JCI The Journal of Clinical Investigation

Altered responses of human macrophages to lipopolysaccharide by hydroperoxy eicosatetraenoic acid, hydroxy eicosatetraenoic acid, and arachidonic acid. Inhibition of tumor necrosis factor production.

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J Clin Invest. 1997;99(6):1445-1452. https://doi.org/10.1172/JCI119303.

Research Article

The regulation of allergic and autoimmune inflammatory reactions by polyunsaturated fatty acids and their metabolic products (eicosanoids) continues to be of major interest. Our data demonstrate that arachidonic acid 5,8,11,14-eicosatetraenoic acid (20:4n-6) and its hydroxylated derivatives 15(s)-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) and 15(s)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) regulate agonist-induced tumor necrosis factor alpha (TNF) production, a cytokine that plays a role in inflammatory diseases. Although 20:4n-6 and 15-HETE caused a reduction in production of TNF in mononuclear leukocytes stimulated with phytohaemagglutinin, pokeweed mitogen, concanavalin A, and Staphylococcus aureus, 15-HPETE was far more active. 15-HPETE was also found to dramatically depress the ability of bacterial lipopolysaccharide to induce TNF production in monocytes and the monocytic cell line Mono Mac 6. These fatty acids depressed the expression of TNF mRNA in Mono Mac 6 cells stimulated with LPS; 15-HPETE was fivefold more active than 20:4n-6 and 15-HETE. While 15-HPETE treatment neither affected LPS binding to Mono Mac 6 cells nor caused a decrease in CD14 expression, the fatty acid significantly reduced the LPS-induced translocation of PKC (translocation of alpha, betaI, betaII, and epsilon isozymes), suggesting that 15-HPETE acts by abrogating the early signal transduction events. The findings identify another molecule that could form the basis for development of antiinflammatory pharmaceuticals.

Altered Responses of Human Macrophages to Lipopolysaccharide by Hydroperoxy Eicosatetraenoic Acid, Hydroxy Eicosatetraenoic Acid, and Arachidonic Acid

Inhibition of Tumor Necrosis Factor Production

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Abstract

The regulation of allergic and autoimmune inflammatory reactions by polyunsaturated fatty acids and their metabolic products (eicosanoids) continues to be of major interest. Our data demonstrate that arachidonic acid 5,8,11,14-eicosatetraenoic acid (20:4n-6) and its hydroxylated derivatives 15(s)-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) and 15(s)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) regulate agonist-induced tumor necrosis factor alpha (TNF) production, a cytokine that plays a role in inflammatory diseases. Although 20:4n-6 and 15-HETE caused a reduction in production of TNF in mononuclear leukocytes stimulated with phytohaemagglutinin, pokeweed mitogen, concanavalin A, and *Staphylococcus aureus,* 15-HPETE was far more active. 15-HPETE was also found to dramatically depress the ability of bacterial lipopolysaccharide to induce TNF production in monocytes and the monocytic cell line Mono Mac 6. These fatty acids depressed the expression of TNF mRNA in Mono Mac 6 cells stimulated with LPS; 15-HPETE was fivefold more active than 20:4n-6 and 15-HETE. While 15-HPETE treatment neither affected LPS binding to Mono Mac 6 cells nor caused a decrease in CD14 expression, the fatty acid significantly reduced the LPS-induced translocation of PKC (translocation of α, βI, βII, and ϵ isozymes), suggesting that 15-HPETE acts by abrogating the early signal transduction events. The findings identify another molecule that could form the basis for development of antiinflammatory pharmaceuticals. (*J. Clin. Invest.* 1997. 99:1445–1452.) Key words: polyunsaturated fatty acids • mitogens • Mono Mac 6 cells • CD14 • protein kinase C

Introduction

Arachidonic acid and leukotrienes regulate phagocyte function. Arachidonic acid has been shown to stimulate the neutrophil respiratory burst (1–3), degranulation (4, 5), adhesion, and CD11b/CD18 expression (6) and prime neutrophils for increased responses to f-met-leu-phe and PMA (7). In addition, arachidonic acid stimulates the macrophage respiratory burst (8). Products of the metabolism of arachidonic acid such as leukotriene B_4 (LTB_4) and 5-oxo-6,8,11,14-(E,2,2,2)-eicosatetraenoic acid are also well established stimulators of phagocytic cells (9–13). Consequently, lipoxygenase products have been implicated as mediators of the allergic and autoimmune inflammatory reaction (12, 13). Thus, one major focus of pharmaceutical development has been the production of antiinflammatory agents, based on strategies to inhibit the generation of lipoxygenase products. Recently, the possibility that lipoxygenase-derived products can inhibit the inflammatory response has also been recognized, where 15(s)-hydroxy-5,8,11,13-eicosatetranoic acid (15-HETE)[1] was shown to cause inhibition of superoxide production and degranulation of neutrophils activated with f-met-leu-phe, platelet-activating factor, and LTB_4 (14), and to inhibit LTB_4-induced neutrophil transmigration across endothelium (15). Such molecules, together with the possibility of making synthetic derivatives with desirable properties, can often be considered as avenues to develop appropriate antiinflammatory agents for potential use in inhibiting allergic and autoimmune inflammation.

To further clarify the possible role of lipoxygenase products in the regulation of the inflammatory response, we examined the effects of 15-HETE and 15(s)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) on the production of tumor necrosis factor alpha (TNF), a proinflammatory cytokine that plays an important role in exacerbated inflammation and tissue damage. The results show that 15-HPETE is more active than either arachidonic acid or 15-HETE in inhibiting production of TNF and TNF mRNA by human macrophages stimulated by the major microbial product lipopolysaccharide. Evidence suggests that 15-HPETE inhibits the ability of LPS to induce TNF production at a postreceptor binding level involving inhibition of LPS-induced protein kinase C (PKC) translocation.

Methods

Preparation and culture of mononuclear leukocytes and monocytes. Peripheral blood mononuclear leukocytes (MNL) were prepared from the blood of healthy volunteers by centrifugation on hypaque-ficoll solution (16). The MNL were cultured in 96-well microtiter

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Received for publication 18 June 1996 and accepted in revised form 30 January 1997.

J. Clin. Invest.

0021-9738/97/03/1445/08 $2.00
Volume 99, Number 6, March 1997, 1445–1452

1. *Abbreviations used in this paper:* 15-HETE, 15(s)-hydroxy-5,8,11,13-eicosatetraenoic acid; 15-HPETE, 15(s)-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 20:4n-6, 5,8,11,14-eicosatetraenoic acid; LTB_4, leukotriene B_4; MNL, mononuclear leukocytes; PKC, protein kinase C; TNF, tumor necrosis factor alpha.

plates (U-bottom; Linbro, ICN-Flow, Sydney, Australia). To each well containing 100 μl of 2×10^6 MNL/ml was added 100 μl of either 1 μg/ml PHA, 25 μg/ml concanavalin A, 1/100 dilution of pokeweed mitogen (670-5360; Wellcome Pharmaceuticals and Reagents Pty. Ltd., Sydney, Australia; Calbiochem-Behring, Sydney, Australia; and Gibco Laboratories, Grand Island, NY, respectively) or 5×10^7 *S. aureus*/ml. The cultures were supplemented with 2.5% human group AB serum, and then incubated at 37°C in the presence of 5% CO_2-air and high humidity. The incubation times were varied and are as indicated in Results. After incubation, the cell-free supernatants were collected for determination of TNF.

