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Stimulation of receptor-coupled phospholipase A_2 by interferon-γ

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The biomolecular mechanisms that mediate signal transduction by type II (γ) interferon (IFN) are poorly understood. IFN-γ is a potent growth inhibitory cytokine also endowed with antiviral, immunomodulatory, and differentiating activities on various cell targets, including neural cells. IFN-γ induced a rapid and transient activation of phospholipase A_2 in LAN-5, a human neuroblastoma cell line. A consequence of phospholipase A_2 activation was the release of arachidonic acid and the generation of lysophospholipids from membrane phospholipids. Treatment of pre-labeled LAN-5 cells with a receptor-saturating concentration of IFN-γ led to a time-dependent release of [^{3}H]arachidonic acid into the culture media and generation of [^{32}P]lysophosphatidylcholine. Pretreatment of cultures with the phospholipase A_2 inhibitor, bromophenacyl bromide, markedly inhibited both [^{3}H]arachidonic acid release and lysophosphatidylcholine production induced by IFN-γ treatment. Pretreatment of LAN-5 cells with nordihydroguaiaretic acid, a lipoxygenase inhibitor, or with indomethacin, a cyclooxygenase inhibitor, amplified the release of [^{3}H]arachidonic acid and production of lysophosphatidylcholine induced by non-saturating concentrations of IFN-γ. In parallel, and with the same time-dependent effect, a significant decrease in phosphatidylcholine labeling was observed in IFN-γ-treated cells, further indicating that a potential signal transduction mechanism of IFN-γ is the hydrolysis of membrane phosphatidylcholine by phospholipase A_2.

Interferon-γ; Phospholipase A_2; Indomethacin; Nordihydroguaiaretic acid (NDGA); Neuroblastoma

1. INTRODUCTION

IFN-γ, a lymphokine produced by activated T lymphocytes and NK cells, exerts a variety of biologic effects, including antiviral, immunomodulatory, and antiproliferative activities [1,2]. Moreover, IFN-γ interacts with cells other than those of the immune system, such as those of the nervous system, and may have a role in the interaction between the nervous and immune systems [3], and exhibits differentiation-promoting effects on neuronal cells [4]. IFN-γ exerts its biological functions via a cell-surface receptor, recently shown to be an ubiquitous protein occurring in different molecular forms on human cells [5]. Recent studies suggest that the signal transduction mechanism triggered by IFN-γ may involve a role for a GTP-binding protein [6] and/or that of protein tyrosine/serine kinases [7], including protein kinase C [8] and Ca^{2+}/calmodulin-dependent kinase pathways [9]. However, the intracellular mechanism(s) that mediate IFN-γ-induced responses have yet to be fully elucidated, particularly as it relates to the induction of cell differentiation. Increasing evidence supports a role for arachidonic acid (AA) metabolites in the control of neuronal cell differentiation and, although IFN-γ initiates its action through binding to a specific receptor distinct from that recognizing IFN-α/β [10], very recently it has been shown that IFN-α induced the transient activation of phospholipase A_2 (PLA_2) in 3T3 fibroblasts [11]. Since stimulation of PLA_2 is a common source of second messengers for receptor-mediated signaling [12,13], we tested whether activation of PLA_2 followed IFN-γ stimulation of human neuroblastoma cells.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

All lipid standards and inhibitors were purchased from Sigma (St. Louis, MO, USA). Human recombinant IFN-γ was obtained from Genzyme Corp. (Boston, MA, USA). IFN-γ (1,000 U/μl) was kept in aliquots in phosphate-buffered saline (PBS) (Flow Laboratories, Milan, Italy) plus 0.1% bovine serum albumin (BSA) at −80°C.

2.2. *Cell cultures*

The LAN-5 NB cell line was a gift from R. Seeger [14]. Cells were maintained in the logarithmic phase of growth in 75-cm^2 plastic culture flasks (Falcon Plastic, Oxnard, CA, USA) in RPMI 1640 medium (Flow) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Seromed, Biochrom KG, Berlin, Germany), sodium penicillin G (50 IU/ml), and streptomycin sulfate (50 μg/ml) (complete medium) at 37°C in a 5% CO_2–95% air humidified incubator. Cells were split following treatment with 1 mM EDTA in Hank's salts solution (Flow), washed, counted, and re-plated in fresh complete medium.

