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Brain Research 803 (1998) 44–53

BRAIN RESEARCH

Research report

Nerve growth factor stimulates diacylglycerol de novo synthesis and phosphatidylinositol hydrolysis in pheochromocytoma cells

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Accepted 26 May 1998

Abstract

Induction of neurite outgrowth by treating pheochromocytoma cells (PC12 cells) with nerve growth factor (NGF) is associated with major increases in cellular levels of diacylglycerol (DAG), an essential and probably limiting precursor in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) syntheses. To identify the sources of this DAG we examined the effects of NGF treatment on the conversion of [^{3}H]oleic acid (OA) or [^{3}H]glycerol to [^{3}H]glycerolipids, and the turnover of these products in PC12 cells. In kinetic studies on [^{3}H]OA incorporation, most of the radioactivity in the cells initially was free [^{3}H]OA; then it appeared predominantly as [^{3}H]DAG and, eventually, as large amounts of [^{3}H]phospholipids (PLs). In NGF pre-treated cells, the increases in the levels of [^{3}H]DAG (which were most prominent) and PLs were similar to those in unlabeled DAG and PLs. These effects of NGF could be partially blocked by an inhibitor (triacsin C) of long chain acyl-CoA synthetase. NGF pre-treatment also significantly enhanced the incorporation of [^{3}H]glycerol into lipids, a pathway for de novo synthesis of glycerolipids. In studies on the degradation of [^{3}H]OA-labeled lipids, the disappearance of [^{3}H]OA-labeled neutral lipids exhibited an initial rapid phase and a subsequent stable phase. NGF treatment transiently promoted the hydrolysis of [^{3}H]PI to [^{3}H]DAG. These data suggest that the increases in DAG levels observed in PC12 cells exposed to NGF derive mainly from de novo synthesis and, to a lesser and transient extent, from the hydrolysis of [^{3}H]PI.

Keywords: Oleic acid; Glycerol; Diacylglycerol; Phospholipid; Nerve growth factor; PC12 cell

1. Introduction

The biosynthesis of phosphatidylcholine (PC) as well as the total quantities, per cell, of it and other membrane phospholipids (PLs) are markedly increased when pheochromocytoma cells (PC12 cells) are differentiated by treatment with nerve growth factor (NGF) for 2 or 4 days. These increases reflect accelerated conversion of endogenous CDP-choline to PC, mediated by increases in choline phosphotransferase activity and enhanced saturation of this enzyme with its other substrate, diacylglycerol (DAG) [1].

Both the formation and the metabolism of DAG may comprise potentially important regulatory sites for the biosynthesis of membrane compounds [29,33]. The sources of the increased DAG in NGF-treated cells are unknown, but cannot simply be phospholipase C (PLC)-mediated hydrolysis of phospholipids, inasmuch as cellular levels of PC, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) also increase with NGF treatment.

We examined the major possible sources of this increased DAG, again, using PC12 cells differentiated into a sympathetic neuron-like phenotype by exposure to NGF. This model system has been widely used to study, inter alia, nuclear translocation or activation of PI 3-kinase [4,16]; hydrolysis of glycosylphosphatidylinositol [5]; glycolipid composition [2]; syntheses of gangliosides, glycolipids and total lipids [12]; changes in neuronal cells [21]; divergence of signaling pathways [17]; and metabolism via the pentose phosphate pathway [6]. We were especially interested in determining the extent to which the increase in DAG with NGF treatment derives principally from de novo synthesis via acylglycerol, and/or from hydrolysis of particular PLs.

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2. Materials and methods

2.1. Materials

PC12 cells were obtained from ATCC (Rockville, MD, USA). RPMI 1640 medium (with glutamine and without sodium bicarbonate), heat-inactivated horse serum (HS) and fetal bovine serum (FBS), and NGF (2.5S) were all obtained form Gibco BRL (Grand Island, NY, USA). [^{3}H]oleic acid (OA) (7.4 Ci/mmol), [1,2,3-^{3}H]glycerol ([^{3}H]glycerol, 80 Ci/mmol) and [^{32}P] ATP (30 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA). OA, monoacylglycerol (MAG), DAG, triacylglycerol (TAG), phosphatidic acid (PA), PC, PE, PS, PI, a kit for protein determination, deoxyribonucleic acid (DNA standard), 1,6-diphenyl-2,3,5-hexatriene (DPH), ATP and phosphate buffered saline tablet (PBS) were all obtained from Sigma (St. Louis, MO, USA). 2E,4E,7E-Undecatriene-1-triazene (triacsin C) was obtained from Biomol (Plymouth Meeting, PA, USA). 2′-[4-Hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole (Hoechst 33258) was obtained from Aldrich Chemical (Milwaukee, WI, USA). Ultrafluor was obtained from National Diagnostics (Atlantic, GA, USA).

2.2. Cell culture

PC12 cells were cultured according to the method of Greene and Tischler [8]. The medium was RPMI 1640 containing 10% HS and 5% FBS (medium A). Stock cultures were grown in 75 cm^2 tissue culture flasks (Corning Costar, Cambridge, MA, USA) at 37°C and an atmosphere of 95% air/5% CO_2. For experiments, cells at a density of 2×10^5/ml in 2-ml medium A were plated on 35-mm tissue culture dishes coated with mouse collagen IV (Fisher, Bedford, MA, USA) for at least one day. At 24 h prior to treatment, the medium A bathing the cells was replaced with a new RPMI l640 medium containing 1% HS but lacking FBS (medium B). The experimental cells were treated with NGF (50 ng/ml), triacsin C (4.8 nmol/ml) and/or labeled with [^{3}H]OA (5 μCi/ml) or [^{3}H]glycerol (8 μCi/ml) for the periods of time indicated.

