Published in final edited form as:

Mol Neurobiol. 2017 March ; 54(2): 964–976. doi:10.1007/s12035-016-9705-9.

Transcriptional regulation of human transforming growth factor- α in astrocytes

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Abstract

Transforming growth factor- α (TGF- α) is known to play multifunctional roles in the central nervous system (CNS), including the provision of neurotropic properties that protect neurons against various neurotoxic insults. Previously, we reported that TGF- α mediates estrogen-induced enhancement of glutamate transporter GLT-1 function in astrocytes. However, the regulatory mechanism of TGF- α at the transcriptional level remains to be established. Our findings revealed that the human TGF- α promoter contains consensus sites for several transcription factors, such as NF- κ B and yin yang 1 (YY1). NF- κ B served as a positive regulator of TGF- α promoter activity, corroborated by observations that overexpression of NF- κ B p65 increased, while mutation in the NF- κ B binding sites in the TGF- α promoter reduced the promoter activity in rat primary astrocytes. Pharmacological inhibition of NF- κ B with PDTC (50 μ M) or QNZ (10 μ M) also abolished TGF- α promoter activity, and NF- κ B directly bound to its consensus site in the TGF- α promoter as evidenced by electrophoretic mobility shift assay (EMSA). Dexamethasone (DX) increased TGF- α promoter activity by activation of NF- κ B. Treatment of astrocytes with 100 nM of DX for 24 h activated its glucocorticoid receptor and signaling proteins including MAPK, PI3K/Akt and PKA, via non-genomic pathways, to enhance TGF- α promoter activity and expression. YY1 served as a critical negative regulator of the TGF- α promoter as overexpression of YY1 decreased, while mutation of YY1 binding site in the promoter increased TGF- α promoter activity. Treatment for 3 h with 250 μ M of Manganese (Mn), an environmental neurotoxin, decreased astrocytic TGF- α expression by activation of YY1. Taken together, our results suggest that NF- κ B is a critical positive regulator, whereas YY1 is a negative regulator of the TGF- α promoter. These findings identify potential molecular targets for neurotherapeutics that may modulate TGF- α regulation and afford neuroprotection.

Keywords

Transforming growth factor- α ; NF- κ B; YY1; manganese; dexamethasone

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INTRODUCTION

Transforming growth factor- α (TGF- α) belongs to the epidermal growth factor (EGF) family, which are ligands for the EGF receptor (EGFR) (Marquardt et al., 1984). In addition to its wide distribution in mammalian peripheral tissues, TGF- α is also highly expressed in both developing and adult central nervous system (CNS) [Reviewed in (Junier 2000)] (Lee et al., 1995). In pathological conditions, the upregulation of TGF- α expression in neoplastic tissues has been described as a common feature of many human tumors, including breast cancer (Ciardiello et al., 1991), tumor cell lines (Salomon et al., 1995) and chemically transformed rodent epithelial cells (Lee et al., 1995).

In normal neurophysiological condition, both neuronal and glial cells contribute to the production of TGF- α , yet astrocytes have been described as the primary targets for TGF- α (Ma et al., 1994). TGF- α promotes neuronal survival and differentiation (Chalazonitis et al., 1992) as well as proliferation of astrocytes (Kornblum et al., 1999). TGF- α protects against excitotoxicity, attenuating *N*-methyl-D-aspartic acid (NMDA)-induced neurotoxicity in cortical neurons by activation of the ERK1/2 pathway (Petegnief et al., 2003). It has also been reported that TGF- α induces angiogenesis, neurogenesis and neuroprotection after stroke, possibly by a pro-angiogenic effect secondary to the incorporation of bone marrow-derived endothelial cells into blood vessels in the infarct border zone (Leker et al., 2009). TGF- α is also able to alter the glial border and enhance axonal growth after spinal contusion (White et al., 2011). Furthermore, TGF- α increases the number of newborn neurons around the infarction area after stroke by inducing the proliferation of progenitors and protecting existing endogenous progenitors (Cameron et al., 1998; Cooper and Isacson 2004).

Astrocytic TGF- α plays a critical role in neuroprotection against various neurotoxic insults. Astrocyte-derived TGF- α has been shown to be neuroprotective and is upregulated during brain injury (Junier et al., 1991; Yuen and Mobley 1996). TGF- α transforms astrocytes to growth supportive phenotype after spinal cord injury (White et al., 2011). The EGFR, a receptor of TGF- α , is also upregulated primarily by astrocytes surrounding the lesion after spinal cord injury (SCI) (White et al., 2011). Furthermore, TGF- α mediates estrogenic effects in mouse uterine tissue (Nelson et al., 1992), as well as neuroprotective effects of estrogen and tamoxifen, a selective estrogen receptor modulator, against manganese (Mn)-induced cytotoxicity in rat primary astrocytes (Lee et al., 2012).

Despite extensive studies on the neuroprotective roles of TGF- α in neurons and astrocytes, the molecular and cellular mechanisms of its regulation are not completely understood. Human TGF- α expression can be induced by EGF, phorbol ester (Bjorge et al., 1989; Muller and Seifert 1982), protein kinase C activators, angiotensin, bradykinin, epinephrine (Raymond et al., 1989) and estrogen (Ciardiello et al., 1991; Raja et al., 1991). Earlier studies have shown that binding of transcription factors, such as specificity protein 1 (Sp1), activating protein 2 (AP2) and p53 to the TGF- α promoter increased its transcription (Chen et al., 1994; Shin et al., 1995; Wang et al., 1997).

