandin F_{2α} and anti-6-keto-prostaglandin F_{1α} were purchased from Oxford Biomedical Research, Inc. (Oxford, UK). 6-keto-Prostaglandin F_{1α} is a stable metabolite of the prostacyclin (PGI₂) and was used to raise the antibody. Purified mouse monoclonal antibody (1 mg/100 μl) and goat polyclonal antibody (0.5 mg/ml) conjugated to alkaline phosphatase were generously provided by Dr. A. Rot (Sandoz Forschungsinstitut, Vienna, Austria). 2,3,4,4,6-Pentafluorobenzylbromide and N-ethyl-diisopropylamine were from Aldrich Chemie (Milwaukee, WI); bis(trimethylsilyl)trifluoroacetamide, dodecane, acetonitrile, methanol, acetic acid, and chloroform were from Janssen Chimica (Beerse, Belgium), anhydrous pyridine and other reagents or solvents of purest analytical grade available were purchased from Merck (Darmstadt, Germany). The Vybrant cell adhesion kit based on the fluorogenic dye calcein acetoxymethyl ester used to label the neutrophils was from Molecular Probes (Eugene, OR) and the Cytotoxicity detection kit based on lactate dehydrogenase release was purchased from Boehringer Mannheim (Mannheim, Germany).

Human Umbilical Vein Endothelial Cell (HUVEC) Isolation and Culture

HUVECs were isolated according to Jaffe and colleagues.²³ Cords were stored at 4°C just after birth in stock buffer (4 mmol/L KCl, 140 mmol/L NaCl, 10 mmol/L HEPES, 1 mmol/L glucose, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml fungizone, pH 7.3). Umbilical veins were rinsed with 20 ml of phosphate-buffered saline (PBS) containing antibiotics and fungizone at the above cited concentrations. Umbilical veins were then incubated for 35 minutes at 37°C with 4 ml of collagenase type II (Sigma Chemical Co.) 0.05% in PBS. The cells were harvested in M199 plus 20% fetal calf serum (Gibco, Paisley, Scotland), centrifuged 10 minutes at 1000 rpm and seeded on 0.20% gelatin-coated culture dishes (25 cm²; Falcon Plastics, Oxnard, CA). The next day, the cells were washed with medium to eliminate blood cell contamination. Only monolayers of primary cultures that were tightly confluent were used for these studies. Confirmation of their identity as endothelial cells was obtained by detecting factor VIII antigen assessed by immunofluorescence staining.²⁴ Human microvascular endothelial cells (HMEC-1; Centers for Disease Control, Atlanta, GA) were routinely cultivated in 75-cm² flasks in MCDB-131 (Gibco) culture medium supplemented with 15% fetal calf serum, epidermal growth factor (10 ng/ml hEGF), hydrocortisone (1 ng/ml), and L-glutamine (10 mmol/L). When at 90% confluence, the cells were seeded and incubated in hypoxic conditions. Human coronary aortic endothelial cells (HCAECs; Biowhittaker, Heidelberg, Germany) were cultivated in the recommended EGM-2 BulletKit media with 20% fetal calf serum and containing different supplements (Biowhittaker).

Isolation and Labeling of Human Neutrophils

Human PMNs were purified from blood of healthy donors by the procedure of Boyum.²⁵ Briefly, 30 ml of venous anticoagulated blood from normal patients were mixed with 5 ml of 6% dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) and allowed to sedimentate at room temperature for 60 minutes. After hypotonic lysis of erythrocytes performed with NaCl 0.2% for 1 minute, cells were centrifuged 20 minutes at 1000 rpm on Lymphoprep (Nycomed Pharma, Oslo, Norway). For labeling, neutrophils at a density of $\pm 10^7$ cells/ml were incubated with either 20 μCi ⁵¹Cr/ml (specific activity = 250 to 500 mCi/mg chromium; Amersham Laboratories, Buckinghamshire, UK) or labeled with 10 μg/ml calcein-AM in Hanks' balanced salt solution (HBSS) without calcium and magnesium for 60 minutes at 37°C with intermittent mixing every 10 minutes. Labeled neutrophils were washed three times, and diluted to 5×10^6 cells/ml before 500 μl (2.5×10^6 neutrophils) were added in the upper compartment of the migration chamber.

In Vitro Model of Hypoxia

Ischemia was simulated by exposing cells to hypoxia (100% N₂ atmosphere) at 37°C. Endothelial cells were seeded in gelatin-coated Petri dishes (Ø = 35 mm; Falcon Plastics). For incubation, cells were rinsed twice with modified HBSS (140 mmol/L NaCl, 5 mmol/L KCl, 0.4 mmol/L MgSO₄·0.7H₂O, 0.5 mmol/L MgCl₂·0.6H₂O, 3 mmol/L Na₂HPO₄·0.2H₂O, 0.4 mmol/L KH₂PO₄, 5.5 mmol/L glucose, pH 7.35) containing 1 mmol/L CaCl₂, and covered with 0.7 ml of HBSS. Hypoxic conditions were produced with an atmosphere of 100% N₂ in an incubator gas chamber. PO₂ was 130 mmHg in normoxic conditions, dropped to 10 mmHg after 30 minutes hypoxia as described here, and reached the air value (130 mmHg) in <5 minutes after reoxygenation.²⁶ Hypoxia time never exceeded 120 minutes, and cells retained >98% viability as determined on HUVECs by a dye exclusion method.²⁶ HMEC-1 were incubated in hypoxic conditions in CO₂-independent medium (Gibco) containing 2 mmol/L glutamine and 2% fetal calf serum in 100% N₂ atmosphere. Concomitantly, normoxic control cells were maintained in normal atmosphere (21% O₂). When inhibitors such as indomethacin, salicylic acid, NDGA, or cycloheximide were used, the molecules were added to HBSS for the hypoxia incubation. The conditioned media were recovered directly after the hypoxia incubation to avoid any reoxygenation effect.