2.3. *Release of cellular [^{3}H]AA in LAN-5 cells*

LAN-5 cells (5×10^5/well in 24-well plates) were labeled for 24 h at 37°C with [^{3}H]AA (0.1 mCi/ml, Amersham) in RPMI 1640 complete medium. After labeling, monolayer cells were washed three times with complete medium at 37°C. For time-course experiments, RPMI 1640 with IFN-γ (1,000 U/ml) was added at time zero after washing. At the different time points examined, aliquots from quadruplicate wells for each time were measured for [^{3}H]AA released into the culture medium using a β scintillation counter. [^{3}H]AA release was dose-dependent and

maximal between 800 and 1,000 U of IFN-γ per ml. In order to confirm uptake of label into appropriate pools, [^{3}H]AA-labeled phospholipids were analyzed by thin layer chromatography [15]. Typically, about 35–40% of added [^{3}H]AA was incorporated into lipids. Under these condition, the lipid profile of incorporated [^{3}H]AA was phosphatidylcholine (PC) (53 ± 2%), lysoPC (10 ± 2%), phosphatidylethanolamine (PE) (10 ± 3%), lysoPE (9 ± 3%), phosphatidylinositol (PI) (11 ± 2%), and phosphatidyl- serine (PS) (8 ± 2%). For the inhibition experiments, after washing, labeled LAN-5 cells were pretreated for 10 min at 37°C with bromophenacyl bromide (BPB) (10 μM), and then treated with or without 1,000 U/ml IFN-γ, or pretreated for 10 min at 37°C with indomethacin (INDO) (10 μM) or nordihydroguaiaretic acid (NDGA) (10 μM) and then treated with or without 200 U/ml IFN-γ. After 2 min and 30 s at 37°C aliquots were counted for [^{3}H]AA release as above.

2.4. *Production of lysoPC in LAN-5 NB cell line*

LAN-5 cells (1×10^6 cells/25-cm^2 flask) were labeled overnight at 37°C with 20 μCi/ml of carrier-free [^{32}P]orthophosphate (Amersham, 10 mCi/ml) in phosphate-free RPMI 1640 supplemented with 10% dialyzed FCS. After labeling, monolayers were washed three times with RPMI 1640 at 37°C, and treated at 37°C for different times with or without IFN-γ (1,000 U/ml). At indicated time points the medium was aspirated and cells harvested by scraping in ice-cold PBS, and labeled lipids extracted [16]. Chromatographic separation of the major phospholipid subclasses was carried out on Silica Gel LK5 TLC plates (Whatman) [17]. For the separation of lysophospholipids, LK6 TLC plates were developed as described [15]. LysoPS and lysoPI were run together in this system. The ^{32}P-labeled phospholipids were visualized by autoradiography and identified by co-chromatography with unlabeled standards detected by iodine vapour. To test the effects of INDO and NDGA on the production of lysoPC, LAN-5 cells were labeled as described above. After washing, cells were pretreated for 10 min at 37°C with BPB (10 μM) and then stimulated or not with 1,000 U/ml IFN-γ, or pretreated with INDO (10 μM) or NDGA (10 μM) and then stimulated or not with 200 U/ml IFN-γ. After 5 min at 37°C the cells were extracted for lipids analysis and processed as above.

3. RESULTS AND DISCUSSION

It has been previously shown that IFN-γ is able to promote concentration-dependent growth inhibition of a neuroblastoma cell line, LAN-5, together with striking morphological changes and modulation of membrane and cytoskeletal proteins [18]. IFN-γ-induced LAN-5 cells rapidly acquired a pattern similar to that observed in mature ganglioneurocytes [18]. This cell line expresses high levels of the high-affinity IFN-γ receptor ($K_d = 4.53 \pm 0.19 \times 10^{-10}$, $B_{max} = 7{,}164 \pm 377$ sites) in agreement with data in other unrelated models [5].

