THE MONOCYTE-DERIVED NEUTROPHIL ACTIVATING PEPTIDE (NAP/INTERLEUKIN 8) STIMULATES HUMAN NEUTROPHIL ARACHIDONATE-5-LIPOXYGENASE, BUT NOT THE RELEASE OF CELLULAR ARACHIDONATE

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Polymorphonuclear leukocytes (PMNL) are known to play an important role in acute inflammatory processes (1) as well as in some chronic disease conditions such as rheumatoid arthritis (2) and psoriasis (3). A number of neutrophil chemotactic factors appear to be involved in the tissue accumulation of PMNL, including C5a (4), bacterial F-Met peptides (5), the platelet-activating factor PAF (6), and leukotriene B4 (LTB4) (7). In addition to these well-characterized chemotaxins there is now ample evidence that upon stimulation host cells themselves are able to respond with the release of protein-like PMNL chemotactic factors. Although several studies revealed the existence of such biological activity (8-12), until recently the factors responsible for PMNL chemotactic activity were not biochemically purified and characterized.

In previous studies we as well as others have purified a novel neutrophil-activating cytokine obtained from LPS- or mitogen-stimulated human mononuclear cells, which was termed MONAP (13), MDNCF (14), NAF (15), LYNAP (16), or chemotactic monokine (17), all being identical by NH2-terminal amino acid sequencing (14, 15, 17, 18). More recently, this cytokine was tentatively termed neutrophil-activating peptide or interleukin 8 (NAP/IL-8) (19, 20). NAP/IL-8 is a 8.359-kD polypeptide containing 72 amino acid residues of which the complete amino acid sequence was recently determined (18). The amino acid sequence of NAP/IL-8, which also has been cloned now (21), is identical to the deduced amino acid sequence of a fragment of the 3-10C cDNA sequence (22). So far NAP/IL-8 appears to be a polypeptide

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Abbreviations used in this paper: AA, arachidonic acid; C5a, 74-amino acid glycopeptide from the 5th complement component; 5(S),12(S)-DiHETE, 5(S),12(S)-dihydroxy-6E,8Z,10E-eicosatetraenoic acid; 5-HETE, 5(S)-hydroxyeicosatetraenoic acid; 5-LO, arachidonate 5-lipoxygenase; LTB4, leukotriene B4 (5(S),12R)-dihydroxy-6Z,8E,10E-eicosatetraenoic acid); 20-COOH-LTB4, 20-carboxy-LTB4; 20-OH-LTB4, 20-hydroxy-LTB4; trans-LTB4, 5(S),12(R)-dihydroxy-6E,8E,10E-eicosatetraenoic acid; epi-trans-LTB4, 5(S),12(S)-dihydroxy-6E,8E,10E-eicosatetraenoic acid; NAP, mononuclear cell-derived neutrophil-activating 72-amino acid peptide previously termed MONAP, MDNCF, NAF, LYNAP, and now IL-8; PAF, platelet-activating factor; PGB2, prostaglandin B2; RP-HPLC, reversed phase HPLC; TFA, trifluoroacetic acid.

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with 30–40% structural homology to platelet-derived polypeptides, such as platelet factor 4, β-thromboglobulin, connective tissue activating peptide III, as well as other host defense cytokines such as IFN-γ-inducible protein IP-10, 9E3 from Rous sarcoma–induced chicken fibroblasts, and a mitogen called melanoma growth stimulating activity (MGSA) (16, 18, 21, 23). Purified NAP/IL-8 shows potent chemotactic activity for human neutrophils in vitro (13, 14, 16, 17), activates the release of superoxide anions (13, 24), and elicits release of the primary granule constituents from neutrophils (myeloperoxidase, β-glucuronidase, and elastase) (13, 15, 24).

The neutrophil-activating mediators C5a, FMLP, and PAF, all of which are known to bind to ligand-specific membrane receptors on neutrophils, have been shown to activate the arachidonate-5-lipoxygenase with the release of LTB4 by human neutrophils in the presence of exogenous arachidonic acid (AA) (25–27).

The biological similarity of NAP/IL-8 to other clearly characterized neutrophil chemoattractants raises the question whether this novel cytokine is also able to stimulate the neutrophil arachidonate-5-lipoxygenase with the release of neutrophil chemotactic leukotrienes in the presence or the absence of extracellular AA.

In this article we report that NAP/IL-8 at concentrations relevant in vivo is able to stimulate the release of LTB4 in the presence of exogenous AA.

Materials and Methods

Neutrophil Isolation. Human PMNL were isolated using a modification of the method of Henson (28), similar to a method previously described (29). Briefly, venous blood was drawn into 1/6 vol of acidic citrate/dextran (0.085 mol/liter sodium citrate, 0.065 mol/liter citric acid in double-distilled, pyrogen-free water containing 2% (wt/vol) Dextran T 70 (Pharmacia Fine Chemicals, Uppsala, Sweden), centrifuged, and the supernatant containing plasma, platelets, and mononuclear cells was removed. The sediment containing E and PMNL was mixed with gelatine solution (2.5% (wt/vol) in 0.9% NaCl) and E were allowed to sediment for 30 min at 37°C. PMNL-rich supernatants were collected and contaminating red cells were lysed by 0.85% (wt/vol) NH4Cl for 7 min at room temperature followed by two washes with PBS. Contaminating platelets were separated by threefold low speed centrifugation of the final neutrophil preparation through 2% (wt/vol) BSA in PBS (30). Finally, PMNL were suspended in CaCl2/MgCl2-free PBS and stored at 4°C until further use. Viability of the final PMNL preparation usually was better than 97% by using the trypan blue dye exclusion test. Eosinophil contamination did not exceed 5% of the final granulocyte preparation. PMNL preparations with higher eosinophil contamination were not used in these experiments.