2.3. Procedures of NGF treatment and [^{3}H]OA or [^{3}H]glycerol labeling

In NGF pre-treatment studies, cells were exposed to NGF for 0, 0.5, 1, 2 or 4 days, and then labeled with [^{3}H]OA for an additional 1 h, or with [^{3}H]glycerol for an additional 20 h. In some cases, cells were pre-treated with NGF for 1 day, and then exposed to both [^{3}H] OA and triacsin C for an additional 1 h. In kinetic studies on [^{3}H]OA incorporation, cells were briefly and simultaneously treated with NGF and labeled with [^{3}H]OA for 0, 10, 20, 40 or 60 min. In studies on the degradation of [^{3}H]OA-labeled lipids, cells were first pre-labeled with [^{3}H]OA for 1 h, washed once with medium B to stop labeling, and then treated with NGF for 0, 0.5, 1, 2 or 4 h.

2.4. Cell collection and lipid extraction

To harvest cells, the medium was aspirated and the cells were then washed once with cold PBS (4°C, 2 ml), scraped from the dish into ice-cold methanol (−20°C, 1 ml), and collected into a test tube. After sonication with a cell disrupter (Ultrasonic, Plainview, NY, USA), 0.1 ml of suspension was picked up for protein and DNA analyses, and then chloroform (1.8 ml) and distilled water (0.9 ml) were successively added to 0.9 ml of the remaining suspension for extracting lipids by the method of Van Veldhoven and Bell [32]. The suspension was vortexed, centrifuged at 4000 rpm 5 min, and separated into two phases. The aqueous phase was aspirated and the organic phase was dried using a speed vacuum concentrator (Savant Instruments, Farmingdale, NY, USA).

2.5. Labeled neutral lipid assay

The residue from the organic phase was reconstituted in a mixed solution (50 μl) of chloroform and methanol (1:1, by volume). An aliquot (20 μl) of the labeled neutral lipid extract was then purified by one dimensional thin layer chromatography (TLC) for about 45 min, on a pre-adsorbent silica gel G plate (Analtech, Newark, DE, USA), using a system consisting of petroleum ether/diethyl ether/acetic acid glacial (70:30:2, by volume) as the mobile phase [22]. OA and neutral lipid (MAG, DAG and TAG) standards were used to identify the corresponding bands after staining the plate with iodine vapor; their relative mobilities (R_f values) were 0.64, 0.12, 0.39 and 0.88, respectively. The powders obtained from the OA, MAG, DAG and TAG bands were scraped from the plate and collected into vials containing ultrafluor (15 ml). The associated radioactivities were counted by liquid scintillation spectrometry (Beckman, Irvine, CA, USA).

2.6. Labeled PL assay

An aliquot (20 μl) of the labeled phospholipid extract was purified by TLC [31] for about 150 min on a silica gel G plate using a mobile phase containing chloroform/triethylamine/ethanol/water (30:30:34:8, by volume). PL (PC, PE, PS and PI) standards were used to identify the corresponding bands under long wave UV light after spraying the plates with 0.1% DPH in petroleum ether. Their R_f values were 0.09, 0.54, 0.30 and 0.42, respectively. The radioactivities associated with the different PL bands were counted by liquid scintillation spectrometry.

2.7. Labeled PA assay

An aliquot (5 μl) of the labeled PA extract was purified by TLC [22] for about 90 min on a LK6D silica gel plate

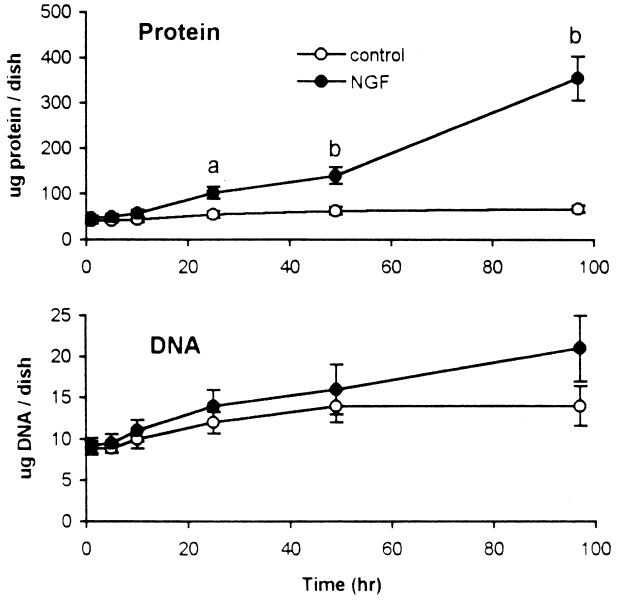

Fig. 1. Effects of NGF treatment on protein and DNA contents in PC12 cells. After incubation in medium B for 24 h, PC12 cells were treated with 50 ng NGF/ml medium B for 1–97 h. The cells were harvested and measured for protein and DNA contents as described in Section 2. Values represent the mean ± SD of protein or DNA contents (μg protein or μg DNA/dish; $n = 4–10$). Compared with control, a: $P < 0.05$, b: $P < 0.01$.

(Whatman, Clifton, NJ, USA) using a mobile phase containing chloroform/methanol/acetic acid glacial (60:15:5, by volume). PA standard was used to identify the corresponding band after staining the plate with iodine vapor. The R_f value of PA was 0.42. The radioactivity in the powder from the PA band was counted as above.

2.8. DAG and PL mass assay

PC12 cells cultured in the coated dishes in the presence or absence of 50 ng/ml of NGF for 2 or 4 days were collected and sonicated in 0.4 ml of ice-cold methanol. The samples were then transferred to new tubes and mixed with two volumes of chloroform/methanol (1:1, by volume). The neutral lipids were extracted by the method of Bligh and Dyer [3], and DAG contents were assayed by the method of Preiss et al. [19]. DAG was converted to [^{32}P]PA using DAG kinase and [^{32}P]ATP, and then the [^{32}P]PA was purified by TLC as described above [22]. The actual level of DAG was determined by comparing [^{32}P]PA in the experimental samples with the [^{32}P]PA generated by standards containing known amounts of 1,2-dioleoylglycerol.

To measure the amounts of PC, PE, PS and PI, cellular PLs were extracted and purified by TLC as described above [31]. The samples scraped from individual PL bands were digested with 70% perchloric acid at 150°C for at least 2 h. Phosphate in the samples was assayed according to the method of Svanborg and Svennerholm [28], using dipalmitoyl PC as standard.