Glucocorticoids (GCs) exert various peripheral effects, such as anti-inflammation and immunosuppression, but the brain is also the major neural target for the action of GCs.

Endogenous GCs can bind to both the glucocorticoid receptor (GR) and the mineral-corticoid receptor (MR), and activate the GR with higher affinity during stress or at the circadian peak (De Kloet et al., 1998; Reul and de Kloet 1985). The synthetic GCs can selectively bind to the GR and exert much higher potency, leading to their extensive utilization in the clinics to treat various pathological conditions. Particularly, dexamethasone (DX) possesses > 25 times the potency of endogenous hydrocortisone, and it is widely used in clinics, including the treatment of rheumatic problems, a number of skin diseases, severe allergies and brain swelling (Newton 2000). At the cellular level, treatment of DX increases the expression of astrocytic glutamate transporter-1 (GLT-1), which plays a key role in preventing glutamate-induced excitotoxic neuronal death (Zschocke et al., 2005). TGF- α also increases GLT-1 expression via the EGFR, and estrogenic compounds increase GLT-1 via upregulation of TGF- α in astrocytes (Lee et al., 2009). These observations indicate that both glucocorticoids and estrogenic agents upregulate GLT-1 expression and enhance glutamate uptake in astrocytes. Moreover, enhancement of GLT-1 expression by these compounds is mediated via upregulation of TGF- α in astrocytes. Therefore, it is crucial to study the mechanisms by which DX increases TGF- α expression to identify molecular target for putative neuroprotective therapeutics.

Chronic exposure to high levels of manganese (Mn) induces neurological disorders referred to as manganism which shares the pathological symptoms with PD (Aschner et al., 2009). We have previously reported that Mn reduces TGF- α mRNA and protein levels in rat primary astrocytes (Lee et al., 2012). But the molecular mechanism of Mn in downregulation of TGF- α at the transcriptional levels remains to be established.

In the present study, we addressed the molecular mechanism of transcriptional regulation of TGF- α using rat primary astrocytes. We used DX as it exerts neuroprotective effects (Jeanneteau et al., 2008), along with dibutyryl cAMP (dbcAMP), a known NF- κ B activator (Zelenaia et al., 2000), and Mn as a neurotoxin to delineate transcriptional mechanisms and cellular signaling pathways involved in the regulation of TGF- α promoter activity and mRNA expression. Our findings indicate that NF- κ B is a critical positive transcription factor of the human TGF- α promoter, and DX increases TGF- α transcription via the GR followed by activation of multiple signaling proteins, such as MARK/ERK, PI3K/Akt and PKA. In contrast, Mn decreased TGF- α promoter activity via activation of transcription factor yin yang 1 (YY1) in rat primary astrocytes.

MATERIALS AND METHODS

Materials

Cell culture media MEM, DMEM, opti-MEM and transfection reagent Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). Pyrrolidine dithiocarbamate (PDTC), 4-*N*-[2-(4-phenoxyphenyl)ethyl]-1,2-dihydroquinazoline-4,6-diamine (QNZ), H89, LY294002, PD98059, were from Tocris Bioscience (Ellisville, MO). DX, dbcAMP and mifepristone were obtained from Sigma-Aldrich (St. Louis, MO). NF- κ B (p65) and I κ B α antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Luciferase reporter assay kit was obtained from Promega (Madison, WI). The QuickChange lightening site-directed mutagenesis kit was obtained from Agilent technologies (Clara, CA).

Astrocytes isolation and culture

Astrocytes from one-day-old Sprague–Dawley rats were isolated and cultured as described previously (Aschner et al., 1992). Briefly, cerebral cortices were digested with Dispase and DNase I and astrocytes obtained on pellet after centrifugation of tissue digest were plated at a density of 1×10^5 cells/ml. After 24 hours of plating, the fresh media was added and the cultures were maintained at 37 °C in a 95% air, 5% CO₂ incubator for 3 weeks in minimum essential medium (MEM) supplemented with 10% horse serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin. The purity of astrocytes was confirmed by staining with astrocyte-specific marker, glial fibrillary acidic protein (GFAP) and these cultures showed > 95%-positive staining. All experiments were performed after 3 weeks of isolation when the cells reached confluency.

Cloning of the TGF- α promoter

The genomic DNA was used to amplify the human TGF- α promoter (–950 to +40 bp) with the following sets of primers: forward, 5'-ATT CTC GAG GCA CCA GGG GCC ACC TCA GAG-3' with *XhoI* site and reverse 5'-GCC AAG CTT GCT CTC CAG CCT CCT GCC CTA-3' with *HindIII* site. The PCR fragment was subcloned into the *XhoI* and *HindIII* sites of the pGL3-basic vector (Promega). The TGF- α promoter-luciferase plasmid construct was confirmed by DNA sequencing.