NAP/IL-8 Assay. NAP/IL-8 was detected in HPLC column fractions by two different methods as recently described in detail (13). When high amounts of NAP-IL-8 were expected an PMNL enzyme release assay was used. Briefly, PMNL (10⁷ cells/ml PBS) were preincubated with cytochalasin B (5 μg/ml, Sigma Chemical Co., Munich, FRG) and thereafter stimulated with column fractions at appropriate dilutions in complete PBS (cPBS; PBS containing 0.1% [wt/vol] BSA, 0.9 mM CaCl₂, and 0.49 mM MgCl₂) for 30 min. In some cases fractions were lyophilized before the degranulation assay. After centrifugation, cell-free supernatants were tested for myeloperoxidase release as described (13). When low amounts of NAP/IL-8 were expected, as well as for determination of chemotactic lipids, an indirect PMNL chemotaxis assay was used as described (13).

Production of NAP/IL-8. A mixture of human mononuclear cells obtained after Ficoll medium separation of venous blood anticoagulated with 1 mM EDTA as described (13) was incubated with LPS (Salmonella Minnesota RE 595, Calbiochem-Behring Corp., Marburg, FRG; 1 μg/ml) together with PHA (10 μg/ml) in RPMI containing 20 mM Heps (cell density: 5 x 10⁶ cells/ml) using plastic tissue culture flasks (Falcon Labware, Oxnard, CA) in
a humidified atmosphere containing 5% CO₂ at 37°C. After 40–48 h of incubation, conditioned media were collected and frozen below −70°C until further use.

**NAP/IL-8 Purification.** NAP/IL-8 was purified to homogeneity as recently described (13). Briefly, supernatants of LPS/PHA-stimulated mononuclear cells were acidified, and after centrifugation the supernatants were chromatographed on a G-75-Sephadex gel column (Pharmacia Fine Chemicals). NAP/IL-8 eluting in the 5–20-kD area was further purified by sequentially performed wide pore RP-8-HPLC (Zorbax PEP-RP-1; Dupont, Bad Nauheim, FRG), TSK-2000 3 SW-HPLC (LKB, Bromma, Sweden; 0.8 × 60 cm), and finally, narrow pore RP-18-HPLC (Nucleosil, 5 μm-octadecyl silica-column; Macherey-Nagel, Düren, FRG), as described (13). Purity of NAP/IL-8 was ascertained by SDS-PAGE under nonreducing conditions in the presence of 6 M urea using a recently optimized method for detection of polypeptides (31). NAP/IL-8 preparations were free of endotoxin as revealed by the Limulus lysate assay (Sigma Chemical Co.).

In experiments where PMNL-5-lipoxygenase stimulating activity should be determined in crude supernatants of stimulated mononuclear cells, these first were chromatographed on a G-75-gel column, as described before, followed by a preparative wide pore RP-8-HPLC separation. Biologically active G-75-gel column fractions (5–20-kD pool) were applied directly to a preparative wide pore RP-8-HPLC column (300-7C8 Nucleosil, 250 × 12.6 mm; Macherey-Nagel) and eluted from the column using a gradient of increasing concentrations of acetonitrile in 0.1% TFA. During the HPLC separation of peptides absorbance was monitored at 215 nm. Integration values obtained by the peak integrator (Spectra Physics SP 4270) were used to determine the amounts of peptides and proteins eluting in a given peak. To convert integration units to protein concentrations, known amounts of ubiquitine (Sigma Chemical Co.) were used for calibration. Nearly 10⁶ integration units corresponded to 1 μg ubiquitine.

RP-8-HPLC-purified, PMNL-5-LO-stimulating peptides were further purified by wide pore CN-propyl-RP-HPLC (5 μm, 250 × 4.6 mm; J. T. Baker Chemical Co., Gross Gerau, FRG). Peptides eluted with a gradient of increasing concentrations of n-propanol containing 0.1% TFA. If necessary, fractions containing PMNL-5-LO-stimulating activity off CN-propyl-RP-HPLC were finally purified with a narrow pore RP-18-HPLC column using a gradient of increasing concentrations of acetonitrile as described (13).

**Activation of PMNL-5-Lipoxygenase.** Purified neutrophils (10⁷ cells) suspended in 1 ml PBS (without CaCl₂ and MgCl₂) were preincubated with exogenously added arachidonic acid at the desired concentration (prepared by solubilization of an aliquot of an arachidonic acid stock [1 mg/ml ethanol] in 20 μl 0.1 mol/l NaHCO₃ solution after blowing up the ethanol with argon and dilution with 0.5 ml PBS) for 5 min at 37°C. Thereafter, 40 μl of a stock of CaCl₂ (90 mmol/liter) and MgCl₂ (49 mmol/liter) followed by a prewarmed (37°C) solution of NAP/IL-8 in PBS was added. NAP/IL-8 stored as a stock solution (100 μg/ml) in acetonitrile containing 0.1% TFA below −70°C, was freshly prepared by lyophilization and solubilization of the residue in PBS just before experiments were started. Cells were incubated for the desired time at 37°C and the vials thereafter were cooled to 0°C in an ice-water bath. After centrifugation at 4°C, supernatants were collected and 100 ng PGB₂ (Sigma Chemical Co.) in 10 μl methanol, as well as the radical scavenger 4-hydroxy-2,2,6,6-tetramethyl-piperidinoxy free radical (2 μg) (Sigma), was added. Samples were stored below −70°C until HPLC analysis of eicosanoids.