2.9. Protein and DNA measurements

The contents of protein and DNA in cells were measured by the bicinchoninic acid method (Perkin-Elmer, Notwalk, CT, USA) and by the Hoechst fluorescent spectrometric method [13] (Hoefer Scientific Instrument, San Francisco, CA, USA), respectively.

2.10. Data analysis

Data were expressed as means ± SD. Student's *t*-test, two-way analysis of variance, or multiple comparison tests were used for comparisons between groups. Metabolic kinetic and regression analyses of the data were performed following linear and non-linear models.

3. Results

3.1. Increases in cellular levels of DAG and individual PLs in NGF-treated PC12 cells

Protein synthesis was stimulated intensively by NGF treatment, while DNA level increased only slightly (Fig.

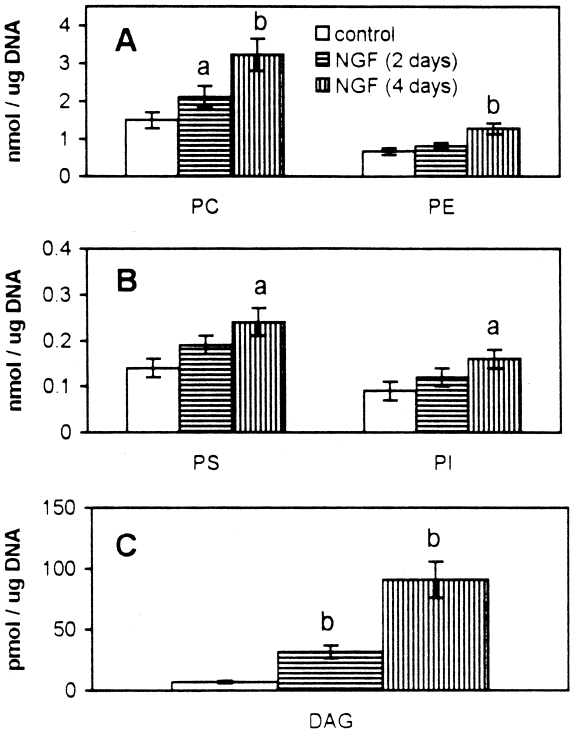

Fig. 2. Increases in glycerolipid levels in PC12 cells treated with NGF. PC12 cells cultured in the collagen-coated dishes were treated with 50 ng/ml of NGF for 2 or 4 days. Cellular DAG and individual PLs were purified, and the amounts of them were measured as described in Section 2. Values represent the mean ± SD of (A) PC and PE, (B) PS and PI (nmol/μg DNA; $n = 6$), and (C) DAG (pmol/μg DNA; $n = 4$). Compared with control (average values of 2 and 4 days), a: $P < 0.05$, b: $P < 0.01$.

1), indicating that the NGF primarily increased not the number of cells but their protein contents and, probably, their size. Thus, data on the effects of NGF on cellular lipids are expressed as dpm/μg DNA instead of as dpm/μg protein.

During the incubation period of 4 days, in untreated control cells, neither cellular levels of protein, DNA (Fig. 1) and glycerolipids (Fig. 5) nor cell shape and size were obviously changed. Exposure of PC12 cells to 50 ng/ml of NGF time-dependently increased cellular levels of individual PLs, by 20–40% and 65–115%, after 2 and 4 days, respectively (Fig. 2A and B), a period concurrent with continued neurite outgrowth (as observed previously by Araki and Wurtman [1] and again in the present study). Among these individual PLs, levels of PC, a direct product of DAG by the Kennedy cycle [33], increased by 115% (Fig. 2A); whereas PI, produced from a different biosynthetic pathway [33], increased by 65% (Fig. 2B). The most significant increases were found in cellular DAG levels; these were 3.5 and 12 times higher, in the cells treated with NGF, after 2 and 4 days, respectively, than those in the untreated control cells (Fig. 2C).

3.2. Utilization of [^{3}H]OA by PC12 cells after 10–60 min of incubation

To observe the synthesis rates of individual glycerolipids, we measured [^{3}H]OA incorporation into glyc-