Site-directed mutagenesis

The point mutations were carried out following the instructions provided in the site-directed mutagenesis kit using pGL3-TGF- α construct as original template. The primers used were for NF- κ B mutations as follows: in –53 bp position, 5'-CTC CCG CGC CGG GAG CAG GCT CTG CCT AGT CTG C-3' (forward); 5'-GCA GAC TAG GCA GAG CCT GCT CCC GGC GCG GGA G-3' (reverse), in –218 bp position, 5'-TCT GTG CCC TCA GGA GAG CAT CTC CCA TCG GGG CG-3' (forward); 5'-CGC CCC GAT GGG AGA TGC TCT CCT GAG GGC ACA GA-3' (reverse) and in –507 bp position, 5'-TAA CGG GTG CCC GAG CGG CTT CGC TCC GCC GCC T-3' (forward); 5'-AGG CGG CGG AGC GAA GCC GCT CGG GCA CCC GTT A-3' (reverse). Similarly, the following primers were used for YY1 binding site mutation in –800 bp position, 5'-CGT CAC AGA GAC CTA CTT TTT ATC AGT CCC-3' (forward); 5'-GGG ACT GAT AAA AAG TAG GTC TCT GTG ACG-3' (reverse) and for Sp1 site mutation in –128 bp position, 5'-CGC CTT CCT ATT TCA GTC CTG CGG GCA GCG C-3' (forward), 5'-GCG CTG CCC GCA GGA CTG AAA TAG GAA GGC G-3' (reverse). Clones were sequenced to confirm the mutations.

Luciferase assay

Cells were grown in 24-well plates for 2-3 days before transfecting overnight with wild type or various mutants TGF- α promoter luciferase vectors. The co-transfection with expression vectors was done in some cases. After transfection, the cells were treated with the designated compounds in opti-MEM for indicated time periods. Luciferase activity was measured with the Bright-Glo luciferase kit (Promega) according to the manufacturer's instructions, and normalized to the protein content as determined by Bradford protein assay (Bio-Rad). The

normalization was also verified by co-transfection with firefly reporter pGL4.75 plasmids (Promega) carrying the *Renilla* luciferase reporter gene.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using the LightShift Chemiluminescent kit from Thermo Scientific (Rockford, IL, USA) following the manufacturer's instructions. Briefly, 5 µg of nuclear extract was incubated for 20 min on ice with biotin labeled oligos containing NF-κB binding sites (–218 bp position) of the TGF-α promoter. The DNA-protein complexes were resolved in 8% non-denaturing DNA polyacrylamide gels and transferred to nylon membrane. The complexes were detected with the Chemiluminescent Nucleic Acid Detection Module from Thermo Scientific. The primers pairs used were: (TGF-α –218) 5'-CTG TGC CCT CAG GGG GGC ACC CCC ATC GG –3' and 5'-CCG ATG GGG GTG CCC CCC TGA GGG CAC AG –3'. These oligos were HPLC purified and biotin labeled from Operon Technologies. To confirm the specificity of the binding, the unlabeled control oligos were used in excess during the reaction.

Quantitative real time PCR (qPCR)

The total RNA was extracted using Trizol reagent and equal amounts of RNA (2 µg) was transcribed to cDNA with high capacity cDNA reverse transcription kit (Applied biosystems, USA). For qPCR, following primers were used: 5'-GAG CCC TCG GTA AGT ATG TTT AG-3' (TGF-α forward) and 5'-CAT AGT GGA GGT GAC TTG TTA GAG-3' (TGF-α reverse); 5'-TCC CTC AAG ATT GTC AGC AA-3' (GAPDH forward) and 5'-AGA TCC ACA ACG GAT ACA TT-3' (GAPDH reverse). The reactions were carried out in a total volume of 25 µl, containing a 1 µg of cDNA template of each sample, 0.4 µM of primers and RT2 SYBR Green qPCR Master Mix (SABiosciences/Qiagen). The PCR was done in CFX96 real time PCR detection system (Bio-Rad) with one cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. All samples were normalized relative to GAPDH. A web-based PCR array data analysis (SA Biosciences/Qiagen) was used to analyze the data and in some cases the PCR products were also run on 1% agarose gel and visualized with ethidium bromide staining.

Western blot

At the end of the treatment, cells were washed twice with ice-cold phosphate buffered saline (PBS) and cell lysates were prepared by adding radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail. The protein concentration was determined by BCA assay and 30 µg of total protein were run on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane for western blot analysis. The primary antibodies were used at 1:1000 dilution and horse radish peroxidase (HRP)-conjugated secondary antibodies (Promega) were used at 1:5000 dilution. The blots were developed with enhanced chemiluminescence detection kit (Pierce).

Statistical analysis

The data were described as a mean ± SEM and analyzed using GraphPad Prism software (GraphPad Inc., La Jolla, CA). Statistical difference between groups was determined by

Student's *t*-test, one-way or two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test with a statistical significance at $p < 0.05$. Each experiment was carried out in at least three independently prepared astrocytes. The number of samples for each experiment was at least three for western blot and real-time PCR analyses, and four for glutamate uptake and luciferase assays.

RESULTS

NF- κ B is a critical positive regulator of TGF- α

Sequence analysis of the promoter region of human TGF- α revealed consensus sites for several potentially critical transcription factors, such as NF- κ B, Sp1 and YY1 (Fig. 1A and B). We previously reported that NF- κ B positively regulates TGF- α promoter activity, which mediates tamoxifen-induced upregulation of GLT-1 in astrocytes (Karki et al., 2013). Thus, in the present study, we investigated the promoter region for the consensus sites that are involved in NF- κ B-induced positive regulation of TGF- α promoter activity. We identified three NF- κ B binding sites (–53, –218 and –507) in the TGF- α promoter sequences (Fig. 1).

