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Effect of Interferon on Phospholipid Methylation by Peripheral Blood Mononuclear Cells

P. Bougnoux, E. Bonvini, Z.L. Chang, and T. Hoffman

Biological Research and Therapy Branch, Frederick Cancer Research Facility, National Cancer Institute, Frederick, Maryland 21701

The effect of human interferon (IFN) preparations on the metabolic pathway leading to the synthesis of phosphatidylcholine (PC) by a stepwise addition of methyl groups to phosphatidylethanolamine (PE) was investigated in human peripheral blood mononuclear (PBMN) cells. An inhibition of the synthesis of PC via this pathway was regularly observed with both α- (recombinant or natural) and β-IFN. This inhibition was apparent within the first 5 min of treatment, reached its maximum between 15 min and 1 hr, and persisted at the same level until 6 hr, the last time point examined. Each of the transmethylated products of PE underwent a similar inhibition, as measured by the turnover rate of individual products. The intracellular pool of the methyl donors, methionine and *S*-adenosylmethionine (SAM), was shown to be unaffected. The methyltransferase activity of IFN-pretreated cell extracts was unchanged. These findings support the hypothesis that IFN induces a functional change in phospholipid methylation at the level of organized membrane-bound phospholipid methyltransferase enzymes in intact cells.

Key words: interferon, phospholipids, methylation, membrane fluidity, phosphatidylcholine

Evidence that interferon (IFN) induces alterations in the plasma membrane [1,2] which lead to changes in cell function has been accumulating recently. For example, mouse L cells have a greater net negative charge on their cell surface after treatment with IFN [3]. Human fibroblasts treated with IFN demonstrate increased plasma membrane rigidity as observed by electron spin resonance (ESR) spectroscopy [4]. Hela cells treated with IFN show impaired redistribution of membrane components in response to lectin treatment [5]. Additional biochemical observations support this hypothesis. In mouse sarcoma cells, the relative proportion of saturated to unsaturated fatty acids of cellular phospholipids (PL) was increased after treatment by β-IFN [6]. Chang et al. [7] observed a change in the density of plasma membranes from IFN-

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treated L cells, indicative of an increase in the protein/lipid ratio, another factor determining membrane rigidity.

Besides changes in the core (fatty acid) portion of phospholipids, each individual lipid head group within the plasma membrane exerts an effect on total fluidity of the membrane. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the main PL in mammalian membranes, where PC acts as a "fluidizer" and PE as a "rigidifier" [8]. Although PC is mainly synthetized via the cytidyl-diphosphate (CDP)-choline pathway [9], further alteration of the membrane PC/PE composition is accomplished via transmethylation of PE [10]. A relationship between the synthesis of PC through this pathway and an increased fluidity of the membrane of erythrocytes has been established by Hirata [11] and applied to a wide range of receptor-mediated biologic phenomena in other cell types.

We have examined the possibility that alterations in the phospholipid transmethylation pathway might be one of the biochemical events leading to IFN-induced changes in the cell membranes. We describe inhibition of PL transmethylation in peripheral blood mononuclear (PBMN) cells treated with different IFN preparations. We examined a number of possible mechanisms to account for this effect.

METHODS

Cell Preparation

PBMN cells were isolated from venous blood or from plateletpheresis packs from normal subjects using Ficoll-Hypaque density gradients [12]. Cells at the interphase were washed three times with phosphate-buffered saline (PBS, pH 7.4), and resuspended in methionine-free RPMI 1640 (Gibco, Grand Island, NY) containing 30 mM N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid (HEPES) and 2% fetal bovine serum (FBS).

Interferons

The human interferon preparations used were: leukocyte (α)-recombinant IFN, specific activity: 2×10^4 U/mg protein, kindly provided by Dr. S. Petska of the Roche Institute for Molecular Biology, Nutley, NJ [13]; α-natural IFN (1×10^8 U/mg protein, Interferon Sciences Inc., New Brunswick, NJ); and fibroblast (β-) IFN (3×10^7 U/mg protein, HEM Research, Rockville, MD).