A consequence of membrane PLA_2 activation is the release of AA from membrane phospholipids, typically from phosphatidylcholine (PC) or phosphatidylinositol (PI) [12]. Arachidonic acid can then be metabolized to prostaglandins, thromboxanes, and leukotrienes via cyclooxygenase- or lipoxygenase-catalyzed reactions, respectively [19]. Treatment of [^{3}H]AA-labeled LAN-5 cells with a receptor-saturating concentration of IFN-γ (1,000 U/ml) led to a rapid and time-dependent release of [^{3}H]AA into the culture media (Fig. 1A). Pretreatment of cultures with the PLA_2 inhibitor, bromophenacyl bromide (BPB, 10 μM) markedly inhibited both basal and stimulated [^{3}H]AA release (Fig. 1B), clearly

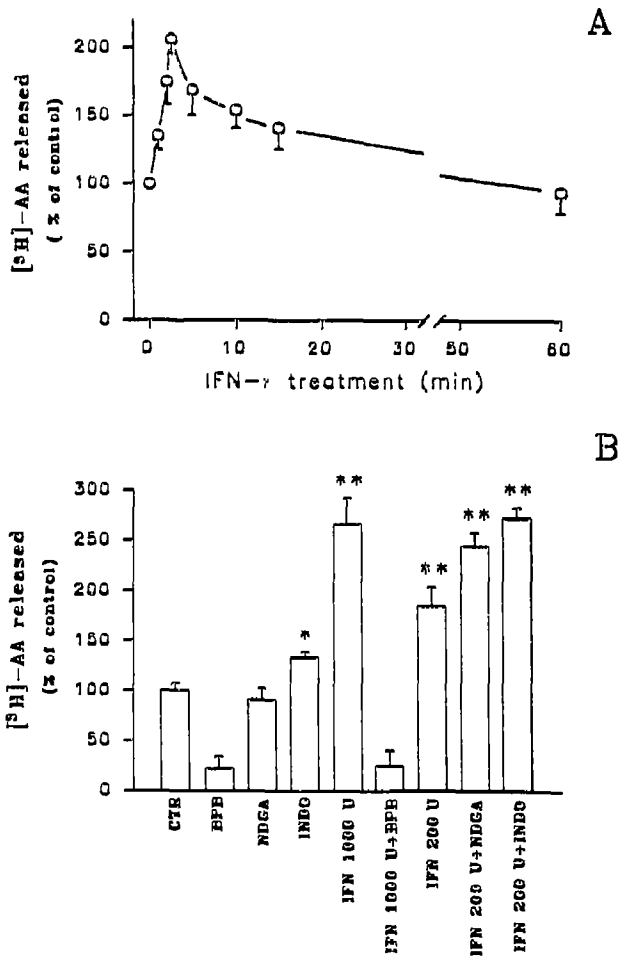

Fig. 1. Stimulation of release of cellular [^{3}H]AA by IFN-γ in LAN-5 cells. (A) Time-dependent release of [^{3}H]AA by IFN-γ treatment. (B) Effects of inhibitors of PLA_2, cyclooxygenase, and lipoxygenase enzymes (BPB, INDO, and NDGA, respectively) on the release of [^{3}H]AA induced by treatment with IFN-γ for 2.5 min. The results in A are representative of five independent experiments, and those in B represent three independent experiments each done in duplicate ($^*P < 0.05$; $^{**}P < 0.001$, evaluated by Student's t-test for unpaired data).

indicating a constitutive activation of PLA_2 in our model as well as an increase in AA metabolism in response to IFN-γ. The possibility of an AA release by phospholipase C activation and subsequent deacylation of diacylglycerol in response to IFN-γ has been previously ruled out [20].