Chemotaxis Assay

A 24-well chemotaxis polystyrene chamber (Ø = 16 mm; Corning, New York, NY) was used to study neutrophil migration. The chamber contains a lower compartment for the chemotactic stimulus separated by a filter from the upper compartment containing the labeled neutrophil suspension. The lower compartments were filled with 500 µl of either control solution (HBSS containing 1 mmol/L CaCl₂ and normoxia HUVEC-conditioned media) or HBSS containing the molecules to be tested for their chemotactic activity (hypoxia HUVEC-conditioned media, PAF, rhIL-8, or rhIL-1β). The filter was a sterilized polycarbonate membrane (Nunc, Roskilde, Denmark) of 10-mm diameter with pore size of 3 or 8 µm (diameter). Except in Figure 1, all experiments were performed with the chromium-51 (⁵¹Cr)-labeling technique and porous membrane of 8 µmol/L pore size. The upper compartment was filled with 500 µl of a neutrophil suspension (5 × 10⁶ cells/ml). Experiments with PGF_{2α} dimethyl amide were performed after neutrophils have been pre-incubated with this molecule (1 µg/ml) for 30 minutes before the chemotaxis assay. Assays were run in several independent experiments performed in triplicate for each condition. After the neutrophil suspensions were added to the upper compartments, the chamber was placed in an incubator at 37°C with humidified room air (95% air/5% CO₂) for 120 minutes. After the incubation, the filter was removed and neutrophils that have completely migrated through the microporous membrane were lysed by adding 0.5 ml of NaOH 1 N for 60 minutes to the solution in the lower compartment of the migration cham-

Figure 1. Effect of rhIL-8 and HUVEC-conditioned media on neutrophil chemotaxis. Conditioned media from HUVECs exposed to 120 minutes of normoxia, 120 minutes of hypoxia, HBSS, or HBSS containing 1 µg/ml of IL-8 were added to the lower compartment of the chemotaxis chamber. HBSS-CaCl₂ (0.5 ml) (1 mmol/L) containing 2.5 × 10⁶ of labeled PMNs was added in the upper compartment and incubated for 120 minutes in a 5% CO₂ incubator at 37°C. Results are expressed in number of PMNs that have totally migrated through the membrane, either for ⁵¹Cr-labeled PMNs (**A**) or for the fluorogenic dye calcein-AM-labeled PMNs (**B**). Two different pore size polycarbonate membranes (3 or 8 µm Ø) were tested and results are expressed as means ± 1 SD for one representative experiment performed in triplicate. *, #, ***, or ###: Significantly different from the normoxic HUVEC-conditioned media with *P* < 0.05 or *P* < 0.001 for respectively 8 (*)- or 3 (#)-µm pore size membrane using analysis of variance 1 and Scheffé's contrasts.

ber. Finally, the radioactivity of this lysate was measured in a gamma counter (1275 Minigamma; LKB Wallac, Turku, Finland). The number of neutrophils that had completely migrated in the lower compartment of the chamber (adherent or not) was then calculated from the radioactive-labeled neutrophils input. For calcein-labeled neutrophils, adherent neutrophils were lysed by adding 100 µl of 0.1% (v/v) Triton X-100 (in 50 mmol/L Tris-HCl, pH 7.4) and fluorescence emission at 530 nm from 485 nm excitation was read on a spectrofluorometric plate reader (Fluo star BMG Lab Technologies, Champigny sur Marne, France). Cell number was calculated from the calcein-labeled neutrophils input.

For scanning electron microscopic observations, after a wash to remove the nonadherent neutrophils, neutrophils adherent to the filter were fixed with glutaraldehyde (Ladd Research Industries, Inc., Burlington, VT) 2% in 0.1 mol/L cacodylate buffer (Sigma) pH 7.4, for 10 minutes at room temperature, then for 20 minutes at 4°C. Samples were then dehydrated for 5 minutes in 25, 50, 75, 95, and 100% ethanol at room temperature. They were finally prepared by critical-point drying, mounted on an aluminum stub to observe the lower side of the polycarbonate membrane, and covered with a thin layer of gold (20 to 30 nm). The examination was carried on in a scanning electron microscope (XL-20; Philips, Eindhoven, the Netherlands).

IL-8 Assay

IL-8 in endothelial cell homogenates and in HUVEC-conditioned media was quantitated using a sensitive capture enzyme-linked immunosorbent assay²⁷ in 96-multiwell immunoassay plates (Flow Laboratories, Amsterdam, The Netherlands). Mouse monoclonal antineutrophil activating protein-1/IL-8 IgG was used at 5 μ g/ml to coat wells of microtiter plates for 16 hours at 4°C in a humidified chamber. After washing four times with 100 μ l of phosphate-buffered saline (PBS) containing Tween 20 (0.05%) (Bio-Rad, Richmond, CA), 100 μ l of conditioned media from HUVECs or purified human recombinant IL-8 at concentrations ranging from 0.02 to 10 ng/ml was added and incubated for 2 hours at 37°C. Plates were rinsed four times with PBS containing Tween-20, as above, and goat anti-IL-8 IgG conjugated to alkaline phosphatase (5 μ g/ml) was added and incubated for an additional 2 hours at 37°C. Next, substrate, *p*-nitrophenylphosphate (Bio-Rad) at 1 mg/ml was added and plates were further incubated to allow color development. The enzymatic reaction was stopped after 11 minutes with 50 μ l NaOH (2 N) and absorbance was read at 405 nm in a BioRad 3550 Multiplate Reader (Bio-Rad). Optical density values were converted to nanograms of IL-8 using a standard calibration curve and results are expressed in ng/mg of proteins of the corresponding cultures.

Hydroxyeicosatetraenoic Acid Assay

11-HETE and 15-HETE were quantified by gas chromatography-mass spectrometry after extraction and derivatization as described by Murphy and Clay.²⁸ Cells seeded at confluence in gelatin-coated Petri dishes ($\varnothing$ = 60 mm) were rinsed twice with HBSS and then 1.5 ml of HBSS was added for the incubation under hypoxia as described above. Positive control were performed by stimulating the endothelial cells with histamine (Aldrich Chemie) (5 μ mol/L) for 30 minutes. After incubation, 4 μ l of acetic acid, ethanol (final concentration 15%), and 1 μ l of internal standard containing respectively 8.2 and 13.6 ng/ μ l of 11-HETE and 15-HETE were added to the conditioned media. Concentrations were estimated using the corresponding HETE (internal standards) in which oxy-

gen atoms (O¹⁶) of acid function were enzymatically replaced by oxygen atoms (O¹⁸) in presence of H₂¹⁸O.²⁸ Briefly, ¹⁸O-HETEs were prepared by incubation of the native compounds with butyryl cholinesterase (30 U) (Boehringer) reconstituted in 100 μ l of H₂¹⁸O (Aldrich Chemie) for 10 minutes at 37°C. The reaction was initiated by adding 10 μ l of 5-,11-,12-,15-HETEs methyl ester solution (Cayman Chemical Co.) dissolved in methanol. Preliminary experiments indicated that a 24-hour incubation at 37°C was sufficient to effect 90% hydrolysis of the methyl ester at these concentrations of enzyme and substrate. The incubation in H₂¹⁸O was stopped by adding 1 ml of methanol. After a short vigorous mixing, 1 ml of H₂O was added and the solution was acidified with HCl (1 N) to pH 3.5.