β-IFN from mice was obtained by infection of a mouse fibroblast cell line with Newcastle disease virus (obtained from M. Pauker, Medical College of Pennsylvania, Philadelphia, PA). Its specific activity was 6×10^7 U/mg protein. Each IFN preparation was used at 800 U/ml.

Reagents

L-[methyl-^{3}H]methionine (80 Ci/mmol), *L*-[methyl-^{14}C]methionine (57 mCi/mmol), *S*-[methyl-^{3}H]adenosyl-*L*-methionine (78.5 Ci/mmol), and *L*-[^{35}S]methionine (1,070 Ci/mmol) were purchased from New England Nuclear (Boston, MA).

Phospholipid standards were obtained from Gibco. *L*-Methionine and *L*-cysteine were from Sigma (St. Louis, MO). *S*-Adenosylmethionine and *S*-adenosylhomocysteine were from Boehringer (Indianapolis, IN). Silica gel G and cellulose thin-layer chromatography (TLC) plates were from Analtech (Newark, DE).

Assay of PL Methylation

Cells in polypropylene tubes were labeled with 4 μM [methyl-^{3}H]methionine for 3 hr at 37°C, in a 5% CO_2 atmosphere either in the presence or absence of IFN. The reaction was stopped by removing the medium, followed by the addition of 10% cold trichloroacetic acid (TCA), containing 10 mM methionine. The 27,000 g pellet was washed twice with TCA, and extracted with 6 ml of *n*-butanol, saturated with 0.1 N HCl. After centrifugation, the butanol extract was washed twice with 2 ml of 0.1 N HCl [14]. An aliquot was counted in 10 ml of Aquasure (New England Nuclear, Boston, MA) and the remaining butanol phase dehydrated over sodium sulfate and then evaporated to dryness. The radioactivity incorporated by the cells was approximately 1% of the total radioactivity contained in the incubation medium and was distributed as follows: 15% in the acid-soluble material, 20% in the butanol phase, 10% in the HCl washing, and 55% in the pellet.

Separation and Identification of the Products

Cell lipids were redissolved in 50 μl of $CHCl_3/CH_3OH$ 1:1 containing 50 μg of a mixture of standards as carrier [lysophosphatidylcholine (LPC), PC, phosphatidyldimethylethanolamine (PDE), phosphatidylmonomethylethanolamine (PME), PE] and applied under nitrogen on "pre-adsorbent" silica G plates (250-μm thickness, 20 × 20). The plates were developed in saturated tanks in two steps with the following solvents: (1) acetone/petroleum ether (1:3 v/v) and (2) chloroform/methanol/acetic acid/water (100:50:13:5 v/v) [15]. The plates were stained with iodine vapor, followed where indicated by ninhydrin spray (0.4% in water-saturated butanol). Each sample was processed identically and run on one plate under identical conditions. With this system, the Rf values were: LPC:0.08, PC:0.22, PDE:0.53, PME:0.66, and PE:0.7. The radioactivity was quantified with a TLC scanner [16] (Imaging Proportional Counter, Bioscan, Washington, D.C.), and was found to be distributed in five peaks, identifiable after calibration of the scanner and comparison with the stained plates: PME:10%, PDE:8%, PC:50%, LPC:15%, and an unidentified, polar fraction which stayed at the origin using this solvent system.

