Induction of c-fos and c-jun mRNA Expression by Basic Fibroblast Growth Factor in Cultured Rat Müller Cells

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PURPOSE. Exogenous basic fibroblast growth factor (bFGF) induces bFGF gene expression in cultured rat Müller cells. To elucidate the mechanism that links exogenous bFGF to transcriptional regulation of bFGF gene expression in these cells, the authors examined mRNA expression of the proto-oncogenes c-fos and c-jun in response to exogenous bFGF in cultured rat Müller cells.

METHODS. Müller cells from 1- to 3-day-old Sprague-Dawley rats were isolated and cultured in essential modified Eagle's medium + 10% fetal calf serum. Cultured cells were identified by immunocytochemical analysis using antibodies against vimentin, carbonic anhydrase C, and glial fibrillary acidic protein.4 This technique shows that Müller cells proliferate and show a pronounced increase in immunocytochemical labeling for glial fibrillary acidic protein4 as well as in the light damage model.4,5 The protection afforded by exogenous bFGF and glial fibrillary acidic protein gene expression in the inner nuclear layer, suggesting that Müller cells play a role in this induction. In addition, Gao and Hollyfield5,7 have observed upregulation of bFGF mRNA in Müller cells in inherited retinal degeneration and in retinas exposed to bright light, indicating that degenerative processes of photoreceptors induce bFGF upregulation in retinal Müller cells. These findings suggest that upregulation of bFGF in Müller cells functions, in part, to enhance photoreceptor survival.

These findings provide further insight into the roles of Müller cells and exogenous bFGF in protecting against photoreceptor degeneration. (Invest Ophthalmol Vis Sci. 1998;39:565–573)
Figure 1. Induction of c-fos gene expression by basic fibroblast growth factor (bFGF) in Müller cells. (A) Temporal expression of c-fos mRNA in cultured rat Müller cells in response to human recombinant bFGF. A major c-fos transcript was detected in all lanes. Migration of 28S and 18S rRNA is indicated at the right (upper panel). The 18S rRNA served as a control for RNA loading (lower panel). The time after bFGF treatment is indicated at the top of each lane. (B) Data from three independent experiments were normalized, averaged, and presented as relative to the control level (mean ± SD, n = 3). (C) Dose-response effects of human recombinant bFGF on the induction of c-fos gene expression. The dose of bFGF treatment is indicated at the top of each lane. (D) Data from three independent experiments were normalized, averaged, and presented as relative to the control level (mean ± SD, n = 3). Each significant difference from control is indicated by asterisks (*P < 0.05; **P < 0.01).

Müller cells. The induction appears to occur mainly through activation of protein kinase C (PKC). However, the mechanism that links exogenous bFGF to transcriptional regulation of endogenous bFGF in Müller cells has not yet been determined. One of the earliest cellular changes elicited by many growth factors is the induction of immediate early genes. Two prominent members of the immediate early gene family are the proto-oncogenes c-fos and c-jun. The c-fos and c-jun genes are inducible and have a variety of agents capable of initiating cell growth, differentiation, and development. It is known that a heterodimeric complex formed by a fos-jun combination binds to and stimulates the transcription of second-response genes that contain the transcription factor AP-1 in their promoters. We think that fos-jun is the link between exogenous bFGF and the transcriptional regulation of bFGF mRNA expression in Müller cells. This hypothesis arose from the following observations: The expression of c-fos or c-jun mRNA has been seen previously in the retina, focal injury to rat retina has been shown to induce the expression of c-fos mRNA in the inner nuclear layer, and this coincides with the elevation of bFGF gene expression near the injury site in the inner nuclear layer; intravitreal injection of other growth factors, such as epidermal growth factor or transforming growth factor-α, induced c-fos gene expression in the inner nuclear layer, and the AP-1 sequence regulated by fos-jun has been found in the bFGF promoter. To further understand the role of retinal Müller cells and exogenous bFGF in protecting against photoreceptor degeneration, we examined the expression of mRNA from the proto-oncogenes c-fos and c-jun in response to exogenous bFGF in cultured rat Müller cells. We show here that bFGF rapidly induces c-fos and c-jun gene expression and that this induction can be blocked by PKC inhibitors.

Materials and Methods

Animals and Cell Culture

All animals used in this study were cared for and handled according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Müller cells were isolated and cultured according to an established protocol with modification. Briefly, Müller cells from Sprague-Dawley rats of postnatal days 1 to 3 were isolated and cultured with essential modified Eagle’s medium and 10% fetal calf serum. Confluent cultures were passaged no more than four times. Medium was replenished 24 hours before treatment. As described previously, cells were identified by immunocytochem-
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Induction of c-fos and c-jun by bFGF. A major c-jun transcript was detected in all lanes. The 18S rRNA served as a control for RNA loading. The time after bFGF treatment is indicated at the top of each lane. (D) Data from three independent experiments were normalized, averaged, and compared with the control level (mean ± SD, n = 3). Each significant difference from control is indicated by asterisks (*P < 0.05; **P < 0.01).