HETEs were extracted from the media by adsorption-elution on octadecyl (C18) mini columns (Amersham, UK) according to the manufacturer's procedure after addition of fixed and known amounts of O¹⁸-HETEs as internal standards. HETEs were eluted with a petroleumbenzine/chloroform solution (50:50; v/v) and samples were stored at -70°C until derivatization. Hydrogenation of HETEs was performed in methanolic solution with platinum dioxide as a catalyst and bubbling with hydrogen for 3 minutes at room temperature. Solutions were then filtered on 0.22- μ m Aerobisc nylon filter (Cayman Chemical Co.) before derivatization. Hydrogenated HETEs were derivatized according to the method of Leis and colleagues²⁹ with minor modifications. HETEs were esterified with 2,3,4,4,6-pentafluorobenzylbromide and trimethylsilylated with bis(trimethylsilyl)trifluoroacetamide.

Analysis of HETEs was performed on a Hewlett-Packard 5988 quadrupole mass spectrometer (MS) interfaced to Hewlett-Packard 5890 gas chromatograph (GC) equipped with a HP5-MS column (Hewlett-Packard, Palo Alto, CA). The column was kept at 190°C for 1 minute, then programmed to 280°C with an increase of 3°C per minute. Negative ion chemical-ionization mass spectrometry shows one intensive peak at *m/z* corresponding to M-pentafluorobenzyl ion. This ion was used for selective-ion monitoring at *m/z* = 399 or *m/z* = 403 for, respectively, HETEs (O¹⁶) and HETEs (O¹⁸) and the different HETEs were identified by their retention time that is, respectively, 19.3 and 19.8 minutes for 11- and 15-HETE. The cells were lysed with 1 ml of NaOH 0.5 N. The lysates were collected and assayed for protein content according to Lowry and colleagues³⁰ and results were expressed as ng/min/mg of proteins.

Rat Liver Ischemia

The method used has been described in a recent paper from our group.³¹ Briefly, female Wistar rats weighing 200 to 250 g were starved for 18 hours before liver perfusion and were anesthetized with ether inhalation for 5 minutes. The abdomen was opened, the hepatic vein rapidly cannulated, and the perfusion of the liver remaining in the abdomen started within 1 minute. The perfusion solution was modified Krebs-Henseleit bicarbonate buffer (pH 7.4) (in mmol/L): NaCl, 137; KCl, 5.4; MgSO₄, 0.7; H₂O,

0.81; glucose, 11; Na₂HPO₄, 0.2; H₂O, 0.34; NaHCO₃, 24.4; and KH₂PO₄, 0.35. The buffer was kept at 42°C to obtain 37°C in the liver perfusion. The buffer was continually gassed with either O₂/CO₂ (19:1) for normoxic or N₂/CO₂ (19:1) for hypoxic buffer (PO₂ = 10 to 15 mmHg). Livers were perfused for 5 minutes at a constant flow rate of 8 ml/min⁻¹ in a circulating mode to equilibrate the tissue before one of the following conditions was imposed. Controls and ischemic livers have been, respectively, perfused with normoxic and hypoxic buffer. Circulation was then stopped for 20 minutes. Livers were then washed 1 minute with the perfusion liquid at 8 ml/min⁻¹ and these liquids were rapidly frozen at -70°C until use in the chemotaxis assay.

PGF_{2α} Assay

PGF_{2α} released in endothelial cell-conditioned media or in perfusion liquids collected after rat liver normoxia or ischemia was quantitated using an enzyme immunoassay kit (Cayman Chemical Co.) as described by the manufacturer.

Statistical Analysis

All values were expressed as means ± 1 SD. Data were analyzed using one-way (analysis of variance 1) or two-way analysis of variance (analysis of variance 2). Scheffé's contrasts were used to discriminate significant differences between group means. Data were considered statistically significant if *P* was <0.05.

Results

Effect of Endothelial Cell-Conditioned Media on Neutrophil Chemotaxis

The effect of conditioned media from HUVECs incubated for 120 minutes in normoxia or hypoxia was tested on neutrophil chemotaxis and compared to neutrophil migration triggered with 1 μg/ml of rhIL-8 (Figure 1). We compared neutrophil chemotaxis for neutrophils labeled with ⁵¹Cr (Figure 1A) or calcein-AM (Figure 1B) and estimated neutrophil numbers recovered after a complete migration through a polycarbonate membrane with a pore size diameter of either 3 or 8 μm. Baseline migration of neutrophils observed for HBSS alone represents migration of unstimulated neutrophils. rhIL-8 triggered a significant neutrophil chemotaxis (Figure 1).

Conditioned media from endothelial cells incubated in normoxia for 120 minutes did not stimulate neutrophil chemotaxis. However, neutrophil migration induced by hypoxic HUVEC-conditioned media ranged from a two-fold to fourfold increase. Incubation in hypoxia for 120 minutes was based on preliminary data obtained from a time course experiment showing that hypoxic HUVEC-conditioned media triggered a significant neutrophil migration for 120 minutes of hypoxia (data not shown). The reproducible effect of HUVEC-conditioned media on neu-

trophil chemotaxis has been observed in 10 independent experiments performed in triplicate using endothelial cells from different umbilical veins and neutrophils isolated from different healthy donor blood samples. After 120 minutes of hypoxia, >98% of the endothelial cells retain viability as assessed by ethidium bromide/acridine orange or by erythrosin B staining (data not shown) whereas neutrophil plasma membrane integrity was tested by a LDH release assay and <5% of LDH observed after a 120-minute incubation in HBSS. The neutrophil responsiveness after purification was also observed when migration was triggered either by IL-8 chemokine (1 μg/ml) or PAF (10⁻⁶ mol/L) (data not shown).

