

Activation of Cytokine Production by Secreted Phospholipase A₂ in Human Lung Macrophages Expressing the M-Type Receptor¹

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Secreted phospholipases A₂ (sPLA₂) are enzymes released in plasma and extracellular fluids during inflammatory diseases. Because human group IB and X sPLA₂s are expressed in the lung, we examined their effects on primary human lung macrophages (HLM). Both sPLA₂s induced TNF- α and IL-6 release in a concentration-dependent manner by increasing their mRNA expression. This effect was independent of their enzymatic activity because 1) the capacity of sPLA₂s to mobilize arachidonic acid from HLM was unrelated to their ability to induce cytokine production; and 2) two catalytically inactive isoforms of group IB sPLA₂ (bromophenacyl bromide-inactivated human sPLA₂ and the H48Q mutant of the porcine sPLA₂) were as effective as the catalytically active sPLA₂s in inducing cytokine production. HLM expressed the M-type receptor for sPLA₂s at both mRNA and protein levels, as determined by RT-PCR, immunoblotting, immunoprecipitation, and flow cytometry. Me-indoxam, which decreases sPLA₂ activity as well as binding to the M-type receptor, suppressed sPLA₂-induced cytokine production. Incubation of HLM with the sPLA₂s was associated with phosphorylation of ERK1/2, and a specific inhibitor of this pathway, PD98059, significantly reduced the production of IL-6 elicited by sPLA₂s. In conclusion, two distinct sPLA₂s produced in the human lung stimulate cytokine production by HLM via a mechanism that is independent of their enzymatic activity and involves activation of the ERK1/2 pathway. HLM express the M-type receptor, but its involvement in eliciting cytokine production deserves further investigation. *The Journal of Immunology*, 2005, 174: 464–474.

Phospholipases A₂ (PLA₂)³ are enzymes that release fatty acids from the sn-2 position of phospholipids. Known enzymes include high m.w. cytosolic PLA₂s and low m.w. secreted PLA₂ (sPLA₂) (1). These two classes of enzymes have different cellular sources, subcellular locations, and Ca²⁺ requirements for their enzymatic activities (2). Among the PLA₂ isoforms that have been characterized and cloned, at least 12 different groups of sPLA₂s have been described in reptile and bee venom as well as in mammalian tissues (3).

Extracellular sPLA₂ levels are increased in various systemic inflammatory diseases (adult respiratory distress syndrome, septic shock, and acute pancreatitis) (4–6), autoimmune diseases (rheumatoid arthritis, Crohn's disease, and ulcerative colitis) (7–9), and allergic disorders (bronchial asthma and allergic rhinitis) (10, 11). In most of these diseases, sPLA₂ levels increase in plasma and inflammatory fluids (synovial fluid, bronchoalveolar lavage, and nasal lavage), indicating that sPLA₂s are released systemically as well as at sites of tissue inflammation. These observations implicate sPLA₂ in inflammation.

Some groups of sPLA₂s release arachidonic acid (AA), the precursor of eicosanoids, from cell membranes or extracellular phospholipids (2). Thus, sPLA₂s may participate in the synthesis of proinflammatory eicosanoids by using their own enzymatic activity. However, there is evidence that some of the biological effects exerted by sPLA₂s cannot be fully explained by their catalytic activity (12). For example, some effects are elicited by sPLA₂s whose enzymatic activity has been irreversibly inactivated (13–15). In addition, intra-articular or intradermal injection of catalytically inactive sPLA₂s induces inflammation (16, 17).

Another mechanism by which sPLA₂s may induce biological responses is based on the ability of certain sPLA₂s to bind to heparan sulfate-containing proteoglycans of cell membranes (18, 19). In addition, sPLA₂s may perturb plasma membrane symmetry by a scramblase-dependent mechanism (20). Finally, some sPLA₂s interact with specific membrane receptors (21). In particular, it has been suggested that activation of the M-type receptor (M-type-R) may be involved in some in vitro sPLA₂-induced effects (13, 22–24). The M-type-R is a 180-kDa protein cloned from various species, including humans (25). However, not all sPLA₂ isoforms bind to this receptor, and binding is strictly species specific (21).

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³Abbreviations used in this paper: PLA₂, phospholipases A₂; AA, arachidonic acid; BPB, bromophenacyl bromide; bvGIII, bee venom group III; GIB, group IB; GX, group X; hGIII-cd, human recombinant group III sPLA₂ catalytic domain; HLM, human lung macrophage; mGIB, mouse GIB; mGX, mouse GX; MR, mannose receptor; M-type-R, M-type receptor; pGIB, porcine GIB; WT, wild type.

Thus, it has yet to be demonstrated that sPLA₂s induce activation of M-type-R in humans.

Macrophages play a pivotal role in the modulation of immune and inflammatory responses by producing a number of mediators, including cytokines and chemokines (26). Interestingly, most inflammatory diseases associated with increased sPLA₂ levels are characterized by overproduction of cytokines such as TNF- α , IL-1, and IL-6 (27), three major cytokines produced by macrophages. These cells are widely represented in the lung, liver, bone marrow, and synovia (26), which means that macrophages are a potential target for sPLA₂s released during inflammation.

We previously showed that the basic sPLA₂ from *Naja mossambica mossambica* venom and the human group IIA sPLA₂ (hGIIA) stimulate IL-6 production from human lung macrophages (HLM) by a mechanism that appears to be unrelated to their enzymatic activities (28). Two other sPLA₂ isoforms, human group IB (hGIB) and human group X (hGX) sPLA₂s, have been detected in the human lung, where they are coexpressed with M-type-R (25, 29, 30). Interestingly, in the mouse, GX has been characterized as the most potent AA-mobilizing sPLA₂ in macrophages (31) and as a high affinity ligand for M-type-R (32). However, given the stringent species specificity of sPLA₂/M-type-R interactions, it is important to investigate the effects and mechanisms of action of these sPLA₂s in primary human cells.

In this study we show that hGIB and hGX induce the production of TNF- α and IL-6 by HLM via a mechanism independent of their enzymatic activities. Furthermore, we report that HLM express M-type-R and provide evidence supporting the role of this or other receptors in cytokine production.

Materials and Methods

Reagents and buffers

GIB (from porcine pancreas (pGIB)) and group III (from *Apis mellifera* venom (bvGIII)) sPLA₂s, LPS (from *Escherichia coli* serotype O26:B6), bromophenacyl bromide (BPB), human serum albumin, BSA, Percoll, PIPES, L-glutamine, antibiotic-antimycotic solution (10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 25 μ g/ml amphotericin B), and Triton X-100 were obtained from Sigma-Aldrich. RPMI 1640 and FCS were purchased from ICN. AA was obtained from BIOMOL. [³H]AA (100 Ci/nmol) was purchased from DuPont NEN Products. The rabbit Ab against phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and the MEK-1 inhibitor PD98059 were obtained from Cell Signaling. The rabbit Ab anti-ERK2 (C-14), guinea pig, and rabbit control Abs were obtained from Santa Cruz Biotechnology. The sPLA₂ inhibitor Me-iodoxam (99.9% pure) was prepared as previously described (33). Recombinant hGIB and human group X (hGX) sPLA₂s were prepared as previously described (34–36). Recombinant hGIII sPLA₂ catalytic domain (hGIII-cd) was produced in S2 cells as previously described for hGIID and mouse GIIC sPLA₂s (36). The detailed protocol will be described elsewhere (S. Bezzine and G. Lambeau, unpublished observations). The H48Q mutant of pGIB (pGIB-H48Q) (37) and the BPB-inactivated hGIB (15) were prepared as previously described. Guinea pig Ab to the M-type-R of rabbit origin was prepared by immunizing the guinea pig with the purified protein as previously described (38). Rabbit antiserum directed against a soluble mouse M-type-R that was produced as described for the rabbit soluble M-type-R (39) was prepared by immunizing the rabbit with 100 μ g of purified protein as previously described (38). A small aliquot of rabbit serum was withdrawn before immunization (pre-immune serum) and was used as the negative control. The anti-mannose receptor (anti-MR) Ab (PAM-1) was a gift from Dr. S. Sozzani (Istituto Mario Negri, Milan, Italy). All other reagents were obtained from C. Erba (Milan, Italy).

PIPES buffer is 25 mM PIPES, 110 mM NaCl, and 5 mM KCl. Lysis buffer for Western blotting is 20 mM Tris (pH 7.5), 5 mM EDTA, 1 mM PMSF, 2 mM benzamide, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 mM NaF, 150 mM NaCl, 1 mM Na₃VO₄, 1% Nonidet P-40, and 5% glycerol.