Monocytes were purified from the MNL fraction by adherence to cytodex microcarriers (beads) as described previously (17). In brief, this involved a 2.5-h incubation of the MNL with the beads in medium 199 containing 10% heat-inactivated human AB serum. The nonadherent cells were discarded, the beads containing the adherent cells were washed three times to remove additional nonadherent cells, and then the adhered cells were released by vortex agitation for two periods of 1 min each. The monocytes of > 95% purity were cultured as described above for the MNL using *Escherichia coli* K235 LPS obtained from Sigma Chemical Co. (St. Louis, MO) as a stimulant for inducing TNF production.

Mono Mac 6 cells. Mono Mac 6 cells were kindly provided by Dr. H.W.L. Ziegler-Heitbrock (18). The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. All media and reagents were screened for LPS by use of the Limulus Amebocyte Lysate Assay.

Measurement of cytokines. TNF was measured as previously described (19) using ELISA. Monoclonal antibody to TNF alpha, rabbit anti–TNF polyclonal antisera, and human recombinant TNF (derived from *E. coli*) were kindly provided by Dr. G. Adolf (Ernst-Boehringer Institut, Vienna, Austria). These were used to set up quantitative ELISA for measuring cytokines in supernatants. The ELISA was conducted by a series of steps that involved coating of wells of microtiter plates with an immunoglobulin G fraction of goat anti–mouse immunoglobulin G (Cappel Laboratories, Malvern, PA). This was followed by the addition of the monoclonal antibody to TNF. Serial dilutions of the supernatants or a known amount of cytokine standard was added. The amount of cytokine bound was estimated by adding rabbit anti–TNF antisera followed by a sheep anti–rabbit immunoglobulin–horseradish peroxidase conjugate (Silenus, Melbourne, Australia), and then the substrate 2,2[1]-azino-di[3-ethylbenzthiazoline sulfonate-(6)] (Boehringer Mannheim, Sydney, Australia). After incubation at 37°C for 45 min, the A_{414} was measured in a Titertek Multiskan Spectrophotometer (ICN-Flow). The readings were related to a standard curve of human recombinant TNF for quantitating the cytokines.

Preparation of HPETE and HETE derivatives of 5,8,11,14-eicosatetraenoic acid. Arachidonic acid was purchased from Sigma Chemical Co. The 15-HETE and 15-HPETE were prepared as previously described (20) and outlined below. 15-HPETE was obtained by incubating 5,8,11,14-eicosatetraenoic acid (20:4n-6) (50 mg in 1 ml ethanol) with soybean lipoxidase (7 mg, type 1B; Sigma Chemical Co.) in 100 ml of 0.1 mol/liter sodium borate, pH 9.0, for 20 min at room temperature. The reaction mixture was acidified with 5 ml 1 mol/liter citric acid and fatty acid derivatives were extracted three times with 4 ml diethylether. The sample was evaporated under N_2 and taken up in 3 ml hexane/diethylether (9:1 vol/vol) before application to a silicic acid column (4 g) equilibrated in the same solvent. Unoxidized 20:4(n-6) was eluted with 120 ml of this solvent and 15-HPETE was eluted with 120 ml hexane/diethylether (1:1 vol/vol). A portion (5 mg) of 15-HPETE was reduced to 15-HETE with 2 mg sodium borohydride for 2 h at 4°C and recovered by the addition of 2.5 ml water, acidification with formic acid, and extraction three times with 4 ml of diethylether. Fatty acids and their derivatives (20:4n-6, 15-HPETE, and 15-HETE) were stored in chloroform at −20°C and purity checked by thin layer chromatography and where possible by gas-liquid chromatography. The HPETE and HETE derivatives consisted of at least 95% 15-HPETE and 15-HETE, respectively, with other isomers making up the rest. There was no evidence of 20:4n-6 in the preparations.

Solubilization of fatty acids. To overcome fatty acid insolubility in aqueous solution, we prepared mixed DPC–fatty acid micelles in HBSS by sonication as previously described (3). Briefly, the appropriate amounts of fatty acid and DPC, both dissolved in redistilled chloroform, were added to 10-ml glass tubes in a 1:4 ratio (by mass) and the solvent completely evaporated under nitrogen at 30°C. HBSS (1 ml) was then added and the mixture was sonicated for 2×1 min at 4°C (Ystrom Systems Ultrasonicator, Technic Inc., Westwood, NJ) (power setting, 8; tune setting, 4). Control incubations contained micelles of DPC alone appropriately diluted. This solubilization technique gave reproducible results. Fatty acids were checked for purity by silica gel thin-layer chromatography in diethylether/hexane/glacial acetic acid (60:40:1, vol/vol). The plates were visualized with iodine. There was no evidence of oxidation products of commercial fatty acids with storage or after solubilization. The mononuclear cells were exposed to the fatty acids for 30 min before the addition of the stimulus.

cDNA probes. The human TNF cDNA probe was an 820-bp EcoRI fragment cloned into a PUC vector obtained from Genentech Inc. (South San Francisco, CA). The G3PDH cDNA control probe was a 1.1-kb fragment obtained from Clontech (Palo Alto, CA).

RNA isolation and hybridization. Mono Mac 6 cells (10^6 in total volume of 1 ml) were induced as described in the figure legends. RNA for slot blots was isolated and prepared by the RNAZOL™ B method for isolation of RNA (Cinna/Biotecx, Friendswood, TX) (21). The cells were pelleted and lysed by resuspension in 0.4 ml of RNAZOL™ B/10^6 cells. To extract the RNA-phenol, 50 μl of chloroform was added, vigorously shaken for 15 s, and placed on ice for 5 min. The tubes were centrifuged at 12,000 *g* for 15 min at 4°C. The upper phase was carefully removed and the RNA precipitated by addition of an equal volume of isopropanol by incubation on ice for 15 min. The RNA was pelleted by centrifugation at 12,000 *g* for 15 min at 4°C.

For preparing a series of threefold dilutions, the RNA samples were diluted in a diluent solution containing formaldehyde, saline sodium citrate (Ajax Chemicals, Sydney, Australia), SDS (ICN, Sydney, Australia), and diethylpyrocarbonate (DEPC, Sigma Chemical Co.) and heated at 65°C for 5 min. The samples were applied to a Gene Screen Plus nylon membrane using a slot blot vacuum manifold (Hoefer Scientific Instruments, San Francisco, CA). The filters were baked for 2 h at 80°C, and then prehybridization was performed for 16–24 h in a solution containing formamide, dextran-sulphate, SDS, SSC, Denhardt's solution, and denatured herring sperm DNA (DuPont-NEN, Boston, MA).

DNA probes were oligolabeled using [α-^{32}P]dCTP as described (Amersham International, Little Chalfont, UK) and hybridization was allowed to proceed for 24 h at 42°C. Slot blots were washed in 2× SSC, 1% SDS for 10 min at room temperature, and then repeated at 42°C for 30 min, and then 0.2% SSC, 0.1% SDS at 65°C for 15 min. Autoradiographs were prepared by exposure with intensifying screens at −70°C to XAR films (Eastman Kodak Co., Rochester, NY). The extent of hybridization was quantified by scanning with a laser densitometer (LKB, Bromma, Sweden) and the relative intensities of the threefold dilution bands were calculated as the specific density of the band of interest (background subtracted) divided by the specific density of the G3PDH reprobing of the same band (background subtracted).