Experimental Protocols

We first investigated the temporal course of induction of c-fos and c-jun gene expression at a bFGF concentration of 10 ng/ml, the concentration that induced maximal upregulation of bFGF gene expression in cultured Müller cells.8 Previous investigation of the temporal course for the induction of bFGF gene expression showed that bFGF mRNA expression increased as early as 2 hours after bFGF exposure, and this increase reached a maximum at 8 hours.8 Here, we further studied earlier time points for bFGF induction by exogenous bFGF to compare them with the induction of c-fos and c-jun gene expression by bFGF. We studied time points 10, 30, and 45 minutes and 1, 1.5, 2, and 3 hours after exposure to bFGF. The dose-dependent effect of bFGF (0.01, 0.1, 1, 10, and 100 ng/ml) on c-fos and c-jun was then studied.

We have shown in a previous study that bFGF gene expression in cultured rat Müller cells was induced by bFGF through the activation of PKC.5 To determine whether the induction of c-fos and c-jun gene expression was mediated by PKC, we examined whether direct activation of the PKC pathway by the PKC activator phorbol 12-myristate 13-acetate (PMA; 1, 10, 100, 500 nM) induces c-fos and c-jun gene expression in cultured Müller cells. To further confirm that bFGF-induced c-fos and c-jun gene expression were mediated through PKC activation, we used the PKC inhibitors H-7 and GF109203X. It has been demonstrated in vitro and in intact cells that GF109203X is a potent and more specific inhibitor of PKC.23 H-7 (30 μM) or GF109203X (1 μM) were added to dishes of Müller cells 2 hours before bFGF (10 ng/ml) or PMA (100 nM) exposure and were observed for an additional 30 minutes. As controls, Müller cells were treated for 2.5 hours with H-7 (30 μM) alone, for 2.5 hours with GF109203X (1 μM) alone, for 30 minutes with PMA (100 nM) alone, or for 30 minutes with bFGF (10 ng/ml) alone. Untreated control cultures were also examined.

We also investigated the cyclic adenosine monophosphate (cAMP) pathway and bFGF-induced c-fos and c-jun gene expression in cultured Müller cells. SQ22536 is a cell-permeable adenylate cyclase inhibitor that inhibits cAMP production induced by prostaglandin E1, in platelet lysates (IC50 = 82 μM) and in intact platelets (IC50 = 1 μM). It has been successfully used as a probe for the involvement of adenylate cyclase in a variety of receptor systems, including those for prostaglandins24 and neurotransmitters.25 The dose we used in this study was 100 μM, which is sufficient for blocking adenylate cyclase activity. SQ22536 was added to dishes of Müller cells 2 hours before bFGF exposure and
FIGURE 3. Induction of c-fos and c-jun gene expression by the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) in Müller cells. (A) Dose–response effects of PMA on the induction of c-fos gene expression. The concentration of PMA is indicated at the top of each lane. (B) Data from three independent experiments were normalized, averaged, and compared with the control level (mean ± SD, n = 3). (C) Dose–response effects of PMA on the induction of c-jun gene expression. (B) Data from three independent experiments were normalized, averaged, and compared with the control level (mean ± SD, n = 3). Each significant difference from control is indicated by asterisks (*P < 0.05; **P < 0.01).

was followed up for an additional 30 minutes. As controls, Müller cells were treated for 2.5 hours with SQ22536 (100 µM) alone, for 30 minutes with bFGF (10 ng/ml) alone, or for 30 minutes with forskolin (5 µM, cAMP activator) alone. Untreated control cultures were also examined.

The PKC inhibitors H-7 and GF1092303X, the adenylate cyclase activator forskolin, and the adenylate cyclase inhibitor SQ22536 were purchased from BIOMOL (Plymouth Meeting, PA). Human recombinant bFGF (R&D Systems, Minneapolis, MN) was used in all experiments. We used phosphate-buffered saline for dissolving bFGF, forskolin, and SQ22536 and used dimethyl sulfoxide solution for H-7 and GF1092303X. Addition of phosphate-buffered saline or dimethyl sulfoxide alone to culture medium did not significantly alter c-fos and c-jun mRNA levels.