Further evidence of PLA_2 activation in response to IFN-γ was obtained by measuring [^{3}H]AA release in LAN-5 cells treated with subsaturating amounts of IFN-γ (200 U/ml) after pretreatment with NDGA (10 μM), a lipoxygenase inhibitor, or INDO (10 μM), a cyclooxygenase inhibitor. Both inhibitors led to a marked amplification of the release of [^{3}H]AA induced by non-saturating IFN-γ treatment (Fig. 1B) producing a relative amount of [^{3}H]AA released identical to that induced by saturating concentrations of IFN-γ. Pretreatment of LAN-5 cells with 50 μM BW577C, an

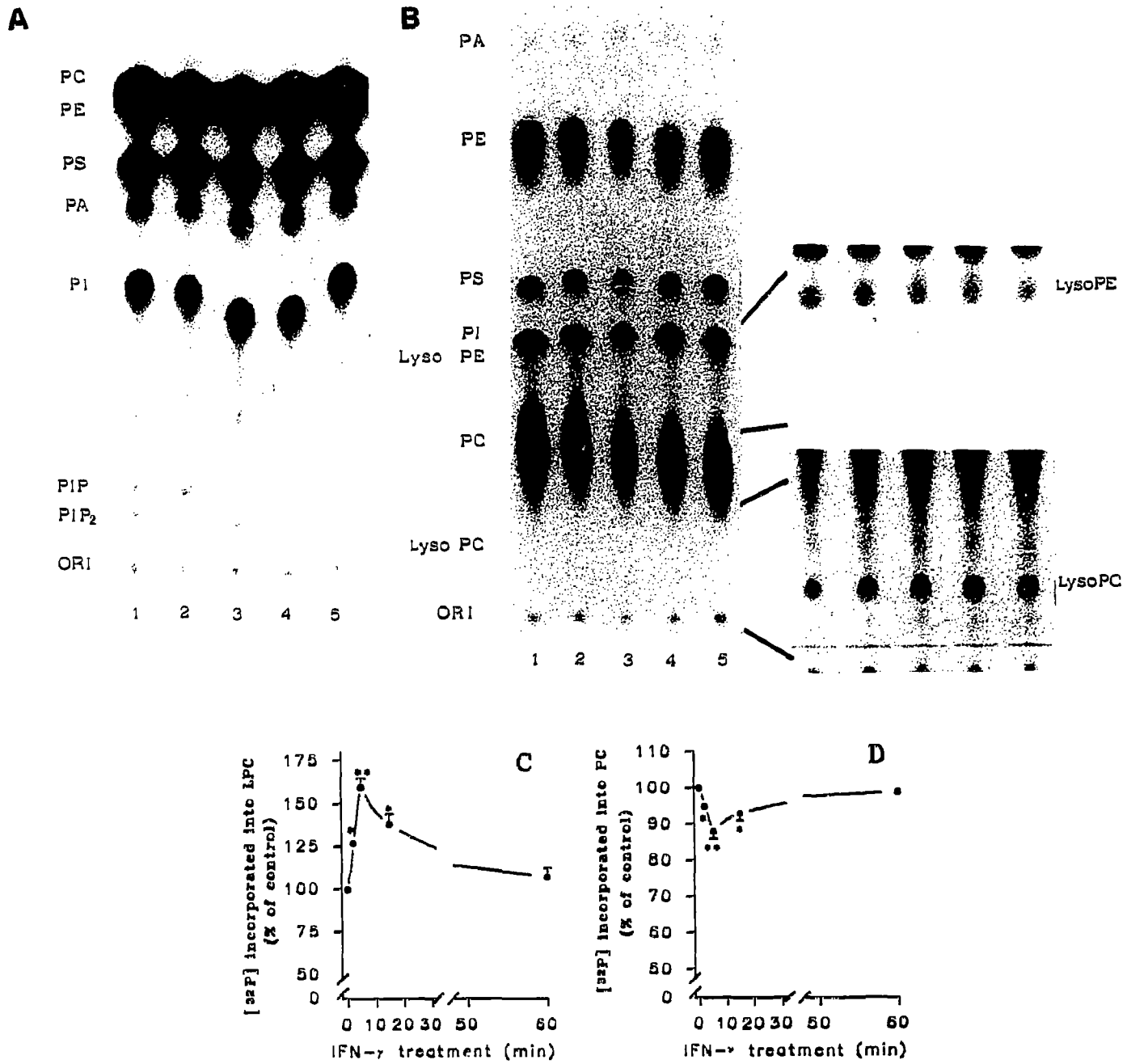

Fig. 2. (A) Chromatographic separation of the major phospholipid subclasses. (B) Separation of lysophospholipids. The TLC plates were exposed to Kodak X-AR-5 films for 1 day (A and B, magnification ×1) or for 3 days (B insets, magnification ×1.5). Lane 1, untreated cells; lanes 2–5, cells treated with IFN-γ for 2.5, 5, 15 and 60 min, respectively. Quantification of ^{32}P incorporated into phospholipids was determined by scraping the labeled phospholipids off the plates and liquid scintillation counting (C and D). Radioactivity applied at the origin of the TLC plates (15,000 cpm per track, A and B; 30,000 cpm per track, insets) was used as a measure of total ^{32}P-labeled phospholipids. Results are the average ± S.D. of three independent experiments