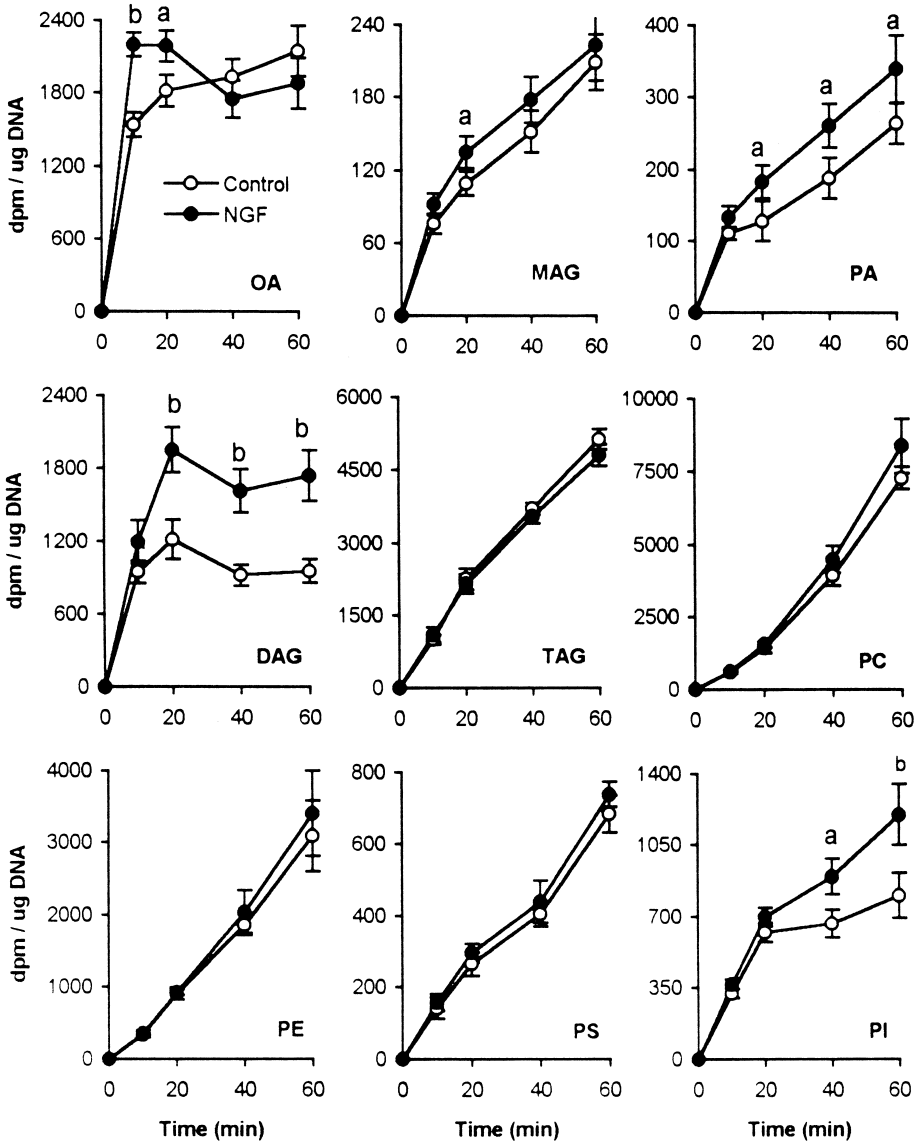

Fig. 3. Time-dependent incorporation of [^{3}H]OA into lipids. After incubated in medium B for 24 h, PC12 cells were simultaneously exposed to 50 ng NGF and 5 μCi [^{3}H]OA/ml medium B for 0, 10, 20, 40 or 60 min. The cells were harvested and assayed for lipid levels and DNA contents as described in Section 2. Values represent the mean ± SD of [^{3}H]lipid levels (dpm/μg DNA; $n = 6$). Compared with control, a: $P < 0.05$, b: $P < 0.01$.

erolipids in PC12 cells briefly exposed to NGF for 10–60 min. In untreated control cells, levels of free [^{3}H]OA, [^{3}H]PA and [^{3}H]PI increased sharply after 10 min of incubation, and then rose more slowly; [^{3}H]DAG levels peaked after 20 min and then were stable, while [^{3}H]PC levels increased slowly for 20 min and then more rapidly (Fig. 3). In the cells exposed briefly to NGF, free [^{3}H]OA peaked after 10 min at levels about 40% higher than those in control cells, and then decreased to those seen in controls; levels of [^{3}H]PA and [^{3}H]DAG were about 40% and 80% higher, respectively, than those in control cells after 20 min; [^{3}H]PI levels were about 35% higher after 40 min; and [^{3}H]PC levels were only 15% higher than those in control cells after 60 min (Fig. 3). These data indicate that the increases were found initially in free [^{3}H]OA levels, then in levels of [^{3}H]PA and [^{3}H]DAG, and eventually in [^{3}H]PLs. Moreover a brief exposure to NGF could promote this process.

Fig. 4 illustrates the dynamic relationships between [^{3}H]OA, [^{3}H]DAG and [^{3}H]PC. In control cells the levels of [^{3}H]OA, [^{3}H]DAG and [^{3}H]PC after 10 min of incubation were 1540, 950 and 600 dpm/μg DNA, respectively, and after 40 min they were 1930, 920 and 3930 dpm/μg DNA, respectively (Fig. 4A). In cells treated briefly (10–60 min) with NGF, these levels after 10 min of incubation were 2200, 1200 and 610 dpm/μg DNA, and after 40 min, 1750, 1610 and 4500 dpm/μg DNA, respectively (Fig. 4B). The ratios of [^{3}H]OA, [^{3}H]DAG and [^{3}H]PC levels in NGF-treated cells to their levels in control cells, after 10 min of incubation, were 1.4, 1.2 and 1.0; after 40 min, they were 0.9, 1.8 and 1.1, respectively. The changes in the specific activities (Fig. 4C) of [^{3}H]DAG and [^{3}H]PC also followed the above pattern, further confirming the findings based on total radioactivity measurements. These data show that [^{3}H]OA served as a source material; that [^{3}H]DAG was an important precursor for [^{3}H]PC formation; and that the major increases in [^{3}H]DAG levels caused by short-term exposure to NGF resulted from acceleration of its de novo synthesis involving the combination of fatty acyl-CoA with glycerol phosphate. It should be pointed out that [^{3}H]PA is a common precursor for both [^{3}H]DAG and [^{3}H]PI syntheses [33], so the changes in [^{3}H]PA levels did not exactly follow the pattern of [^{3}H]DAG.

3.3. Utilization of [^{3}]OA or [^{3}H]glycerol by PC12 cells pre-treated with NGF from 0.5 to 4 days

To explore the effects of long-term treatment with NGF on the synthesis rates of glycerolipids, we exposed PC12 cells to NGF from 1 to 4 days, and then labeled them with [^{3}H]OA. The levels of [^{3}H]OA-labeled lipids in untreated control cells exposed to the [^{3}H]OA for 1 h were, in descending order, [^{3}H]PC > [^{3}H]TAG > [^{3}H]PE > [^{3}H]DAG (Fig. 5); NGF pre-treatment not only enhanced the incorporation of [^{3}H]OA into most of the lipids ([^{3}H]DAG levels showed the greatest increase), but also changed the above order to [^{3}H]PC > [^{3}H]DAG > [^{3}H]TAG > [^{3}H]PE (Fig. 5). After 2 days of NGF pre-treatment (Fig. 5B) the levels of [^{3}H]OA-labeled PC and PE were 5700 and 80 dpm/μg DNA higher than (or 1.7- and 1.0-fold) those of control, respectively, and after 4 days of NGF pre-treatment (Fig. 5C) these levels of [^{3}H]PC and [^{3}H]PE were now 8710 and 933 dpm/μg DNA higher than (or 2.0- and 1.4-fold) those of control, respectively. Taking the data on the cellular levels of non-radioactive glycerolipids (Fig. 2) and the data on the levels of [^{3}H]OA-labeled lipids (Fig. 5) together, one could conclude that NGF treatment not only increased the actual amount of cellular lipids in the PC12 cells, but also their rates of syntheses.