RNA Extraction and Northern Blot Analysis
Cultured rat Müller cells were lysed and homogenized in a solution of 5.5 M guanidinium thiocyanate (5.5 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosine, pH 7.0), and total RNA was isolated using a gradient method with cesium trifluoroacetate (CsTFA; Pharmacia, Piscataway, NJ). Total RNA (20 µg each sample) was electrophoresed on 1% agarose formaldehyde gels and transferred down the wick in 20× SSC (1× SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0) to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL). Blots were ultraviolet irradiated to immobilize RNA and then prehybridized for 4 hours in a hybridization solution containing 50% formamide, 5× Denhardt’s solution, 5× SSPE (1× SSPE = 0.15 M NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4), 200 µg/ml denatured salmon sperm DNA, and 5% sodium dodecyl sulfate at 50°C. Random-primed, 32P-labeled cDNA probes (c-fos, 2.2-kb rat cDNA, a gift from Tom Curran; c-jun, 1.8-kb rat cDNA, a gift from Robert Tjian; bFGF, 477-bp rat cDNA, a gift from A. D. Baird; and 18S rRNA, 1.1-kb cDNA, a gift from D. Schlesinger) were added to the hybridization buffer (106 cpm/ml) and hybridized at 50°C overnight. Blots were then washed twice in 2× SSC, 0.1% sodium dodecyl sulfate at room temperature for 5 minutes and twice in 0.1× SSC, 0.1% sodium dodecyl sulfate at 65°C for 10 minutes. After the posthybridization wash, blots were exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA), and the data were digitized by scanning the phosphor screen with a phosphor imager system (Molecular Dynamics). In all northern blots, c-fos mRNA was detected as a major band at approximately 2.2 kb, c-jun mRNA at 2.7 kb, and bFGF mRNA at 7.0 kb. Data were digitized from this major band and analyzed using software (ImageQuant, Molecular Dynamics). Hard copies of blots were obtained by exposing the blots to...
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RESULTS

Induction of c-fos and c-jun Gene Expression by Basic Fibroblast Growth Factor

Figures 1A and 1B show that c-fos mRNA increased (9.6-fold) as early as 10 minutes after bFGF exposure. This increase peaked at 30 minutes (17.4-fold) and rapidly declined thereafter. Figures 1C and 1D show c-fos gene expression after cells were treated at increasing concentrations of bFGF for 30 minutes (0.01, 0.1, 1, 10, and 100 ng/ml). Induction of bFGF gene expression was first detected at a low concentration of 0.1 ng/ml (Fig. 1D). With 1.0 ng/ml, c-fos mRNA increased to a level of 10-fold, and a maximum of induction (15-fold) was reached with 10 ng/ml bFGF. The expression declined slightly from the maximum to 14-fold at 100 ng/ml. The temporal pattern and concentration dependence of c-fos gene expression were similar to those of c-jun (Fig. 2).

Induction of c-fos and c-jun Gene Expression by bFGF Through the Protein Kinase C Pathway

Figure 3 shows c-fos and c-jun gene expression after the cells were treated with increasing concentrations of PMA for 30 minutes (1, 10, 100, and 500 nM). Induction of c-fos and c-jun gene expression began at a low concentration of 1 nM (c-fos 3.8-fold; c-jun 2-fold). With 10 nM PMA, c-fos mRNA increased to a level of 9-fold, whereas c-jun increased to 4.5-fold. Maximum increases in the induction were reached with 100 nM PMA.

As seen in Figures 4 and 5, one question is whether the induction of c-fos and c-jun gene expression by bFGF was mediated by PKC. In Figure 4, cells were treated with PMA (100 nM) for 30 minutes, bFGF (10 ng/ml) for 30 minutes, or H-7 alone for 2.5 hours. Cells were also treated with H-7 (30 µM) for 2 hours before bFGF (10 ng/ml, 30 minutes) or PMA (100 nM, 30 minutes) exposure. PMA induced c-fos gene expression.
expression by 17.3-fold and \textit{c-jun} gene expression by 7.6-fold, whereas bFGF induced a 16.5-fold increase of \textit{c-fos} gene expression and a 7.1-fold increase of \textit{c-jun} mRNA gene expression (Fig. 4). Pretreatment with H-7 blocked the \textit{c-fos} and \textit{c-jun} induction by bFGF and PMA, whereas H-7 alone did not alter the baseline levels of \textit{c-fos} and \textit{c-jun} mRNA (Fig. 4).

To further confirm that bFGF-induced \textit{c-fos} and \textit{c-jun} gene expression was mediated through PKC activation, GF109203X, a more specific PKC inhibitor, was used. Figure 5 shows that GF109203X (1 \textmu M for 2.5 hours) alone did not change the baseline levels of \textit{c-fos} and \textit{c-jun} mRNA, whereas a 2-hour pretreatment with GF109203X inhibited the induction of \textit{c-fos} and \textit{c-jun} gene expression by bFGF (10 ng/ml for 30 minutes).