To confirm the role of NF- κ B in the regulation of TGF- α promoter activity, we mutated the NF- κ B binding sites and tested the effect of NF- κ B p65 on the wild-type (WT) or triple NF- κ B mutant TGF- α promoter activity by overexpression of the p65 subunit. As shown in Fig. 2A, NF- κ B p65 increased TGF- α promoter activity by 3-fold, whereas triple NF- κ B mutant led to significantly lower TGF- α promoter activity compared to WT TGF- α , and completely abrogated the effect of NF- κ B p65 (Fig. 2A), indicating that these NF- κ B binding sites are critical for the activation of the TGF- α promoter. Next, we determined the most critical NF- κ B binding sites for the positive regulation of TGF- α promoter activity by comparison of NF- κ B single, double or triple mutants with WT TGF- α . The results showed that individual mutation of all three consensus sites (–53, –218 and –507) of the NF- κ B significantly decreased TGF- α promoter activity ($p < 0.001$, Fig. 2B). Interestingly, all double NF- κ B mutants showed the similar degree of promoter activity to the single mutants (Fig. 2B). The triple NF- κ B mutant with all three site mutations did not show additive decrease in the TGF- α promoter activity compared with any of the three double mutants or single mutants. We also determined if NF- κ B modulates TGF- α mRNA expression levels, and the results showed that overexpression of NF- κ B p65 in astrocytes increased TGF- α mRNA levels (Fig. 2C), whereas inhibition of NF- κ B by overexpression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ B α) which sequesters NF- κ B in the cytosol to prevent NF- κ B activation, decreased TGF- α mRNA levels in astrocytes (Fig. 2D).

The critical role of NF- κ B in TGF- α promoter activity was further confirmed by employing PDTC and QNZ which are known pharmacological inhibitors of NF- κ B. Treatment of astrocytes with PDTC and QNZ significantly decreased ($p < 0.001$) TGF- α promoter activity (Fig. 3A), suggesting that NF- κ B is responsible for the basal level of its activity. The specificity of these inhibitors was partly demonstrated by the observation that they did not induce any change in the activity of NF- κ B triple mutant. Since transcription factors modulate promoter activities upon their bindings to the consensus sites, we also determined if NF- κ B binds to the TGF- α promoter region. The EMSA showed the direct binding of NF-

κ B to one of NF- κ B consensus sites (–218 was chosen as a representative one) in the TGF- α promoter (Fig. 3B).

Pharmacological modulation of TGF- α promoter activity via NF- κ B

Pharmacological compounds were applied to determine if they modulate the regulation of TGF- α gene transcription via NF- κ B. We employed dbcAMP and DX to test if NF- κ B mutations in the TGF- α promoter abolish the stimulatory effects of these compounds on TGF- α promoter activity. We chose dbcAMP as a selective activator of NF- κ B pathway (Zelenia et al., 2000) and DX, a glucocorticoid, as a general activator of many transcriptional pathways, including NF- κ B (Khan et al., 2013). The results showed that triple NF- κ B mutant completely abolished the enhancing effects of dbcAMP on TGF- α promoter activity (Fig. 4A), suggesting that dbcAMP-induced increase in TGF- α promoter activity is mediated via the NF- κ B pathway. In regard to DX, there was still an increase in the TGF- α promoter activity in the NF- κ B mutant, but this increase was significantly reduced compared to the WT promoter ($p < 0.001$, ~2.5-fold increase in wild type vs ~1.5-fold increase in mutant, Fig. 4B). To confirm that DX induced the increase in TGF- α promoter activity by activation of NF- κ B, we tested if DX increases NF- κ B reporter activity in astrocytes. As shown in Fig. 5A, DX (0–200 nM) increased NF- κ B reporter activity in a concentration-dependent manner. Moreover, the enhancing effect of DX on TGF- α promoter activity was completely abolished in the presence of the NF- κ B inhibitors, PDTC or QNZ (Fig. 5B), indicating that DX increases TGF- α promoter activity via activation of NF- κ B. PDTC or QNZ alone significantly decreased the basal levels of TGF- α promoter activity (Fig. 5B), indicating that TGF- α promoter is constitutively activated by NF- κ B in astrocytes. The significant increase in promoter activity in triple NF- κ B mutant with DX suggests that, in addition to NF- κ B, other pathways are also involved in mediating the stimulatory effects of DX on TGF- α promoter activity.

Transcription factor Sp1 is a positive regulator, whereas YY1 is a negative regulator of the TGF- α promoter

It has been reported that Sp1 directly binds and elevates the expression of TGF- α in HeLa cells (Chen et al., 1992; Chen et al., 1994). Results from the present study corroborated a positive regulatory role for Sp1 on TGF- α in astrocytes, given that mutation in the Sp1 binding site (–128) of the TGF- α promoter significantly decreased its promoter activity ($p < 0.01$, Fig. 6A). Overexpression of Sp1 increased both TGF- α promoter activity ($p < 0.01$, Fig. 6B) and mRNA levels ($p < 0.05$, Fig. 6C), indicating that Sp1 is a critical positive regulator of TGF- α promoter activity in astrocytes.