Measurement of Turnover

An application of the method described by Schrimke for membrane proteins [17] was developed for measurement of the turnover of methylated products of PE. Cells were first labeled in the presence of IFN or medium (control) for 3 hr with 1 μM [methyl-^{14}C]methionine, as described above for [methyl-^{3}H]methionine labeling, washed twice, and resuspended in medium at 37°C with IFN. After 1 hr, [methyl-^{3}H]methionine was added and the cells were incubated for an additional 3 hr. The lipids were extracted, separated, and identified as above. For quantification of the radioactivity of each of the two isotopes of the methyl group of methionine incorporated into the intermediate products of transmethylation, each lane was scraped off in horizontal bands of 1.5 cm, and the lipids were eluted with methanol/water (1:1) and counted in a two-channel β-counter. For each methylated product of phospholipids, the ratio between ^{3}H and ^{14}C was considered as an index of the turnover. Spillover of ^{14}C into the ^{3}H channel was negligible at the ratio $^3H/^{14}C$ obtained (40–120).

Assay of Phospholipid N-Methyltransferase (EC 2.1.1.17)

N-methylation of unlabeled PE, PME, and PDE was assayed by measuring the incorporation of the ^{3}H-methyl group from *S*-[methyl-^{3}H]adenosyl-*L*-methionine ([^{3}H]SAM) into butanol-extractable phospholipids by an adaptation of the technique described by Bremer [18] and Vance and Schneider [19]. The source of enzymatic activity was supernatant remaining after sedimentation at 800 g of osmotically lysed, freeze-thawed PBMN cells, which had been incubated with either α-recombinant IFN or medium for 2 hr. The protein concentration of the cell extract was measured by a dye-binding assay [20].

The reaction was performed in polypropylene tubes at 37°C in a final volume of 500 μl. PL-methyl acceptor substrate, prepared using 0.3 mg/ml PE and 0.1 mg each of PDE and PME was evaporated to dryness under nitrogen and brought into solution by sonication (15 sec, twice) in 3 ml of 5 mM Tris-HCl buffer (pH 9.2) containing 0.06% Triton X-100. The incubation mixture contained 50 μl of 1.25 M Tris-HCl buffer (pH 9.2), 300 μl of PL substrate, 50 μl of 10 mM cysteine in the same buffer, and 50 μl of 0.68 μM [^{3}H]SAM in 0.9% saline. The reaction was started by addition of 50 μl of either the cell extract (160 μg cell protein), boiled cell extract, or saline and was stopped after 15 or 45 min by adding 2.5 ml of 0.1 N HCl, saturated with butanol. Lipids were extracted with 2 ml *n*-butanol, washed with water, quantified, separated, and identified as described. The distribution of the radioactivity was localized in only three peaks with the Rf values of the standards and was 65% PC, 12% PDE, 16% PME. [^{3}H]SAM was not retained using this extraction procedure and did not migrate in the chromatographic system described. Under the above conditions, the amount of reaction product was proportional with time.

Assay of Methionine and SAM Incorporation by PBMN Cells

IFN-treated or control cells were labeled with [^{35}S]methionine to follow methionine metabolism other than transmethylation. Labeled cells were washed twice with medium and 1 ml of cold 15% TCA was added. The concentration of intracellular free methionine, SAM, and their metabolites was determined by measuring the radioactivity of the TCA-soluble cellular material. The acid-insoluble fraction was heated for 5 min in a boiling water bath, washed with 10% TCA, dissolved in aqueous SDS, and the radioactivity counted for estimation of [^{35}S]methionine incorporation into protein.

RESULTS

Effect of IFN on Phospholipid Methylation of PBMN Cells

Treatment of PBMN cells with recombinant IFN-α resulted in an average decrease of 60% (56–75%) in the incorporation of the methyl group from methionine into phospholipids (Table I). This effect was consistently observed during a 6-hr time frame (data shown for three experiments encompassing time points up to 3.5 hr). In subsequent experiments, designed to evaluate possible "early" effects, inhibition was observed as early as 5 min, with maximal inhibition achieved at 15 min (Fig. 1).