Triacsin C, an inhibitor of long chain acyl-CoA synthetase, partially blocked the incorporation of [^{3}H]OA into [^{3}H]DAG (by 37–66%), [^{3}H]TAG (by 49–53%) and [^{3}H]PC (by 17–39%), causing free [^{3}H]OA to accumulate

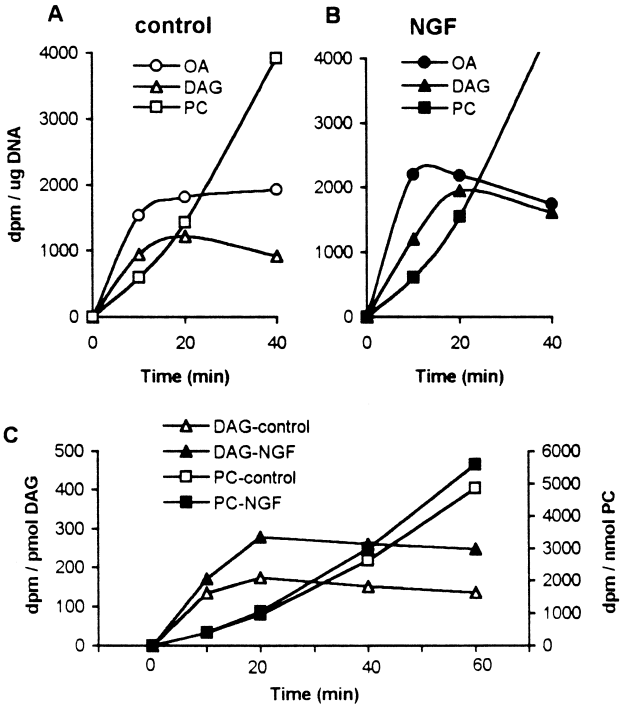

Fig. 4. Comparison of time-dependent courses between the levels of [^{3}H]OA, [^{3}H]DAG and [^{3}H]PC in PC12 cells. The experimental conditions and methods were same as described in Fig. 3. After incubation in medium B for 24 h, PC12 cells were simultaneously treated with 50 ng/ml of NGF and labeled with 5 μCi/ml of [^{3}H]OA for 0, 10, 20, 40 or 60 min. The cells were then harvested and assayed for unlabeled and [^{3}H]OA-labeled lipid levels and DNA contents as described in Section 2. Values are total radioactivities (A and B; dpm per DNA) and specific activities (C; dpm per lipid) in control and NGF-treated cells, and represent means of 4–6 determinations.

by 138–172% in both control cells and NGF pre-treated cells (Fig. 6).

Because the increases in [^{3}H]OA-labeled lipid levels could have resulted either from increased de novo syntheses of glycerolipids, or in the reacylation of lipids, we examined the incorporation of [^{3}H]glycerol, a 'backbone' of glycerolipids, into control cells and cells pre-treated with NGF. The increases in [^{3}H]glycerol incorporation caused by NGF were similar to the increases in [^{3}H]OA incorporation, indicating that NGF pre-treatment also promoted the incorporation of [^{3}H]glycerol into lipids by stimulating their de novo syntheses (Fig. 7). The percent