Flow cytometry analyses. After pretreatment with 15-HPETE or vehicle at 37°C/30 min, the Mono Mac 6 cells were examined either for the expression of CD14 antigen or ability to bind FITC-labeled LPS. To examine for the expression of CD14 antigen, the cells were treated with a monoclonal antibody to CD14 (FMC32; Silenus, Victoria, Australia) or isotype-matched control monoclonal antibody, washed in isoton II + 1% BSA, and then treated with FITC-conjugated goat anti–mouse IgG (Organon Teknika-Cappel, Durham, NC) each at 4°C for 30 min. After two washes in Isoton II + 1% BSA, the cells were fixed with 1% paraformaldehyde. The fluorescence intensity of the cell population was analyzed by flow cytometry on a FAC-

Scan® (Becton Dickinson & Co., Mountain View, CA). Binding of LPS to Mono Mac 6 was examined by the addition of FITC-labeled LPS (Sigma Chemical Co.) and incubating at 4°C for 30 min. After incubation, the cells were processed as above for flow cytometric analysis. To ensure that LPS binding was to the CD14 receptor, another set of experiments was set up in which the cells were treated with the anti–CD14 monoclonal antibody (after the HPETE or vehicle treatment), and then examined for ability to bind FITC-labeled LPS. For the flow cytometric analyses, ~ 10,000 cells were counted. The data was processed with a software Lysis II program (Becton Dickinson & Co.).

PKC extraction and assay. The cells (3×10^7) were washed in HBSS and incubated with different concentrations of fatty acids or stimuli for the time indicated in Results. After incubation, cells were washed again with HBSS and sonicated in buffer containing 20 mM Tris, pH 7.5, 5 mM EGTA, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml benzamidine, 1 μg/ml pepstatin A, 15 mM 2-mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride, and then centrifuged at 100,000 *g* for 30 min. The pellet was resuspended in sonication buffer with 2% Triton X-100. After 30 min on ice, the extract was centrifuged at 100,000 *g* for 30 min. The supernatant was adsorbed onto DE 52 (Whatman Ltd, Maidstone, UK) and washed twice in Hepes. PKC was eluted with 0.2 M NaCl. PKC activity was assayed by using Histone type III-S (Sigma Chemical Co.) as substrate according to the method of Hardy et al. (22). The reactions were terminated by spotting aliquots of assay mixtures onto P81 paper (Whatman Ltd.). After three washes in 85 mM orthophosphoric acid, the radioactivity was determined by liquid scintillation spectrometry. For immunoblotting (23), Laemmli buffer was added to DE52-purified samples. Proteins (20 μg) were separated by SDS-polyacrylamide gel (10%) electrophoresis, and then transferred to nitrocellulose membranes and probed with isotype-specific PKC antibodies. The immune complexes were detected by enhanced chemiluminescence. Laser densitometry was performed on a personal laser densitometer (Molecular Dynamics, Sunnyvale, California) and quantification achieved using Image Quant Software (Molecular Dynamics).

[γ-^{32}P]ATP was obtained from Bresatec (Adelaide, South Australia). Monoclonal antibodies for PKC isoenzymes were obtained from Boehringer Mannheim (Mannheim, Germany). Enhanced chemiluminescence and reinforced nitrocellulose membranes were from DuPont-NEN and Schleicher and Schuell (Dassel, Germany), respectively. All other reagents unless indicated were from Sigma Chemical Co.

Statistical analysis. Differences between the various treatments were analyzed by either the Student's *t* test for paired data or Bonferroni's test for multiple comparisons.

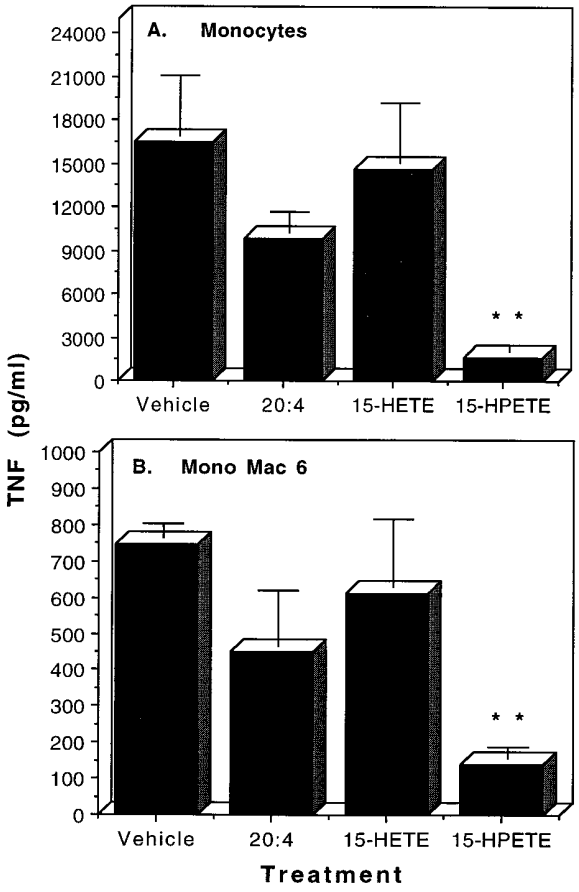

Figure 1. The effect of 20:4n-6, HETE, and HPETE on LPS-induced TNF production in (*A*) monocytes and (*B*) Mono Mac 6 cells. Leukocytes were pretreated for 30 min with the fatty acids, and then challenged with LPS (1 μg/ml). Production of TNF was measured after 24 h of incubation. The results are presented as mean±SEM of triplicate cultures. Similar results were seen in two other experiments. Statistical analyses $^{**}P < 0.01$ compared with vehicle treatment control. For monocytes, 15-HPETE vs. 20:4n-6, $P < 0.05$; 15-HPETE vs. 15-HETE, $P < 0.01$. For Mono Mac 6, 15-HPETE vs. 20:4n-6, $P < 0.01$; 15-HPETE vs. 15-HETE, $P < 0.001$.

Results

In initial studies, the effects of arachidonic acid and its oxygenated derivatives on the ability of MNL to produce TNF were examined. MNL were pretreated for 30 min with either 10 μg/ml of arachidonic acid, 15-HETE, or 15-HPETE, and then stimulated with either PHA, pokeweed mitogen, concanavalin A, or *S. aureus.* Supernatants were collected after 72 h of culture and the amount of TNF present quantitated by ELISA. The results from five experiments showed that the 15-HETE had similar activity to arachidonic acid in its capacity to inhibit TNF production (40–50% inhibition), but 15-HPETE was significantly more active (> 96% inhibition) than arachidonic acid and HETE (data not presented) when examined at the equivalent concentration of 10 μg/ml.

Further studies were undertaken using purified macrophages and a macrophage cell line, Mono Mac 6. The results showed that 15-HPETE was significantly more effective than arachidonic acid ($P < 0.05$) and 15-HETE ($P < 0.01$) in inhibiting the LPS-induced TNF production (Fig. 1). Studies on cell viability by the trypan blue dye exclusion method and lactate dehydrogenase release showed that these fatty acids at these concentrations had no effect on cell viability (data not presented).