To ascertain whether interferon *per se* was responsible for the observed effect, human PBMN cells were also treated with a "mock" preparation of recombinant α-IFN, consisting of a supernatant prepared from the identical strain of bacteria without insertion of the gene for human IFN. No change was observed under these conditions

(Table II). Treatment of PBMN cells with human β-IFN under similar conditions as described for α-recombinant IFN also resulted in decreased incorporation of methyl groups from methionine into PL, whereas β-IFN from mice was without effect. Sixty units per milliliter of β-IFN inhibited methylation to the same degree as other doses tested, that is, up to 4,000 U/ml. Phospholipid methylation was examined in cells treated with graded concentrations of α-(natural) IFN. Inhibition was observed at IFN concentrations as low as 1–10 U/ml and was maximal in a range between 100 and 1,000 U/ml (Fig. 2). The effects described above were observed only when IFN was present throughout the labeling period. When cells were labeled and washed prior to the addition of IFN, the rate of decay of incorporated [^{3}H]methionine into PL was unaffected (data not shown).

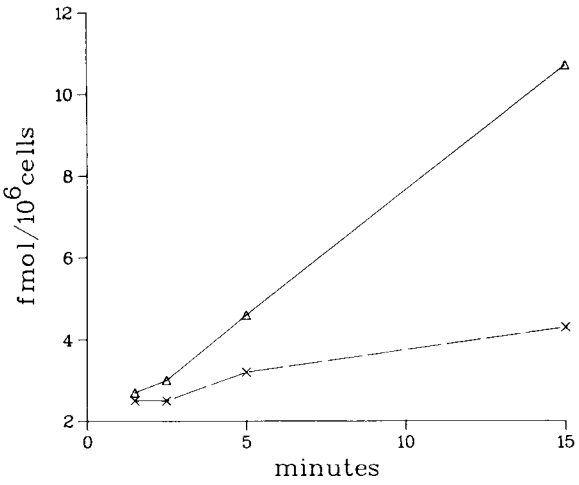

Fig. 1. Early effect of IFN on PL methylation. PBMN cells (5×10^6) were labeled with 4 μM [methyl-^{3}H]methionine in the presence (x---x) or absence (△——△) of α-recombinant IFN. The reaction was stopped by adding 10% cold TCA and the lipids were extracted as described in Methods.

TABLE I. Effect of Interferon Treatment on Phospholipid Methylation by PBMNC

	Labeling time (min)	[^{3}H]Methyl incorporation (fmol/10^6 cells)	
		Medium-treated	IFN-treated[a]
Exp. 1	60	20.1	9.4 (53%)[c]
Exp. 2	90	37.2 ± 0.2[b]	16.1 ± 0.1 (56%)
	210	174.7 ± 5	42.2 ± 1 (76%)
Exp. 3	180	74.7	28.5 (62%)

[a]α-IFN, 800 U/ml.
[b]Standard deviation of duplicates.
[c]% inhibition.

Effect of Interferon on PME, PDE, PC, and LPC Turnover

Alterations in the amount of phospholipids in the cell membrane may be achieved by changes in the rate of synthesis of either the intermediate products, since two enzymes have been described [21], or by change in their rate of degradation. We therefore examined the effect of IFN on the rate of synthesis and degradation of each of the methylated products of PE utilizing a successive "double label" technique. The turnover rate for PME and PDE was the same, but was slower for PC and LPC. When the experiment was carried out in presence of 800 U/ml of IFN, a decreased turnover rate was observed for each product (Fig. 3).

Methyltransferase Activity of Extracts of IFN-Treated or Control PBMN Cells

In order to establish whether or not the effect of IFN was attributable to a direct effect on cell transmethylases, the methylation of purified PE, PME, and PDE by