The biological relevance of neutrophil chemotaxis induced by HUVEC-conditioned media is suggested by the fact that similar results were obtained with two other endothelial cell lines, ie, HMEC-1 and HCAEC. A very good correlation was observed between the PGF_{2α} concentration measured by enzyme immunoassay and the chemoattractant activity of the conditioned media (Figure 2). The PGF_{2α} release was dependent of the vascular origin because we measured 14,171 ± 888, 4765 ± 698, and 6148 ± 649 pg of PGF_{2α}/mg of proteins in the hypoxic-conditioned media for, respectively, the HUVECs, the HMEC-1, and the HACEC versus 5210 ± 1058, 2066 ± 485, and 2927 ± 400 pg of PGF_{2α}/mg of proteins in the normoxic-conditioned media of the same cell types (*n* = 3). The number of PMNs recovered at the end of the chemotaxis assay was clearly proportional to the amount of PGF_{2α} released in the conditioned media.

Results presented in Figure 3 showed that the oriented neutrophil migration in response to conditioned media from HUVECs exposed to hypoxia for 120 minutes was 3.7-fold higher than the migration observed in response to normoxic HUVEC-conditioned media after baseline migration has been subtracted (six independent experiments performed in triplicate, *n* = 18). The neutrophil migration was higher than the one observed in response to 5 ng/ml of exogenous rhIL-1β (Figure 3A). A 45-minute reoxygenation period of HUVECs in a normal atmosphere after the 120 minutes of hypoxia did not increase nor decrease the chemotactic activity present in the conditioned medium compared to hypoxia alone (data not shown).

These biochemical data were supported and illustrated by morphological observations in phase contrast microscopy (Figure 3B) and in scanning electron microscopy (Figure 3C). Figure 3B illustrates the number of neutrophils that have completely migrated and are recovered on the bottom of the well containing the insert used for the migration assay. Many more neutrophils were seen when hypoxic HUVEC-conditioned media was placed in the lower compartment of the chamber (photo B) when compared to normoxic HUVEC-conditioned media (photo A). Similarly, numerous neutrophils were observed in response to 5 ng/ml of exogenous rhIL-1β (photo C). Figure 3C showed more adherent neutrophils to the lower side of the polycarbonate filter when hypoxic HUVEC-conditioned medium (photo B) or 5 ng/ml of rhIL-1β (photo C) was added than when normoxic

Figure 2. A: Quantification by an enzyme immunoassay of PGF_{2α} concentration present in conditioned media collected after 120 minutes of normoxia (open bars) or hypoxia (hatched bars) for HUVECs, HMEC-1, and HCAEC. Results are expressed in percentage of normoxic control and expressed as means $\pm$ 1 SD for one experiment performed in triplicates. **B:** Effect of these different endothelial cell-conditioned media on neutrophil chemotaxis. Conditioned media from HUVECs, HMEC-1, or HCAEC exposed to normoxia (open bars) or hypoxia (hatched bars) for 120 minutes were added to the lower compartment of the chemotaxis chamber. Results are expressed in percentage of normoxic control as means $\pm$ 1 SD for one experiment performed in triplicate. ***, Statistically significantly different from the time-matched normoxic HUVEC-conditioned media with $P < 0.001$ using analysis of variance 1.

HUVEC-conditioned medium was used (photo A). Furthermore, neutrophils are more spread after migration in response to IL-1 β than in response to hypoxic conditioned media (photo C versus B). The results confirm and illustrate that endothelial cells incubated in hypoxic conditions release chemotactic factor(s) for neutrophils.

Effect of Hypoxia on the Release of IL-8 and IL-1

Among the chemokines, IL-8 exhibits a strong chemotactic activity on neutrophils and is known to be secreted by endothelial cells in several conditions.¹⁶ The effect of hypoxia on the synthesis and release of IL-8 by HUVECs was thus investigated at the protein level by enzyme-linked immunosorbent assay. When HUVECs were ex-

Figure 3. A: Effect of HUVEC-conditioned media or rhIL-1 β on neutrophil migration. Conditioned media from HUVECs exposed to normoxia or hypoxia for 120 minutes, 5 ng/ml of rhIL-1 β , or HBSS alone were added to the lower compartment of the chemotaxis chamber. Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD for six independent experiments for hypoxia and four independent experiments for IL-1 performed in triplicate. * or **, Statistically significantly different from HBSS with $P < 0.05$ or $P < 0.01$. NS, Nonsignificantly different from HBSS using analysis of variance 2 and Scheffé's contrasts. **B:** Micrographs in phase contrast microscopy of neutrophils that have migrated through the membrane observed on the bottom of the lower compartment. Conditioned media from HUVECs exposed to normoxia (A) or hypoxia (B) for 120 minutes, or 5 ng/ml of rhIL-1 β (C) were added to the lower compartment of the migration chamber (original magnification, $\times 115$). **C:** Micrographs in scanning electron microscopy of neutrophils adherent to the lower side of the polycarbonate membrane. Conditioned media from HUVECs exposed to normoxia (A) or hypoxia (B) for 120 minutes or 5 ng/ml of rhIL-1 β (C) were added to the lower compartment of the migration chamber (original magnification, $\times 600$).

posed to hypoxic conditions for up to 2 hours, no increase in IL-8 release or in IL-8 synthesis in cell homogenate was observed (data not shown). As no IL-8 synthesis increase or release is observed after hypoxia, this chemokine does not seem to be a candidate that could account for the early chemotactic activity present in the hypoxic HUVEC-conditioned medium. The putative role of IL-8 and other peptide chemokines have been further investigated and ruled out based on several evidences: boiled HUVEC-conditioned media as well as preincubation of the hypoxic-conditioned media with a neutralizing anti-IL-8 antibody (200 μ g/ml) for 30 minutes did not prevent or inhibit the oriented neutrophil migration induced by hypoxic-conditioned media (Figure 4). The specific effect of the neutralizing antibody was tested on the migration triggered by rhIL-8 and 87% inhibition of the chemotaxis was obtained (data not shown). IL-1 is also

Figure 4. Effect of a PAF receptor antagonist (WEB 2086, 10^{-5} mol/L), of the neutralizing antibody anti-rhIL-8 (200 $\mu\text{g}/\text{ml}$) or boiled conditioned media on the neutrophil migration triggered by the hypoxia HUVEC-conditioned media. The neutralizing anti-IL-8 antibody (200 $\mu\text{g}/\text{ml}$) was added to the conditioned media for 30 minutes whereas the WEB 2086 was added to the neutrophil suspension 30 minutes before the assay. Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD for one experiment performed in triplicate. ***, Significantly different from normoxic-conditioned media with $P < 0.001$. NS, Nonsignificantly different from hypoxic-conditioned media using analysis of variance 1 and Scheffé's contrast.

known to have a chemotactic activity for neutrophils but neither IL-1 α nor IL-1 β could be detected in the incubation medium of normoxic or hypoxic HUVECs for up to 2 hours (data not shown).