Examination of the time course of TNF mRNA production in Mono Mac 6 cells showed that TNF mRNA was not detected in noninduced cells, but after stimulation with LPS, TNF mRNA was first observed at 30 min, peaked at 90 min, and had fallen to low levels by 180 min (Fig. 2). The effects of arachidonic acid and its derivatives on TNF mRNA were examined. The data in Fig. 3 show that 15-HPETE markedly inhibited the LPS-induced expression of TNF mRNA. When comparing the effects of arachidonic acid, 15-HETE, and 15-HPETE on the level of TNF mRNA, it was evident that while all three inhibited the LPS-induced TNF mRNA levels, the relative effects of these paralleled those of the effects on TNF release by these cells. The results show that 15-HPETE was significantly more active than either 15-HETE ($P < 0.001$) or

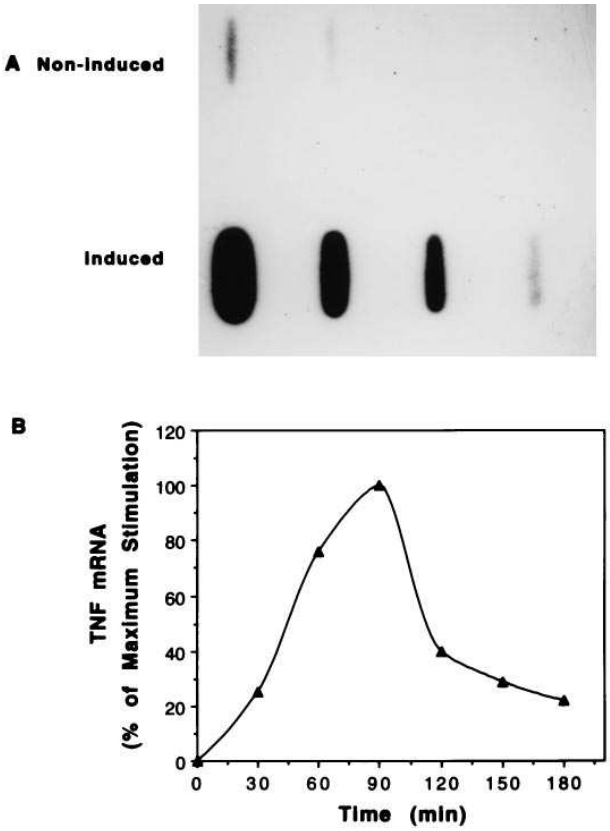

Figure. 2. Steady state mRNA levels of TNF in Mono Mac 6 cells. (*A*) Slot blots showing levels of TNF mRNA from cells incubated at 37°C for 90 min in either the presence or absence of 1 μg/ml of LPS. The mRNA was analyzed at threefold dilutions (left to right). (*B*) The time course of TNF mRNA accumulation in cells induced by LPS. At selected intervals after LPS addition (zero time), RNA was extracted and quantified. Each point is the mean of three experiments.

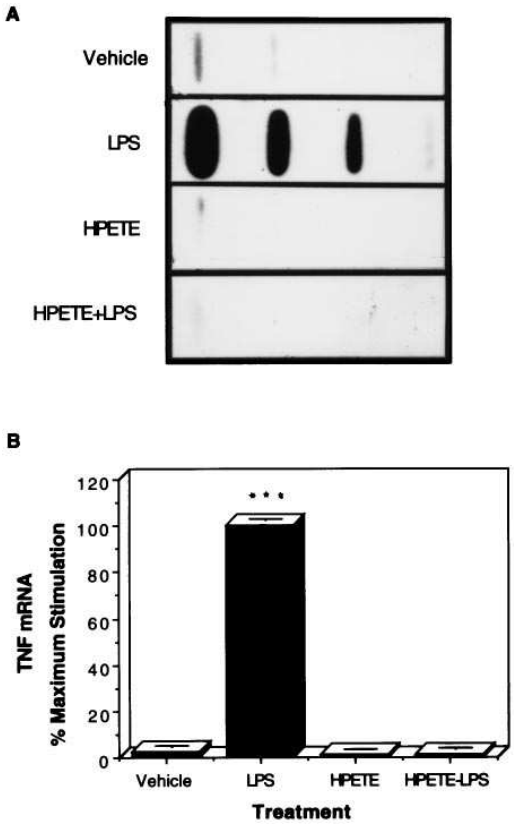

Figure 3. The effects of HPETE on TNF mRNA expression in Mono Mac 6 cells stimulated with 1 μg/ml LPS for 90 min. (*A*) Slot blots showing the effects of 30-min pretreatment with 5 μg/ml HPETE on TNF mRNA expression (presented as threefold dilutions from left to right). (*B*) The results are the mean±SEM of three experiments. These are expressed as a percentage of the LPS response (100%). ***$P < 0.001$; significantly different from the vehicle control and HPETE treatments.

arachidonic acid ($P < 0.001$) (Fig. 4). A concentration-related effect was seen between 0.5 and 2.5 μg/ml (Fig. 5 *A*). The minimal fatty acid concentration to significantly affect TNF mRNA was 300 ng/ml for 15-HPETE compared with 5 μg/ml for arachidonic acid and 15-HETE (data not presented).

The effects of 15-HPETE on TNF mRNA expression were dependent on the duration of the pretreatment (Fig. 5 *B*). Maximal inhibition occurred with 60-min preincubation with the fatty acid. However, inhibitory effects of 15-HPETE were still evident even without pretreatment.

The effects of HPETE on agonist-induced TNF mRNA production were examined over a range of LPS concentration of 0.1, 0.5, 1.0, and 1,000 ng/ml. The responses in relation to TNF mRNA production to all the LPS concentrations were similarly inhibited by 5 μg/ml of HPETE. The relative percent inhibition of LPS-induced TNF mRNA production expressed as mean±SEM of six determinations from two to four different experiments were 94.0±3.1, 92.8±3.3, 94.5±3.0, and 97.3±1.3 for 0.1, 0.5, 1.0, and 1,000 ng/ml of LPS, respectively.

It is possible that one of the targets of 15-HPETE is an LPS receptor such as the CD14 antigen. The results showed that 15-HPETE caused no change in the expression of CD14 on Mono Mac 6 (Fig. 6 *A*). Furthermore, there was no change in the

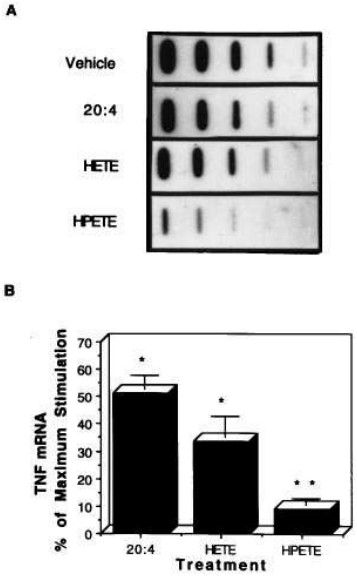

Figure 4. Effects of 20:4n-6, HETE, and HPETE on LPS-induced TNF mRNA expression in Mono Mac 6 cells. (*A*) Slot blots (presented as threefold dilution) showing the effects of 30-min pretreatment of cells with vehicle or 5 μg/ml of 20:4n-6, HETE, and HPETE on 1 μg/ml LPS-induced TNF mRNA expression after 90-min incubation at 37°C. (*B*) Comparisons between the effects of 20:4n-6, HETE, and HPETE (at 5 μg/ml), shown as percent maximum stimulation standardized against the LPS-vehicle treatment. Each point was significant compared with vehicle control. *$P < 0.05$, **$P < 0.01$. The HPETE effect was significantly different from the effects seen with both 20:4n-6 and HETE, $P < 0.001$. (Percent inhibition of LPS-vehicle control values for 20:4n-6, HETE, and HPETE were, respectively, 48.8, 66.5, and 90.7%.)