**RP-HPLC Quantitation of Nonradioactive Eicosanoids.** RP-HPLC analysis of the supernatants was performed with a C₁₈ Nucleosil-ODS 2-HPLC-column (4.6 × 250 mm) (Bischoff, Leonberg, FRG). Supernatants were extracted by the use of a precolumn cartridge (RP-18-silica) similar to the method described by Powell (32) by pressing the total volume of the supernatant (~2 ml) containing 10% (vol/vol) methanol through the precolumn, which previously was equilibrated with 0.1% (wt/vol) ammoniumphosphate, pH 4.5 (solvent A), followed by washing the precolumn with 1 ml solvent A and then 2 ml solvent A, containing 40% (vol/vol) HPLC grade methanol (solvent B).

The HPLC-column was equilibrated with 50% A and 50% B and the lipids focused onto
the precolumn eluted by a gradient of increasing amounts of methanol with the following protocol: time 0 min: 50% A, 50% B; time 6 min: 60% A, 40% B; time 12 min: 60% A, 40% B; time 28 min: 70% A, 30% B; time 50 min: 70% A, 30% B; time 52 min: 100% A; time 65 min: 100% A. A flow rate of 1.0 ml/min at 50°C was maintained and the effluent was monitored at 270 nm (leukotrienes) or 237 nm (monohydroxy acids).

Peak areas were integrated using a Spectra Physics computing integrator (SP 4270) and the amounts of leukotrienes were calculated by calibration of the column with known amounts of authentic samples of 20-COOH-LTB₄ (Paesel, Frankfurt, FRG), 20-OH-LTB₄ (Paesel), LTB₄ (Paesel), and 5-HETE (a generous gift of Dr. Bartmann, Hoechst-AG, Frankfurt, FRG). All values were corrected for recovery by the use of the internal standard PGB₂.

Experiments with the Use of [³H]AA. In some cases PMNL-5-LO activation experiments were performed in the presence of [³H]AA (Amersham, Braunschweig, FRG). [³H]AA (specific activity, 201.5 Ci/mmol) dissolved in ethanol (250 μCi/ml) was treated with 10 μl 0.1 mol/liter NaHCO₃, pH 9, and the ethanol was blown up under a stream of argon. The residue was dissolved in PBS and the [³H]AA solution was immediately used thereafter at desired concentrations.

Some experiments were performed identically to that described under "Activation of PMNL-5 Lipoxygenase," whereby 2.5 μCi [³H]AA was added just before CaCl₂/MgCl₂ was given to the cell suspension (10⁷ PMNL) to minimize incorporation of [³H]AA into the cellular membrane. In other experiments, 10⁷ PMNL were incubated with 5 μCi [³H]AA in 2 ml of PBS containing 0.9 mmol/liter CaCl₂ and 0.5 mmol/liter MgCl₂ for 1 h at 37°C. Thereafter cells were washed three times with PBS and suspended in 1 ml PBS. To 10⁷ prelabeled and prewarmed (37°C) PMNL in 1 ml PBS, 40 μl CaCl₂ (90 mmol/l) containing 50 mmol/liter MgCl₂ was added followed by 0.5 ml of the stimulus in PBS or PBS alone with subsequent incubation for 30 min at 37°C. After centrifugation supernatants were frozen below ~70°C under argon or applied directly to RP-HPLC. Radioactivity was monitored by on-line counting, whereby the column eluate was mixed with liquid scintillant (2 ml/min; Rialuma; J. T. Baker Chemical Co.) and counted in a 1 ml flow cell.

Results

Detection of a PMNL-5-Lipoxygenase-activating Factor in LPS/PHA-stimulated Mononuclear Cell Supernatants. When human PMNL were stimulated with crude supernatants of LPS/PHA-stimulated mononuclear cells in the presence of 5 × 10⁻⁶ mol/liter exogenous AA, significant amounts of 20-COOH-LTB₄, 20-OH-LTB₄, both trans-isomers of LTB₄, as well as LTB₄, were detectable in supernatants analyzed by RP-HPLC (data not shown). When partially purified (G-75-gel filtration) mononuclear cell supernatants were applied to a preparative wide pore RP-8-HPLC column, LTB₄ synthesis-inducing activity eluted in a single fraction (Fig. 1), corresponding to a UV peak absorbing at 215 nm. This fraction also contained potent PMNL chemotactic activity (data not shown). IL-1, which is also present in supernatants of LPS-stimulated mononuclear cells, eluted several minutes later than PMNL chemotactic activity (Fig. 1). In these fractions as well as in others no significant LTB₄ synthesis-inducing activity could be detected, even when 200-μl aliquots of fractions were tested.