Isolation and purification of HLM

Macrophages were obtained from the lung parenchyma of patients undergoing thoracic surgery as previously reported (28). The macrophage sus-

pension was enriched (75–85%) by flotation over Percoll density gradients. The cells were suspended (10⁶ cells/ml) in RPMI 1640 containing 5% FCS, 2 mM L-glutamine, and 1% antibiotic-antimycotic solution and incubated in 24-well plates. After 12 h, the medium was removed, and the plates were gently washed with RPMI 1640. More than 98% of adherent cells were macrophages, as assessed by α -naphthyl acetate esterase staining (40). In the experiments with freshly isolated macrophages, the enriched macrophage suspension was layered onto additional Percoll gradients to reach a purity ranging between 95 and 99%, as assessed by HLA-DR/CD71 double staining with flow cytometry.

Cell incubations

Adherent macrophages were incubated (37°C, 6–24 h) in RPMI 1640 containing various concentrations of hGIB, pGIB, pGIB-H48Q, bvGIII, hGIII-cd, or hGX. Commercial preparations of sPLA₂ were repurified by size exclusion chromatography (15) before use. All sPLA₂ preparations were routinely checked for LPS contamination (*Limulus* amoebocyte test; ICN) and discarded if the LPS concentration was above the detection limit of the assay (0.125 endotoxin unit/ml). In selected experiments, the cells were preincubated (4°C, 20 min) with guinea pig anti-rabbit M-type-R Ab (10 μ g/ml) or PAM-1 (10 μ g/ml) in the presence of 5 μ g/ml purified human IgG to block nonspecific binding sites and were then stimulated with hGIB or hGX (1 μ g/ml). In other experiments, HLM were incubated (37°C, 30 min) with various concentrations (5–50 μ M) of PD98059 before stimulation with hGIB or hGX (10 μ g/ml). At the end of incubation, supernatants were removed, centrifuged twice (1000 \times g, 4°C, 5 min) and stored at –80°C for subsequent determination of TNF- α and IL-6. The cells remaining in the plates were lysed with 0.1% Triton X-100 for determination of the total cellular content of proteins (28).

ELISA and RT-PCR for TNF- α and IL-6

Cytokine release in the supernatant of macrophage cultures was measured in duplicate determinations using commercially available ELISA kits for TNF- α and IL-6 (Euro Clone). The linearity range of the assay was between 20 and 800 pg/ml (TNF- α) and between 6 and 200 pg/ml (IL-6). The results were normalized for the total protein content in each well.

In the experiments for semiquantitative RT-PCR, adherent macrophages (4 \times 10⁶ ml) were incubated (37°C, 3 h) in RPMI 1640 alone or with hGIB, bvGIII, and hGX (10 μ g/ml). At the end of the incubation, RNA was isolated by the TRIzol technique (Invitrogen Life Technologies). RT-PCR was performed with target-specific primers for TNF- α and IL-6 as previously described (41). RNA was normalized for the constitutive marker gene β -actin. All PCR products together with a DNA ladder as a size standard were separated on 2.5% agarose gel, stained with ethidium bromide, and photographed. Verification of all PCR products was obtained by DNA sequencing.

Real-time quantitative PCR was performed according to the following protocol. Adherent macrophages (4 \times 10⁶/2 ml) were incubated (37°C) in RPMI 1640 alone or with hGX (10 μ g/ml) for different time periods (3–12 h). At the end of the incubation, RNA was isolated using the SV96 total RNA isolation system (Promega) according to the manufacturer's instructions and was treated with RNase-free DNase I. RT was performed as previously described (41). Real-time quantitative PCR was performed on the iCycler (Bio-Rad) using the PE-SYBR Green PCR kit (Applied Biosystems). Target-specific primers for TNF- α and IL-6 were synthesized using Beacon Designer 2 software (TNF- α : forward, 5'-AGGCAGTCA GATCATCTTCTCG-3'; reverse, 5'-GGAGGAGAGACGGTAGTTCT3'; IL-6: forward, 5'-TTCGGTCCAGTTGCCTTC-3'; reverse, 5'-TCGT TCTGAAGAGGTGAGTG-3') and were produced and purified by Custom Primers (Invitrogen Life Technologies). After amplification was completed, a melting curve was performed increasing the temperature to 95°C at 0.2°C/sec. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of PCR product. Thus, each specific PCR product generates a specific signal and therefore a product-specific melting peak. Real-time PCR data were analyzed with iCycler iQ analysis software (Bio-Rad), and sPLA₂-induced mRNA expression of TNF- α and IL-6 was presented as the fold increase vs untreated cells. The results were normalized for the constitutive marker gene β -actin.

Determination of the enzymatic activity of sPLA₂s

The enzymatic activity of sPLA₂s was determined as previously described (40, 41) in [³H]AA-labeled HLM. In these experiments, adherent macrophages were incubated (24 h, 37°C) with [³H]AA (1 μ Ci/10⁶ cells) to label the endogenous pools of AA at equilibrium (40). AA-labeled macrophages were then incubated (1 h, 37°C) with 10 μ g/ml hGIB, bvGIII, or hGX. Radioactive AA released from the cells was isolated by TLC and quantitated as previously described (40, 41).

RT-PCR for M-type-R

Total RNA from adherent HLM (5×10^6) purified from three different donors was extracted using the SV96 total RNA isolation system (Promega). RT was performed as described above. cDNAs from human kidney and placenta were used as control cDNA. A cDNA fragment of the M-type-R was amplified using specific primers (forward, 5'-AGAGATGTGAAACCAAGA-3'; reverse, 5'-TCACAGTCTTCTCTCC-3'), designed according to the published sequences (GenBank accession no. U17033). The amplification protocol consisted of 35 cycles: denaturation for 45 s at 94°C, annealing for 45 s at 60°C, and extension for 1 min and 30 s at 72°C. A final extension at 72°C for 10 min was performed. A PCR fragment of the expected size (325 bp) was amplified from all cDNAs. The sequences of the various PCR fragments were identical with the previously published sequence (GenBank U17033), except at position 204, where a silencing mutation C to A was found.

Immunoprecipitation and immunoblot for M-type-R

Freshly isolated macrophages (3×10^6) were immediately lysed in lysis buffer. Cell lysates were kept on ice for 20 min, then microfuged for 15 min at 4°C. Supernatants were collected as protein extracts containing lysed cell components without nuclei and diluted in electrophoresis sample buffer (NOVEX; Invitrogen Life Technologies) to a final volume of 100 μ l. Protein extracts (20 μ l) were separated on 4–12% bis-Tris gels (NuPAGE; NOVEX) under nonreducing conditions together with a biotinylated protein ladder (Cell Signaling) and transferred to a nitrocellulose membrane (Bio-Rad). Membranes from 293 cells transfected with rabbit M-type-R or soluble purified mouse M-type-R were used as positive controls. After immersion overnight in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20, pH 7.6, containing 5% milk (Bio-Rad), membranes were washed with 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20, pH 7.6, then incubated (22°C, 2 h) with guinea pig anti-rabbit M-type Ab (1/3000 dilution), with rabbit anti-mouse M-type Ab (1/500 dilution), or with irrelevant Abs (guinea pig IgG and rabbit IgG). The membranes were then washed and incubated (22°C, 1 h) with HRP-conjugated goat anti-guinea pig IgG Ab (Cappel Laboratories, ICN) and goat anti-rabbit IgG Ab (Amersham Biosciences), respectively. An HRP-conjugated, anti-biotin Ab was used for detection of the m.w. markers. Membrane-bound Abs were visualized with the ECL Western blotting detection reagent (Amersham Biosciences) and developed under a chemiluminescent detection system (ChemidocXRS; Bio-Rad).

For immunoprecipitation, freshly isolated macrophages (5×10^6) were immediately lysed in 1 ml of lysis buffer. Cell lysates were kept on ice for 10 min and then microfuged for 20 min at 4°C. Lysates were precleared with Gammabind G Sepharose beads (Amersham Biosciences) for 1 h at 4°C to remove any nonspecific binding. The clarified lysates were then incubated (1 h, 4°C) with guinea pig anti-rabbit M-type Ab or control guinea pig Ab prebound to Gammabind G Sepharose beads (1 h, 4°C). Immunoprecipitated proteins were eluted by boiling in electrophoresis sample buffer to a final volume of 120 μ l. Electrophoresis, transfer, and immunoblotting with rabbit anti-mouse M-type-R Ab were performed as described above.