YY1 is a negative regulator of TGF- α in astrocytes, as shown in Fig. 7. YY1 is a ubiquitous multifunctional transcription factor that can act as a transcriptional repressor or activator (Hyde-DeRuyscher et al., 1995). Sequence analysis revealed that the human TGF- α promoter has one putative YY1 consensus binding site (–800 position) (Fig. 1). Since we found that YY1 contributes to the regulation of GLT-1 (Karki et al., 2014) and GLAST (Karki et al., 2015), we tested if YY1 also plays a role in TGF- α promoter activity. The results showed that mutation of the YY1 binding site increased TGF- α promoter activity ($p < 0.001$, Fig. 7A), whereas overexpression of YY1 reduced its promoter activity, and the

YY1 mutant abrogated its repressive effect on TGF- α ($p < 0.05$, Fig. 7B), indicating that YY1 is a critical negative regulator of the TGF- α promoter. YY1 Mutation itself significantly increased TGF- α promoter activity, even under conditions of YY1 overexpression (Fig. 7B). Furthermore, overexpression of YY1 by YY1 plasmid construct transfection significantly decreased astrocytic TGF- α mRNA levels (Fig. 7C).

Mn represses TGF- α promoter activity via activation of YY1

Mn increased YY1 promoter activity (Fig. 8A) and mRNA levels (Fig. 8B) in astrocytes, as previously described (Karki et al., 2014). In contrast, Mn decreased TGF- α mRNA (Fig. 8C) and protein levels (Fig. 8D). In addition, mutation of YY1 binding sites on the TGF- α promoter completely abrogated Mn-induced reduction of TGF- α promoter activity (Fig. 8E), indicating that YY1 is a critical mediator of Mn-induced repression of TGF- α . YY1 mutation significantly increased TGF- α promoter activity even in the presence of Mn (Fig. 8E), indicating that YY1 binding site at -800 in the TGF- α promoter is a critical repression site for TGF- α . The Mn-induced increase in YY1 expression requires ~3 hours, whereas Mn-induced reduction in TGF- α expression requires ~6 hours, indicating that Mn modulates YY1 prior to TGF- α (Fig. 8).

Intracellular signaling pathways involved in the regulation of TGF- α

Since DX is a synthetic ligand of the GR, a nuclear receptor, its cellular effects take place via nuclear translocation of the ligand-receptor complex after DX binds to the GR (Lowenberg et al., 2008). Thus, we tested if DX-induced increase in TGF- α expression occurs via the activation of the GR. As shown in Fig. 9A, DX increased TGF- α mRNA levels in a concentration-dependent manner, but in the presence of mifepristone, a GR antagonist, the DX-induced TGF- α mRNA expression was abolished (Fig. 9B). Since the TGF- α promoter does not contain GRE, the effect of DX on TGF- α is possibly induced via non-genomic pathways. Accordingly, we tested if activation by phosphorylation of intracellular kinases is required for DX-induced enhancement of TGF- α promoter activity and mRNA levels by employing the specific inhibitors of several intracellular kinases, such as MAPK/ERK, PI3K/Akt and PKA. The results showed that DX-induced enhancement of TGF- α promoter activity was abolished in the presence of PD98059 (MAPK/ERK inhibitor), LY294002 (PI3K/Akt inhibitor) and H89 (PKA inhibitor) (Fig. 9C). Similarly, the DX-induced increase in TGF- α mRNA levels was also completely abolished in the presence of these kinase inhibitors (Fig. 9D and E).

DISCUSSION

We demonstrate for the first time that the TGF- α promoter contains three putative NF- κ B consensus sites, which play a critical role in regulating TGF- α promoter activity. DX, a selective agonist of the GR, significantly increased TGF- α expression via activation of NF- κ B and nongenomic signaling pathways, such as MAPK/ERK, PI3K/Akt and PKA. Furthermore, the transcription factor YY1 serves as a critical negative regulator of TGF- α . Mn repressed TGF- α via activation of YY1, since that overexpression of YY1 decreased TGF- α promoter activity, whereas mutation of YY1 binding site on the TGF- α promoter

increased its promoter activity and reversed the Mn-induced repression of TGF- α promoter activity.

TGF- α is increased by a steroid hormone estrogen (Ciardiello et al., 1991; Lee et al., 2012; Raja et al., 1991), and has been shown to exert neuroprotective effects in several experimental animal models, including spinal cord injury (White et al., 2011) and ischemic infarction (Leker et al., 2009). The properties of TGF α for induction of angiogenesis, neurogenesis and neuroprotection after stroke implicate its potential as a putative target for pharmacological modulation and treatment of neurodegenerative diseases. Accordingly, understanding the molecular mechanisms involved in modulation of TGF- α expression and identifying molecular targets that enhance TGF- α expression are deemed invaluable. Notably, astrocytes play a critical role in TGF- α -induced neuroprotection, supported by previous findings that treatment of TGF- α in spinal cord injury afforded neuroprotection by activating EGFR (White et al., 2011). Thus, better characterization of the molecular mechanisms of astrocytic TGF- α regulation is deemed timely and meritorious.