each done in duplicate ($^{*}P$ <0.05; $^{**}P$ <0.001 evaluated by Student's t-test for unpaired data). The percentage of unstimulated levels were: 45 ± 4.5, PC; 22 ± 2.5, PE; 13 ± 1.5, PI; 10 ± 2, PS; 4.5 ± 0.75, PA; 3 ± 0.25, lysoPC; 1.1 ± 0.3, PIP; 0.6 ± 0.2, PIP2; 0.5 ± 0.15, lysoPE; 0.3 ± 0.1, lysoPS + lysoPI. The positions of phospholipid standards are marked on the margins: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4 phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; lysoPE, lysophosphatidylethanolamine; lysoPS, lysophosphatidylserine; lysoPI, lysophosphatidylinositol; lysoPC, lysophosphatidylcholine.

inhibitor of both lipoxygenase and cyclooxygenase enzymes, also resulted in amplification of the signal generated by non-saturating IFN-γ treatment (data not shown). It is noteworthy that a significant increase in

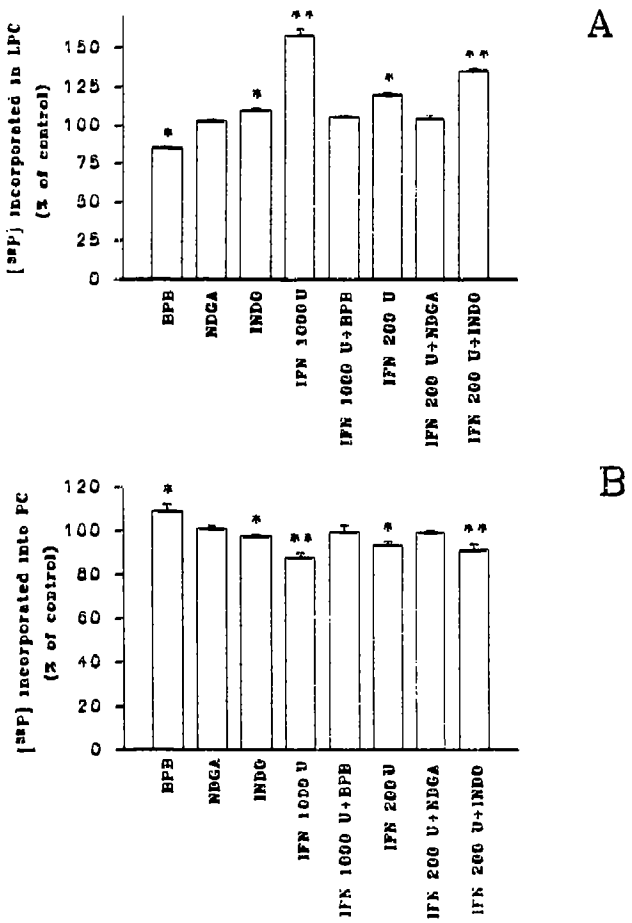

Fig. 3. Effects of inhibitors of PLA_2, cyclooxygenase, and lipoxygenase enzymes (BPB, INDO, and NDGA, respectively) on the production of lysoPC (A) and hydrolysis of PC (B). The data are means ± S.D. of three independent experiments each done in duplicate ($^*P< 0.05$; $^{**}P<0.001$, evaluated by Student's t-test).

the release of [^{3}H]AA was observed in untreated cultures by INDO alone, indicating a possible negative feedback exerted by some prostanoids in basal conditions.

