Cyclooxygenase-1 Behaves as a Delayed Response Gene in PC12 Cells Differentiated by Nerve Growth Factor*

(Received for publication, May 16, 1997, and in revised form, June 3, 1997)

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Treatment of PC12 cells with nerve growth factor (NGF) results in a differentiation program characterized by expression of immediate early and delayed response genes. In this program, morphological changes such as neurite extension are accompanied by phenotypic changes in enzyme expression, including an increased capacity for prostaglandin synthesis. Cyclooxygenase (COX), the enzyme responsible for prostaglandin production, exists as two isoforms: constitutive COX-1 and inducible COX-2. We report that COX-1 behaves as a delayed response gene in PC12 cells exposed to NGF. Six hours following NGF treatment, COX-1 mRNA levels were elevated in PC12 cells, reaching nearly 5-fold above basal levels at 12 h. This increase was blocked by cycloheximide and was accompanied by concomitant increases in COX-1 protein and enzyme activity. COX-1 protein remained elevated for at least 10 days and localized to the cytoplasm and neurites of NGF-differentiated PC12 cells. Moreover, basic fibroblast growth factor, but not epidermal growth factor, caused similar increases in COX-1, which is consistent with expression characteristics of other delayed response genes in PC12 cells. This is the first example of neurotrophic factor regulation of cyclooxygenase and may have important implications for determination of the differentiated phenotype in PC12 cells.

Nerve growth factor (NGF) is a member of the neurotrophin family of growth factors and has long been known to maintain sympathetic neurons and certain populations of sensory neurons in the periphery (1–3). Phenotypic changes in response to NGF have been studied in PC12, a cell line derived from rat pheochromocytoma (4). A neuronal phenotype is induced by NGF exposure, which promotes differentiation and survival of these cells (5). NGF also induces several delayed response gene products including VGF (6, 7), GAP-43 (8), and transin (9). Like NGF, basic fibroblast growth factor (bFGF) induces PC12 cell differentiation (10), whereas epidermal growth factor (EGF) is mitogenic in PC12 cells (11). These differential effects have been used to characterize the signal transduction pathway leading to the differentiated phenotype. NGF and bFGF both cause a prolonged activation of Ras resulting in a prolonged increase of extracellular signal-regulated kinase activity. In contrast, EGF causes transient activation of Ras and extracellular signal-regulated kinase (12). One proposed mechanism of delayed response gene induction is through the prolonged activation of Ras leading to the phosphorylation of the cAMP regulatory element-binding protein for up to several hours (13, 14) that, along with immediate early gene products, leads to subsequent delayed response gene expression.

Cyclooxygenase (COX), the enzyme responsible for the conversion of arachidonic acid to prostaglandin H₂, exists as two isoforms, COX-1 and COX-2. In non-neuronal cells COX-2 behaves similarly to other immediate early genes in that it is rapidly and transiently induced by various growth factors and cytokines without requiring new protein synthesis (15, 16). It is also rapidly induced in central nervous system neurons through synaptic activation (17). COX-1, on the other hand, is constitutively expressed in a variety of cell types (16), although a few examples of COX-1 regulation in non-neuronal cells have been reported (18–20). Regulation of COX by neurotrophic factors has not been previously demonstrated in neuronal cells; however, NGF-induced differentiation of PC12 cells leads to elevated prostaglandin levels (21). We report for the first time that NGF induces COX-1 in PC12 cells, that this induction is inhibited by cycloheximide, and that bFGF (but not EGF) also induces COX-1, suggesting that COX-1 behaves as a delayed response gene in these cells.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, human recombinant bFGF, human recombinant EGF, fetal bovine serum, horse serum, penicillin-streptomycin, and Trasylol reagent were obtained from Life Technologies, Inc. A monoclonal antibody to COX-2 was purchased from Transduction Labs (Lexington, KY) and was found to have cross-reactivity to both ovine (Caymen Chemical, Ann Arbor, MI) and rat COX-1 antigen (a gift from Phyllis Whiteley, Roche Bioscience, Palo Alto, CA). Initial experiments were performed using NGF provided by Dr. Eugene Johnson who also kindly supplied the neutralizing antibody to NGF. Later experiments were performed using 2.5 ng NGF purchased from Life Technologies, Inc. No differences in response were noted between the two sources of NGF. Texas red-conjugated goat anti-mouse polyclonal antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). [α-32P]dCTP and PGE₂ RIA kits were purchased from NEN Life Science Products. Arachidonic acid was purchased from New Check Prep (Elyssian, MN). All other products were from Sigma.

Cell Culture—PC12 cells were grown on plastic tissue culture dishes in Dulbecco’s modified Eagle’s medium with 5% heat-inactivated horse serum and 5% fetal bovine serum in the presence of penicillin-streptomycin. For most studies, cells were plated at a density of 10⁵ cells/cm² and were grown to 70–80% confluency prior to the start of each experiment.