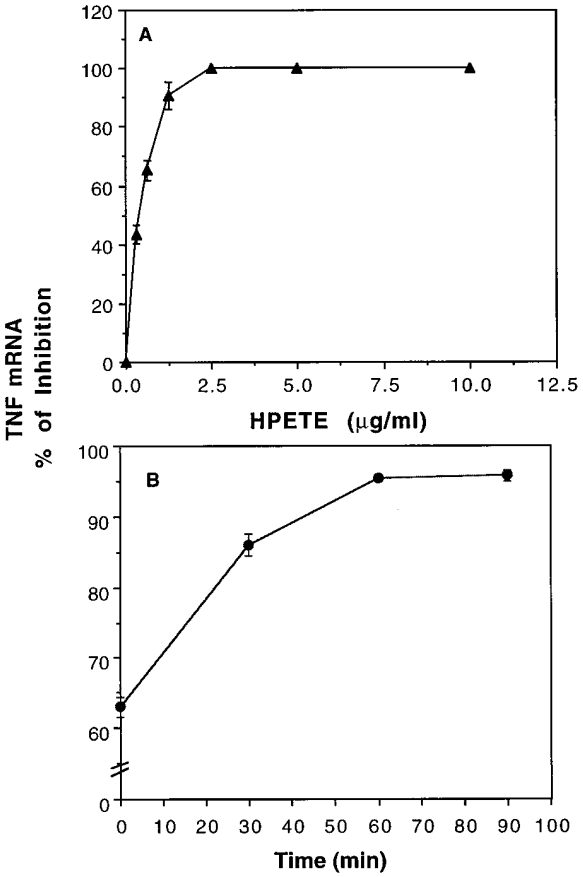

Figure 5. (*A*) The concentration-related effects of HPETE on LPS-induced TNF mRNA in Mono Mac 6 cells. The data are expressed as percent inhibition compared with the vehicle-treated cells and are presented as the mean±SEM of three experiments. (*B*) The effect of HPETE pretreatment time on LPS-induced TNF mRNA production in Mono Mac 6 cells. Cells were pretreated with 5 μg/ml of HPETE for the times indicated, and then stimulated with 1 μg/ml LPS for TNF mRNA for 90 min at 37°C. The mRNA was analyzed, quantitated, and expressed as percent inhibition induced by HPETE of the LPS-induced reponse. Mean±SEM of three experiments.

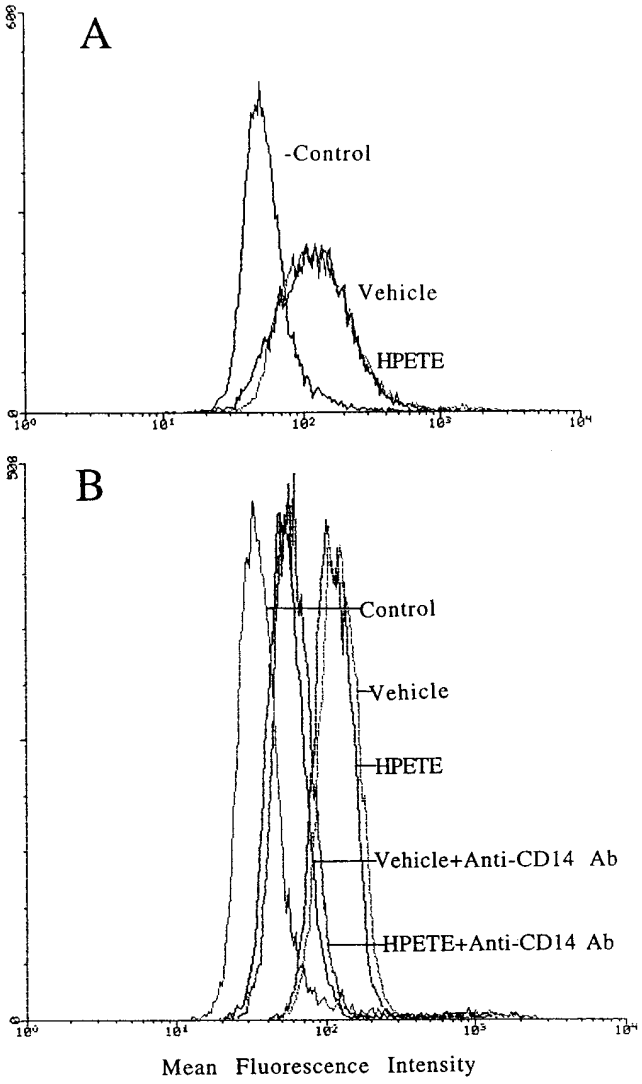

Figure 6. The effect of HPETE on CD14 expression and LPS binding to Mono Mac 6. Flow cytometric analyses of Mono Mac 6 cells stained with either (*A*) anti–CD14 monoclonal antibody and FITC-labeled rabbit anti–mouse IgG conjugate, or (*B*) FITC-labeled LPS. Also shows the effects of pretreating cells with 50 μg/ml anti–CD14 monoclonal antibody on the binding of FITC-labeled LPS at 1 μg/ml.

ability of the 15-HPETE–treated cells to bind FITC-labeled LPS (Fig. 6 *B*). This LPS binding was inhibited to a similar extent in vehicle and 15-HPETE–treated cells by the addition of anti–CD14 antibody (Fig. 6 *B*).

Mono Mac 6 cells treated with either 1 ng/ml or 1 μg/ml LPS showed translocation of PKC from the cytosol to a particulate fraction (Fig. 7). Treatment of the cells with the fatty acid totally abrogated both the 1 ng/ml and 1 μg/ml LPS-induced translocation of PKC as determined by enzyme assays using Histone type III-S as substrate (Fig. 7). The data in Fig. 8 *A* show that pretreatment with 15-HPETE inhibited the LPS-induced translocation of the α, βI, βII, and ϵ isozymes of PKC. There was some translocation of PKC induced by 15-HPETE (Figs. 7 and 8, *A* and *C*), but when LPS was also added, this membrane-bound PKC decreased. This raised the possibility that the combined action of 15-HPETE and LPS resulted in increased degradation of translocated PKC. Examination of total PKC in the cell showed that there was essentially no difference between untreated Mono Mac 6 cells and those treated with either 15-HPETE alone, LPS alone, or the combination of 15-HPETE and LPS (Fig. 8, *B* and *D*).

Discussion

Receptor ligation on various cell types by a host of endogenous or exogenous mediators leads to the activation of phospholipase A_2 and release of arachidonic acid from membrane phospholipids (24). This cellular activation is associated with the stimulation of the lipoxygenase enzyme system that leads to the oxygenation of arachidonic acid and the generation of 5-HPETE, 12-HPETE, and 15-HPETE, respectively. These then either spontaneously or catalytically convert to the corresponding HETE forms. 5(S) HETE can also be converted into its 5-oxo derivative in a reaction catalyzed by a specific 5(S) hydroxyeicosanoid dehydrogenase (25). The metabolism of

