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Cyclooxygenase-2 Expression Inhibits Trophic Withdrawal Apoptosis in Nerve Growth Factor-differentiated PC12 Cells*

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Cyclooxygenase-2 (Cox-2), an enzyme responsible for catalyzing the committed step in prostanoid biosynthesis, is the product of an immediate early gene capable of being up-regulated by diverse stimuli. Significantly Cox-2 mRNA is absent from rat pheochromocytoma (PC12) cells, both basally and following stimulation with a range of agonists. Using PC12 cells engineered to stably express isopropyl-1-thio-β-D-galactopyranoside-inducible Cox-2 (PCXII-4), we have investigated the putative effects of Cox-2 expression on differentiation, proliferation, and trophic withdrawal apoptosis. Cox-2 bioactivity had no effect on nerve growth factor-induced differentiation, epidermal growth factor-induced proliferation, or aromatic L-amino acid decarboxylase expression. However, trophic withdrawal apoptosis, induced by the removal of nerve growth factor following differentiation, was markedly reduced in the PCXII-4 when compared with control cells, as assessed by annexin V staining, DNA laddering, and Hoechst 33258 staining. The specificity of this effect was confirmed using two pharmacologically distinct nonsteroidal anti-inflammatory drugs, indomethacin and NS398. Investigations showed that the activity of the pro-apoptotic protease caspase-3 was reduced in PCXII cells. This study demonstrates that Cox-2-derived prostaglandins exert cytoprotective effects in trophic factor withdrawal apoptosis and provides evidence that this is, at least in part, due to suppression of caspase-3 activity.

Cyclooxygenase (Cox)1 catalyzes the first, rate-limiting step in the formation of prostaglandin and thromboxane eicosanoids from phospholipase A2-released arachidonic acid (1). Also known as prostaglandin endoperoxide H synthase, the enzyme is responsible for both the cyclooxygenase reaction in which arachidonate is converted to prostaglandin G2 and the peroxidase reaction in which this intermediate undergoes a bi-electron reduction to prostaglandin H2. Prostaglandins are involved in a wide range of physiological and pathophysiological responses. Two forms of the prostaglandin endoperoxide H synthase have been identified, encoded by distinct genes but exhibiting structural and enzymatic similarities. Cox-1 is the “constitutive” isoform, whereas Cox-2 is the “inducible” form of the enzyme. Cox-2, normally absent from cells, is rapidly expressed in response to a wide variety of cytokines (2), growth factors (3), and ligands of G protein-coupled receptors (4). Constitutive expression of Cox-2 has, however, been described in the brain, kidney, and testes (5–7). Further, the constitutive expression of Cox-2 in colon carcinoma has been shown to represent a causal factor in the transformation of this tissue (8).

The cDNA encoding Cox-2 was originally described as 12-O-tetradecanoylphorbol-13-acetate-inducible sequence 10 (9), one of a number of primary response genes rapidly and transiently induced by 12-O-tetradecanoylphorbol-13-acetate in Swiss 3T3 cells rendered quiescent by confluence. Early studies identified the expression of 12-O-tetradecanoylphorbol-13-acetate-inducible sequence genes to be cell type-specific. 12-O-Tetradecanoylphorbol-13-acetate-inducible sequence 10/Cox-2 mRNA was notably absent from rat pheochromocytoma (PC12) cells stimulated with a range of growth factors and ion channel agonists (10), leading to the conclusion that this inability to express 12-O-tetradecanoylphorbol-13-acetate-inducible sequence 10 resulted from either a developmental “shut-down” of the gene or a mutation that rendered successful transcription impossible. We have generated PC12 cell lines stably expressing isopropyl-1-thio-β-D-galactopyranoside ( IPTG)-inducible Cox-2 (PCXII). Because PC12 cells are used as a cell culture model of nerve growth factor (NGF)-induced neuronal differentiation and have facilitated studies into the signaling pathways leading to the differentiated phenotype (11), this cell line represents an ideal tool for investigations into the effects of Cox-2 overexpression on cellular differentiation. Further, PCXII cells also facilitate studies into the effect of Cox-2-derived prostaglandins on the trophic withdrawal apoptosis, which occurs upon NGF removal subsequent to differentiation. This cell culture model of programmed cell death has been used extensively to provide insights into the mechanisms underlying the abnormal neuronal apoptosis characteristic of various neurodegenerative pathologies (12).