We found consensus sites of several putative transcription factors in the human TGF- α promoter sequences (Fig. 1). NF- κ B contributes to TGF- α promoter activity as a major positive regulator in astrocytes (Fig. 2). NF- κ B is a dimeric transcription factor, usually consisting of two of five subunits such as p50, RelA/p65, c-Rel, RelB and p52 to form the active transcriptional dimer with a major dimer consisted of p50 and p65. NF- κ B dimers are present in the cytoplasm of most cells in an inactive form, sequestered by I κ B. Upon its activation, it is translocated into the nucleus where it regulates the expression of various genes (review in (Kaltschmidt et al., 2005)). NF- κ B regulates the expression of genes involved in a broad range of biological processes, including synaptic plasticity (Ahn et al., 2008), neurogenesis (Denis-Donini et al., 2008) and differentiation (Tchoghandjian et al., 2014). Although NF- κ B activation can contribute to inflammation and apoptosis after brain injury (Dvorianchikova et al., 2009), its activation is also essential for neuronal survival (Bhakar et al., 2002; Middleton et al., 2000), protecting these cells from insults and neurodegeneration. It has been suggested that the cell type and the timing of NF- κ B activation may determine the fate of neurons (Sarnico et al., 2009). We found three critical NF- κ B binding sites (-53, -218, -507) in the TGF- α promoter sequences (Fig. 1). Each site is equally crucial to TGF- α regulation, supported by the results that all three single mutants showed similar levels of reduction (~50%) in TGF- α promoter activity. Interestingly, double or triple NF- κ B mutants did not decrease further TGF- α promoter activity, suggesting that inactivation of any NF- κ B binding site already reduces TGF- α promoter activity to the lowest level and all three NF- κ B sites are equally important in the regulation of TGF- α promoter activity.

In the mammalian CNS, glucocorticoids (GCs) play an essential role in the hippocampus throughout the lifespan. During development, GCs serve as differentiating agents for hippocampal granule neurons (Banker 1980), and GRs are distributed throughout the brain (De Nicola et al., 1998). GCs, including the synthetic DX, are known to stimulate the production of neurotrophic factors, such as basic fibroblast growth factor (bFGF) and nerve growth factor (NGF), which are vital for the survival of neurons (Mocchetti et al., 1996). Although long-lasting exposure to exceedingly high doses of corticosterone exerts

deleterious effects in the brain, slightly elevated plasma corticosterone concentrations exert neuroprotection against excitotoxic insults (Abraham et al., 2000; Abraham et al., 1997). DX exerts neuroprotection in experimental setting of multiple sclerosis by reduction of iNOS expression levels in microglia (Golde et al., 2003).

Astrocytes, in addition to neurons and microglia, also express GRs (Chou et al., 1991; Vielkind et al., 1990). Treatment of astrocytes with GCs results in activation of intracellular signaling pathways that lead to the induction of long range calcium wave propagation (Simard et al., 1999) and alterations in gene transcription patterns. This indicates that GCs may regulate the expression of astrocyte-derived growth factors to protect neurons in the hippocampus. GRs also promote expression of glutamine synthetase (GS), which recycles glutamate, catalyzing its conversion into the amino acid glutamine in astrocytes (Laping et al., 1994). Although GC-mediated regulation of GS (Vardimon et al., 1999) and glutamate transporter GLT-1 expression (Zschocke et al., 2005) has been reported, the regulatory mechanism of GCs on TGF- α expression yet to be studied. Our findings indicate that DX-induced activation of the GR results in an increase of TGF- α promoter activity and mRNA levels via NF- κ B.

The human TGF- α promoter contains at least one Sp1 binding motif at -128 position (Fig. 1). It has been reported that the rat TGF- α promoter contains Sp1 binding site, playing a critical role in its promoter activity (Chen et al., 1994). The present study indicates that Sp1 contributes to TGF- α promoter activity as a positive regulator in astrocytes.

YY1 is a zinc finger transcription factor that can either activate or repress several genes (Yao et al., 2001); it plays an important role in cellular proliferation, differentiation and apoptosis. Growing evidence suggests that YY1 contributes to the pathogenesis of cancer and inflammation (Gordon et al., 2006). TNF- α -induced activation of YY1 represses Fas expression, indicating that YY1 contributes to TNF- α -induced cell survival (Huerta-Yepez et al., 2006). YY1 expression is markedly increased in idiopathic pulmonary fibrosis and in mouse models of lung fibrosis (Lin et al., 2011). In the CNS, YY1 also plays a critical role in regulating development and function and accordingly, its involvement in neurological diseases has been suggested (He and Casaccia-Bonnel 2008). Our previous findings revealed that YY1 serves as a negative transcriptional regulator of the astrocytic glutamate transporters EAAT1 and EAAT2 (Karki et al., 2014). We found that the human TGF- α promoter contains one putative YY1 binding site in -800 position of its promoter sequences (Fig. 1). Our finding suggests that Mn represses TGF- α promoter activity via activation of YY1. This result offers a transcriptional mechanism that is consistent with our previous findings, namely that Mn decreased TGF- α mRNA and protein levels in primary astrocytes (Lee et al., 2012). Chronic exposure to high levels of Mn causes manganism, which shares pathological symptoms with PD, but the molecular and cellular mechanisms involved are not completely established. YY1 appears to be a critical factor for Mn-mediated neurotoxicity, as it plays an important role in Mn-induced repression of GLT-1 (Karki et al., 2014), GLAST (Karki et al., 2015) and TGF- α (Fig. 8), all of which are closely associated with neuroprotection.