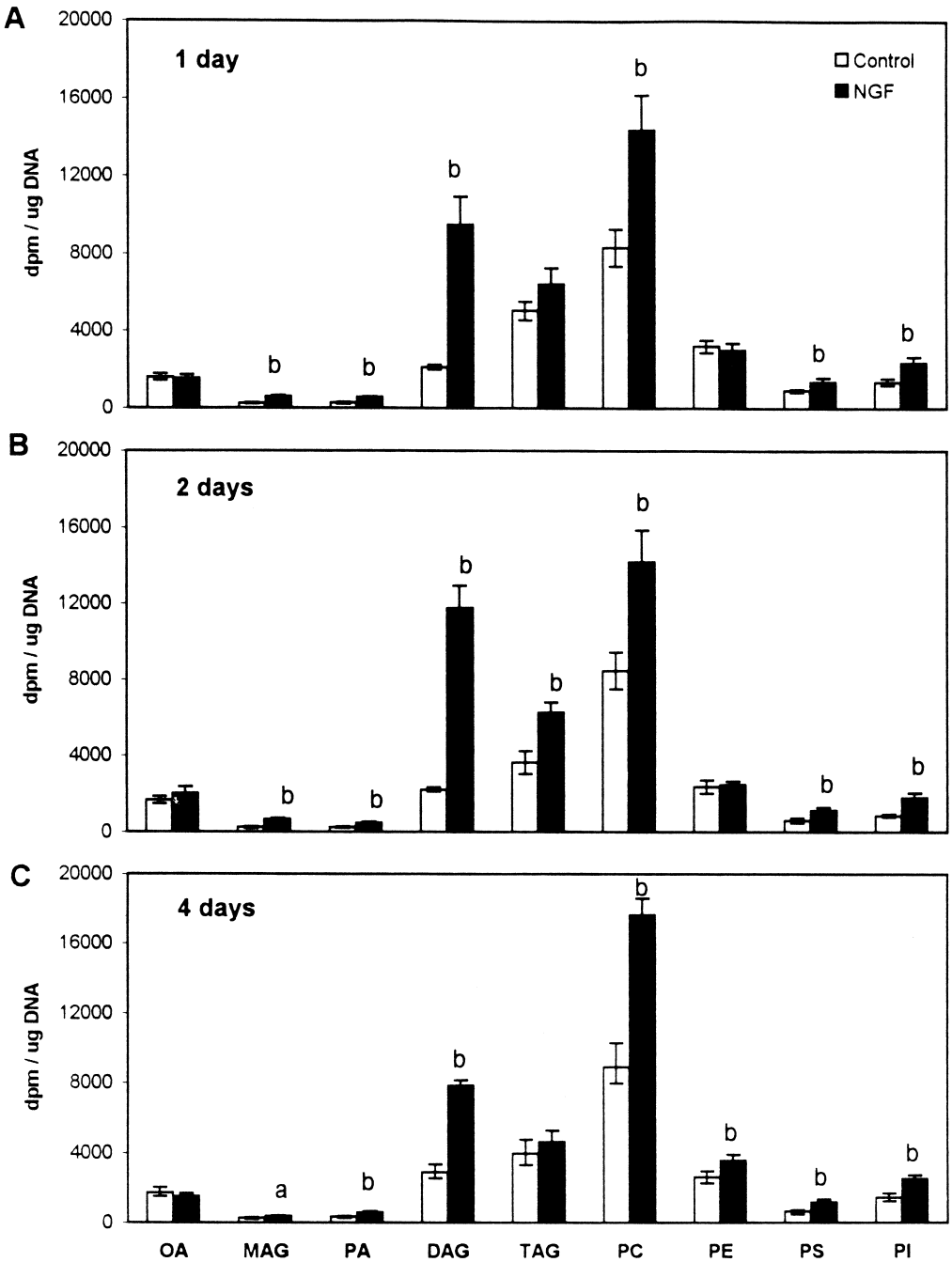

Fig. 5. Incorporation of [^{3}H]OA into neutral lipids and phospholipids in control and NGF pre-treated PC12 cells. After incubation in medium B for 24 h, PC12 cells were pre-treated with 50 ng NGF/ml medium B for 1 (A), 2 (B) or 4 (C) days, and then exposed to both 50 ng NGF and 5 μCi [^{3}H]OA/ml medium B for an additional 1 h. The cells were then harvested and assayed for [^{3}H]OA-labeled lipid levels and DNA contents as described in Section 2. Values represent the mean ± SD of [^{3}H]lipid levels (dpm/μg DNA; $n = 6–10$). Compared with control (independent values of 1, 2 or 4 days), a: $P < 0.05$, b: $P < 0.01$.

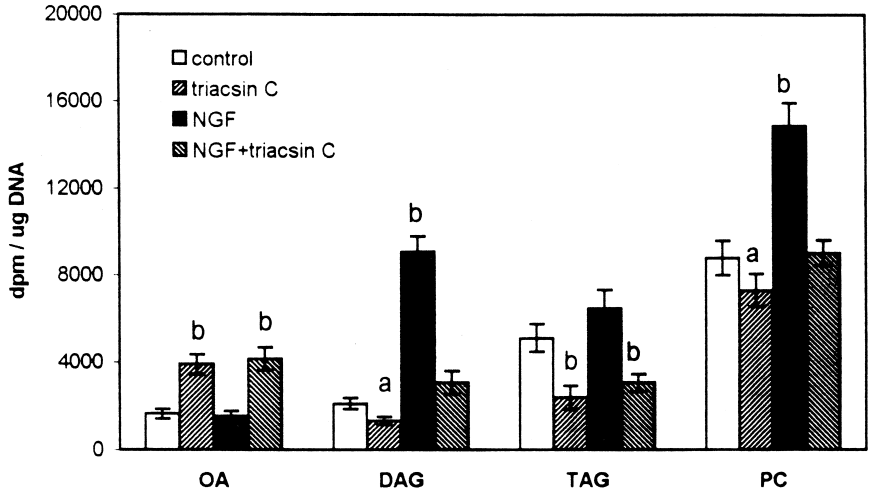

Fig. 6. Effects of triacsin C on incorporation of [^{3}H]OA into lipids in both control cells and NGF pre-treated cells. After incubation in medium B for 24 h, PC12 cells were pre-treated with 50 ng NGF/ml medium B for 0 or 1 day, and then exposed to 50 ng NGF, 4.8 nmol triacsin C and 5 μCi [^{3}H]OA/ml medium B for an additional 1 h. The cells were then harvested and assayed for [^{3}H]OA-labeled lipid levels and DNA contents as described in Section 2. Values represent the mean ± SD of [^{3}H]lipid levels (dpm/μg DNA; $n = 6$). Compared with control, a: $P < 0.05$, b: $P < 0.01$.

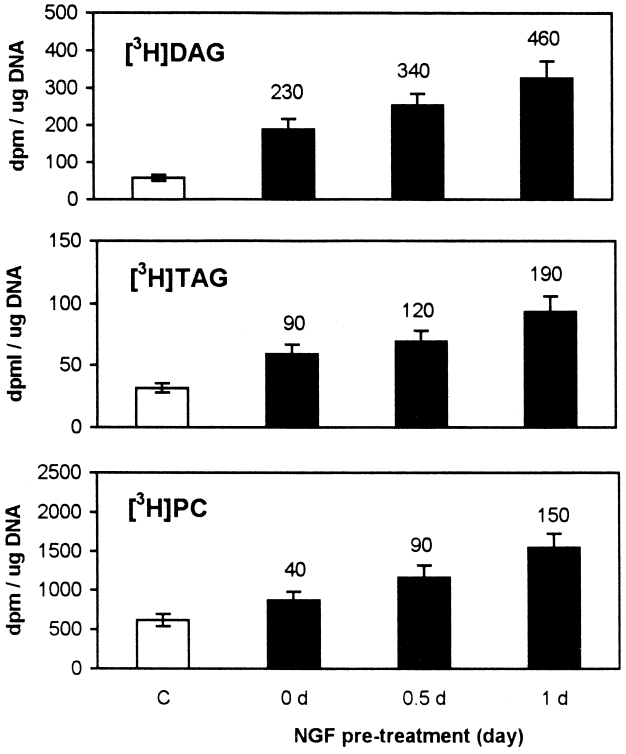

Fig. 7. Effects of NGF pre-treatment on incorporation of [^{3}H]glycerol into lipids in PC12 cells. After incubation in medium B for 24 h, PC12 cells were pre-treated with 50 ng NGF/ml medium B for 0, 0.5 or 1 day, and then exposed simultaneously to 50 ng NGF and 8 μCi [^{3}H]glycerol/ml medium B for an additional 20 h. The untreated control cells (open bars) were exposed only to [^{3}H]glycerol. The cells were then harvested and assayed for [^{3}H]glycerol-labeled lipid levels and DNA contents as described in Section 2. Values represent the mean ± SD of [^{3}H]lipid levels (dpm/μg DNA; $n = 6$). Numbers above bars show percent increase over control.

increases in [^{3}H]glycerolipid levels were, in descending order, [^{3}H]DAG (230–460%) > [^{3}H]TAG (90–190%) > [^{3}H]PC (40–150%).