Effect of Hypoxia on HETE Synthesis

Phospholipase A_2 is strongly activated in hypoxic endothelial cells¹⁸ leading to the release of arachidonic acid that is mainly metabolized to prostaglandins but monohydroxylated derivatives such as HETEs can also be synthesized. The ubiquitous hydroxylated fatty acids derived from arachidonic acid (HETEs) or linoleic acid (HODEs) exhibit diverse biological effects including chemotaxis.³² We thus studied the effect of hypoxia on the synthesis of these mediators. HUVECs mainly synthesize 11-HETE and 15-HETE by a specific lipoxygenase pathway.³³ As observed in Figure 5, HUVECs incubated either in normoxic or hypoxic conditions for 120 minutes release low and similar amount of 11-HETE and 15-HETE. Although histamine at 5 $\mu\text{mol}/\text{L}$ was able to strongly stimulate the synthesis of both HETEs, hypoxia did not have any significant effect on HETEs release.

PAF is also a strong chemoattractant for neutrophils. However, previous results have shown that PAF was no longer detected in the conditioned medium of HUVECs incubated for 120 minutes under hypoxic conditions nor associated to plasma membranes of HUVECs incubated

Figure 5. 11- and 15-HETE synthesis by HUVECs incubated in normoxic (filled bars) or hypoxic (hatched bars) conditions for 120 minutes or stimulated with histamine (5 $\mu\text{mol}/\text{L}$) for 30 minutes (open bars). Results are expressed in ng of HETE/minute/mg of proteins. The number of tests performed in two independent experiments is indicated between brackets.

120 minutes under hypoxia.²¹ PAF production was assessed by gas chromatography-mass spectrometry as well as using the rabbit platelet aggregation assay thus excluding the presence of an oxidatively modified phospholipid that is analog of PAF.³⁴ To rule out a role of PAF, the effect of WEB 2086, a specific PAF receptor antagonist, was investigated. Preincubation of neutrophils with WEB 2086 at 10^{-5} mol/L for 30 minutes before the assay was also ineffective to prevent the neutrophil migration induced by hypoxic HUVEC-conditioned media (Figure 4). These results confirm that PAF, a mediator reported to be membrane-associated in endothelial cells,³⁵ is not involved in the neutrophil chemotaxis observed in our experimental model.

Identification of the Chemotactic Factor

To determine the nature of the chemoattractant(s) released by HUVECs during hypoxia, different inhibitors of the hypoxia-induced activation of HUVECs were used. These molecules were added to HUVECs during the hypoxia incubation or preincubated with the cells for 4 hours before hypoxia incubation. The conditioned media were then tested for their chemotactic activity for neutrophils. For each inhibitor used, we first verified that they did not by themselves interfere with the migration of neutrophils. None of them at the concentration used here reduced the number of neutrophils that migrated toward a standard chemoattractant, rhIL-1 β (data not shown).

As shown in Figure 6, indomethacin, a cyclooxygenase inhibitor (10^{-5} mol/L), and preincubation of the HUVECs in the presence of salicylic acid (10^{-6} mol/L) for 4 hours before the incubation in hypoxia, completely inhibited the release of chemoattractant molecules for neutrophils. Previous studies showed that hypoxia in the same experimental conditions increases the release of prostaglandins synthesized by HUVECs.¹⁸ The presence of indomethacin almost completely inhibited the prostaglandin production: 10^{-5} mol/L of indomethacin inhibited PGI_2 synthesis by 100%, $\text{PGF}_{2\alpha}$ by 94%, PGE_2 by 96%, and PGD_2 by 100%. Taken together, these results suggest that a cyclooxygenase product was responsible, at least

Figure 6. A: Effect of inhibitors or antibodies on the neutrophil migration triggered by the hypoxic HUVEC-conditioned media. Conditioned media from HUVECs incubated under normoxia (hatched bars) or hypoxia for 2 hours in the presence (open bars) or in the absence (filled bars) of indomethacin (10^{-4} mol/L), NDGA (10^{-4} mol/L), or cycloheximide (10^{-6} mol/L). Salicylic acid (10^{-6} mol/L) was pre-incubated with HUVECs for 4 hours before hypoxia incubation. Rabbit polyclonal antisera ($10 \mu\text{L}$) neutralizing PGF_{2α} and 6-keto-PGF_{1α} were added separately or together to the hypoxic HUVEC-conditioned media just before the migration assay. **B:** Effect of rabbit polyclonal anti-PGF_{2α} antibody (Ab) or pre-immune serum (PIS) on the PGF_{2α}-induced neutrophil migration. Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD. The number of tests performed in three independent experiments is indicated between brackets. * or **, Significantly different from hypoxic HUVEC-conditioned medium with $P < 0.05$ or $P < 0.01$. NS, Nonsignificantly different from hypoxic HUVEC-conditioned medium using analysis of variance 2 and Scheffé's contrasts.

in part, for the chemotactic activity present in the hypoxic HUVEC-conditioned medium.