Our findings suggest that DX increases TGF- α expression via activation of a nongenomic pathway. The effect of DX on upregulation of TGF- α takes place after its binding to the GR, followed by activation of multiple intracellular signaling proteins, such as MAPK/ERK, PI3K/Akt and PKA. The classical mechanism of the DX-GR action is the genomic pathway characterized by the nuclear translocation of the DX-GR complex, followed by the complex's binding to the promoter region where the GRE cis-element is located, resulting in modulation of gene transcriptions of interest (Stahn and Buttgereit 2008). However, non-genomic pathways, which involve activation of intracellular signaling proteins after activation of the GR by GCs including DX, have also been reported to regulate several genes (Solito et al., 2003). The molecular mechanisms of nongenomic steroid actions, such as the allosteric actions of steroids on membrane-bound neurotransmitter receptors including the GABA_A receptor have been reported (Baulieu et al., 2001; Lambert et al., 2001). The specific membrane-bound steroid receptors have been suggested (Gametchu 1987) to be coupled either to PKC- or PKA-dependent signaling pathways via G proteins (Chen and Qiu 1999) or to other signaling systems. It has also been suggested that nongenomic steroid actions may activate MAPK and other signaling cascades possibly via stimulation of the classical intracellular receptors (Croxtall et al., 2000). Finally, we have proposed a model for DX action on upregulation of TGF- α expression, and Mn effect on downregulation of TGF- α via activation of YY1 (Fig. 10). DX activates GR in the cytosol, which activates intracellular signaling proteins such as ERK, PI3K and PKA, followed by the activation of transcription factors including NF- κ B which ultimately leads to an increase in TGF- α expression. Mn activates YY1 to repress TGF- α , which might contribute to its neurotoxic effects in the brain.

In conclusion, our findings underscore the potential role of astrocytes in upregulating TGF- α as a target for neuroprotection. Moreover, understanding the molecular mechanisms of TGF- α expression at the gene transcription level may lead to the development of novel therapeutic strategies for neurodegenerative disorders.

Acknowledgements

This study was supported in part by grants NIGMS SC1089630 (EL), R01 ES024756 (EL), UL1 TR000445 (EL), R01 ES10563 (MA) and R01 ES07331 (MA).

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Main Points

1. NF- κ B is a critical positive regulator of the human TGF- α .
2. YY1 is a negative regulator of TGF- α and mediates manganese-induced repression of TGF- α .
3. Dexamethasone increases TGF- α expression via the non-genomic signaling pathway and NF- κ B.

Fig. 1.

Nucleotide sequence of the human TGF- α promoter. (A) Nucleotide sequence of the 5'-terminal region of the human TGF- α gene. The numbering is based on the transcription start site designated as +1. Putative transcription factor binding sites are underlined and their names are indicated below. (B) The consensus sites of various transcription factors in the TGF- α promoter region are schematically shown.

Fig. 2.

NF-κB positively regulates TGF-α expression. (A) Astrocytes were co-transfected with 0.5 μg of wild type (WT) or triple mutant (TM) and 0.1 μg of either empty vector (EV) pcrRSV or p65, followed by the measurement of TGF-α promoter activity. (B) Cells were transfected overnight with 0.5 μg of WT, single (-53, -218 and -507 position), double (-53/-507, -53/-218 and -218/-507 positions) or triple NF-κB mutants (all three mutant NF-κB sites in -53, -218 and -507 positions), followed by the luciferase assay to determine TGF-α promoter activity. (C) Cells were transfected overnight with 2 μg of EV pcrRSV or p65 followed by TGF-α mRNA analysis by qPCR. The overexpression of p65 was confirmed by western blotting. (D) Cells were transfected overnight with 2 μg of EV pCMV or IκBα, followed by TGF-α mRNA analysis by qPCR. The overexpression of IκBα was confirmed by western blotting. (* $p < 0.05$, *** $p < 0.001$, # $p < 0.05$, ### $p < 0.001$; significantly different from the control; N.S., not significant; Student's *t*-test, one-way and two-way ANOVA followed by Tukey's *post hoc* test; N=3).

Fig. 3.

The NF- κ B pathway plays a critical role in the regulation of TGF- α promoter activity. (A) Astrocytes were transfected overnight with 0.5 μ g of WT or TM TGF- α luciferase plasmid, followed by 24 h treatment with 10 μ M of PDTC or QNZ. The luciferase assay was carried out to determine TGF- α promoter activity. (B) EMSA was performed with biotin-labelled oligos harboring -218 NF- κ B binding site of TGF- α promoter as described in the Methods section. Epstein-Barr Nuclear Antigen DNAs and nuclear extract from the kit were used as positive control. A shift containing NF- κ B complex is shown with arrow head. (^{###} $p < 0.001$, $*p < 0.05$, $***p < 0.001$; significantly different from controls; Student's *t*-test or ANOVA followed by Tukey's *post hoc* test; N=3).

Fig. 4.