3.4. Disappearance of [^{3}H]OA pre-labeled lipids from NGF-treated PC12 cells

After pre-labeling PC12 cells with [^{3}H]OA until 96% of intracellular radioactivity was found in [^{3}H]glycerolipids, a chase study was performed. The disappearance of free [^{3}H]OA and of [^{3}H]OA-labeled neutral lipids exhibited a rapid phase during the 2 h after cessation of [^{3}H]OA labeling, followed by a slow phase (Fig. 8). By 4 h after the cessation of [^{3}H]OA labeling, the half lives ($T_{1/2}$) of [^{3}H]OA, [^{3}H]MAG, [^{3}H]DAG and [^{3}H] TAG, as calculated according to the exponential equation of $Y_{1/2} = Y_{\max} e^{-KtT_{1/2}}$ were 0.9, 2.0, 0.95 and 1.0 h, respectively. During the 4-h incubation after cessation of [^{3}H]OA labeling, [^{3}H]PS levels were increased by 50% over baseline, probably reflecting continued synthesis of this phospholipid from [^{3}H]PC or [^{3}H]PE by base exchange. [^{3}H]PC and [^{3}H]PE levels were unchanged; and [^{3}H]PI levels decreased gradually (Fig. 8). These results affirm that, as precursors for PL syntheses, fatty acids and neutral lipids disappear rapidly from the cells. In contrast, PLs, as biomembrane constituents, exhibit relatively slower turnovers.

Fig. 8 also shows that NGF transiently accelerated the turnover of newly-formed [^{3}H]PI. By 0.5 h after the cessation of [^{3}H]OA labeling, [^{3}H]PI levels in NGF-treated cells were 32% lower than those in control cells ($P < 0.01$), while [^{3}H]DAG levels in NGF-treated cells were 23% higher than those in the controls ($P < 0.05$). These tran-

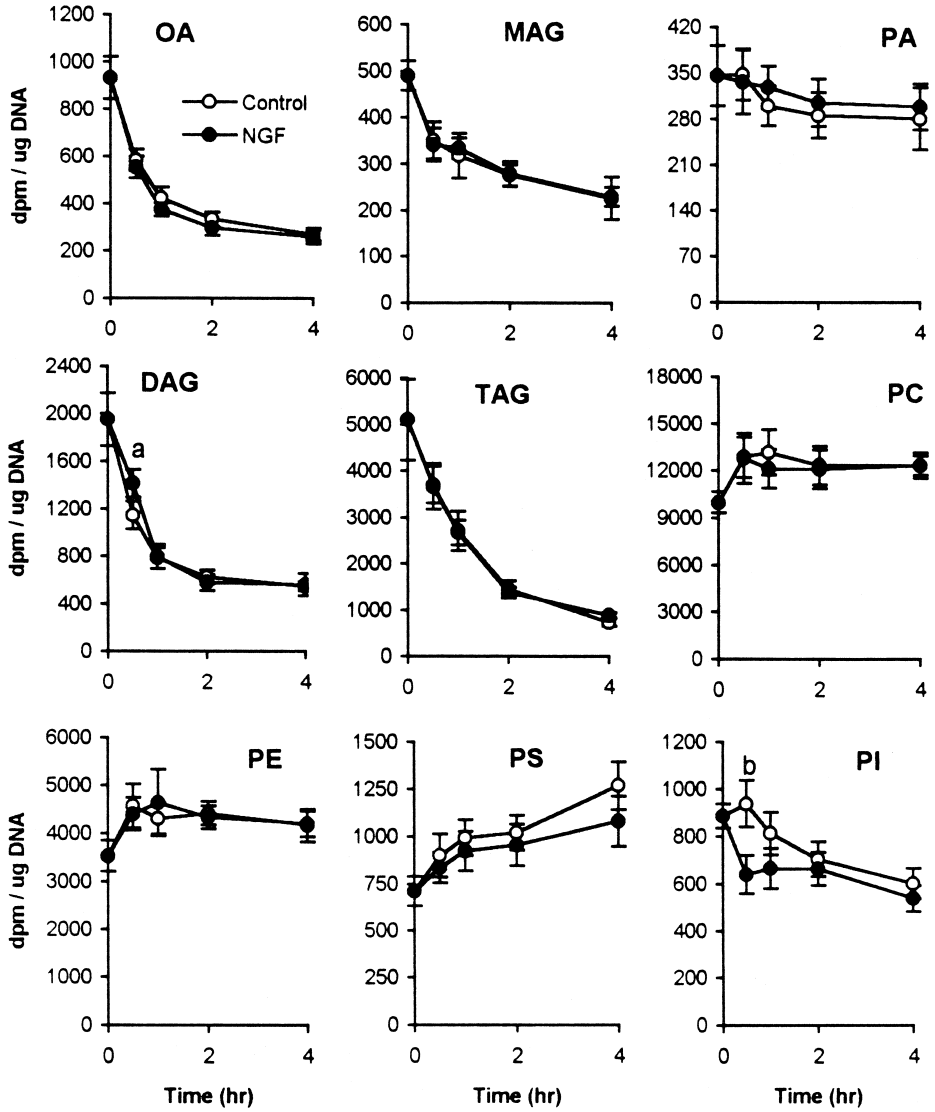

Fig. 8. Degradation of [^{3}H]OA-labeled lipids in PC12 cells treated with NGF and in control cells. After incubation in medium B for 24 h, PC12 cells were pre-labeled with 5 μCi [^{3}H]OA/ml medium B for 1 h, then washed once with medium B without radioactivity, and subsequently treated with 5 ng NGF/ml medium B for 0, 0.5, 1, 2 or 4 h. The cells were extracted, and [^{3}H]OA-labeled lipids and DNA were measured as described in Section 2. Values represent the mean ± SD of [^{3}H]lipid levels (dpm/μg DNA; $n = 4–6$). Compared with control, a: $P < 0.05$, b: $P < 0.01$.

sient differences between the untreated and treated cell groups suggest that initially, the increased production of [^{3}H]DAG could derive in part from accelerated hydrolysis of [^{3}H]PI.