Evidence that NAP/IL-8 Is a PMNL-5-Lipoxygenase Activator. Fractions containing 5-LO-stimulating activity were purified to homogeneity by additional CN-propyl-HPLC off RP-8-HPLC-purified material (Fig. 2). Major activity eluted in a peak absorbing at 215 nm, which demonstrates an intense line at 10 kD upon SDS-PAGE with an additional faint band from a 5-kD trace contaminant (Fig. 2). The SDS-PAGE profile was indistinguishable from that obtained from authentic NAP/IL-8 that has been purified to homogeneity. We also have detected the minor contaminant
Figure 1. Preparative wide pore RP-8-HPLC-profile of G-75-gel chromatography purified supernatants of LPS/PHA-stimulated human mononuclear cells. Supernatants of human mononuclear cells (4 × 10^8 cells containing 30% monocytes) were partially purified on a G-75-gel column and biologically active fractions (5-20 kD area) were separated on a preparative wide pore RP-8-HPLC column using the program indicated in Materials and Methods. Elution of the peptides was monitored at 215 nm. 20 μl aliquots of each fraction were tested for LTB4 production in PMNL using the protocol described in Materials and Methods. The total amount of LTB4 produced by 10^7 PMNL (sum of LTB4, 20-OH-LTB4, and 20-COOH-LTB4) within 10 min incubation is indicated (shaded bars). Note the appearance of a single LTB4 synthesis-inducing peak and the absence of such activity in fractions containing IL-1 (the elution position of authentic IL-1β is indicated by the arrow).

Representing possibly a shorter fragment of NAP/IL-8 in NAP/IL-8 preparations purified by NAP/IL-8 affinity chromatography followed by RP-18-HPLC (Schröder, J.-M., and M. Sticherling, unpublished results).

When human PMNL were exposed to 6 × 10^{-8} mol/liter NAP/IL-8 in the presence of 8 × 10^{-6} mol/liter AA, several intense UV-absorbing peaks were detected, as shown in a typical chromatogram (Fig. 3 A). In the absence of NAP/IL-8, nearly no UV-absorbing peaks could be seen in the leukotriene/HETE area (Fig. 3 B) when PMNL were incubated with 8 × 10^{-6} mol/liter AA only.

Peaks absorbing at 270 nm were identified to be 20-COOH-LTB4 (10.9 min), 20-OH-LTB4 (12.7 min), trans-LTB4 (21.1 min), epi-trans-LTB4 (21.8 min), and LTB4 (22.9 min). A peak absorbing at 237 nm was identified to be 5-HETE (35.5 min). This was proven by further experiments with authentic samples that were applied to the same column. These eluted from the column exactly at the same retention
time and demonstrated a single peak when co-injected with the expected eicosanooids either upon RP-HPLC or SP-HPLC (data not shown). Furthermore, all 270-nm absorbing peaks were collected, separately pooled, and analyzed for their UV spectrum in a double-beam spectrophotometer. The presence of triple absorption peaks near 260, 270, and 280 nm for fractions eluting at 10.9, 12.7, 21.1, 21.8, and 22.9 min, revealed that all these fractions contain material with a conjugated triene structure. The peak eluting at 35.5 min demonstrated a UV maximum at 237 nm, which is typical for a conjugated cis, trans-diene as 5-HETE is too. In addition, fractions were lyophilized and analyzed for PMNL chemotactic activity using the Boyden chamber method at final leukotriene concentrations calculated by UV absorbance at 270 nm to be between 10 and 0.1 ng/ml. Half-maximal chemotactic response has been found at 0.4 ng/ml for the peak eluting at 22.9 min, which fits well with the ED50 found in our laboratory for authentic LTB4.

The presence of chemotactic activity in fractions corresponding to the retention times of authentic LTB4 and 20-OH-LTB4 at concentrations known to be active in authentic material gives additional evidence that these both major metabolites are indeed 20-OH-LTB4 and LTB4 and not 5-(S),12(S)-DiHETE, or its metabolites, which are known to lack PMNL chemotactic activity. Moreover, straight phase HPLC of pooled "LTB4"-peaks, obtained after RP-HPLC of NAP/IL-8-stimulated PMN, revealed retention time identical to that of authentic LTB4, and only traces of 5(S), 12(R)-DiHETE (data not shown).
FIGURE 3. RP-HPLC analysis of the monoHETE and diHETE derivatives in supernatant fluids isolated from 10^7 PMNL. (A) PMNL were treated with 6 x 10^{-8} mol/liter NAP/IL-8 for 15 min at 37°C in the presence of 8 x 10^{-6} mol/liter exogenous AA. AA metabolites were monitored by UV absorption at 270 nm (diHETEs) and 237 nm (monoHETEs). Compounds I-VII were identified to be 20-COOH-LTB4 (I), 20-OH-LTB4 (II), Δ²-trans-LTB4 (III), 12-epi-Δ²-trans-LTB4 (IV), LTB4 (V); and 5-HETE (VI). Shaded bars indicate PMNL chemotactic indices of an aliquot of peak fractions at 1:100 dilution, except 5-HETE, which was tested at 1:2 dilution as described in Materials and Methods. The chemotactic index of authentic LTB4 at 1 μg/ml has been found to be 3.8 (buffer control, 1.0) for PMNL. (B) PMNL were treated with 8 x 10^{-6} mol/liter AA for 5 min. at 37°C. Note the attenuation as in A. Determination of PMNL chemotactic activity in column fractions identical to that collected in A at the same dilution did not reveal any significant chemotactic activity (data not shown). (C) PMNL were exposed to 6 x 10^{-6} mol/liter NAP/IL-8 in the presence of 6 x 10^{-7} mol/liter AA containing [³H]AA (specific activity in this experiment, 3 Ci/mmol).