To identify the nature of the prostaglandin(s) responsible for the chemotactic activity, neutralizing antibodies directed against PGF_{2α} or against PGI₂ were tested. They were added for 30 minutes to the conditioned media before the chemotaxis assay. Anti-PGF_{2α} antibodies inhibited 76% of the chemotactic activity present in the hypoxic HUVEC-conditioned medium; this inhibition was statistically significant (Figure 6A). This activity was also reduced by 79% by anti-PGI₂ antibodies but because of the rather important standard deviation, this inhibition did not reach statistical significance. The two antibodies added together inhibited the chemotactic activity by 95%. As a control for the efficiency of these antibodies, their ability to block pure PGF_{2α}- and PGI₂-stimulated chemotaxis was established previous to their use as shown for PGF_{2α} in Figure 6B. According to the manufacturer (Oxford Biomedical Research, Inc.), the specificity of both antibodies is very high and the cross-reac-

Figure 7. Effect of exogenous prostaglandins on neutrophil migration. PGD₂ (2.1 ng/ml), PGE₂ (3.4 ng/ml), PGF_{2α} (8.7 ng/ml), and 6-keto-PGF_{1α} (34.5 ng/ml) were tested separately or together on neutrophil migration. Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD for one experiment performed in triplicate. ** or ***, Significantly different from HBSS with $P < 0.01$ or $P < 0.001$. NS, Nonsignificantly different from HBSS using analysis of variance 1 and Scheffé's contrasts.

tivity with other prostanoid derivatives is $<1\%$. Moreover, pre-immune serum had no effect (Figure 6B). These results suggest that PGF_{2α} and to a lesser extent PGI₂ were responsible for the chemotactic activity for neutrophils present in hypoxic HUVEC-conditioned medium. On the other hand, NDGA, a lipoxygenase inhibitor, did not affect the chemotactic activity (Figure 6A). Neither did cycloheximide (Figure 6A) nor boiling the media for 10 minutes before the chemotaxis assay (Figure 4) inhibit the induced neutrophil migration, indicating that a protein or a *de novo* protein synthesis is not required for this activity. To confirm the possible role of prostaglandins, pure exogenous molecules at the same concentration as the one assayed in the hypoxic HUVEC-conditioned medium were tested for their chemotactic activity for neutrophils (Figure 7). PGF_{2α} at 8.66 ng/ml demonstrated a strong significant chemotactic activity (76,977 migrated neutrophils). To a lesser extent and not significantly, PGI₂ at 34.66 ng/ml was also chemotactic (52,097 migrated neutrophils). PGD₂ at 2.10 ng/ml and PGE₂ at 3.37 ng/ml did not show any chemotactic activity. A mixture of the four PGs at these concentrations demonstrated a strong chemotactic activity (104,252 migrated neutrophils), higher than (but not statistically different from) PGF_{2α} alone and of the same magnitude as 5 ng/ml of rhIL-1 β (97,343 migrated neutrophils). These results suggest that PGF_{2α} is the most efficient chemoattractant molecule among the PG family members tested. We then confirmed the role of these molecule by performing a dose-response curve for neutrophil migration in response to different concentrations of PGF_{2α} (Figure 8A) or flupro-

Figure 8. A: Effect of different concentrations of PGF_{2α} on neutrophil migration. Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD for $n = 3$. ***, Significantly different from HBSS with $P < 0.001$. NS, Nonsignificantly different from HBSS using analysis of variance 1 and Scheffé's contrasts. **B:** Effect of different concentrations of fluprostenol, a PGF_{2α} analog, on neutrophil migration. Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD for one experiment performed in triplicate. * or ***, Significantly different from HBSS with $P < 0.01$ or $P < 0.001$. NS, Nonsignificantly different from HBSS using analysis of variance 1 and Scheffé's contrasts.

stenol (Figure 8B), a stable and synthetic analog of PGF_{2α} acting as a PGF_{2α} agonist.³⁶ Both molecules induced a dose-dependent migration of neutrophils. The concentrations for which the maximal effects were obtained were 100 ng/ml and 500 ng/ml for, respectively, the PGF_{2α} and fluprostenol. However, a concentration as low as 5 ng/ml (same range as the concentration determined in hypoxic HUVEC-conditioned media) was sufficient to induce a statistically significant migration. These results confirm that PGF_{2α} triggers neutrophil chemotaxis *in vitro*, in a dose-dependent manner. Neutrophil migration triggered by PGF_{2α}, IL-1 β , or IL-8 was really because of chemotaxis as demonstrated by the fact that when equal amounts of these chemoattractants were added in the upper and lower compartments of the chamber, no migration was observed (Table 1).

Neutrophil Migration Is Blocked by a PGF_{2α} Antagonist

The specific effect of PGF_{2α} onto its F2-prostaglandin (FP) receptors was addressed using PGF_{2α} dimethyl amide, a specific PGF_{2α} antagonist.^{37,38} Preincubation of neutrophils for 30 minutes in the presence of this molecule (1 μ g/ml) before the chemotaxis assay, dose-dependently inhibited the neutrophil migration in response to PGF_{2α} (10 ng/ml) (Figure 9A) but was without any effect on the migration triggered by iloprost (30 ng/ml), a stable and biologically active prostacyclin analog (Figure 9B). These data show that when the FP receptors on neutrophils are blocked by PGF_{2α} dimethyl amide, no migration can be observed in response to PGF_{2α}.

We then confirmed the role of PGF_{2α} present in the hypoxic endothelial cell-conditioned media. Preincubation of neutrophils with a F2-prostaglandin receptor antagonist, PGF_{2α} dimethyl amide, completely abolished their migration triggered by hypoxic HUVEC-conditioned media (Figure 10). These results indicate that PGF_{2α} is the main molecule responsible for the chemoattractant activity for neutrophils present in hypoxic HUVEC-conditioned media and that its effect on neutrophils is specific ie, mediated through the FP receptor.

Table 1. Effect of IL-8, IL-1 β , and PGF_{2α} on Neutrophil Migration when the Chemoattractant Is Present Only in the Lower Compartment (Column 3) or in Both Compartments of the Migration Chamber (Column 5)

Treatment	Molecule added to the lower compartment	PMN Number $\pm$ SD	+ Molecule added to the upper compartment	PMN Number $\pm$ SD
HBSS		20,757 $\pm$ 4640		
IL-8	1000 ng/ml	126,298 $\pm$ 17,253*	1000 ng/ml	14,634 $\pm$ 4216 [#]
IL-8	100 ng/ml	137,760 $\pm$ 14,350*	100 ng/ml	13,925 $\pm$ 3944 [#]
IL-8	10 ng/ml	23,893 $\pm$ 2776	10 ng/ml	5,717 $\pm$ 1888
IL-1 β	5 ng/ml	124,842 $\pm$ 41,387*	5 ng/ml	31,808 $\pm$ 10,416 [#]
PGF _{2α}	100 ng/ml	201,114 $\pm$ 8978*	100 ng/ml	12,170 $\pm$ 7026 [#]
PGF _{2α}	1 ng/ml	36,848 $\pm$ 3937	1 ng/ml	11,237 $\pm$ 2789
PGF _{2α}	100 ng/ml	381,024 $\pm$ 24,680*	1 ng/ml	354,256 $\pm$ 11,614
PGF _{2α}	100 ng/ml	369,226 $\pm$ 39,801*	10 ng/ml	46,778 $\pm$ 2128 [#]
PGF _{2α}	100 ng/ml	354,956 $\pm$ 5216*	100 ng/ml	7,728 $\pm$ 2237 [#]

*Statistically significantly different from HBSS; [#], statistically significantly different from the corresponding test containing the chemoattractant only in the lower part of the migration chamber with $P < 0.001$ using analysis of variance 1 and Scheffé's contrasts.