Flow cytometric analysis of the M-type-R

Surface expression of M-type-R was examined by indirect immunofluorescence and flow cytometry (FACS; BD Biosciences). Freshly isolated macrophages were suspended in PBS at a concentration of 5×10^6 /ml. Fifty microliters of cell suspension were incubated (4°C, 20 min) with saturating amounts of primary Abs (guinea pig anti-rabbit M-type Ab, guinea pig purified control IgG (Cappel Laboratories, ICN), rabbit anti-mouse M-type Ab, or rabbit preimmune serum) in the presence of 20% FCS to coat non specific binding sites. After washing with PBS, cell pellets were stained (4°C, 20 min) with secondary Abs (FITC-conjugated goat anti-guinea pig IgG (Cappel Laboratories, ICN) or PE-conjugated donkey anti-rabbit IgG (Abcam). To quench high spontaneous autofluorescence of HLM, cell pellets were washed twice with PBS, suspended in 0.2 ml of saturated Crystal Violet solution (Certistain; Merck), and incubated for 5 min at 22°C (42). Cell-Quest software (BD Biosciences) was used for acquisition and analysis according to a one-color procedure (43). The cut-off point between positive and negative cells and background fluorescence was assessed by analyzing macrophages incubated with isotype-matched Abs. Fluorescence intensity was analyzed by recording the mean fluorescence intensity expressed in linear units.

Vesicle binding studies

The binding of hGX to phospholipid vesicles loaded with sucrose was quantified as described previously (44). Briefly, vesicles of 20 mole %

dioleoyl phosphatidylserine in dioleoyl phosphatidylcholine (diether phospholipids prepared as described previously (44)) were prepared by extrusion in 176 mM sucrose and 5 mM MOPS, pH 7.4, then transferred into a binding buffer (5 mM MOPS (pH 7.4), 2 mM CaCl₂, and 0.1 M KCl). Vesicles were doped with a trace amount of tritiated phosphatidylcholine so that phospholipid concentrations could be determined, and vesicle pelleting could be verified (44). Binding mixtures contained 0.1 ml of binding buffer, 1 μ l of 5 mM Me-iodoxam in DMSO (50 μ M Me-iodoxam) or 1 μ l of DMSO vehicle only, various concentrations of phospholipid (see Fig. 8), and 0.5 μ g of recombinant hGX. After ultracentrifugation of pellet vesicles, an aliquot of each supernatant was analyzed by scintillation counting to verify that the vesicles pelleted (44), and an aliquot of each supernatant was analyzed by hGX immunoblot. Immunoblotting was conducted using a 16% SDS-PAGE gel; the protein was transferred to a polyvinylidene difluoride membrane, and the blot was probed with a 1/10,000 dilution of anti-hGX antiserum (33), followed by detection with ECL (Amersham Biosciences).

Phosphorylation of ERK1/2

Freshly isolated macrophages were suspended in PIPES buffer containing 1 mM CaCl₂ and 1 g/L glucose. The cells (10^6 /sample) were incubated with hGX (10 μ g/ml). The reactions were stopped at various time points (1–120 min) by adding ice-cold PIPES buffer, and the samples were microfuged for 30 s. Cell pellets were immediately lysed in lysis buffer, and Western blot for phosphorylated ERK1/2 was performed as previously described (15). To verify equal protein content of each sample, membranes were stripped with stripping buffer (7 M guanidine hydrochloride in distilled water) and then reblotted with anti-ERK2 Ab.

Statistical analysis

The data are expressed as the mean \pm SE of the indicated number of experiments. The *p* values were determined by *t* test for paired samples (45).

Results

Effects of sPLA₂s on TNF- α and IL-6 release from HLM

We first examined hGIB- and hGX-induced release of TNF- α and IL-6 from HLM. Macrophages were incubated (6 h, 37°C) with increasing concentrations (0.1–10 μ g/ml) of hGIB or hGX and with bvGIII, an isoform that does not induce TNF- α and IL-6 production from human blood monocytes (41). Both hGIB and hGX induced a concentration-dependent release of TNF- α and IL-6 (Fig. 1). The effects of both sPLA₂s became significant at 1 μ g/ml. In contrast, bvsPLA₂-III did not induce secretion of IL-6 and TNF- α even at 20 μ g/ml or when incubation was prolonged up to 24 h (data not shown).

Because the GIII used in these experiments was not of human origin, we also evaluated human GIII (hGIII)-induced cytokine release from HLM. The full-length hGIII consists of three separate domains with the central catalytic domain of 141 residues that is similar in structure to bvGIII (46). Because we have expressed the catalytic domain of hGIII (hGIII-cd) as a recombinant protein displaying PLA₂ enzymatic activity, we tested this protein on macrophages. As for bvGIII, incubation (6 h, 37°C) of HLM with hGIII-cd (up to 20 μ g/ml) did not induce IL-6 (1.52 ± 0.66 ng/mg protein vs 1.40 ± 0.60 in unstimulated cells) or TNF- α (2.10 ± 0.78 ng/mg protein vs 1.93 ± 0.80 in unstimulated cells) release.

To ascertain whether hGIB and hGX activate cytokine mRNA transcription in HLM, we initially explored IL-6 and TNF- α mRNA expression in these cells by semiquantitative RT-PCR. Fig. 2A depicts the β -actin- (first row), IL-6- (second row), and TNF- α - (third row) specific RT-PCR amplification products from an experiment representative of three in which macrophages were cultured for 3 h in medium alone or in the presence of 10 μ g/ml hGIB, bvGIII, and hGX. Both hGIB and hGX increased mRNA expression of IL-6 and TNF- α compared with that in unstimulated cells. In contrast, bvGIII, which did not induce cytokine release, did not influence IL-6 or TNF- α mRNA expression. To compare cytokine mRNA induction by sPLA₂s, real-time quantitative PCR

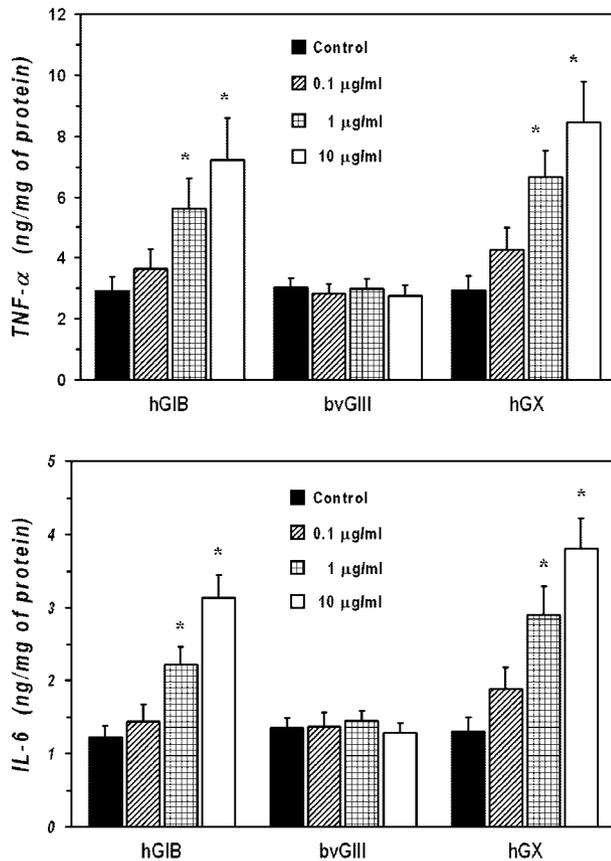


FIGURE 1. Effect of sPLA₂s on TNF- α and IL-6 release from HLM. Cells were incubated (37°C, 6 h) with hGIB, bvGIII, and hGX. TNF- α (upper panel) and IL-6 (lower panel) release was determined in the supernatants by ELISA. The data are the mean \pm SE of six experiments. *, $p < 0.05$ vs control.

was performed in three experiments in which HLM were stimulated with hGX (10 μ g/ml). Human GX increased TNF- α and IL-6 mRNA expression (Fig. 2B). The maximum effect was observed after 3 h for TNF- α expression and after 6 h for IL-6, suggesting that the two cytokines have different transcription kinetics in HLM. These experiments demonstrate that hGIB and hGX induce IL-6 and TNF- α expression and release from HLM.