Supernatant fluids were separated by RP-HPLC and analyzed for radioactivity as described in Materials and Methods. Note the presence of radioactive peaks at elution time nearly identical to that of UV-absorbing peaks in A. VII indicates the elution time of [³H]AA.

In other experiments PMNL were stimulated with NAP/IL-8 in the presence of 6 x 10^{-7} mol/liter [³H]AA (specific activity in these experiments: 3 Ci/mol). Now radioactive peaks were detectable in the position of authentic LTB4, 20-OH-LTB4, and 5-HETE (Fig. 3 C), confirming the results shown in Fig. 3 A that these peaks indeed derive from AA metabolites.

Dose Response of NAP/IL-8-induced LTB4 Production. When 10^7 PMN were stimulated with different doses of NAP/IL-8 in the presence of 8.2 x 10^{-6} mol/liter exogenous AA for 5 min, a dose-dependent increase of LTB4 production was observable (Fig. 4). Maximal total LTB4 production (sum of 20-COOH-LTB4, 20-OH-LTB4, and LTB4) was observed at NAP/IL-8 concentrations >6 x 10^{-8} mol/liter. Half-maximal release occurred at 3.6 x 10^{-8} mol/liter. When 10^7 PMNL were incubated with 10^{-6} mol/liter FMLP as control stimulus instead of NAP/IL-8, in the presence of 8.2 x 10^{-6} mol/liter exogenous AA, 162 ± 63 ng total pro-
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FIGURE 4. Appearance of metabolites in supernatant fluids of PMNL stimulated with different NAP/IL-8 concentrations. 10⁷ PMNL were incubated in the presence of 8 × 10⁻⁶ mol/liter AA with different concentrations of NAP/IL-8 for 5 min at 37°C. Supernatants were analyzed by RP-HPLC for AA metabolites. Amounts of 20-COOH-LTB₄ (X), 20-OH-LTB₄ (O), LTB₄ (Δ), as well as the total amount of LTB₄ produced (sum of 20-COOH-LTB₄, 20-OH-LTB₄, and LTB₄) (●) were estimated via determination of the peak integral as described in Materials and Methods. When NAP/IL-8 was used at concentrations below 3 × 10⁻⁹ mol/liter LTB₄ and 20-OH-LTB₄ were estimated via the number of half-maximal effective doses to elicit PMNL chemotaxis in putative 20-OH-LTB₄- or LTB₄-containing fractions as described in Materials and Methods. Results of five experiments ± SD are shown.

Reduced LTB₄ was detected (n = 11). NAP/IL-8–induced LTB₄ production, therefore, is in the same order as that found with FMLP as stimulus.

Time Course of the AA Metabolite Profile from NAP/IL-8–stimulated PMNL. 5-Lipoxygenase activation in NAP/IL-8–stimulated PMNL occurs very rapidly. Fig. 5 shows that already 1 min after challenge with NAP/IL-8 one of the major arachidonate metabolites is LTB₄. Also, both isomers, trans-LTB₄ as well as epi-trans-LTB₄, are detectable, however, at low amounts. At that time nearly no 20-COOH-LTB₄ is observable; however, larger amounts of 20-OH-LTB₄ are present. LTB₄ declines very rapidly and is nearly absent after 40 min of incubation of the cells with NAP/IL-8. Maximal total LTB₄ production (sum of 20-COOH-LTB₄, 20-OH-LTB₄, and LTB₄) occurred 40 min after stimulation with NAP/IL-8. An additional finding is the detection of larger amounts of 5-HETE only within 10 min after challenge, with a rapid decline thereafter. After a 40-min stimulation with NAP/IL-8, no UV-absorbing peak at the position of 5-HETE is observable (Fig. 5).

Availability of Exogenous AA for Leukotriene Production by NAP/IL-8–stimulated PMNL. The detection of 5-lipoxygenase products in NAP/IL-8–stimulated PMNL is dependent upon the presence of exogenous AA (Fig. 6). When the total amount of produced LTB₄ is calculated by summing up the amounts of produced 20-COOH-LTB₄, 20-OH-LTB₄, and LTB₄ for determination of half-maximal total
Figure 5. Appearance of AA metabolites in supernatants of NAP/IL-8-stimulated PMNL as a function of reaction time. 10^7 PMNL were incubated with 6 x 10^-8 mol/liter NAP/IL-8 in the presence of 8 x 10^-6 mol/liter AA for various time periods (0–40 min). Supernatants were separated by RP-HPLC and the amounts of diHETE derivatives (20-COOH-LTB4 (□), 20-OH-LTB4 (△), LTB4 (△), sum of trans-LTB4 isomers (○), 5-HETE (●), and the total amount of LTB4 produced (sum of 20-COOH-LTB4, 20-OH-LTB4, and LTB4) (X) were estimated as described in Materials and Methods. Results of a typical experiment out of four experiments are shown.