Figure 9. Effect of PGF_{2α} dimethyl amide (PDA), a PGF_{2α} antagonist, on neutrophil migration triggered by PGF_{2α} (10 ng/ml) (A) or by iloprost (30 ng/ml) (B). Neutrophils were preincubated with 100 or 1000 ng/ml of PDA for 30 minutes before the migration assay. Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD for one experiment performed in triplicate. ***, Significantly different from HBSS with $P < 0.001$. ###, Significantly different from PGF_{2α} with $P < 0.001$. NS, Nonsignificantly different from iloprost using analysis of variance 1 and Scheffé's contrasts.

PGF_{2α} Is also Released by Ischemic Rat Liver

To address the physiological relevance of these results, we tested the chemotactic activity of perfusion liquids collected from rat liver perfused in normoxia or ischemia. The neutrophil migration triggered by the ischemic perfusion liquid was 2.5-fold higher than the migration obtained in response to the normoxic control (Figure 11). Ischemia stimulated the synthesis and/or the release of PGF_{2α} because 2500 pg PGF_{2α}/ml were quantitated in the perfusion liquid after ischemia versus 320 pg PGF_{2α}/ml for the normoxic control (data not shown). Fur-

Figure 10. Effect of PGF_{2α} dimethyl amide (PDA), a PGF_{2α} antagonist, on neutrophil migration triggered by HUVEC-conditioned media. Conditioned media from HUVECs exposed to normoxia or hypoxia for 120 minutes were added to the lower compartment of the migration chamber. Before the assay, neutrophils were or were not preincubated for 30 minutes with PGF_{2α} dimethyl amide (1000 ng/ml). Results are expressed in number of PMNs that have totally migrated through the membrane as means $\pm$ 1 SD for one experiment ($n = 6$). ** or ***, Statistically significantly different from normoxic HUVEC-conditioned media with $P < 0.05$. ###, Significantly different from hypoxic HUVEC-conditioned media using analysis of variance 1 and Scheffé's contrasts.

thermore, when neutrophils were preincubated for 30 minutes in the presence of the PGF_{2α} antagonist, PGF_{2α} dimethyl amide, before the assay, the migration induced by the ischemic perfusion liquid was almost totally abolished (Figure 11). This experiment has been repeated twice with similar results. The inhibitory effect of the PGF_{2α} antagonist was only observed on the migration induced by the ischemic perfusion liquid whereas little inhibition was observed on the migration triggered by normoxic perfusion liquid. These results suggest that the synthesis and/or the release of PGF_{2α} by the liver was induced by ischemia and that this molecule was, in these conditions, the main mediator involved in neutrophil chemotaxis when assayed *in vitro*.

Discussion

Ischemia-reperfusion injury often initiates an acute inflammatory response in which neutrophils are major participants. Experimental evidence indicates that the interplaying inflammatory reactions are exacerbated by reperfusion and that accumulating neutrophils contribute to the reperfusion damage. However, this process is initiated during the ischemic period. To recruit neutrophils from the circulation, chemotactic factors are likely to be released already during the ischemia period. Indeed chemoattractants have been shown to be released both *in vitro*⁷ and *in vivo*³⁹ by ischemic hearts.

This study shows that endothelial cells incubated in low oxygen tension as well as ischemic-perfused rat liver release neutrophil chemoattractant activity higher in magnitude than the one observed in the presence of 5

Figure 11. Effect of $\text{PGF}_{2\alpha}$ dimethyl amide (PDA), a $\text{PGF}_{2\alpha}$ antagonist on neutrophil migration triggered by rat liver perfusion liquids. Perfusion liquids from rat liver exposed to normoxia or ischemia for 20 minutes were added to the lower compartment of the migration chamber. Before the assay, neutrophils were or were not preincubated for 30 minutes with $\text{PGF}_{2\alpha}$ dimethyl amide (1000 ng/ml). Results are expressed in number of PMNs that have totally migrated through the membrane as means for one experiment.

ng/ml of rhIL-1 β . Neutrophil chemotactic activity detected in hypoxic-conditioned media of HUVECs is significant after 120 minutes of exposure to hypoxia. The time course of release is similar to the one observed for the release of prostaglandins by hypoxia-activated endothelial cells¹⁸ after an elevation in the cytosolic calcium concentration⁴⁰ induced in hypoxic conditions. The effect does not seem to be endothelial cell source-specific because similar results were obtained with two other endothelial cell lines from different vascular beds (HMEC-1 and HCAEC).

Using the HUVECs submitted to hypoxia as a model, we attempted to identify chemotactic factor(s) responsible for the chemoattractant activity in the early hypoxic events. A lipidic mediator is most likely responsible for the chemotactic effect because a *de novo* protein synthesis was not required (preincubation of HUVECs with cycloheximide is without any effect on neutrophil chemotaxis) and because the hypoxic HUVEC-conditioned media kept neutrophil activity after boiling. Furthermore, IL-1 α or IL-1 β was not detected, the synthesis of IL-8 was unchanged under hypoxia and pre-incubation of the conditioned media with a neutralizing anti-rhIL-8 did not inhibit the oriented neutrophil migration. IL-8 expression has been reported to increase in hypoxic endothelial cells²⁷ and *in vivo* in the myocardium after ischemia-reperfusion.¹¹ Reasons for this apparent discrepancy are unclear but duration of hypoxia or ischemia and experimental settings can be advanced. Karakurum and colleagues²⁷ found that IL-8 synthesis by endothelial cells is only significantly induced after longer periods of hypoxia (6 hours). Oz and colleagues⁴¹ reported that the increase of IL-8 release by primary cultures of saphenous vein

endothelium incubated under hypoxia takes at least 4 hours.