Role of sPLA₂ enzymatic activity in cytokine production from HLM

We conducted two sets of experiments to understand whether the differential ability of sPLA₂s to induce cytokine production from macrophages could be related to their enzymatic activity. We first determined the release of AA induced by hGIB, bvGIII, and hGX from intact macrophages prelabeled with [³H]AA. In three experiments, at a concentration of 10 μ g/ml, hGX and bvGIII, but not hGIB, induced the release of a significant amount of AA from HLM (percentage of total cellular [³H]AA released in 1 h: hGIB, $3.1 \pm 0.7\%$; bvGIII, $6.0 \pm 1.1\%$ ($p < 0.05$ vs unstimulated); hGX, 6.6 ± 1.1 ($p < 0.05$ vs unstimulated); unstimulated, 2.9 ± 0.5). These results are in line with earlier studies showing that hGX and bvGIII, which bind to the extracellular face of mammalian cell membranes, but not hGIB, efficiently release [³H]AA when added exogenously to intact mammalian cells (36, 41, 44). Our data demonstrate that the capacity of each sPLA₂ to mobilize AA in macrophages is unrelated to their ability to induce cytokine production. In fact, hGIB induced TNF- α and IL-6 production, but did not mobilize AA, whereas bvGIII efficiently released AA, but did not

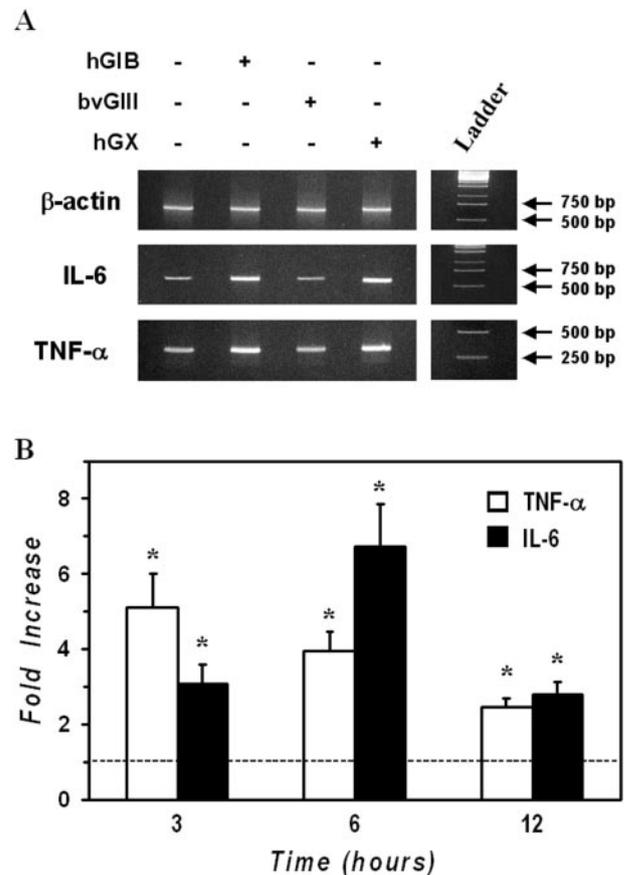


FIGURE 2. Effect of sPLA₂s on IL-6 and TNF- α mRNA expression in HLM. *A*, Semiquantitative RT-PCR, showing β -actin-, IL-6-, and TNF- α -specific amplification products from a representative experiment in which HLM were cultured for 3 h with RPMI 1640 alone or with 10 μ g/ml hGIB, bvGIII, or hGX. A 100-bp DNA ladder was used as the standard. Adequate normalization of RNA for each sample was confirmed by the equality of RT-PCR amplification products for β -actin gene expression. *B*, Real-time quantitative RT-PCR analysis of TNF- α and IL-6 mRNA expression in HLM cultured with 10 μ g/ml hGX for different time periods. RT-PCR was performed with SYBR Green technology as described in *Materials and Methods*. Human GX-induced expression of TNF- α and IL-6 was expressed as the fold increase vs that in untreated cells. The results were normalized for β -actin. The data are the mean \pm SE of three experiments. *, $p < 0.05$ vs untreated cells.

influence cytokine synthesis. Human GX induced both AA release and IL-6 and TNF- α production. It is possible that hGIB catalyzes the release of fatty acids other than AA from macrophage membranes and that such fatty acids may be linked to cytokine production. However, this hypothesis seems unlikely, because hGIB displays essentially no preference toward sn-2 fatty acyl chains with different degrees of unsaturation (36).

To explore further the role of enzymatic activity in sPLA₂-induced cytokine production, HLM were incubated (6 h, 37°C) with increasing concentrations of two catalytically inactive forms of GIB: an hGIB inactivated by exposure to BPB for 4 h at 37°C, which inhibits >98% of enzymatic activity (15), and an active site variant of pGIB in which the catalytic residue histidine 48 was replaced by glutamine (H48Q). This mutant (pGIB-H48Q) has minimal changes in its three-dimensional structure, stability, and M-type-R binding capacity, but it has essentially no catalytic activity (<0.01% the enzymatic activity of the wild-type (WT) protein, as determined by *E. coli* sPLA₂ activity assay) (36, 37, 47). Fig. 3 shows that both BPB-inactivated hGIB and pGIB-H48Q

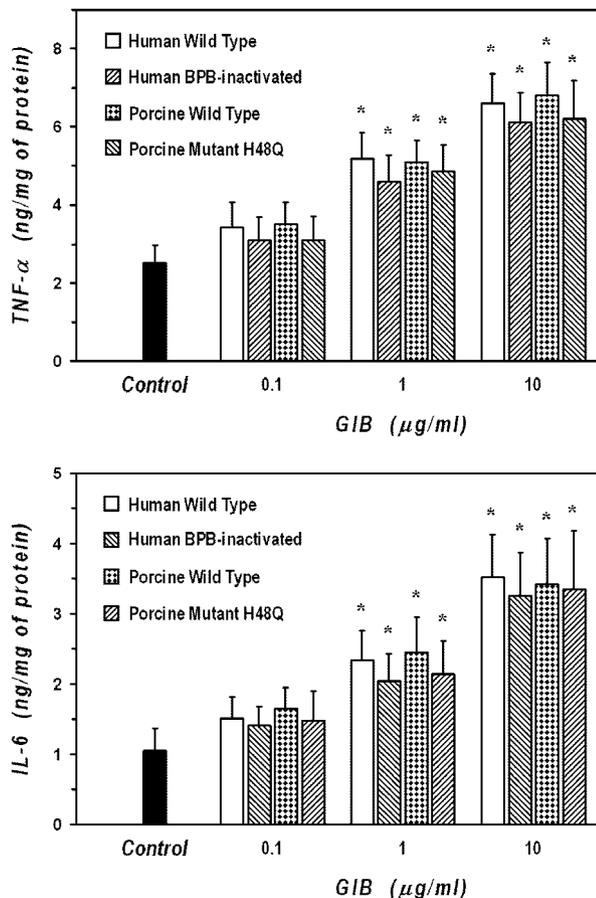


FIGURE 3. Effect of enzymatically active sPLA₂s and BPB-inactivated and mutant H48Q on TNF- α and IL-6 release from HLM. Cells were incubated (37°C, 6 h) with hGIB (human WT), BPB-inactivated hGIB, pGIB (porcine WT), and the H48Q mutant of pGIB. TNF- α (upper panel) and IL-6 (lower panel) release was determined in the supernatants by ELISA. The data are the mean \pm SE of four experiments. *, $p < 0.05$ vs control.

retain the ability to induce the release of TNF- α (upper panel) and IL-6 (lower panel) from HLM compared with the catalytically active, WT sPLA₂s of both human and porcine origins.

Thus, two distinct approaches demonstrate a lack of correlation between enzymatic activity and the cytokine-inducing ability of the sPLA₂s. It is also evident that catalytic activity is not required for sPLA₂-induced cytokine production in HLM.

Expression of M-type-R on HLM

Mouse GIB (mGIB) and mouse GX (mGX) are high affinity ligands for the mouse M-type-R (32, 34). In contrast, bvGIII does not bind to the rodent or human M-type-R (34). Although conclusive data on the binding of human sPLA₂ isoforms to the human receptor are lacking, the different abilities of the various sPLA₂s to induce cytokine release from HLM suggest that the M-type-R could be involved in this process. This receptor has been cloned from different mammalian species (21), and its mRNA has been detected in human monocytes (48). Because the expression of the M-type-R on tissue macrophages has not yet been studied, we investigated its presence on HLM using multiple experimental strategies.