Figure 6. Production of AA metabolites by NAP/IL-8-stimulated PMNL as a function of AA concentration. 10^7 PMNL were preincubated with various amounts of AA (3 x 10^-7 to 8 x 10^-6 mol/liter) followed by stimulation with 6 x 10^-8 mol/liter NAP/IL-8 for 5 min at 37°C. Supernatants were analyzed by RP-HPLC and the amounts of 20-COOH-LTB4 (△), 20-OH-LTB4 (○), LTB4 (△), and the total produced amounts of LTB4 (●) were estimated as described in Materials and Methods. At concentrations of AA below 8 x 10^-7 mol/liter the amounts of LTB4 and 20-OH-LTB4 produced were estimated via the number of ED50 doses in the chemotaxis assay as described in Fig. 5 and Materials and Methods. The mean of five experiments ± SD is shown.
LTB₄ release, a concentration nearly $3 \times 10^{-6}$ mol/liter exogenous AA is necessary (Fig. 6). When single metabolites were analyzed it appeared that with decreasing amounts of exogenous AA the ratio of 20-COOH-LTB₄/20-OH-LTB₄/LTB₄ increases. Using exogenous AA at concentrations below $8 \times 10^{-7}$ mol/liter UV-absorbing peaks at the positions of leukotrienes and 5-HETE became so small that UV detection alone is not sufficient for identification of eicosanoids. In these experiments fractions corresponding to 20-OH-LTB₄ as well as LTB₄ were collected, lyophilized, and analyzed for the number of ED₅₀ doses by using the Boyden chamber chemotaxis assay. In the system used, the ED₅₀ for LTB₄ and 20-OH-LTB₄ corresponded to $1.3 \times 10^{-9}$ mol/liter and $9.5 \times 10^{-9}$ mol/liter, respectively. Significant amounts of PMNL chemotactic lipids eluting at the position of authentic 20-OH-LTB₄ and LTB₄ were detected when cells were stimulated with NAP-IL-8 using AA concentrations higher than $3 \times 10^{-7}$ mol/liter.

Further support was obtained by the use of $^3$H-labeled AA in these experiments. Using a radioactivity detector after HPLC separation of eicosanoids, radioactivity peaks could be detected at the position of 20-COOH-LTB₄, 20-OH-LTB₄, LTB₄, and 5-HETE only when exogenous AA at concentrations higher than $2 \times 10^{-7}$ mol/liter (Fig. 3 C) was present. When $10^7$ PMNL were stimulated with NAP/IL-8 in the presence of $2.5 \mu$Ci $[^3]H]$AA corresponding to $6.2 \times 10^{-5}$ mol/liters AA, no differences were observed in the buffer control in the radioactivity profile after HPLC separation of incubation supernatants (Fig. 7, A and B). Similar results were ob-

**Figure 7.** Stimulation of PMNL with NAP/IL-8 in the presence of $[^3]H]$AA. (A) $10^7$ PMNL were incubated with $6 \times 10^{-6}$ mol/liter NAP/IL-8 in the presence of $2.5 \mu$Ci $[^3]H]$AA (specific activity, 212 Ci/nmol) for 10 min at 37°C. Supernatants were separated by RP-HPLC as described in Materials and Methods and radioactivity was monitored by the use of on-line radiodetection with continuous mixing with scintilator fluid. Radioactivity eluting from the column is shown as a function of retention time. Arrows indicate elution position of authentic $[^3]H]$AA metabolites ($[^3]H]-20-COOH-LTB₄$ (I), $[^3]H]-20-OH-LTB₄$ (II), $[^3]H]-LTB₄$ (III), $[^3]H]-5-HETE$ (IV)). (B) Shows the control experiment, where instead of NAP/IL-8 PBS was used. In both cases a representative run out of six experiments is shown.
tained when PMNL pretreated with 5 μg/ml cytochalasin B were used (data not shown). The major peaks seen in the radiochromatograms that represent only 0.1% conversion of [3H]AA may be attributed to 12-HETE and 15-HETE, possibly in part produced by some contaminating platelets or eosinophils, or may have been nonenzymatically formed by autoxidation of [3H]AA during the incubation period.

**Influence of NAP/IL-8 Stimulation upon AA Release by PMNL.** PMNL were incubated with 5 μCi [3H]AA for an optimal time period of 1 h and (after washing) were then stimulated for 5 min with 6 × 10⁻⁸ mol/liter NAP/IL-8 before HPLC separation with on-line radioactivity monitoring. The results of such experiments, shown in Fig. 8A, indicate that compared with the control (Fig. 8B), no significant amounts of free [3H]AA or 5-lipoxygenase products were released after stimulation with NAP/IL-8. Using the calcium ionophore A23187 as a control stimulus the typical profile of AA-metabolites including [3H]AA itself are observable (Fig. 8C).