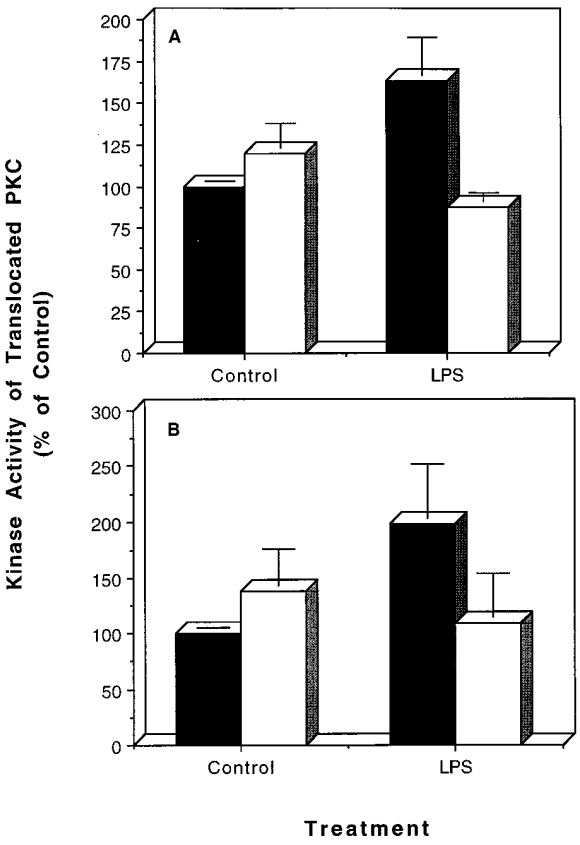

Figure 7. The effect of HPETE treatment on LPS-induced PKC translocation in Mono Mac 6 cells. Mono Mac 6 cells were pretreated with 5 μg/ml of HPETE (□) or vehicle alone (■) for 30 min at 37°C, and then treated with either 1 μg/ml (*A*) or 1 ng/ml (*B*) LPS, and the ability of the agonist to induce translocation of PKC was examined. The activity of PKC was assayed as described in Methods. The data represent the mean±range of duplicate assays of one experimental run and are representative of two other experiments.

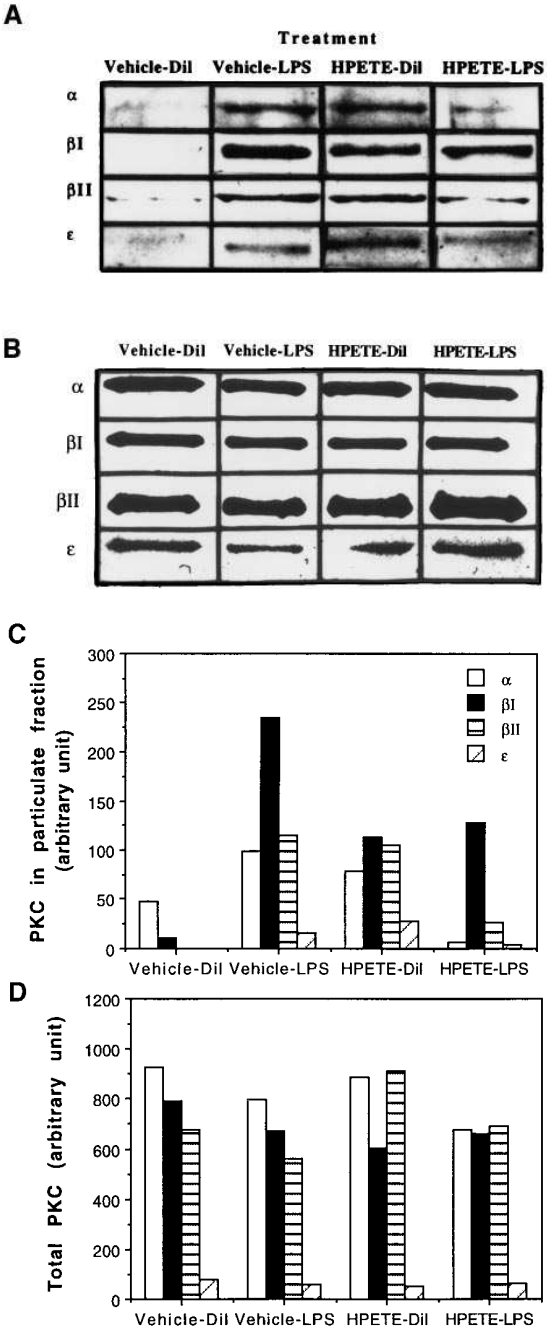

Figure 8. The effect of HPETE treatment on the LPS-induced translocation of PKC isozymes. Mono Mac 6 cells were preincubated with 5 μg/ml HPETE for 30 min, and then treated with 1 μg/ml LPS. After 5 min, PKC fractions were prepared and immunoblotted with isozyme-specific antibodies as described in Methods. (*A*) Translocation of PKC isozyme. (*B*) Total PKC isozyme. (*C* and *D*) Quantification of (*A*) and (*B*), respectively, by direct laser densitometry as described in Methods. The data are representative of three experiments; the percent inhibition (±SEM) of LPS-induced translocation of the PKC isozymes, α, βI, βII, and ϵ by HPETE were 91.4±3.2, 44.1±10.00, 62.7±10.8, and 68.7±7.7, respectively.

arachidonic acid via the cyclooxygenase pathway generates prostaglandins such as PGE_2 and thromboxanes such as TxA_4.

These arachidonic acid metabolic products have a wide range of biological properties that form the basis of physiological vs. pathophysiological responses in health vs. disease states, respectively (26). Some of the well defined biological activities of the leukotrienes are chemotactic activity (LTB_4), smooth muscle contraction (LTC_4), SRS-A–like action (LTD_4), and proinflammatory activity (LTE_4). In the case of prostaglandins, PGD_2, PGE_1, and PGE_2 cause broncho- and vasodilation and PGF_2 is a broncho- and vasoconstrictor. The thromboxanes, A_2 and B_2, contribute to pathogenesis through their properties as platelet activators and vasodilators. Furthermore, because of the role of eicosanoids in the regulation of processes of the inflammatory response, there have been widespread attempts to treat a range of autoimmune and allergic inflammatory diseases by altering the production of these eicosanoids.

Arachidonic acid has been shown previously to have a wide range of biological effects on leukocytes. These include effects on neutrophils (1–7), with respect to activation of NADPH oxidase, increased expression of CR3, degranulation, enhanced cytotoxic properties, and increased adhesion. Activation of the macrophage respiratory burst by arachidonic acid has also been reported (8, 27). These functions are associated with the proinflammatory properties of this polyunsaturated fatty acid

and most likely exacerbate the effects induced by its metabolic product, LTB_4, of the lipoxygenase pathway. The antiinflammatory activity has been associated with products of the arachidonic acid metabolism in the cyclooxygenase pathway.

We now describe a novel activity of the arachidonic acid metabolic product, 15-HPETE, of the lipoxygenase pathway in relation to antiinflammatory properties. Arachidonic acid, 15-HETE, and 15-HPETE all depressed TNF production by human MNL in response to various stimulators, PHA, concanavalin A, pokeweed mitogen, and *S. aureus.* Further investigations conducted with purified monocytes and a monocytic cell line, Mono Mac 6, showed that arachidonic acid, 15-HETE, and 15-HPETE all inhibited the LPS-induced TNF production. While arachidonic acid and 15-HETE had comparable activity, 15-HPETE was significantly more active. The effects of these fatty acids were reflected in the TNF mRNA expression and 15-HPETE was significantly more inhibitory than either arachidonic acid or 15-HETE. The minimal concentration of fatty acid required to cause significant inhibition of LPS-induced TNF mRNA expression was 5 μg/ml for arachidonic acid and 15-HETE and < 1 μg/ml for 15-HPETE. The inhibition by 15-HPETE was in fact evident at 300 ng/ml.

These concentrations of AA and its metabolites are found in pathophysiological fluids. Eissen (28) showed plasma free AA levels in human malaria patients to be > 100 μM (~ 33.3 μg/ml). Yasuda et al. (29) reported that brain free AA levels were 50 μM (~ 16.7 μg/ml) rising to 500 μM (~ 167 μg/ml) under ischemic conditions. Walenga et al. (30) found the HETE levels increase to 40 μM (~ 13.3 μg/ml) in blood stimulated with various agents. HPETE has also been reported to reach levels that we found to be active in inhibiting TNF production, 0.7 μM (~ 0.23 μg/ml) (31). Under certain conditions or diseases where a reduced or compromised concentration of reductases occurs, 15 HPETE accumulates, as seen in HIV infections (32). Indeed, there are pathological conditions where HPETE levels of ~ 20 μM (~ 6.7 μg/ml) have been measured (33).