We first evaluated the expression of M-type-R-specific mRNA by RT-PCR. A cDNA of the predicted size for M-type-R was amplified from mRNA of HLM (Fig. 4A). Sequencing the PCR fragments from macrophage cDNA revealed that they were identical

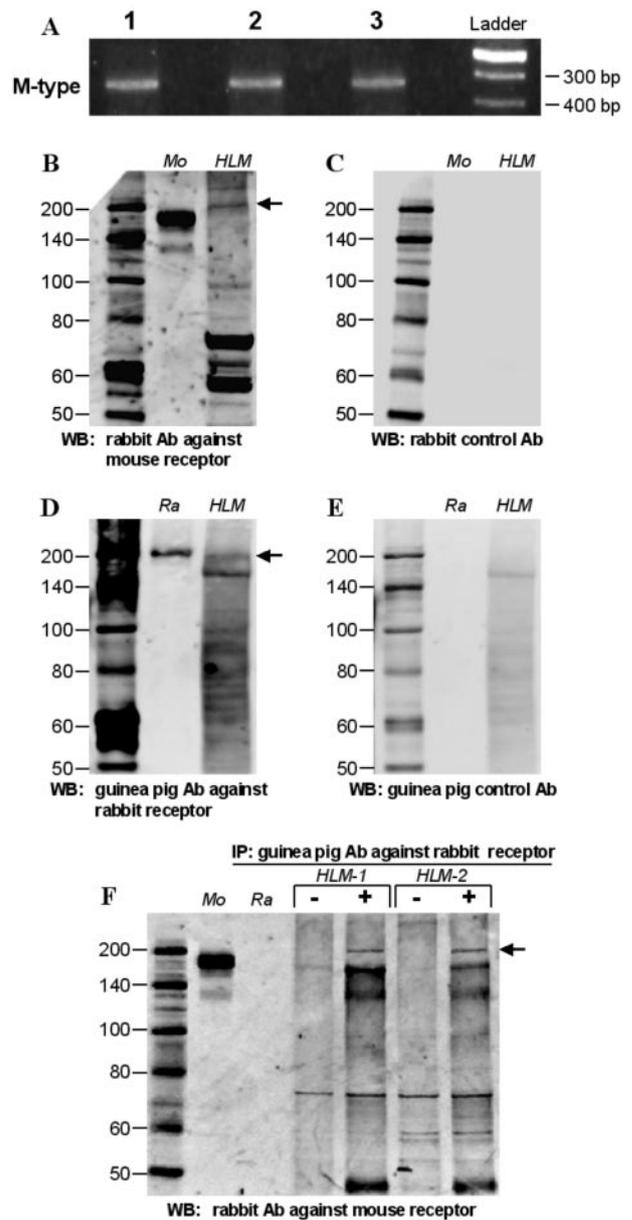


FIGURE 4. Expression of the M-type-R for sPLA₂ in HLM. A, M-type-R specific RT-PCR amplification products from HLM purified from three different lung preparations. The amplification protocol is described in *Materials and Methods*. A 100-bp DNA ladder was used as the standard. B–E, Western blot (WB) of the M-type-R protein in macrophage cell lysates. Total protein extracts together with a biotinylated protein ladder were separated and transferred to nitrocellulose as described in *Materials and Methods*. Membranes were blotted with rabbit Ab against mouse M-type-R (B), rabbit control Ab (C), guinea pig Ab against rabbit M-type-R (D), or guinea pig control Ab (E), then incubated with HRP-conjugated goat anti-rabbit IgG Ab (B and C) or goat anti-guinea pig IgG Ab (D and E). An HRP-conjugated anti-biotin Ab was used for detection of the M_r markers. Mouse (Mo) or rabbit (Ra) M-type-R proteins served as standards. F, Immunoprecipitation (IP) of the M-type-R protein in macrophage cell lysates. IP with control guinea pig Ab (lanes –) or with guinea pig Ab against the rabbit M-type-R (lanes +), extraction of immunoprecipitated cellular proteins, electrophoresis, and transfer to nitrocellulose were performed as described in *Materials and Methods*. Membranes were blotted with rabbit Ab against mouse M-type-R, then incubated with HRP-conjugated, goat anti-rabbit IgG Ab in the presence of an HRP-conjugated, anti-biotin Ab used for detection of the M_r markers. Mouse (Mo) or rabbit (Ra) M-type-R proteins served as standards.

with the published sequence of the membrane-bound, human M-type-R (25) and to the PCR fragments amplified in parallel from human kidney and placental cDNAs.

To determine whether the mRNA was translated into the M-type-R protein, proteins from whole macrophage lysates were separated, transferred to nitrocellulose and immunoblotted with two distinct sets of polyclonal Abs raised against rabbit and mouse M-type-R. The anti-mouse M-type Ab (Fig. 4B), but not the irrelevant Ab (Fig. 4C), detects an immunoreactive band with an M_r of ~180 kDa (arrow), which corresponds to that of the soluble mouse M-type-R used as standard. Similarly, in Fig. 4D, the anti-rabbit M-type-R Ab, but not the irrelevant Ab (Fig. 4E), recognized a band (arrow) that comigrated with the rabbit M-type-R. Nonspecific labeling between 55 and 70 kDa represents cross-reactivity of primary Abs. To further verify the specificity of the immunoreactive protein recognized by the two Abs, we immunoprecipitated the M-type-R from two additional macrophage lysates using the anti-rabbit M-type Ab. After electrophoresis and transfer, the membranes were immunoblotted with the anti-mouse M-type Ab. Fig. 4F shows that the anti-rabbit M-type-R Ab (lanes +), but not the irrelevant Ab (lanes -), immunoprecipitated a protein (arrow) that was recognized by the anti-mouse M-type Ab and comigrated with the mouse M-type-R. It should be noted that the Ab did not detect the rabbit M-type-R.

Finally, to confirm that the M-type-R was expressed on the macrophage surface, we performed flow cytometric analysis with the same Abs as those used for immunoblotting. HLM were incubated with the anti-rabbit M-type Ab from guinea pig or with the guinea pig purified control IgG and then stained with FITC-conjugated anti-guinea pig IgG Ab from goat. Fig. 5A shows that M-type-R on HLM is detected by anti-rabbit M-type Ab. Expression of M-type-R on macrophages was also detected by the rabbit serum obtained after immunization with mouse M-type-R, but not with the preimmune serum (Fig. 5B). These experiments demonstrate that HLM synthesize the mRNA for the human M-type-R and translate it into a protein that is expressed on the cell surface.

Effect of Me-indoxam on sPLA₂-induced cytokine production

Indoxam, a site-directed inhibitor of sPLA₂ enzymatic activity (49), also decreases the binding of pGIB and mGX to the mouse M-type-R (32, 50). The x-ray structure of the complex of hGX and Me-indoxam, a molecule closely related in structure to indoxam, shows that a portion of the inhibitor bound in the active site slot is exposed on the surface of the enzyme (M. H. Gelb, unpublished observations). Furthermore, mutagenesis studies have shown that residues on the sPLA₂ surface near the active site slot are involved in M-type-R binding (51). In the next group of experiments, we evaluated the effect of Me-indoxam on TNF- α and IL-6 release from HLM. Me-indoxam (0.01–10 μ M) did not affect the basal secretion of TNF- α and IL-6 from HLM (data not shown). Preincubation (15 min, 37°C) of hGX (1 μ g/ml) with various concentrations of Me-indoxam before the addition to HLM dose-dependently inhibited TNF- α (Fig. 6, upper panel) and IL-6 (Fig. 6, lower panel) release. The IC₅₀ values of Me-indoxam were 253 \pm 72 and 320 \pm 87 nM on TNF- α and IL-6 release, respectively. These values are consistent with the affinity of Me-indoxam for hGX based on the finding that this inhibitor blocks the enzymatic activity of this sPLA₂ with IC₅₀ values in the 1–2 μ M range (36).

