RESEARCH ARTICLE

Staurosporine Induced Apoptosis Rapidly Downregulates TDP-43 in Glioma Cells

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Abstract

TDP-43 is a ubiquitously expressed DNA/RNA binding protein that has recently attracted attention for its involvement in neurodegenerative diseases. While TDP-43 has been found to participate in various important cellular activities including stress and apoptosis, little is known about its role in cancer cells. Here we report that staurosporine (STS) induced apoptosis in U87 glioma cells is associated with rapid downregulation of TDP-43 at both mRNA and protein levels. The latter is dependent on activation of caspase 3. More importantly, we have shown that knockdown of TDP-43 by specific siRNA dramatically enhanced cytotoxicity of STS. These results suggest that normal level of TDP-43 may be protective for cancer cells under apoptotic insult.

Keywords: Apoptosis - caspase-3 - glioma cells - staurosporine - TDP-43

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Introduction

TAR-DNA-binding protein 43 (TDP-43) is a highly conserved heterogeneous nuclear ribonucleoprotein (hnRNP) which consists of 414 amino acids. It is encoded by the TARDBP gene on chromosome 1 (Ou et al, 1995). Recently, TDP-43 has attracted much interest as it was the protein that is the main component of tau-negative inclusions in frontotemporal lobar degeneration (FTLD) (Neumann et al., 2006). Similar TDP-43 proteinopathies are also found in ALS patients, indicating that there is a close relation between TDP-43 and neurodegenerative diseases (Neumann et al., 2006; Forman et al, 2007; Buratti et al, 2008; Sreedharan et al., 2008).

Outside of the central nervous system, TDP-43 is widely expressed in all tissues of mammals (Buratti et al., 2001; Ayala et al., 2005). Under physiological conditions, functions of TDP-43 are associated with various pathways by binding to DNA, RNA or other nuclear proteins (Buratti et al., 2001; Buratti et al, 2004; Ayala et al., 2005; Mercado et al, 2005). It was reported that TDP-43 can recognize the intronic UG tract of CFTR pre-mRNA, thusly promoting skipping of exon 9 in CFTR mRNA (Wang et al, 2004; Ayala et al, 2006; Buratti et al, 2008), suggesting its role in RNA splicing regulation. TDP-43 also can interact with many other splicing related protein (Buratti et al., 2005; Freibaum et al, 2010) via the C-terminal glycine-rich domain of the protein (Wang et al., 2004; Ayala et al., 2005).

Accumulating amount of evidence have showed that TDP-43 is a stress responsive factor. TDP-43 is directed to stress granules (SG) in response of osmotic or oxidative stressor in many types of cells including neurons (Dewey et al., 2011). Formation of SG is significantly delayed without TDP-43 (McDonald et al., 2011). Translocation of TDP-43 from nucleus to SG in cytoplasm is mediated by RNA Recognition Motif 1 (RRM1) and glycine-rich region of TDP-43, which harbors the majority of pathogenic mutations (Bentmann et al., 2012).

TDP-43 plays a key role in cell cycle and apoptosis of neurons. Ayala et al. (2008) reported that TDP-43 regulates cyclin-dependent kinase6 (CDK6) and several other factors which control cell cycle and proliferation. Sreedharan et al. (2008) found that mutant forms of TDP-43 are more prone to cause neural apoptosis in chick embryo. It was also reported that the 25-kDa fragment of TDP-43 generated by caspase cleavage can induce neuronal death through a toxic gain-of-function (Zhang et al., 2009; Wils et al., 2010). Pathological TDP-43 can lead to presenile apoptosis in neurons.

While TDP-43 has been recognized as an important protein involving fundamental cellular activities, research has been mainly focused in neuronal cells and the central nervous system (CNS). In the present study, we investigated the role of this protein in cancer cells by using human glioma cells in relation to apoptosis induced by activation of caspase 3. Our results showed that TDP-43 is also crucial in tumour cell apoptosis and therefore may be a potential target for cancer treatment.

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Materials and Methods

Cell cultures and treatments

Human glioma cell line U87 was purchased from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, Grand Island, NY), penicillin (100 units/mL) and streptomycin (100 μg/mL) (Gibco-BRL, Grand Island, NY) incubated in humidified atmosphere and 5% CO_2 at 37°C. Cells were passaged every 3-4 days at 80% confluence. Cell passages before 40th generation were used for experiment. Cells were seeded into 6/12/24/96-well plates at least 12h prior to treatment. STS, Z-VAD-FMK were all dissolved in DMSO to make a stock solution and stored at -20°C. Before treatment, drug was thawed and diluted in DMEM with 10% FBS to indicated final concentrations.