The other product of PLA_2 action is a lysophospholipid. To further prove PLA_2 activation LAN-5 cells were labeled overnight with [^{32}P]orthophosphate and treated with IFN-γ for various times. Labeled extracts were then analyzed by thin layer chromatography for IFN-γ-stimulated production of lysophospholipids and degradation of precursor phospholipids (Fig. 2). Within 2 min of IFN-γ treatment, lysoPC was increased by 30%, with a maximum increase of 70% after 5 min, relative to the amount from unstimulated cells. By 60 min after IFN-γ treatment, lysoPC returned to near basal levels (Fig. 2B,C). In parallel, with the same time-dependent effect, a significant decrease in PC labeling was observed in IFN-treated cells (Fig. 2B,D), indicating a hydrolysis of PLA_2 of membrane PC as the signal transduction mechanism of IFN-γ. Lysophosphatidylethanolamine (lysoPE) and lysophosphatidylinositol were not elevated in extracts of IFN-γ-treated cells (Fig. 2B insets), and, accordingly, no differences were observed in PE and PI labeling between treated and untreated cultures (Fig. 2A).

The transient nature of the PLA_2 response is compatible with its potential participation in signaling by IFN-γ. The lasting activation of PLA_2, still observed after 15 min (Figs. 1 and 2), could be accounted for by a consequent platelet activating factor activation of PLA_2 by release of intracellular Ca^{2+} stores [21]).

In agreement with what was shown for [^{3}H]AA release, pretreatment of LAN-5 cells with BPB blocked both basal and IFN-γ-induced hydrolysis of PC with consequent inhibition of the production of lysoPC (Fig. 3A,B). Significantly, pretreatment of control cells with INDO gave a slight but reproducible PLA_2 stimulation (Fig. 3), confirming the hypothesis that, in neuroblastoma cells, some prostanoids may play a role as negative regulators of PLA_2 activation, or simply amplify constitutive PLA_2 activation. It is noteworthy that INDO pretreatment was also able to enhance PC hydrolysis induced by non-saturating concentrations of IFN-γ, indicating that, besides the expected accumulation of the hydrolysis products, a prostanoid(s) might also exist in the IFN-γ-stimulated PLA_2 activation working as a negative PLA_2 regulator. The higher increase of INDO-sustained AA release in IFN-γ-treated cells, with respect to untreated cultures, suggests that this control may be exerted at the level of receptor desensitization. Thus, inhibition of PLA_2 and of AA metabolism through cyclooxygenase and lipoxygenase systems affects early signals generated by IFN-γ binding to its receptor in the neuroblastoma model. This conclusion is supported by experiments in which the treatment of LAN-5 cells with 500 U/ml of IFN-γ induced the expression of 2′,5′-oligoadenylate synthetase [22], and this induction was completely abolished by pretreatment with BPB (data not shown). However, the formation of the putative regulatory AA metabolite could not occur via cyclooxygenase or lipoxygenase pathways. Thus, substrate AA derived from IFN-γ receptor activation might be redirected from cyclooxygenase- and lipoxygenase-catalyzed transformation to another AA metabolic pathway (i.e. epoxygenase-catalyzed AA conversions would not be inhibited by INDO and NDGA) [23].

In conclusion, our results suggest that stimulation of receptor-coupled PLA_2 and AA metabolism may participate in signaling IFN-γ-generated responses. Since prostanoids, particularly prostaglandins, are able to induce differentiation of neuroblastoma, both in vitro [24,25] and in vivo [26], an intriguing question is whether PLA_2 activation is necessary and sufficient for induction of neuroblastoma cell maturation by IFN-γ. To this purpose we are investigating whether pretreatment of LAN-5 cells with INDO or NDGA can prevent

the maturative effects of IFN-γ. Moreover, the possibility that the addition of specific AA-derived autocoids can revert the blockade induced by INDO and/or NDGA is currently being probed.

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