To examine whether Me-indoxam blocks cytokine production by a mechanism unrelated to binding to hGX, we tested the effect of this compound on LPS-induced TNF- α and IL-6 release. Under the same experimental conditions in which it completely suppressed hGX-induced cytokine release, Me-indoxam did not affect LPS-induced release of TNF- α (1 μ g/ml LPS, 14.58 \pm 3.25 ng/mg

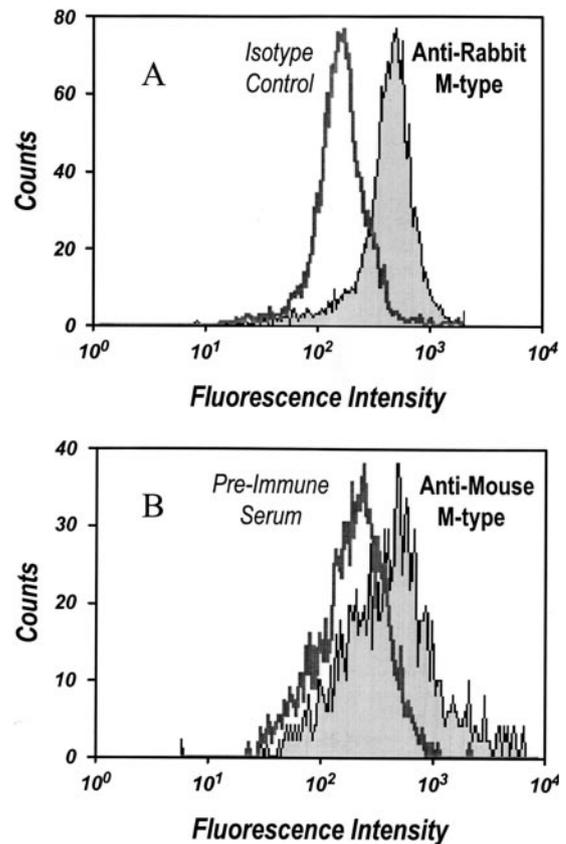


FIGURE 5. FACS analysis of M-type-R expression on HLM. Freshly isolated HLM were incubated (4°C, 20 min) with guinea pig anti-rabbit M-type-R Ab or guinea pig isotype-matched Ab (A) and with rabbit anti-mouse M-type-R Ab or rabbit preimmune serum (B). Cell pellets were stained (4°C, 20 min) with secondary Abs (FITC-conjugated, goat anti-guinea pig IgG (panel A) or PE-conjugated donkey anti-rabbit IgG (B)).

protein; 10 μ M Me-indoxam plus 1 μ g/ml LPS, 15.13 \pm 4.06) and IL-6 (1 μ g/ml LPS, 8.54 \pm 1.62 ng/mg protein; 10 μ M Me-indoxam plus 1 μ g/ml LPS, 7.93 \pm 1.86). Thus, Me-indoxam blocks sPLA₂-promoted cytokine production, but not that promoted by LPS. Importantly, the results with Me-indoxam also indicate that hGX-induced cytokine production is not due to the contamination by endotoxin that may be present in the recombinant protein produced by bacterial expression.

As noted above, Me-indoxam not only blocks the binding of sPLA₂s to the M-type-R, but it also blocks the catalytic activity of various sPLA₂s, i.e., GIB, GIIA, GV, and GX (36). To discriminate among these possibilities, we determined whether Me-indoxam was able to block cytokine production induced by the catalytically inactive pGIB-H48Q mutant. In these experiments, WT hGIB, WT pGIB, and pGIB-H48Q (1 μ g/ml) were preincubated (15 min, 37°C) with an optimal concentration (10 μ M) of Me-indoxam before adding them to HLM. Me-indoxam completely suppressed the release of TNF- α and IL-6 induced by all three proteins (Fig. 7).

The blockade of hGIB- and pGIB-induced cytokine production by Me-indoxam occurs in the low micromolar range (10 μ M). This is consistent with Me-indoxam binding to the active site of these sPLA₂s, given that this compound inhibits their enzymatic activity with an IC₅₀ of 0.2–6 μ M depending on the in vitro enzymatic activity assay used (36). The fact that Me-indoxam also inhibits the effect of the pGIB-H48Q mutant implies that it is still able to bind Me-indoxam despite its mutation in the active site. This is consistent with our finding that the H48Q mutant of hGIIA binds to

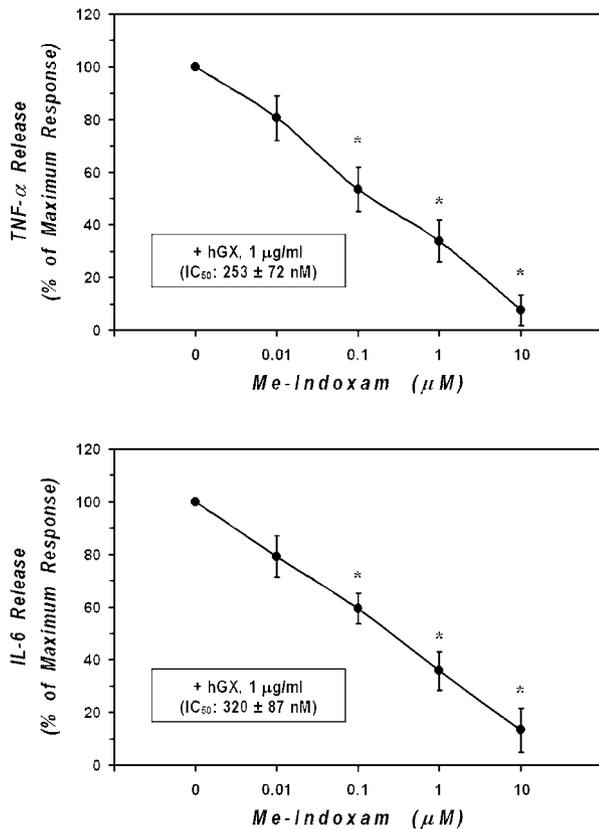


FIGURE 6. Effect of Me-indoxam on hGX-induced TNF- α and IL-6 release from HLM. Human GX (1 μ g/ml) was incubated (37°C, 15 min) with or without Me-indoxam before being added to HLM. TNF- α (upper panel) and IL-6 (lower panel) release was determined in the supernatants by ELISA. The values are expressed as the percent inhibition of the maximum response induced by hGX alone. Cytokine release induced by hGX in the absence of Me-indoxam was 5.47 ± 1.23 and 2.37 ± 0.48 ng/mg protein for TNF- α and IL-6, respectively. The data are the mean \pm SE of four experiments. *, $p < 0.05$ vs hGX alone.

Me-indoxam, although with a lower affinity than the WT enzyme (S. Bezzine and G. Lambeau, unpublished observations). Importantly, the observation that Me-indoxam blocks cytokine production induced by the catalytically inactive pGIB-H48Q mutant indicates that this effect is due to the ability of Me-indoxam to block sPLA₂ binding to a receptor, rather than to inhibition of sPLA₂ enzymatic activity.

The group II subfamily of the sPLA₂s may regulate some cellular functions through perturbation of the plasma membrane (20). Although this effect has not been reported for GIB and GX, it is conceivable that cytokine production induced by these sPLA₂s is related to their ability to bind and perturb the macrophage membrane. Our observation that Me-indoxam blocks sPLA₂-induced TNF- α and IL-6 release suggests that cytokine production is not simply due to sPLA₂ binding to the macrophage membrane, because interaction of Me-indoxam with the active site of sPLA₂ should not prevent binding of the protein to membranes (52). However, we addressed this issue by examining whether Me-indoxam altered the affinity of hGX to phospholipid vesicles. We used mixed diether phospholipid vesicles so that binding experiments could be conducted in the presence of calcium (44). Vesicles were pelleted by ultracentrifugation, and the amount of hGX remaining in the supernatant was determined by immunoblot analysis (enzymatic assay was not used because of the inhibition by Me-indoxam). Previous studies showed that hGX binds to these vesicles

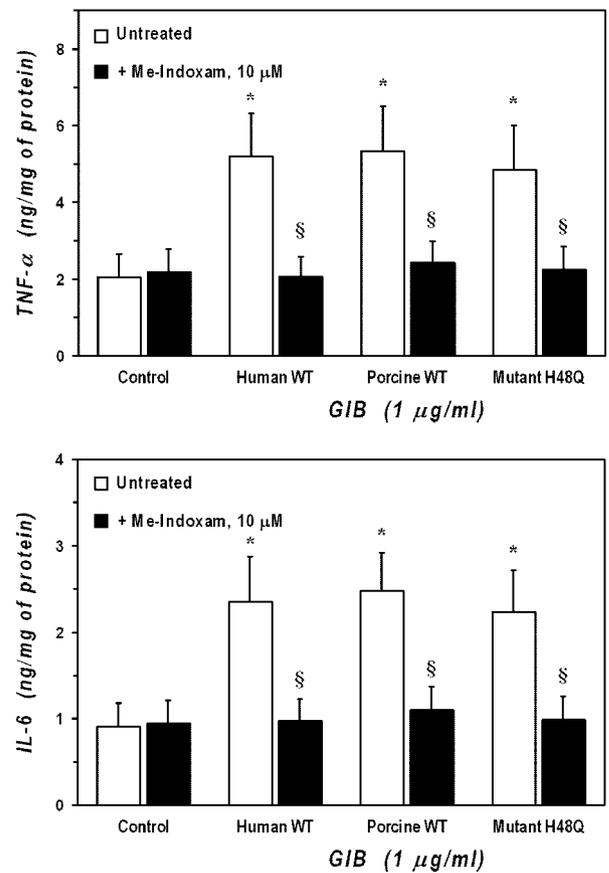


FIGURE 7. Effect of Me-indoxam on TNF- α and IL-6 release induced by hGIB, pGIB, and mutant H48Q of pGIB from HLM. Human GIB, pGIB, and H48Q mutant of pGIB (1 μ g/ml) were incubated (37°C, 15 min) with or without 10 μ M Me-indoxam before being added to HLM. TNF- α (upper panel) and IL-6 (lower panel) release was determined in the supernatants by ELISA. The data are the mean \pm SE of four experiments. *, $p < 0.05$ vs control; §, $p < 0.01$ vs respective untreated.