**cAMP Pathway in \textit{c-fos} and \textit{c-jun} Gene Expression in Cultured Müller Cells**

Figure 6 shows that SQ22536 (100 \textmu M), an adenylate cyclase inhibitor, did not affect the baseline levels of \textit{c-fos} and \textit{c-jun} mRNA at concentrations of 100 \textmu M for 2.5 hours. Pretreatment with SQ22536 for 2 hours before bFGF (10 ng/ml for 30 minutes) did not block bFGF-induced \textit{c-fos} and \textit{c-jun} gene expression. Direct stimulation of adenylate cyclase with forskolin (5 \textmu M for 30 minutes) induced \textit{c-fos} gene expression by 12.7-fold and \textit{c-jun} gene expression by 7.5-fold. These results provide evidence that, although direct stimulation of cAMP can upregulate \textit{c-fos} and \textit{c-jun} gene expression, CAMP may not be involved in bFGF-induced \textit{c-fos} and \textit{c-jun} gene expression in cultured Müller cells.

**Induction of bFGF Gene Expression by Basic Fibroblast Growth Factor**

Figure 7A shows bFGF gene expression after cells were treated with bFGF at a concentration of 10 ng/ml and studied at 10, 30, and 45 minutes and 1, 1.5, 2, and 3 hours. Quantitative data from three separate experiments (Fig. 7B) revealed that the induction of bFGF expression started (1.4-fold) as early as 1 hour after bFGF exposure, and this
Increase reached 2.3-fold at 2 hours and 3.2-fold at 3 hours. No increases in bFGF mRNA were observed at 2 hours after bFGF treatment. These findings indicate that the induction of proto-oncogene c-fos and c-jun mRNA occurs before that of bFGF gene expression.

**DISCUSSION**

We have demonstrated that exposure of cultured rat Müller cells to human recombinant bFGF induces c-fos gene expression in a dose- and time-dependent manner. This is consistent with the studies of some other cell types. A range of extracellular signals is able to induce the rapid and transient accumulation of c-fos mRNA. An increased cytoplasmic level of the c-fos message can often be detected within 5 minutes, peaks within 30 minutes, and returns to baseline values within 60 minutes. Our data showed that the increase in c-fos mRNA in Müller cells occurred rapidly and transiently (peaking 30 minutes after bFGF stimulation), which is characteristic of immediate early gene induction. The pattern of c-jun induction by bFGF was similar to that of c-fos, except that the expression of c-jun peaked later, at 45 minutes instead of 30 minutes after bFGF stimulation. This parallel induction is not surprising because c-fos and c-jun are known to associate as a heterodimer through the formation of a leucine zipper to act as a transcription modulator.

Basic FGF has been immunolocalized to Müller cells and retinal Müller cells possess cell surface FGF-binding sites. Müller cells are major glial cells in the retina that participate in maintaining the normal structure and function of the retina. We have shown previously that exogenous bFGF induces upregulation of bFGF gene expression in retinal Müller cells. This suggests that bFGF acts on FGF receptors of Müller cells to induce the synthesis and release of endogenous bFGF. We have also demonstrated that the induction of bFGF by exogenous bFGF occurs mainly
Signal pathways linking the binding of bFGF to c-fos or c-jun induction in retinal Müller cells are unclear. Because PMA, the PKC activator, induced c-fos and c-jun gene expression in this study, PKC could be involved in translating the signal to the nucleus. In addition, the PKC inhibitors, H-7 and GF109203X, blocked c-fos and c-jun induction by bFGF in retinal Müller cells. Although similar findings have been reported in other systems, growth factor induction of c-fos or c-jun are not always dependent on PKC. We have also shown in this study that forskolin, an adenylate cyclase activator, induces c-fos and c-jun gene expression. This suggests that the induction of c-fos and c-jun in retinal Müller cells could be mediated by multiple pathways. We also treated Müller cells with SQ22536, an analog of adenosine that has been shown to inhibit the activity of adenylate cyclase. However, this adenylate cyclase inhibitor did not block bFGF-induced upregulation of c-fos and c-jun gene expression in the present study, indicating that bFGF-induced gene expression of c-fos and c-jun occurs mainly through the PKC pathway.

The roles of c-fos and c-jun in Müller cell physiology remain unknown. Evidence is accumulating, however, that c-fos encodes a transcription factor that regulates the expression of the late-response genes containing AP-1 binding sites in their regulatory region. This AP-1 sequence has been found in the bFGF promoter and upregulates bFGF expression. As shown in this study (Fig. 7), bFGF mRNA transcription was significantly induced as early as 2 hours after bFGF exposure. This delay relative to c-fos and c-jun (10-30 minutes) may indicate that these proto-oncogenes play a role in bFGF upregulation in retinal Müller cells.

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