EXPERIMENTAL PROCEDURES

Materials—Rat tail collagen I and 2.5% NGF were obtained from Collaborative Biomedical Products. Anti-2.5% NGF antibody and Hoechst 33258 were purchased from Sigma. All cell culture media, sera, genetin (G-418), and IPTG were from Life Technologies, Inc. Hygromycin was obtained from Roche Molecular Biochemicals. Anti-aromatic L-amino acid decarboxylase (AADC) antiserum was obtained from Bio-
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Cox-2 expression in PC12 cells was confirmed by the addition of IPTG to the culture media. Western blot analysis using a polyclonal anti-Cox-2 antibody was carried out on cell lysates of putative clones to confirm that expression of this enzyme was IPTG-inducible. From 16 clones analyzed, 5 were found to exhibit IPTG-inducible Cox-2, and clones 1 and 4, hereafter referred to as PCXII-1 and -4, were selected for further analysis. Because PCXII-1 and -4 were found to behave similarly with respect to differentiation, mitogenesis, and apoptosis, the results from the latter are presented here. Western blot analysis (Fig. 1A) confirmed that, as has been reported previously (10), Cox-2 is not endogenously expressed in these cells, whereas after addition of IPTG Cox-2 was inducibly expressed. The level of expression of Cox-2 was maximum after 17 h of IPTG treatment (Fig. 1A). In PCXII-1 and -4, Cox-2 expression persisted for up to 2 days following the addition of IPTG (Fig. 1B), and, upon removal of IPTG, induction ceased, and the level of Cox-2 expression decreased in 4 days (Fig. 1B). The induction of Cox-2 expression in PCXII-4 cells was paralleled by elevated levels of PGE2 production, as assessed by enzyme-linked immunosorbent assay (Fig. 1C). Taken together these results confirm the IPTG inducibility and bioactivity of vector-encoded Cox-2.

Neuronal Differentiation—Although it has been reported that prostaglandin levels are elevated at very early time points post-NGF addition (16) and that Cox-1 behaves as an NGF-dependent delayed response gene in PC12 cells, localizing in both the cytoplasm and neurites (14), indomethacin was found to have no observable effect on NGF-induced differentiation (16).
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As can be seen NGF (50 ng/ml) treatment of PC-MT and PCXII-4 cells for 12 days led to the induction of the neuronal phenotype as exemplified by neurite extension (Fig. 2, A and C). No IPTG-inducible differences in neurite length between PC-MT cells and either PCXII cell line were apparent (Fig. 2, A and B versus C and D, and data not shown). These findings indicate that Cox-2 bioactivity neither facilitates nor interferes with the neurite extension.

**EGF-dependent Mitogenesis**—PC12 cells respond to different growth factors in distinct ways; whereas NGF and basic fibroblast growth factor induce the neuronal phenotype, EGF induces mitogenesis in these cells (11). Cox-2 has been implicated in the proliferative response elicited by a number of growth factors including EGF (17–19). It has also been reported, however, that intestinal epithelial cells stably expressing Cox-2 exhibit a cellular phenotype (inhibition of cell proliferation) similar to parental cells following treatment with the growth inhibitory cytokine transforming growth factor-β (20), and, therefore, the influence of Cox-2 on cell growth exhibits a degree of cell type specificity. In view of these data, we next assessed the mitogenic responses of PC-MT and PCXII-4 cell lines following stimulation with EGF. As can be seen (Fig. 3), EGF proved to be a potent mitogen for both cell lines; however, the induction of Cox-2 expression had no effect on the proliferative responses of PCXII-4 cells when compared with the control cell line. These results indicate that the growth stimulatory effects of EGF on PC-MT and PCXII-4 cell lines are similar and that the expression of IPTG-inducible Cox-2 neither negated nor enhanced these effects.

**Western Analysis of AADC Expression**—It has been shown that the addition of exogenous PGE$_2$ to PC12 cells leads to the induction of AADC, an enzyme directly involved in the synthesis of neurotransmitter molecules such as dopamine, norepinephrine, and serotonin (21). Further, the observation that Cox-1 is localized both in the cytoplasm and neurites in NGF-differentiated PC12 cells (14) has increased speculation that Cox-derived prostanoids may somehow regulate AADC expression. In view of these findings, the levels of AADC in PCXII-4 versus control cells were investigated. Following NFG differentiation, PC-MT and PCXII-4 cells were lysed and subjected to Western analysis using a polyclonal antibody to AADC. As can be seen (Fig. 4), although the induction of Cox-2 in response to IPTG is clear, the levels of AADC protein remained unchanged between PCXII-4 and PC-MT cell lines. These results indicate that the bioactivity of this Cox isoform at least does not appear to influence AADC protein expression.