**FIGURE 8.** Influence of NAP/IL-8-activation upon release of cellular [3H]AA and its metabolites in PMNL. 10⁷ PMNL were preincubated with 5μCi [3H]AA (specific activity, 212 Ci/mmol) for 1 h at 37°C. Cells were washed and stimulated thereafter either with 6 × 10⁻⁸ mol/liter NAP/IL-8, buffer, or calcium ionophore A 23187 (8 × 10⁻⁶ mol/liter) in PBS containing CaCl₂ and MgCl₂. Supernatants were separated by RP-HPLC and analyzed for [3H]AA and its metabolites with on-line radiodetection as described in Materials and Methods. (A) Experiment with the use of NAP/IL-8; (B) the buffer control; and (C) shows as a positive control the influence of Ca-ionophore A 23187 stimulation upon release of cellular [3H]AA and its metabolism.
INTERLEUKIN 8-INDUCED LEUKOTRIENE B4 PRODUCTION

Discussion

Neutrophil chemotactic factors like C5a, FMLP, or PAF have been shown to activate the PMNL-AA-5-lipoxygenase (25-27, 33). Whereas the major product has been identified as the 20-OH-metabolite of LTB4, it has been possible with a short incubation period to identify both LTB4 and 5-HETE as the main lipoxygenase products (26). Our data demonstrate that NAP/IL-8, similar to other PMNL-chemotactic factors, is also able to activate the PMNL-AA-5-lipoxygenase with the formation of LTB4, 20-OH-LTB4, and 20-COOH-LTB4 as its main products at NAP/IL-8 concentrations that elicit PMNL chemotaxis and the release of primary granule constituents (13, 24). The ratio between these lipoxygenase products depends upon the incubation time (Fig. 5) due to rapid ω-oxidation of initially produced LTB4 by PMNL-LTB4 20-hydroxylase (34, 35). In addition to LTB4 and its metabolites, both trans-isomers could be detected at low amounts. This supports the suggestion that unstable LTA4 is formed by NAP/IL-8 activation of PMNL. The latter is known to hydrolyze nonenzymatically into biologically inactive trans-isomers of LTB4 (36).

5-HETE, known as a product of 5-lipoxygenase activation, is detectable in larger amounts only within 10 min of incubation. I and others have obtained similar results using other more physiological stimuli for cellular activation, such as C5a, FMLP, or PAF (25-27). The absence of 5-HETE after a 20-min incubation might be the result of incorporation into the cellular membrane (37) or formation of its δ-lactone (38).

The total amounts of LTB4 produced by NAP/IL-8 stimulation of PMNL in the presence of 8.2 × 10⁻⁶ mol/liter exogenous AA are lower than those seen with the so far most powerful stimulus, calcium ionophore. In fact, NAP/IL-8 virtually does not induce synthesis of LTB4 in the absence of or in the presence of very low concentrations (<10⁻⁷ mol/liter) of exogenous AA. Therefore, substrate availability appears to be a limiting factor for leukotriene production by NAP/IL-8 stimulation of PMNL. These results are in accordance with previous reports where only in the presence of exogenous AA could LTB4 be detected after stimulation with PMNL chemotactic factors (25, 39, 40).

The amount of exogenously added AA required before LTB4 synthesis commenced has been found to be a point of controversy. Apparently, excessive amounts of exogenous AA (30–250 μM) are necessary in order to obtain significant production of LTB4 or its metabolites following PMNL stimulation with chemotactic factors (25, 26, 40). Moreover, it has been demonstrated that AA itself is able to activate PMNL-5-LO at high concentrations when given in ethanolic solution (41), possibly due to membrane perturbation, increased trans-membrane penetration of AA (42), or induction of additional cellular AA release by the vehicle (43).

In our studies we have avoided the use of ethanol. Also, instead of albumin as a vehicle (25), which could bind larger amounts of AA and therefore might decrease the concentration of freely available substrate AA, we used sodium arachidonate. In addition it was found that chemotaxin-stimulated neutrophils purified by the use of Ficoll or Percoll can produce substantially lower amounts of 5-LO metabolites (unpublished results). Using a gelatin sedimentation technique for isolation of cells, PMNL of normal volunteers stimulated with 10⁻⁵ mol/liter FMLP in the presence of 8.2 × 10⁻⁶ mol/liter AA produced 162 ± 63 ng total LTB4 (sum of LTB4, 20-OH-LTB4, and 20-COOH-LTB4) per 10⁷ cells within 5 min of incubation. This is
far above the amounts previously seen and may be attributed to the different stimulation conditions used in our experiments.

Results obtained from experiments where PMNL were prelabeled with [3H]AA indicate that NAP/IL-8 does not stimulate the release of membrane bound [3H]AA, even at high concentrations of NAP/IL-8 (1.5 \times 10^{-7} \text{ mol/liter}). These findings are somewhat different from those seen with the use of FMLP as stimulus. Recent studies with [3H]AA prelabeled PMNL have shown that 10^{-7} \text{ mol/liter} FMLP is able to release [3H]DiHETEs when cells were preincubated with cytochalasin B (33), which is known to enhance the production of LTB$_4$ up to fourfold, possibly by inhibiting reacylation of AA into the cellular membrane (26).

We also have studied the effect of a cytochalasin B pretreatment of PMNL upon the NAP-IL-8-induced release of leukotrienes and did not observe any significant differences to experiments performed in the absence of cytochalasin B even when NAP/IL-8 at 8 \times 10^{-8} \text{ mol/liter} was used (data not shown).

It appears that maximal LTB$_4$ formation by PMNL in the absence of exogenously added AA can only be achieved when the stimulus is a powerful activator of cellular AA release and, in addition, an activator of the AA-5-lipoxygenase like the (non-physiological) calcium ionophore A23187.