Immunocytochemistry and Subcloning of PC12 Cells—PC12 cells were plated at a density of 1000 cells/well into 24-well dishes containing 12-mm glass coverslips coated with poly-D-lysine. Cells were treated with 50 ng/ml NGF or vehicle for 3 days, fixed with 4% paraformaldehyde for 10 min, and washed with PBS. Coverslips were incubated in...
NGF induces COX in PC12 cells. In A, PC12 cells were treated for 4 days with the indicated concentrations of NGF. Cell lysates were subjected to Western blot analysis with antibody recognizing both COX-1 and COX-2 in rat. In the lane on the far right, lysate was from cells treated with 50 ng/ml NGF that had been preincubated with 10-fold excess neutralizing anti-NGF antibody (37°C for 30 min). In B, data represent the mean laser densitometric values ± S.E. (n = 4) of COX protein levels relative to controls following treatment as described in A. **, p < 0.02 versus 50 ng/ml of NGF, two-tailed Student's t test.

PBS-T for 10 min followed by a 20-min incubation in −20 °C methanol. Samples were rehydrated in PBS-T for 10 min, blocked for 1 h in 10% normal goat serum, and then incubated in primary antibody (1:1000) for 2 days at 4°C. Following extensive washing with PBS-T, samples were incubated in Texas red-conjugated secondary antibody (1:2000) for 2 h. Coverslips were thoroughly washed, partially dried, and placed on glass slides coated with 50 μl of Prolong purchased from Molecular Probes (Eugene, OR). Cells were viewed by epifluorescent microscopy, and images were captured by a Cohu (San Diego, CA) 4910 CCD video camera attached to a Zeiss Axiosplan microscope and visualized using NIH Image/Adobe Photoshop software. COX was detected in parental PC12 cells; however, signal intensity varied within the population. We subcloned PC12 cells by standard methods to develop cell lines that uniformly expressed COX. Briefly, cells were plated in 96-well dishes at a density of approximately 1 cell/well. Following 2–3 passages, subclones were plated separately onto poly-d-lysine-coated glass coverslips and visualized following NGF treatment as described above. The majority of subclones tested expressed COX uniformly as detected by immunofluorescence, and two subclones (PCX and PCX25) with bright COX signal were chosen for further study.

Western Blot Analysis—To measure COX protein levels, parental PC12, PCX, or PCX25 cells were plated in 6-well dishes. After treatment, cells were washed three times with ice-cold PBS and collected in 200 μl of Laemmli sample buffer. Samples were sonicated, and protein concentrations were determined using the bicinchoninic acid method (Micro-BCA kit) purchased from Pierce. Ten-microgram protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blots were blocked in Tris-buffered saline containing 0.1% Tween 20, 2% BSA, and 3% nonfat dry milk for 1 h and then incubated with primary antibody (1:1000) overnight in the same buffer. Following extensive washing and incubation for 2 h with peroxidase-linked secondary antibody at 1:5000 (Amersham Corp.), signals were visualized by enhanced chemiluminescence (ECL, Amersham Corp.) and exposure to Kodak X-AR film. Laser densitometry and quantification were accomplished with a program from Molecular Dynamics (Sunnyvale, CA).

Cyclooxygenase Activity Determination—Cyclooxygenase activity was measured by incubating cells for 15 min with 30 μM arachidonic acid as described previously (22). PGE2 levels were determined by RIA and normalized to total cellular protein determined by the bicinchoninic acid method. In all accumulation and activity experiments, samples were collected from five or six parallel cultures.

Nerve Growth Factor Induces Cyclooxygenase-1

RESULTS

NGF induced COX protein in a dose-dependent fashion in PC12 cells (Fig. 1A). This induction was 9.3 ± 1.4-fold over basal levels (mean ± S.E.; n = 4) at 50 ng/ml of NGF, a dose typically used to differentiate PC12 cells (25), and was significantly blocked (p < 0.02) when added NGF (50 ng/ml) was preincubated for 30 min with 10-fold excess neutralizing antibody to NGF (Fig. 1B). These experiments were carried out after 4 days of NGF treatment; however, COX protein levels remained elevated for at least 10 days (data not shown).

Initial immunocytochemical analysis revealed expression of COX in parental PC12 cells; however, signal intensity of COX immunofluorescence varied within the population. To obtain a more homogeneous cell population for COX expression, we subcloned PC12 cells and selected two subclones, PCX and PCX25, to carry out the remainder of our experiments. As illustrated in Fig. 2, COX was readily detected in the cytoplasm and neurites of PCX cells after NGF-induced differentiation. A similar pattern was seen in the parental cell line and in PCX25 cells (data not shown).