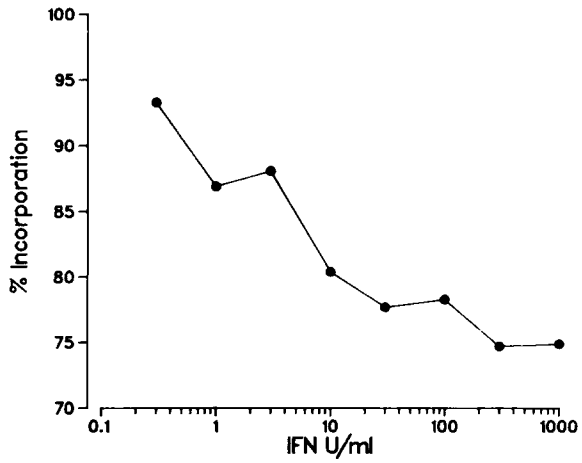

Fig. 2. Dose response of treatment by IFN on PL methylation. Cells (2×10^6/ml) were labeled with 5 μM [methyl-^{3}H]methionine in the presence of varying amounts of α-(natural) IFN for 2 hr. PL methylation was quantified as described in Methods. The data are expressed as a percentage compared to the incorporation of ^{3}H-methyl into total phospholipids of medium-treated cells, which was 80 fmol. Values are means of duplicate determinations which varied less than 5%.

TABLE II. Comparison Between Different IFN Preparations for their Effect on Methyl Group Incorporation into Phospholipids by PBMNC

	[^{3}H]Methyl incorporation (fmol/10^6 cells/hr)	
IFN treatment[a]	Medium	Treated
Recombinant α-IFN	24.8 ± 0.1	10.7 ± 0.1 (57%)[b]
"Mock" α-IFN	19.9 ± 2.0	19.9 ± 0.2 (—)
β-IFN (human)	23.4 ± 0.2	17.2 ± 1.0 (27%)
β-IFN (mouse)	32.2 ± 1.3	32.8 ± 2.4 (—)

[a]All preparations of IFN treated were used at 800 U/ml.
[b]% inhibition.

PBMN cell extracts was directly measured. Most of the methyltransferase activity was found in the "postnuclear" fraction, as previously shown [18,19]. The distribution in phospholipids of the labeled methyl group from [methyl-^{3}H]SAM was very similar to that found after extraction of methionine-labeled living cells. When the cell extract was obtained from cells pretreated for 2 hr with α-recombinant IFN, no appreciable change was observed either at 5 (data not shown) or 45 min (Table III). Similar data were obtained at different time points in two additional experiments.

Measurement of Methionine Incorporation by IFN-Treated and Control Cells

To determine whether the interferon-induced inhibition of PL methylation could be attributed to a change in the uptake of methionine or in the availability of labeled

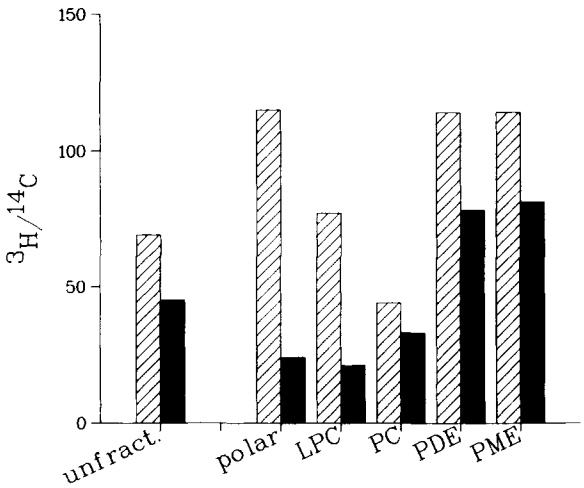

Fig. 3. Effect of interferon on the turnover rate of each intermediate of transmethylation. Cells were labeled at two different times with two different isotopes—^{14}C and ^{3}H—of the methyl group of methionine. The turnover rate of each individual lipid product is represented by the ratio of activity due to the second label (^{3}H) to that due to the first label (^{14}C), as described in Methods. Ratios are given for control (medium-treated) (▨) and α-recombinant IFN-treated (■) cells.