Recent observations indicate that several monokines including TNF enhance the leukotriene generation in neutrophils stimulated with calcium ionophore A23187 (44, 45). More recent reports indicate that granulocyte/macrophage CSF (GM-CSF) has a dramatic effect upon FMLP- or C5a-induced lipid metabolism stimulating the release of substantial amounts of cellular arachidonic acid and LTB$_4$ (41, 46). It would be interesting to know whether also the NAP/IL-8-induced leukotriene production in the absence of exogenous added AA is inducible by GM-CSF priming of PMNL. This could be an explanation for a very recent finding indicating that partially purified IL-1 preparations obtained from LPS-stimulated mononuclear cells, which may contain both NAP/IL-8 and GM-CSF, stimulate LTB$_4$ production in the absence of exogenous AA in PMNL (47).

Furthermore, the suggestion that IL-1 itself is able to stimulate LTB$_4$ production and as stated recently (47), is not supported by our own findings: when supernatants of LPS-stimulated mononuclear cells were separated by preparative reversed-phase HPLC no 5-lipoxygenase products were observed in supernatants of PMNL stimulated with fractions containing IL-1 or with the use of human (h)IL-1$\alpha$ in the presence of exogenous AA. These findings were supported by recent investigations where neither hrIL-1$\alpha$ or hrIL-1$\beta$ were found to be able to activate PMNL function (48, 49). It was concluded that there should exist a neutrophil-activating factor (NAF) present in partially purified IL-1 preparations, which should be responsible for leukotriene production and release of elastase in PMNL (49). The most likely explanation would be that NAP/IL-8 is the cytokine responsible for the effect seen in partially purified IL-1 preparations. NAP/IL-8 is known to elicit release of PMNL primary granule constituents like $\beta$-glucuronidase, myeloperoxidase, or elastase in cytochalasin B–pretreated PMNL (13, 15, 24).

The observation that NAP/IL-8 is able to activate the neutrophil 5-lipoxygenase might be of biological importance: in psoriasis, a noninfectious skin disease with moderate to severe accumulation of neutrophils in the epidermis, LTB$_4$-like material has been found to be present in elevated amounts (50). In various investigations
we were able to show that the major part of neutrophil chemotactic activity in aqueous extracts of lesional psoriatic scales is due to a family of at least seven homologous polypeptides called anionic neutrophil-activating peptide (ANAP) (51), whereby one of the main components, termed $\beta_2$-ANAP, is by NH$_2$-terminal sequencing, identical with NAP/IL-8 (52). Therefore, it is intriguing to speculate that NAP/IL-8 could be a physiologically relevant activator of PMN-5-lipoxygenase when NAP/IL-8, together with exogenous AA, is present in large amounts in diseased tissue such as in psoriatic lesions (52, 53).

In summary, we found that the novel host cell-derived cytokine NAP/IL-8 is able to activate the neutrophil 5-lipoxygenase, similar to other clearly characterized neutrophil chemotactic factors. A limiting step for induction of leukotriene synthesis by NAP/IL-8 in PMNL appears to be the availability of exogenous AA. The lipid mediators LTB$_4$ and 20-OH-LTB$_4$, which both are known to be potent neutrophil chemotactic factors, appear to be of importance in amplifying inflammatory reactions. The in vivo formation of these lipids is regulated by activators of the cellular 5-lipoxygenase and the availability of its substrate AA. Our results document a possible central role of NAP/IL-8 as a proinflammatory mediator.

Summary

LPS and mitogen-stimulated mononuclear cells secrete a cytokine, which is able to activate the PMNL-arachidonate-5-lipoxygenase. This cytokine has been proven to be identical with the recently characterized novel neutrophil-activating peptide NAP/IL-8. NAP/IL-8 is able to activate human PMNL for release of LTB$_4$, $\omega$-oxidized LTB$_4$, and 5-HETE in the presence of exogenous AA. Half-maximal concentration of NAP/IL-8 for release of LTB$_4$ has been found to be near $4 \times 10^{-8}$ mol/liter. Time course studies revealed rapid activation of PMNL, with maximal release of LTB$_4$ within the first 10 min with a decline up to 40 min. High amounts of $\omega$-oxidized LTB$_4$ were detected up to that time. Significant amounts of AA-5-LO-products can be detected only when PMNL were stimulated with NAP/IL-8 in the presence of exogenous AA. The concentration of AA necessary for half-maximal LTB$_4$ release has been found to be $3 \times 10^{-6}$ mol/liter. In the presence of $8 \times 10^{-9}$ mol/liter [$^3$H]AA, NAP/IL-8 ($10^{-9}$ to $10^{-7}$ mol/liter) did not induce the production of LTB$_4$, $\omega$-oxidized LTB$_4$, or 5-HETE. In addition, PMNL prelabeled with [$^3$H]AA did not release either [$^3$H]AA or 5-lipoxygenase metabolites when stimulated with NAP/IL-8 ($10^{-9}$ to $10^{-7}$ mol/liter), indicating that NAP/IL-8 apparently does not activate cellular phospholipases/diacylglycerol-lipases. Apart from FMLP, C5a, and PAF NAP/IL-8 is the fourth clearly characterized neutrophil chemotaxin able to activate the PMNL-5-lipoxygenase. The detection of large amounts of NAP/IL-8, arachidonic acid, as well as LTB$_4$-like material, in lesional material of patients with psoriasis points towards a possibly important role of NAP/IL-8 in amplifying inflammatory processes by induction of LTB$_4$-production.

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