The relative differences of effects between these fatty acids on cytokine production differ from those on the neutrophil and macrophage respiratory burst. Arachidonic acid stimulates both the neutrophil and macrophage respiratory activity (1–3, 8, 27) and increases this response to other agonists (7). Both 15-HETE and 15-HPETE had no effect on this neutrophil response (3) and our recent evidence suggests that this is also so for macrophages (unpublished observations). These differences in effects of arachidonic acid vs. 15-HETE and 15-HPETE are found with respect to other neutrophil functions such as degranulation (34) and migration inhibition activity (35). Thus, the effects on cytokine production constitute the first demonstration that products of arachidonic acid metabolism via the lipoxygenase pathway can depress macrophage function associated with inflammation. Interestingly, the 15-HPETE exhibited significantly more cytokine production inhibitory activity than either 15-HETE or arachidonic acid.

The possible mechanisms by which 15-HPETE may inhibit the LPS-induced TNF production were partly studied. It was evident that these effects are post–LPS-receptor binding. This is supported by the finding that better inhibition of TNF mRNA occurred if the cells were pretreated with the fatty acids rather than added simultaneously with the LPS. Prior stimulation of Mono Mac 6 cells with LPS followed by the addition of 15-HPETE produced very little change in TNF production (data not presented). Furthermore, examination of LPS binding to Mono Mac 6 cells treated with 15-HPETE was normal, as was the expression of the CD14 receptor (involved in LPS binding). LPS dose response curves showed that HPETE inhibited production of TNF equivalently from 0.1 to 1,000 ng/ml. This shows that the inhibition is relevant to both the LBP-CD14 pathway as well as the non–CD14 pathway, the latter most likely to be also involved at the higher LPS concentration.

Further studies suggested that the 15-HPETE–induced inhibition was related to effects on the early signal transduction events. Previously, it has been reported that LPS-induced TNF production by monocytes requires the activation of PKC (36), an observation consistent with our findings (unpublished observations). While 15-HPETE per se translocated PKC to a particulate fraction in Mono Mac 6 cells, pretreatment with the 15-HPETE not only prevented LPS from stimulating the translocation of the PKC isozymes, α, βI, βII, and ϵ, but the fatty acid also caused a loss of membrane-bound PKC isozymes in cells that were subsequently exposed to LPS. The exact mechanism involved is unclear but this was not due to increased degradation of PKC as all cells, treated or untreated with HPETE, were found to contain similar amounts of the various PKC isozymes. Previous studies with neutrophils have shown that 15-HETE added to neutrophils is rapidly incorporated into membrane phospholipids (15). Thus, it seems that this remodeling of membrane phospholipids, while not altering the expression of the LPS receptor, could be responsible for altering the ability to translocate PKC. Our studies on the effects of fatty acids on PKC have shown that these fatty acids not only activate PKC in vitro (22), but also in cells (23). Although the 15-HPETE can translocate and activate PKC, activation of PKC per se is not sufficient to stimulate the production of cytokines since the 15-HPETE did not stimulate TNF production. This is consistent with previous findings on agonist-induced TNF production in macrophages (36).

The data illustrate the potent antiinflammatory potential of products of the arachidonic acid metabolism via the lipoxygenase pathway. In particular, 15-HPETE was a highly effective inhibitor of TNF production by macrophages stimulated with a variety of agonists. The findings signify another immunoregulatory mediator associated with inflammation and cell activation, and support and extend previous reports that showed that a product of the lipoxygenase pathway, namely 15-HETE, downregulates the inflammatory response by inhibiting neutrophil chemotaxis and the neutrophil oxygen radical production (14, 15). Here our work emphasizes the ability of 15-HPETE to perform as a very effective inhibitor of TNF production. Thus, while the tissue/fluid concentrations of HPETE are much less than AA and HETE, HPETE may be just as effective as the former two as it is more active on a molar basis. The major pathogenetic effects of bacterial LPS are mediated through the stimulation of release of pyrogenic cytokines such as TNF, IL-1β, and IL-6 from a variety of tissues, in particular from the mononuclear phagocytic system (37). In this manner, TNF, through its interaction with endothelial cells, causes a general inflammatory response leading to sepsis with severity depending on the persistence of the damage and whether at one or more sites (37). TNF is a major contributor to the pathophysiology of a variety of diseases (37) and many efforts have been made to attempt to understand the pathways that lead to inhibition of its production or activity. The data presented identify another pathway by which regulation of the

production of LPS-induced TNF may be achieved. Of importance is that the identification of specific structural elements of fatty acids (as seen here for HPETE), which are strong inhibitors of cytokine production, is relevant to drug design for anti-inflammatory agents.

Acknowledgments

We are grateful to Brenton Rowan-Kelly for assistance with studies on human mononuclear leukocytes and to Prof. Andy W. Murray for the antisera against the PKC isozymes.

This work was supported by grants from the National Health and Medical Research Council of Australia.

References

1. Badwey, J.A., J.T. Curnutte, and M.L Karnovsky. 1981. Cis-polyunsaturated fatty acids induce high levels of superoxide production by human neutrophils. *J. Biol. Chem.* 256:12640–12643.

2. Curnutte, J.T. 1985. Activation of human neutrophil nicotinamide-adenine dinucleotide phosphate, reduced (triphosphopyridine nucleotide, reduced) oxidase by arachidonic acid in a cell-free system. *J. Clin. Invest.* 75:1740–1743.

3. Poulos, A., B.S. Robinson, A. Ferrante, D.P. Harvey, S.J. Hardy, and A.W. Murray. 1991. Effect of 22-32 carbon n-3 polyunsaturated fatty acids on superoxide production in human neutrophils: synergism of docosahexaenoic acid with f-met-leu-phe and phorbol ester. *Immunology.* 73:102–108.

4. Baggiolini, M., and B. Dewald. 1984. Exocytosis by neutrophils. *In* Regulation of Leukocyte Function. R. Snyderman, editor. Plenum Publishing Corp., New York. 221–245.

5. Bates, E.J., A. Ferrante, D.P. Harvey, M. Nandoskar, and A. Poulos. 1993. Docosahexanoic acid (22:6, n-3) but not eicosapentaenoic acid (20:5, n-3) can induce neutrophil-mediated injury of cultured endothelial cells: involvement of neutrophil elastase. *J. Leukocyte Biol.* 54:590–598.

6. Bates, E.J., A. Ferrante, D.P. Harvey, and A. Poulos. 1993. Polyunsaturated fatty acids increase neutrophil adherence and integrin receptor expression. *J. Leukocyte Biol.* 53:420–426.

7. Hardy, S.J., B.S. Robinson, A. Poulos, D.P. Harvey, A. Ferrante, and A.W. Murray. 1991. The neutrophil respiratory burst response to fatty acids, *N*-formylmethionyl-leucyl-phenylalanine and phorbol ester suggest divergent signaling mechanisms. *Eur. J. Biochem.* 198:801–806.

8. Bromberg, Y., and E. Pick. 1983. Unsaturated fatty acids as second messengers of superoxide generation by macrophages. *Cell. Immunol.* 29:240–252.

9. Samuelsson, B., S.E. Dahlen, J.A. Lindgren, C.A. Rouzer, and C.M. Serhan. 1987. Leukotrienes and lipoxins: structural biosynthesis and biological effects. *Science (Wash. DC).* 237:1171–1176.