with a dissociation constant of 0.1 mM (44). Consistent with this earlier result, the amount of hGX in the supernatant decreased as the phospholipid concentration increased from 0 to 1 mM, and about half the enzyme remained unbound at 0.1–0.2 mM phospholipid (Fig. 8). Moreover, 50 μ M Me-indoxam, which is more than sufficient to saturate the active site of hGX, did not effect the degree of phospholipid vesicle binding.

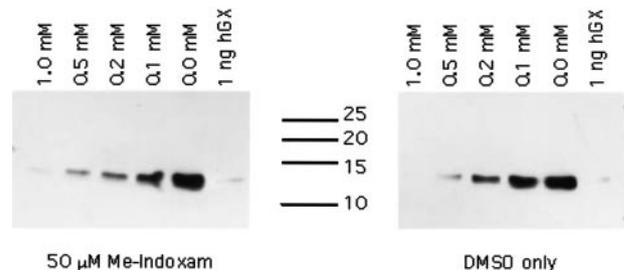


FIGURE 8. Binding of hGX to phospholipid vesicles. Sucrose-loaded vesicles of diether phospholipids were incubated with hGX in the presence (left panel) or the absence (DMSO vehicle only; right panel) of 50 μ M Me-indoxam. Vesicles were sedimented by centrifugation, and the amount of sPLA₂ remaining in the supernatant was analyzed by immunoblotting. Human GX (1 ng) served as a standard. The concentrations given at the top of the blots are the total phospholipid concentrations in the binding mixture.

Effect of anti-MR and anti-M-type-R Abs on sPLA₂-induced cytokine production

The above-mentioned data suggested that a receptor-based mechanism may be responsible for sPLA₂-induced cytokine production. Human macrophages express, in addition to the M-type-R, the MR. MR is closely related in structure to the M-type-R (53), and it has been shown to be involved in the activation of HLM by hGIIA (28). Because an anti-MR (PAM-1) is a blocking Ab that effectively inhibited hGIIA-induced exocytosis in HLM (28), we explored whether PAM-1 or the anti-rabbit M-type-R influenced cytokine production. Fig. 9 shows that neither PAM-1 nor the anti-M-type-R influenced the production of TNF- α induced by both hGIB and hGX. Similar results were obtained when the release of IL-6 was examined (data not shown). These results suggest that sPLA₂s activate cytokine production by binding to sites distinct from those recognized by the Abs on the above receptors or by interacting with as yet unknown membrane targets.

Role of ERK1/2 in sPLA₂-induced cytokine production

Previous studies indicated that sPLA₂s may induce activation of ERK1/2 in various cell types, including mast cells (22), macrophages (54), monocytes (48), and eosinophils (15). This effect is independent from sPLA₂ enzymatic activity and presumably involves the interaction of sPLA₂s with binding sites (22, 48, 55, 56). To explore the intracellular mechanisms responsible for sPLA₂-induced cytokine production, we investigated whether hGX can activate ERK1/2 in HLM. The reactions were stopped at various time points, and the cytosolic extracts were subjected to Western blot with anti-phospho-ERK1/2 Ab. Phosphorylation of ERK1/2 induced by hGX became evident after 1 min and increased up to 120 min (Fig. 10A, upper gel). Reprobing the membrane with the anti-ERK2 Ab (nonphosphorylated form) confirmed equal protein loading (Fig. 10A, lower gel).

To verify that activation of ERK1/2 was required for cytokine production induced by the sPLA₂s, HLM were incubated with a specific inhibitor of the ERK1/2 pathway (PD98059) before stimulation with hGIB and hGX. PD98059 inhibited the release of IL-6 induced by both sPLA₂s in a concentration-dependent fashion, with the maximum effect (~70%) at 50 μ M (Fig. 10B). These results indicate that hGIB and hGX elicit ERK1/2 phosphorylation

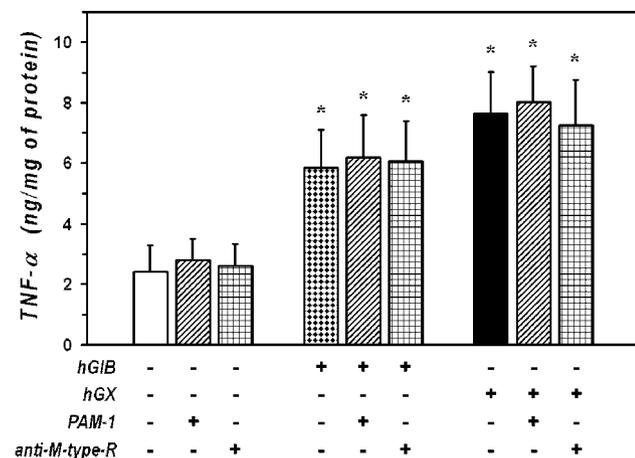


FIGURE 9. Effects of PAM-1 and anti-M-type-R Ab on TNF- α release induced by hGIB and hGX from HLM. Cells were preincubated (4°C, 20 min) with PAM-1 (▨; 10 μ g/ml) or anti-rabbit M-type-R Ab (▩; 10 μ g/ml), then stimulated (37°C, 6 h) with hGIB or hGX (1 μ g/ml). TNF- α release was determined in the supernatants by ELISA. The data are the mean \pm SE of four experiments. *, $p < 0.05$ vs control.

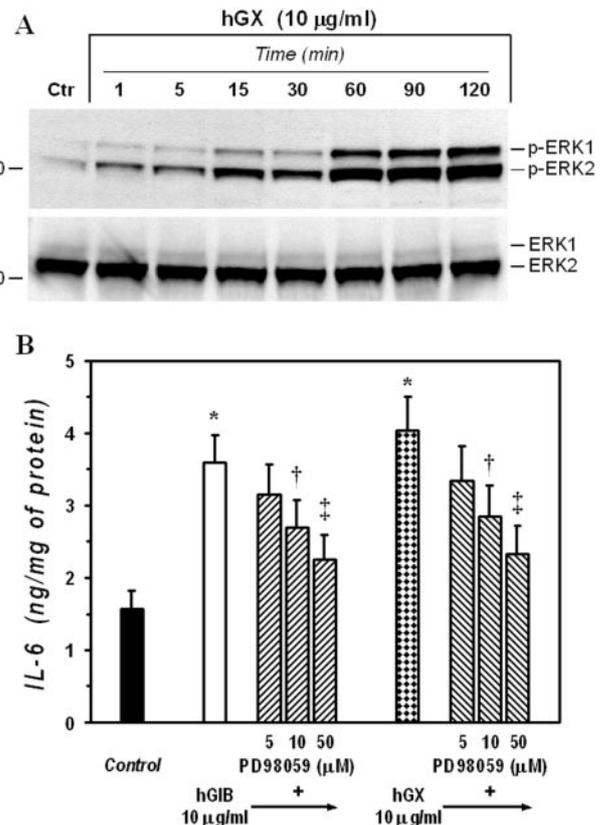


FIGURE 10. Role of ERK1/2 in sPLA₂-induced cytokine production from HLM. *A*, Kinetics of ERK1/2 phosphorylation induced by hGX. HLM were stimulated with hGX (10 μ g/ml) for the times indicated. Reactions were stopped with the addition of ice-cold PIPES buffer. The cells were microfused, lysed, and subjected to Western blot analysis with antiphosphorylated-ERK1/2 Ab (upper gel) as described in *Materials and Methods*. Stripped membrane was reblotted with anti ERK2 Ab (lower gel) to confirm equal protein content of each sample. The Western blot shown is representative of two separate experiments. *B*, Effect of PD98059 on IL-6 release induced by hGIB and hGX from HLM. Cells were preincubated (37°C, 30 min) with PD98059 (5–50 μ M) and then stimulated (37°C, 6 h) with hGIB or hGX (10 μ g/ml). IL-6 release was determined in the supernatants by ELISA. The data are the mean \pm SE of four experiments. *, $p < 0.05$ vs control; †, $p < 0.05$ vs respective sPLA₂; ‡, $p < 0.01$ vs respective sPLA₂.

in HLM and that activation of this pathway is required for cytokine production induced by both sPLA₂s. However, the observation that PD98059 did not completely block IL-6 release suggests that other intracellular pathways may be involved in sPLA₂-induced cytokine production in HLM.