**NGF Withdrawal Apoptosis**—Following differentiation, removal of NGF from PC12 cells leads to cellular apoptosis (12). Apoptosis was observed within 6 h of NGF withdrawal in both cell lines in the absence of IPTG. However, in PCXII-4 cells a marked reduction in annexin V-FITC staining over control cells was apparent when prior NGF-induced differentiation was carried out in the presence of IPTG (data not shown).

To confirm that exogenous Cox-2 expression did indeed confer PC12 cell resistance to NGF withdrawal apoptosis, late stage programmed cell death was evaluated by Hoechst 33258 staining. Evaluation of apoptosis following Hoechst 33258 staining (Fig. 5, A–D) indicated that cellular indications of programmed cell death, including chromatin condensation and nuclear segmentation, were significantly less in PCXII-4 cells differentiated and subjected to trophic factor withdrawal in the

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**FIG. 1. Characterization of PCXII cells.** PC-MT (control cell line containing pRSVNOT alone) and PCXII-1 cells were incubated with IPTG (2.5 μM) for the times indicated (A). PCXII-4 cells were incubated with IPTG (2.5 μM) for the times indicated, following which the cells were placed in media without IPTG for a further 2, 3, or 4 days (B). Cell lysates standardized for protein were subjected to Western blot analysis with anti-Cox-2 antibody (A and B). The position of Cox-2 is indicated. PC-MT and PCXII-4 cells were cultured in 6-well dishes in DMEM, 10% heat-inactivated horse serum, 5% calf serum and on the day of assay growth media was replaced with DMEM, 0.3% bovine serum albumin containing 2.5 mM IPTG. 6 h later excess arachidonic acid (30 μM) was added for 30 min, and PGE$_2$ levels in the combined conditioned media/cell lysate were assessed by enzyme-linked immunosorbent assay as described under "Experimental Procedures" (C). Values represent the means ± S.E. of four independent experiments.
The inclusion of either of two chemically distinct nonsteroidal anti-inflammatory drugs, indomethacin, a broad specificity Cox inhibitor, or NS398, an isoform-specific Cox-2 blocker, for 24 h prior to, as well as during, NGF withdrawal apoptosis eliminated the IPTG-inducible cell protective effect (Fig. 5). Measurement of PGE2 release from PCXII-4 cell during these experiments confirmed that these Cox inhibitors abolished IPTG-dependent increases in prostaglandin release (Fig. 5E). The finding that the inclusion of either indomethacin or NS398 for 24 h prior to the induction of apoptosis was sufficient to abolish the protective effects of this enzyme provides evidence that Cox-2, rather than influencing the differentiation pathway of PC12 cells per se, appears to directly modulate the apoptotic process. To confirm this, PC-MT and PCXII-4 cells were differentiated for 8 days, either in the continuous presence of IPTG as previously or with the addition of IPTG 12 h prior to the induction of apoptosis. Flow cytometric analysis following annexin V-FITC staining (Fig. 6) indicated that induction of Cox-2 expression 12 h prior to the initiation of apoptosis was sufficient to confer cytoprotection comparable with that observed in PCXII-4 cells differentiated in the continuous presence of IPTG (PCXII-4 + IPTG, 8 days versus 12 h, 3.1 versus 4.1% apoptotic cells; Fig. 6, C and D). Moreover, the addition of IPTG for 17 h was also found to be sufficient to attenuate DNA fragmentation in NGF-differentiated PCXII-4 cells (Fig. 7).

Because caspase-3-like proteases have been identified as effectors of NGF withdrawal apoptosis (22) and in view of the observation that short-term Cox-2 expression and bioactivity was sufficient to curtail the death of NGF-differentiated PC12 cells, the activity of caspase-3 in PC-MT and PCXII-4 cells was examined. Cells were differentiated for 7 days, and 12 h prior to the induction of apoptosis, IPTG was added to the culture media. PC-MT and PCXII cells that were manipulated exactly as those subjected to NGF withdrawal but that continued to be cultured in NGF-containing media served as controls. In a separate series of experiments PGE$_2$ (0.01 mM) had no effect on the extent of apoptosis (data not shown).