Pharmacological modulation of TGF- α promoter activity occurs via NF- κ B. (A) Astrocytes were transfected overnight with WT or TM TGF- α luciferase plasmid constructs, followed by treatment with 250 μ M of dbcAMP and determination of TGF- α promoter activity by luciferase assay. (B) After overnight transfection with WT or TM TGF- α luciferase plasmids, cells were treated with 100 nM of DX for 24 h. TGF- α promoter activity was determined by luciferase assay. (* $p < 0.05$, *** $p < 0.001$, ### $p < 0.001$; significantly different from the control or indicated groups; N.S., not significant; two-way ANOVA followed by Tukey's *post hoc* test; N=3).

Fig. 5.

DX enhances TGF- α promoter activity via the NF- κ B pathway in astrocytes. (A) Astrocytes were transfected overnight with 0.5 μ g of NF- κ B reporter plasmid, followed by the treatment with 0-200 nM of DX. NF- κ B reporter activity was determined by luciferase assay. (B) Astrocytes were transfected overnight with 0.5 μ g of TGF- α luciferase plasmid, followed by 24 h treatment with 10 μ M of PDTC or QNZ. The luciferase assay was carried out to determine the TGF- α promoter activity. (* p <0.05, ** p <0.01, *** p <0.001, ## p <0.01, ### p <0.001; significantly different from the control or indicated groups; one-way and two-way ANOVA followed by Tukey's *post hoc* test; N=3)

Fig. 6.

Sp1 positively regulates TGF-α promoter activity. (A) Astrocytes were transfected overnight with 0.5 μg of WT or Sp1 binding site mutant (Sp1m, -128 site of TGF-α promoter) TGF-α luciferase plasmid, followed by the determination of TGF-α promoter activity. (B) Cells were co-transfected overnight with 0.5 μg of TGF-α luciferase plasmid and 0.1 μg of either EV eGFP or Sp1, followed by the measurement of TGF-α promoter activity. (C) Cells were transfected overnight with 2 μg of EV eGFP or Sp1, followed TGF-α mRNA analysis by qPCR. The overexpression of Sp1 was confirmed by western blotting. (* $p < 0.05$, ** $p < 0.01$, ## $p < 0.01$; significantly different from the control; Student's *t*-test or ANOVA followed by Tukey's *post hoc* test; N=3).

Fig. 7.

YY1 negatively regulates TGF-α promoter activity. (A) Astrocytes were transfected overnight with 0.5 μg of WT or YY1 binding site mutant (YY1m, -800 site of TGF-α promoter) TGF-α luciferase plasmid, followed by the determination of TGF-α promoter activity. (B) Cells were co-transfected overnight with 0.5 μg of TGF-α luciferase plasmid and 0.1 μg of either EV pcDNA or YY1, followed by the measurement of TGF-α promoter activity. (C) Cells were transfected overnight with 2 μg of EV pcDNA or YY1, followed by TGF-α mRNA analysis with qPCR. The overexpression of YY1 was confirmed by western blotting. (** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$; significantly different from the control or indicated groups; Student's *t*-test or two-way ANOVA followed by Tukey's *post hoc* test; $N=3$).

Fig. 8.

Mn decreases TGF- α expression via activation of YY1. (A) After transfection with the YY1 promoter construct overnight, astrocytes were treated for 3 h with Mn (250 μ M), followed by luciferase assay. (B) Cells were treated with Mn (250 μ M) for 3 h, followed by qPCR to measure YY1 mRNA levels. (C and D) Cells were treated for 6 h with Mn (250 μ M), followed by measurement of TGF- α mRNA levels with qPCR (C) and TGF- α protein levels with western blot (D). (E) After transfection with the TGF- α promoter constructs (WT and YY1 mutant) overnight, astrocytes were treated for 6 h with vehicle or Mn (250 μ M), followed by luciferase assay. (* p <0.05, ** p <0.01, # p <0.05, ## p <0.01; significantly different from the control or indicated groups; Student's t -test or two-way ANOVA followed by Tukey's *post hoc* test; N=3).

Fig. 9. Multiple signaling pathways are involved in the regulation of TGF-α expression. (A) Astrocytes were treated with indicated concentrations of DX, followed by determination of TGF-α mRNA levels by qPCR. (B) Cells were treated with 10 μM of Mifepristone (Mif), a GR antagonist, and 100 nM of DX, followed by the measurement of TGF-α mRNA levels by qPCR. (C) Cells were transfected overnight with 0.5 μg of TGF-α promoter luciferase plasmid and pretreated with 50 μM of PD98059, 20 μM of LY294002 or 10 μM of H89 for 30 min., followed by treatment with 100 nM of DX for 24 h. The TGF-α promoter activity was measured with luciferase assay. (D and E) Cells were treated with kinase inhibitors and DX as described in (C) and RNA was isolated to run qPCR with GAPDH as an internal control. Equal amounts of PCR products were run on 1% agarose gel. The quantification of TGF-α mRNA levels is shown in (E). (***) $p < 0.001$; significantly different from the control; one-way or two-way ANOVA followed by Tukey's *post hoc* test; N=3)

Fig. 10.

Proposed mechanism of DX-induced TGF- α expression. DX binds GR in the cytosol, which, in turn, activates intracellular signaling proteins, such as ERK, PI3K and PKA. This is followed by the activation of transcription factors, including NF- κ B, leading to increased TGF- α expression. The activation of YY1 by Mn represses TGF- α activity, in turn, leading to neurotoxic effects secondary to the inhibition the neuroprotective effects of TGF- α .