Discussion

We have examined the effects of hGIB and hGX on primary macrophages isolated from human lung parenchyma. Both hGIB and hGX promoted TNF- α and IL-6 production from HLM, as demonstrated by enhanced cytokine mRNA expression and protein levels in the culture medium. This effect was unrelated to sPLA₂ enzymatic activity and required activation of the ERK1/2 pathway. We also provide the first demonstration that HLM express the M-type-R for sPLA₂s and that an inhibitor of sPLA₂/M-type-R interaction effectively blocks sPLA₂-induced cytokine production.

Our observations assume particular relevance because hGIB and hGX are expressed in the human lung (29, 30) together with the M-type-R (25, 30), they are released during lung inflammation (6,

11), and the cytokines they induce play a crucial role in inflammatory diseases of the lung (27).

Various mechanisms could be involved in sPLA₂-induced cytokine production by HLM. We first examined whether this effect could be due to sPLA₂ enzymatic activity. Two results obtained in this study demonstrate that generation of lipid mediators is not the basis for cytokine production in human macrophages treated with sPLA₂s. First, there is no relationship between the ability of sPLA₂s to release AA and their capacity to induce cytokine production. Second, two different isoforms of GIB (BPB-inactivated hGIB and the H48Q mutant of pGIB), which are devoid of catalytic activity, induce cytokine production from lung macrophages. In particular, it is possible that the H48Q mutant has lipolytic activity below the detection level of conventional sPLA₂ enzymatic assays and therefore may produce trace amounts of lipid mediators in macrophage membranes. However, this is not the case, as shown by the very similar dose-response curves of cytokine production by enzymatically active and inactive sPLA₂s (Fig. 3). Taken together, these data exclude sPLA₂ lipolytic activity as a primary mechanism in the activation of cytokine synthesis in macrophages.

Various research groups have shown that mammalian sPLA₂s regulate some cellular functions by mechanisms involving interactions with membrane targets, such as the M-type-R (13, 22, 24). The mRNA for the M-type-R is expressed in human kidney, lung, pancreas, placenta, and skeletal muscle (25, 30). Although the presence of the M-type-R has been reported in human neutrophils and monocytes (23, 48), the resident cells expressing this receptor in human tissues have not been identified. We provide in this study the first demonstration that the M-type-R is expressed in HLM. In fact, we show that these cells express the M-type-R mRNA that is translated into a protein of the appropriate M_r expressed on the macrophage surface, as assessed by immunoblotting, immunoprecipitation, and flow cytometry using anti-M-type Abs from two species.

Interestingly, our results indicate that the two distinct human sPLA₂s (GIB and GX), which can engage the M-type-R in the mouse (32, 34), activate TNF- α and IL-6 production in macrophages. Conversely, two other sPLA₂s (bvGIII and hGIII-cd), which are not able to bind to the mouse M-type-R (34) (G. Lambeau and S. Bezzine, unpublished observations) do not induce cytokine synthesis. These observations prompted the hypothesis that cytokine production by a subset of sPLA₂s involves their binding to the M-type-R. This hypothesis is supported by the data obtained with Me-indoxam showing that, together with the related molecule, indoxam is the only pharmacological agent known to affect the binding of sPLA₂s to M-type-R. Even though the use of these compounds in probing sPLA₂-R events is complicated by the fact that they also inhibit the lipolytic activity of sPLA₂s, Me-indoxam blocks cytokine production induced by both catalytically active and inactive sPLA₂s. Furthermore, Me-indoxam probably blocks cytokine production by specific binding to sPLA₂s based on the observations that the IC₅₀ values for inhibition of cytokine release induced by hGX are comparable to those for inhibition of *in vitro* hGX enzymatic activity (36) and for binding of mGX to the M-type-R (670 nM) (32). However, we cannot exclude that hGIB and hGX activate cytokine production by binding to an unknown HLM membrane target distinct from the M-type-R. Finally, Me-indoxam does not interfere with the basal production of TNF- α and IL-6 from macrophages, and it does not function as a general inhibitor of cytokine synthesis.

Additional support for the hypothesis that sPLA₂s induce activation of HLM by a receptor-based mechanism derives from the data on the involvement of ERK1/2 in cytokine production.

ERK1/2 are major kinases whose activation is required for the synthesis of IL-6 and TNF- α in LPS-stimulated HLM (57). Our results indicate that activation of ERK1/2 induced by sPLA₂s is necessary for cytokine production. These results are line with previous studies showing that sPLA₂s activate ERK1/2 by a nonenzymatic mechanism that presumably involves interaction with membrane targets (15, 22, 48, 55, 56).

Thus, a number of observations in this paper provide indirect evidence that sPLA₂-induced cytokine production involves the M-type-R expressed on HLM or another unknown receptor target: 1) hGIB and hGX, which are ligands for M-type-R in the murine system, induce cytokine release, whereas GIII does not bind to M-type-R and does not induce cytokine secretion; 2) this effect does not require sPLA₂ enzymatic activity; 3) Me-indoxam, which prevents sPLA₂/M-type-R interaction, blocks cytokine release; and 4) sPLA₂s activate the ERK1/2 pathway, which is associated with membrane signaling. The use of siRNA or antisense oligonucleotides to decrease the level of M-type-R would help to determine the role of this receptor in mediating the effects of sPLA₂s.

The M-type-R is homologous to the macrophage MR, the DEC-205 receptor, and the endothelial cell lectin λ receptor, also called endo-180 (53), suggesting that sPLA₂s may act by binding to one of these receptors expressed on HLM. We previously implicated the MR in exocytosis induced by hGIIA (28). However, an mAb against the MR (PAM-1) that blocks hGIIA-induced exocytosis (28) does not affect IL-6 and TNF- α production induced by this sPLA₂ (F. Granata, unpublished observations) and by hGIB and hGX (Fig. 9) making it unlikely that the MR is involved in the effects of sPLA₂ on cytokine production. It will be interesting to explore whether sPLA₂s activate different functions in human macrophages by interacting with distinct receptors.

Finally, it is well established that sPLA₂s must bind to the membrane through the interfacial binding surface to access their active sites to the highly water-insoluble phospholipid substrates (58). This raises the possibility that cytokine production by sPLA₂-treated macrophages results from perturbation of the macrophage membrane. However, the ability of the active site-directed inhibitor, Me-indoxam, to block hGIB- and hGX-induced cytokine production at doses consistent with its affinity for the active site of these sPLA₂s (Figs. 6 and 7) strongly argues against membrane attachment as the basis for cytokine production. In fact, most of the active site-directed inhibitors of sPLA₂s do not cause desorption of the enzyme from the interface (52). However, we also demonstrate that hGX binding to liposomes is not influenced by Me-indoxam at concentrations sufficient to saturate the active site of the enzyme, thereby excluding membrane perturbation as a cause of sPLA₂-induced cytokine production.

In conclusion, our data demonstrate that two human isoforms of sPLA₂ (GIB and GX) expressed in the human lung are potent inducers of TNF- α and IL-6 production in HLM via a mechanism independent of their enzymatic activity that involves activation of the ERK1/2 pathway. Human lung macrophages express the M-type-R for sPLA₂s at both mRNA and protein levels. Our results do not conclusively prove that cytokine production induced by sPLA₂s involves their binding to the M-type-R. However, they suggest that sPLA₂s act by a receptor-operated mechanism, as additional support for a role of sPLA₂s as ligands, not only as enzymes (21). Finally, these results reinforce the role of sPLA₂s as mediators of lung inflammation and identify these molecules as potential targets for pharmacological interventions.

Acknowledgments

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