To determine which isoform of COX was expressed in PCX cells, total RNA was reverse-transcribed and amplified by polymerase chain reaction. NO COX-2 product was detected after 40 cycles of polymerase chain reaction, which is consistent with previously published data (26), whereas a fragment of the expected size for COX-1 was generated with COX-1-specific primers. This fragment was gel-purified and sequenced to verify that COX-1 was present in these cells (data not shown).

We evaluated whether COX-1 was functional in PCX cells by assessing prostaglandin production in NGF-differentiated cells. Four days following the addition of NGF, neuronal PCX
cells showed a 11.5-fold increase ($n = 5$) in endogenous PGE$_2$ accumulation over 4 h relative to naive cells (Fig. 3A). To confirm that this increase in PGE$_2$ was due to increased COX-1 activity, we tested whether exogenous substrate for COX (arachidonic acid) would lead to increases in PGE$_2$ production. Fig. 3B illustrates that COX-1 activity peaks at 2 days with a 9.7-fold induction over basal levels seen prior to NGF treatment ($n = 5$). Fig. 3C shows COX activity in differentiated PCX cells treated with NGF for 4 days to be 9.9-fold over levels in untreated cells at day 4 ($n = 5$), and this increase was inhibited by the non-steroidal anti-inflammatory agent indomethacin, with an IC$_{50}$ of 35 nM, similar to that previously reported for COX-1 (27).

Northern blot analysis revealed that COX-1 mRNA was elevated 6 h following NGF treatment (Fig. 4A) with peak levels reached at 12 h (4.7 ± 0.8-fold over basal levels, $n = 3$; Fig. 4B). COX-1 message remained elevated at 24 and 48 h. No message for COX-2 was detected (data not shown). Only a minimal increase in COX-1 message over basal levels was seen at 3 h suggesting that COX-1 requires new protein synthesis to be expressed. Indeed, pretreatment with cycloheximide (25 mM, applied 30 min prior to NGF) prevented COX-1 expression at 8 h (Fig. 4C), indicating that COX-1 has characteristics of a delayed response gene following NGF treatment.

To further characterize the induction of COX-1 in PC12 cells, we tested whether bFGF or EGF had any influence on COX-1 expression. bFGF causes neuronal differentiation of PC12 cells, whereas EGF acts solely as a mitogen (10, 11). Fig. 5A shows that bFGF caused a similar increase in COX-1 message levels by 6 h as compared with NGF (Fig. 4A); however, EGF had little to no effect on mRNA levels for COX-1. This pattern of induction was replicated at the protein level (Fig. 5B). The time course for the induction of COX-1 by bFGF was similar to that seen following exposure to NGF, and similar results were seen with PCX25 cells (data not shown).
**DISCUSSION**

In many cell systems COX-1 is constitutively expressed (16, 22, 28), but there are some instances where COX-1 is regulated. For example, COX-1 message is induced in mouse osteoblastic MC3T3 cells treated with bFGF (18) and during phorbol ester-induced differentiation of THP-1 cells, a human monocytic leukemia cell line (20). In contrast to the transient induction of immediate early genes like c-fos (29) and certain tetradecanoyl phorbol acetate-induced sequences (26) in PC12 cells, the induction of COX-1 message was delayed and persisted following NGF and bFGF treatment in PCX cells (Figs. 4 and 5). This pattern of expression is similar to other delayed response gene products in PC12 cells such as transin and VGF. Like COX-1, transin mRNA levels increase in response to NGF and bFGF, but not EGF, and this increase in message is blocked by cycloheximide (9). VGF shows similar inductive properties (7). COX-1 induction by NGF and bFGF, but not EGF, is consistent with the idea that COX-1 induction is through the prolonged activation of Ras. Persistent Ras activation by either NGF or bFGF leads to neurite extension, whereas the transient activation of this signaling pathway by EGF does not (12).

The induction of COX-1 by NGF in PC12 cells suggests that it may play a physiologic role in the differentiated phenotype. PC12 cells have been used to investigate the tyrosine hydroxylase pathway involved in the production of the bioactive amines: dopamine, norepinephrine, and serotonin (4). Interestingly, it has been shown that pharmacologic manipulation of PC12 cells with PGE2 leads to an induction of aromatic l-amino acid decarboxylase (30), an enzyme directly involved in the synthesis of these neurotransmitters. COX-1 may be intimately involved in determination of the differentiated phenotype of PC12 cells since prostaglandin levels have been reported to be elevated as early as 15 min following NGF treatment; however, this increase does not affect the morphological phenotype since indomethacin does not hinder NGF-induced neurite extension in PC12 cells (21) or in our PCX subclone (data not shown).

Studies are under way to further understand the mechanism of COX-1 induction in these cells and to address whether other characteristics of the differentiated phenotype depend on COX-1 activity.

**Acknowledgments**—We thank Eugene Johnson for gifts of NGF and neutralizing antibody to NGF, Phyllis Whiteley for recombinant rat COX-1 protein, and Robert Freeman and Larry Tabak for helpful suggestions.

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