4. Discussion

These data show that total levels of cellular glycerolipids (Fig. 2), particularly DAG, and the syntheses of these compounds (Figs. 5–7) are significantly increased by NGF treatment. The de novo synthesis of [^{3}H]PC from [^{3}H]OA (Figs. 5 and 6) or from [^{3}H]glycerol (Fig. 7) involves two steps, i.e., incorporation of [^{3}H]OA or [^{3}H]glycerol into [^{3}H]DAG, and then its combination with CDP-choline to form [^{3}H]PC. NGF rapidly stimulates the first step but affects the second one only more slowly (Figs. 3 and 4), so [^{3}H]DAG initially accumulates. Short-term treatment with NGF transiently promotes the hydrolysis of newly-formed [^{3}H]PI to yield [^{3}H]DAG (Fig. 8). Our data also show that the disappearance of [^{3}H]neutral lipids from cells is faster than that of the more stable [^{3}H]PLs, and is largely unaffected by NGF (Fig. 8).

In our NGF pre-treatment studies of [^{3}H]OA-labeled lipids, the increases in cellular levels of unlabeled DAG were accompanied by increases in the incorporation of [^{3}H]OA into DAG, probably reflecting its de novo synthesis in the PC12 cells. The fact that NGF pre-treatment affected in different extent on the levels of individual glycerolipids could be explained by hypothesizing that some different rates existed between the incorporation of [^{3}H]OA into [^{3}H]DAG (the first step) and the conversion of [^{3}H]DAG to [^{3}H]PC or PE (the second step).

Glycerol is a 'backbone' for de novo syntheses of glycerolipids, and triacsin C is a potent competitive inhibitor of long chain acyl-CoA synthetase [9,30]. Our findings that NGF also increases the incorporation of [^{3}H]glycerol into [^{3}H]glycerolipids (Fig. 7) and that triacsin C, which partially inhibits acyl-CoA synthetase, also blocks this NGF effect (Fig. 6), provide further evidence that the increased cellular DAG (Fig. 2C) and [^{3}H]DAG (Fig. 5) levels derived principally from their de novo syntheses.

The main pathways for PC and PE biosyntheses in mammalian cells include the combination of fatty acyl-CoA with glycerol phosphate or lysophosphatidate to form DAG, and then the combination of this intermediate with CDP-choline or CDP-ethanolamine [29,30]. In some circumstances, a fatty acid is able to react directly with lysophospholipids to form PLs [23]. Besides the de novo synthesis pathway, DAG can also be formed from hydrolysis of PLs by phospholipases [24,26]. Hence, to identify the sources of the increased DAG levels in NGF-exposed cells, using a method involving fatty acid labeling, it was necessary to observe the dynamic relationships between various intermediates in the reactive chain and to identify their precursor–product relationships. Our data from kinetic studies on [^{3}H]OA incorporation show that [^{3}H]DAG levels, expressed either as total radioactivity (Fig. 4A and B) or as specific activity (Fig. 4C), increased much earlier than did those of [^{3}H]PC, suggesting that most of the [^{3}H]DAG derived from de novo synthesis and not from phospholipid breakdown. Such increases in DAG levels via de novo synthesis have also been observed in BC3H-1 myocytes treated with insulin [7]. Furthermore, our findings suggest that enhanced uptake of [^{3}H]OA by cells treated briefly with NGF (manifested as the increase in the free [^{3}H]OA levels, Figs. 3 and 4) may also promote the enhanced de novo synthesis of [^{3}H] DAG.

Much evidence exists that cellular DAG can arise from the hydrolysis of PI or PC by phospholipase C (PLC) or phospholipase D (PLD) [10,11,14,15,18,20,24,27]. DAG formed from PI hydrolysis can temporarily activate protein kinase C (PKC), inducing numerous metabolic changes, while DAG from PC hydrolysis is thought to produce more prolonged PKC activation, resulting in cellular proliferation and differentiation [29]. Thus, our studies on the disappearance of [^{3}H]OA-labeled lipids from PC12 cells were designed to explore whether the phospholipase pathways for DAG formation were also stimulated by NGF. The present data show that NGF does induce, transiently and simultaneously, an accelerated disappearance of [^{3}H]PI and a retarded disappearance of [^{3}H]DAG (Fig. 8), suggesting that PI hydrolysis can be another source of increased DAG.

The rapid disappearance of [^{3}H]OA-labeled neutral lipids suggests either that these compounds are quickly converted to their metabolites, or that much of the [^{3}H]fatty acid in these molecules is replaced by non-radiolabeled fatty acids after cessation of labeling. The facts that [^{3}H]OA-labeled PC or PE levels failed to decline, while those of [^{3}H]OA-labeled PS levels actually increased (Fig. 8) indicate either that these [^{3}H]PLs continue to be formed from [^{3}H]neutral lipids, or that non-radiolabeled fatty acids in them continue to be replaced by [^{3}H]OA, or both. Moreover, the differences in turnover between [^{3}H]PS and [^{3}H]PC or [^{3}H]PE suggest that [^{3}H]PS was being formed from [^{3}H]PC and [^{3}H]PE via base exchange [25,34].

In summary, the present studies show that NGF treatment increases the levels of non-radioactive neutral lipids and PLs in PC12 cells, as well as the synthesis rate of [^{3}H]glycerolipid formed from [^{3}H]OA or from [^{3}H]glycerol. These effects vary with the particular type of lipid studied. The increased [^{3}H]DAG levels seen with NGF treatment derive both from enhanced de novo synthesis and, to a lesser and transient extent, from the hydrolysis of [^{3}H]PI.

Acknowledgements

We gratefully acknowledge the expert technical assistance of Dr. Wataru Araki, Ms. Carol Watkins, Ms. Ingrid U. Richardson, Mr. Jianping Shi and Mr. Jeff Breu. This study was supported by grants from the National Institute of Mental Health (MH-28783) and from the Center for Brain Sciences and Metabolism Charitable Trust.

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