10. Konig, W., W. Schonfeld, M. Raulf, M. Koeller, J. Kneller, J. Scheffer, and J. Brown. 1990. The neutrophil and leukotrienes—role in health and disease. *Eicosanoids.* 3:1–22.

11. Powell, W.S., S. Gravel, R.J. McLeod, E. Milles, and M. Hashefi. 1993. Stimulation of human neutrophils by 5-oxo-6,8,11,14-eicosatetraenoic acid by a mechanism independent of the leukotriene B4 receptor. *J. Biol. Chem.* 268: 9280–9286.

12. Hui, K.P., J. Lötvall, K. Fan Chung, and P.J. Barnes. 1991. Attenuation of inhaled allergen-induced airway microvascular leakage and airflow obstruction in guinea pigs by a 5-lipoxygenase inhibitor (A-63162)[1-3]. *Am. Rev. Respir. Dis.* 143:1015–1019.

13. Griffiths, R.J., E.R. Pettipher, K. Koch, C.A. Farrell, R. Breslow, M.J. Conklyn, M.A. Smith, B.C. Hackman, D.J. Wimberly, A.J. Milici, et al. 1995. Leukotrienne B_4 plays a critical role in the progression of collagen-induced arthritis. *Proc. Natl. Acad. Sci. USA.* 92:517–521.

14. Smith, R.J., J.M. Justen, E.G. Nidy, L.M. Sam, and J.E. Bleasdale. 1993. Transmembrane signaling in human polymorphonuclear neutrophils: 15(S)-hydroxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid modulates receptor agonist-triggered cell activation. *Proc. Natl. Acad. Sci. USA.* 90:7270–7274.

15. Takata, S., M. Matsubara, P.G. Allen, P.A. Janmey, C.N. Serhan, and H.R. Brady. 1994. Remodelling of neutrophil phospholipids with 15(s)-hydroxy-eicosatetraenoic acid inhibits leukotriene B_4-induced neutrophil migration across endothelium. *J. Clin. Invest.* 93:499–508.

16. Ferrante, A., and Y.H. Thong. 1982. Separation of mononuclear and polymorphonuclear leucocytes from human blood by the one-step Hypaque-Ficoll method is dependent on blood column height. *J. Immunol.* 48:81–85.

17. Kumaratilake, L.M.A., and A. Ferrante. 1988. Purification of human monocytes/macrophages by adherence to cytodex microcarriers. *J. Immunol. Methods.* 112:183–190.

18. Ziegler-Heitbrock, H.W.L., E. Thiel, A. Fütterer, V. Herzog, A. Wirtz, and G. Riethmüller. 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer.* 41:456–561.

19. Ferrante, A., R.E.M. Staugas, B. Rowan-Kelly, S. Bresatz, L.M. Kumaratilake, C.M. Rzepczyk, and G.R. Adolf. 1990. Production of tumor necrosis factor alpha and beta by human mononuclear leukocytes stimulated with mitogens, bacteria, and malarial parasites. *Infect. Immun.* 58:3996–4003.

20. Bates, E.J., A. Ferrante, A. Poulos, L. Smithers, D.A. Rathjen, and B.S. Robinson. 1995. Inhibitory effects of arachidonic acid (20:4, n-6) and its monohydroperoxy- and hydroxy-metabolites on procoagulant activity in endothelial cells. *Atherosclerosis.* 116:125–133.

21. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid-guanidinium thiocyanate-phenol-chloroform. *Anal. Biochem.* 162: 156–159.

22. Hardy, S.J., A. Ferrante, B.S. Robinson, D.S. Johnson, A. Poulos, K.J. Clark, and A.W. Murray. 1994. *In vitro* activation of rat brain protein kinase C by polyenoic very-long-chain fatty acids. *J. Neurochem.* 62:1546–1551.

23. Hii, C.S.T., A. Ferrante, Y.S. Edwards, H.Z. Huang, P.J. Hartfield, D.A. Rathjen, A. Poulos, and A.W. Murray. 1995. Activation of mitogen-activated protein kinase by arachidonic acid in rat liver epithelial WB cells by a protein kinase C–dependent mechanism. *J. Biol. Chem.* 270:4201–4204.

24. Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Wash. DC).* 258:607–614.

25. Powell, W.S., F. Gravelle, and S. Gravel. 1992. Metabolism of 5(S)-hydroxy-6, 8, 11, 14-eicosatetraenoic acid and other 5(S)-hydroxyeicosanoids by a specific dehydrogenase in human polymorphonuclear leukocytes. *J. Biol. Chem.* 267:19233–19241.

26. Sinclair, A., and R. Gibson. 1992. Essential fatty acids and eicosanoids: invited papers from the Third International Congress. American Oil Chemists' Society, Champaign, IL. 482 pp.

27. Sakata, A., E. Ida, M. Tominaga, and K. Ongue. 1987. Arachidonic acid acts as an intracellular activator of NADPH-oxidase in Fc receptor-mediated superoxide generation in macrophages. *J. Immunol.* 138:4353–4359.

28. Eissen, E.U. 1993. Significance of plasma free fatty acid levels in human malaria with parasitaemia. *Med. Sci. Res.* 21:405–406.

29. Yasuda, H., K. Kishiro, N. Izumi, and M. Nakanishi. 1985. Biphasic liberation of arachidonic and stearic acids during cerebral ischemia. *J. Neurochem.* 45:168–172.

30. Walenga, R.W., S. Boone, and M.J. Stuart. 1987. Analysis of blood HETE levels by selected ion monitoring with ricinoleic acid as the internal standard. *Prostaglandins.* 34:733–748.

31. Piomelli, D., J.K.T. Wang, T.S. Sihra, A.C. Nairn, A.J. Czernik, and P. Greenland. 1989. Inhibition of Ca^{++}/calmodulin-dependent protein kinase II by arachidonic acid and its metabolites. *Proc. Natl. Acad. Sci. USA.* 86:8550–8554.

32. Sandstrom, P.A., P.W. Tebbey, S. Van Cleave, and T.M. Buttke. 1994. Lipid hydroperoxides induce apoptosis in T cells displaying a HIV-associated glutathione peroxidase deficiency. *J. Biol. Chem.* 269:798–801.

33. Hughes, H., C.U. Smith, J.O. Tsokos-Kuhn, and J.R. Mitchell. 1986. Quantitation of lipid peroxidation products by gas chromatography–mass spectrometry. *Anal. Biochem.* 152:107–112.

34. Bates, E.J., A. Ferrante, L. Smithers, A. Poulos, and B.S. Robinson. 1995. Effect of fatty acid structure on neutrophil adhesion, degranulation and damage to endothelial cells. *Atherosclerosis.* 116:247–259.

35. Ferrante, A., D. Goh, D.P. Harvey, B.S. Robinson, C.S.T. Hii, E.J. Bates, S.J. Hardy, D.W. Johnson, and A Poulos. 1994. Neutrophil migration inhibitory properties of polyunsaturated fatty acids. The role of fatty acid structure, metabolism, and possible second messenger systems. *J. Clin. Invest.* 93: 1063–1070.

36. Shapira, L., S. Takashiba, C. Champagne, S. Amar, and T.E. Van Dyke. 1994. Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNF-α and IL-1β production by human monocytes. *J. Immunol.* 153:1818–1824.

37. Beutler, B. 1992. Tumour necrosis factors. The molecules and their emerging role in medicine. B., Beutler, editor. Raven Press, New York. 1–590.