Caspase-3 Activity—Because caspase-3-like proteases have been identified as effectors of NGF withdrawal apoptosis (22) and in view of the observation that short-term Cox-2 expression and bioactivity was sufficient to curtail the death of NGF-differentiated PC12 cells, the activity of caspase-3 in PC-MT and PCXII-4 cells was examined. Cells were differentiated for 7 days, and 12 h prior to the induction of apoptosis, IPTG was added to the culture media. PC-MT and PCXII cells that were manipulated exactly as those subjected to NGF withdrawal but that continued to be cultured in NGF-containing media served as controls. In a separate series of experiments PGE$_2$ (0.01 mM) had no effect on the extent of apoptosis (data not shown).
was added together with the IPTG to PC-MT cells. 6 h after the removal of NGF, control and apoptotic cells were lysed. Caspase-3 activity was assessed in cell lysates by means of a fluorimetric substrate (Ac-DEVD-AMC) cleavage assay; protease specificity was confirmed in parallel reactions incorporating the caspase-3 inhibitor Ac-DEVD-CHO. As can be seen in Fig. 8, caspase-3 activity levels were similar in control PC-MT and PCXII-4 cells, and this protease was activated in both cell lines following removal of NGF. However, PCXII-4 cells demonstrated consistently lower fold/basal increases in protease activity when compared with PC-MT cells (Fig. 8 and data not shown). When PC-MT cells were treated with PGE₂ (0.01 μM) 12 h prior to the removal of NGF, the apoptosis-dependent fold increase in caspase-3 activity was reduced to levels similar to those found in Cox-2-expressing cells (Fig. 8). Taken together these data indicate that Cox-2 derived prostaglandins exert their cytoprotective effects, at least in part, via suppression of caspase-3 activity.
DISCUSSION

The current study illustrates that a cell culture model of inducible Cox-2 expression enables the investigation of the physiological importance of this enzyme. The PCXII cell culture model permits the consequences of introducing Cox-2, a protein normally absent from PC12 cells, to be examined relatively easily. Expression of Cox-2 in NGF-differentiated PC12 cells was found to result in reduced trophic withdrawal apoptosis following removal of this growth factor. This effect was detected by three independent methods, annexin V staining, DNA laddering, and Hoechst 33258 staining, and was specifically dependent on Cox-2 bioactivity because it was only apparent in PCXII cells in the presence of transcriptionally permissive IPTG and was abolished by two pharmacologically distinct Cox inhibitors. Moreover, the cytoprotective actions of Cox-2 were demonstrated to involve the suppression of caspase-3 activity.

The role of Cox-2 in the cell has been the focus of intense investigation since its discovery. The original hypothesis, that it existed merely to augment the bioactivity of Cox-1, has been expanded by the finding that Cox-2 expression is a causal factor in colon cancer (8). Cell culture models of colon carcinoma have provided evidence that Cox-2 expression leads to enhanced metastatic potential (23), stimulated endothelial motility, and tube formation via increased production of pro-angiogenic factors (24) as well as increased adhesiveness and inhibited butyrate-induced apoptosis (13).

The finding that the anti-apoptotic effects of Cox-2 in differentiated PC12 cells subjected to NGF withdrawal are mediated in part through inhibition of caspase-3 is interesting in view of increasing evidence that supports a role for this protease in neuronal apoptosis (25–27). Caspase-3-deficient mice exhibit abnormal brain cell apoptosis during development, with the result that these animals are characterized phenotypically by ectopic masses of supernumerary cells (25). Using an antibody that specifically recognized active caspase-3 (and not its inactive zymogen), Srinivasan and co-workers (26) carried out elegant studies demonstrating that this enzyme was localized in the somata and neurites of apoptotic neurons in the developing murine nervous system.

In vitro studies have provided evidence that caspase-3 is involved, in a stimulus-dependent manner, in certain apoptotic processes, notably chromatin condensation and DNA fragmen-

FIG. 7. Induction of Cox-2 expression at the time of NGF withdrawal is sufficient to prevent apoptosis visualized by DNA laddering. Genomic DNA was isolated from PCXII-4 cells either before (lane 1) or 17 h after the induction of apoptosis in the presence (lane 2) or absence (lane 3) of IPTG, and DNA fragmentation was visualized by ethidium bromide staining following electrophoresis on a 1.8% agarose gel. Positions of 400- and 800-base pair (bp) markers are shown on the left.
FIG. 8. Effect of Cox-2 expression on caspase-3 activation following NGF withdrawal. PC-MT and PCXII-4 cells were differentiated for 7 days, and IPTG (2.5 mM) was added 12 h prior to the removal of NGF. PC-MT and PCXII cells that were manipulated exactly as those subjected to NGF withdrawal but that continued to be cultured in NGF-containing media served as controls. PGE₂ (0.01 μM) was added together with the IPTG to PC-MT cells, when indicated. 6 h following the removal of NGF control and apoptotic cells were lysed. Caspase-3 activity was assessed in cell lysates by means of a fluorometric substrate cleavage assay as described under “Experimental Procedures.” The experiment was repeated three times with similar results. Shown is a representative experiment.

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