Leukotrienes have also been implicated as mediators of ischemia-reperfusion injury in several experimental models.^{42–44} However, the presence of 5-lipoxygenase in human endothelial cells is controversial and a role of 5-lipoxygenase products as the chemotactic factor released by hypoxic endothelial cells was unlikely because NDGA did not inhibit neutrophil migration. The direct assay of 11-HETE and 15-HETE, two other putative candidates, showed that the synthesis of these molecules was not affected by hypoxia.

Recent experiments have suggested another lipid-derived molecule, PAF as an important candidate for chemotactic activity associated with ischemia.^{7,8} However, a role for this molecule is also unlikely in this work for three reasons. First, PAF is known to remain associated with the endothelial plasma membrane and is not released in the conditioned medium from thrombin- or histamine-stimulated HUVECs.^{45–47} Second, if hypoxia strongly stimulates a transient PAF synthesis in HUVECs, this synthesis is optimal after 90 minutes of hypoxia but PAF could no longer be detected after 120 minutes of hypoxia²¹ while a strong chemotactic activity is still observed. Third, neutrophil preincubation with WEB 2086, a specific and powerful PAF receptor antagonist, is without any effect on neutrophil chemotaxis triggered by hypoxic HUVEC-conditioned media.

In contrast, the inhibition of cyclooxygenase by indomethacin, salicylic acid, and the blocking effect of anti-PG antibodies indicated that one or several prostaglandins were responsible for the chemotactic activity, $\text{PGF}_{2\alpha}$ being the most likely candidate. Moreover, inhibition observed when neutrophils were preincubated with $\text{PGF}_{2\alpha}$ dimethyl amide, a FP receptor antagonist, supports the fact that $\text{PGF}_{2\alpha}$ specifically acts on its receptor on the neutrophil surface. The specificity of this antagonist was tested on neutrophil migration triggered by other chemoattractants and this molecule did not inhibit the neutrophil chemotaxis triggered by either PAF (10^{-6} mol/L) or rhIL-8 ($1 \mu\text{g/ml}$) (data not shown). Taken together, all these results indicate that $\text{PGF}_{2\alpha}$ is the major mediator in hypoxic HUVEC-conditioned media that triggers neutrophil chemotaxis.

In addition, $\text{PGF}_{2\alpha}$ and fluprostenol, a synthetic analog acting as a $\text{PGF}_{2\alpha}$ agonist, are able to sustain directed migration of neutrophils in dose-dependent manner. This dose-response curve indicates that the $\text{PGF}_{2\alpha}$ concentration found in the hypoxic HUVEC-conditioned medium is indeed able to significantly trigger neutrophil migration.

To address the physiological relevance of these results, we studied the chemoattractant activity of perfusion liquids collected from ischemic rat livers. Perfusion liquids from ischemic livers triggered neutrophil migration and the chemotaxis was totally inhibited when neutrophils were preincubated with $\text{PGF}_{2\alpha}$ dimethyl amide. These data indicate that $\text{PGF}_{2\alpha}$ is likely to play an important role as chemoattractant for neutrophils in ischemic tissues *in vivo*. However, even if endothelial cells are well known to synthesize prostanoids, a contribution from other cell

types such as Kupffer cells or hepatocytes has to be considered.

The still controversial role of prostaglandins on neutrophil chemotaxis and functions has been reported in several articles and seems to be dependent on the considered molecule. The inhibitory effect of PGE₂ and PGE₁ on rat neutrophil aggregation and migration would be mediated by an increase of cAMP^{48–50} and/or an activation of PI-3 kinase.⁵¹ PGF_{2α} was described a long time ago as a chemoattractant substance,⁵² but the role of prostanoids on neutrophil migration in hypoxic/ischemic tissues is still unknown. Farber and colleagues⁵³ also showed that HUVECs exposed to low oxygen concentration released a chemotactic activity for neutrophils. However, it occurred much earlier (within 5 minutes) and seemed to be because of a lipoxygenase but not to a cyclooxygenase product. The reason for this discrepancy is unknown.

Several reports have already mentioned the role of prostaglandins in ischemia-reperfusion.^{10,54} Although several prostaglandins like PGE₂^{49,55,56} and PGI₂^{57,58} are known to present inhibitory effects on some neutrophil functions, the chemotactic activity of some prostaglandins for neutrophils has already been assessed in several other experimental models^{59,60} and PGF_{2α} has already been identified as being a chemoattractant for neutrophils.^{52,61} The possibility that the active molecule is actually an isoprostane (8-epi PGF_{2α}) generated by oxygen radical attack on PGF_{2α} that is enzymatically synthesized⁶² cannot be excluded even if we took care to avoid any reoxygenation of the cells.

The recruitment of neutrophils by hypoxic and ischemic tissues is probably a process involving several successive mediators probably originating from several cell types within the tissue. A very early phase could involve molecules like PAF that is known to be quickly synthesized by hypoxic endothelial cells.^{21,63} Then, a rapid but more sustained prostaglandin production takes place. Finally, after this rapid cell activation phase, transcription and *de novo* synthesis of several cytokines like IL-8 and IL-1 could play the same role, keeping neutrophil recruitment going on. Why and what could be the importance to have such a continuum in the neutrophil recruitment response? If the role of phagocytes in hypoxic or ischemic tissues is to participate to remove cell debris and to clean the necrotic/apoptotic zone, one can speculate that the longer and the more severe the hypoxia or ischemia events are, the more neutrophils have to be recruited. On the other hand, we also have to keep in mind that neutrophil chemotaxis is sometimes dissociated from leukocyte activation. It has been shown that prostaglandins inhibit the LTB₄ synthesis and release by neutrophils stimulated by f-MLP. One can imagine that, even if the prostaglandin PGF_{2α} is able to recruit more PMNs, other prostanoids will act on the activation of these inflammatory cells, inhibiting the release of LTB₄ and superoxide anions and thus preventing the exacerbation of tissue damages.⁶⁴ Identification of early event such as the activation of endothelium by low oxygen tension is of great interest to develop new therapeutic strategies.

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