TABLE III. Phospholipid Methyltransferase Activity in Extracts of Medium- or IFN-Treated Cells

Source of cell extract	Butanol phase radioactivity (dpm)	Specific activity (pmol/mg protein/hr)[a]
Medium-treated	72,005 ± 7,984	3.92 ± 0.54
IFN-treated[b]	59,793 ± 1,346	3.09 ± 0.09
No enzyme	13,985	—
Boiled mixture	18,654	0.31

[a]For calculation of specific activity, the nonenzymatic incorporation of methyl group radioactivity (0.11 pmol) was subtracted from the values obtained in the presence of extract and related to weight and time units.

[b]α-recombinant (800 U/ml).

TABLE IV. Effect of IFN on the Distribution of [^{35}S]Methionine in PBMN Cells

	Distribution of radioactivity (cpm × 10^{-2})	
Cell treatment	Acid-soluble material	Acid-insoluble material
Medium	6,012 ± 241	73,080 ± 1,986
Interferon[a]	7,793 ± 451	40,125 ± 882

[a]α-recombinant IFN (800 U/ml).

methyl donors inside the cell, the uptake of [^{35}S]methionine and the formation of [^{35}S]SAM was determined. No appreciable IFN-induced change was observed in the acid-soluble fraction of prelabeled, washed cells (Table IV). IFN caused an inhibition of the incorporation of the ^{35}S-amino acid into the acid-insoluble material, as has been described [22,23]. To identify the resulting distribution of ^{35}S in the methyl donors, the TCA-soluble material was washed with diethylether and separated by TLC on cellulose and silica gel G plates with SAM, SAH, and methionine as standard carriers, using the solvent system of *n*-butanol/acetic acid/water (12:3:5, vol:vol) and staining with ninhydrin. The pattern of distribution of the radioactivity among *S*-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and methionine was unchanged (data not shown).

DISCUSSION

We observed an early decrease in phospholipid methylation in PBMN cells treated with IFN, which was not found using a "mock" α-IFN preparation or mouse β-IFN. This inhibition was consistently observed using IFN preparations from different sources, including natural α- and β-IFN's and a recombinant α-preparation. These findings, as well as the configuration of the dose response make less likely that a common, toxic impurity could account for the observed effect.

Decreased methyl incorporation measured in PLs was not a consequence of increased degradation of methylated PL products, but of an inhibition of their synthesis. This inhibition of synthesis involved each individual intermediate product of transmethylation, with a decreased turnover rate, indicating that the effect of IFN is not restricted to one step in the sequence of transmethylation.

The methyltransferase activity of IFN-pretreated cell extracts in a cell-free assay was not changed, indicating that IFN does not modify the enzymes. Because IFN has been reported to inhibit the cellular uptake of extracellular compounds [24], and because the activity of cell-treated methyltransferase enzymes extracts was not modified, we also verified that the PL methylation inhibition was not correlated with a change in uptake of radiolabeled precursor.

These findings and the observations of a very early effect by IFN support the hypothesis that IFN induces a functional change at the level of well-organized, membrane-bound, PL methyltransferase enzymes. Decreased transmethylation, with a consequent accumulation of PE and a decreased rate of PC synthesis, may in part be responsible for altered membrane physical properties in IFN-treated cells.

IFN, in addition to its antiviral properties, has been shown to affect a wide range of biologic functions. IFN augments Natural Killer activity and makes certain

target cells resistant to lysis [25]; it promotes or inhibits cell differentiation [26]; it causes the enhanced expression of certain histocompatibility antigens [27]; and manifests growth inhibitory and tumoristatic properties [28]. Correlation of the effect of IFN on PL methylation with any of its biologic effects cannot be made from existing data. The exact role of PL methylation on the functional and physical state of the cell membrane is not established, making conclusions regarding the significance of inhibition of this pathway by IFN speculative. Identification of the mechanism whereby IFN inhibits PL methylation could provide insight into this problem as well as providing a molecular basis for some of the effects of IFN. We are currently examining a wider range of cloned and naturally occurring IFN preparations for their effects on this and other pathways of lipid metabolism.

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