MTT staining

Cells were seeded into 24/96-well plate at a concentration of 2.5×10^5/ml 24h. After treatment, culture medium was replaced with MTT staining solution (0.05mg/ml in serum-free medium). Then the cultures were incubated at 37°C with 5% CO_2 for 4h, followed by adding same volume (100ul for 96-well plate, 500ul for 24-well plate) of MTT lysis buffer (50% v/v N, N-dimethylformamide (Sigma), 20% SDS (BIO-RAD), and 0.4% (v/v) glacial acetic acid in distilled water (pH 4.8)). The plates were incubated overnight at 37oC. OD value of each well was measured with a microplate reader (BIO-TEK, Winooski, VT, USA) at 595 nm.

Protein extraction and immunoblotting

After indicated treatment, cells in the plates were washed with ice-cold PBS. 1×sample buffer (62.5 mM Tris-HCl at pH 6.8 and 25°C, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue) was added into the plates to lyse cells. Cell lysate was collected and transferred into 1.5ml tubes followed by heating at 95-100 °C for 5 min. Before loading the samples, protein concentration was measured by a modified DC protein assay (Bio- Rad). Equal amount of protein was used for immunoblotting. SDS-PAGE was performed to separate different sizes of proteins. The protein samples were transferred to nitrocellulose membranes using a wet transfer system (BIO-RAD). The membrane was blocked with 5% nofat milk (BIO-RAD) for 1h at room temperature before incubating with anti-TDP-43 primary antibody at 4 °C overnight. The membranes were washed with TBST (Tris-buffered saline with 0.05% Tween 20) for 3 times, 15min each, followed by incubation of secondary antibody (HRP-conjugated anti-rabbit or mouse IgG) for 1h at room temperature. After 3 times wash with TBST, protein signals were detected by ECL kit (Perkin-Elmer Life Sciences, Boston, MA) using a Kodak image system (Kodak, Rochester, NY). The images were analysed with Image J (NIH, Bethesda, MD, USA).

RNA extraction and qRT-PCR

Total RNA was extracted with Trizol (Invitrogen) according to the manufacture's instruction. 2ug RNA was used for reverse transcription. qRT-PCR was conducted on an Applied Biosystems 7300 Real-Time PCR System using SYBR Green qPCR MasterMix (Invitrogen) in 25 μl of reaction. The TDP-43 primers used are as follows, forward primer: 5' -TGGAGAAGTTCTTATGGTGCAGGTC-3', reverse primer: 5' -GGTATTAGCCTATGGGGGACAC-3' (Invitrogen). β-actin was used as an internal control.

siRNA Transfection

Cells were inoculated into 6/12/96-well plate at a proper seeding concentration 24h before transfection. We used Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to manufacturer's instruction. Any additional treatment was applied at least 24 hours after transfection. siRNA was designed to target to the 3'UTR region of TDP-43 (GenBank accession no. NM_007375). The siRNA sequence was as follows: sense: 5'-CACUACAAUUGAUAUCAAAUU-3', antisense: 5'-UUUGAUAUCAAUUGUAGUGUU-3'. Customized Ambion siRNA was purchased from Life technologies.

Statistical analysis

At least 3 independent experiments were performed for data analysis. Charted data are presented in terms of means with SD. Unpaired, two-tailed Student's t-test was used to compare means of 2 groups, one way ANOVA was used to compare means of 3 or more groups, differences were regarded as statistically significant when $p<0.05$ and were marked with asterisk (*) in figures. Error bars represent the mean±SEM.

Results

Staurosporine (STS) treatment in U87 cells time- and dose-dependently reduced endogenous TDP-43

Staurosporine (STS) is a commonly used agent to induce apoptosis of cancer cells (Bertrand et al, 1994). U87 cells were treated with various concentrations of STS followed by measuring the cell death at 24 hours with MTT staining (Figure 1A). Preliminary study showed that the IC_{50} of STS in U87 cells was approximately 5uM and this concentration was chosen for further experiments. To investigate the levels of TDP-43 in STS induced apoptosis, U87 cells were treated with STS (5uM) for 4h/8h/12h followed by a western blotting on TDP-43 using cell lysates. Immunoblots showed that the amount of TDP-43 began to significantly decline at 8 hours after STS (5uM) treatment (Figure 1B), and further reduced after 12 hours. Figure 1C showed that 1uM of STS was sufficient to induce significant reduction in TDP-43 at 8 hours.

STS treatment down-regulates transcription level of TDP-43

Next we proceeded to explore the mechanism of TDP-43 reduction by STS. First, we investigated the effect of STS on TDP-43 mRNA level in U87 cells. Figure 2A shows that levels of TDP-43 mRNA measured by qRT-PCR reduced concentration-dependently in cells treated with as low as 0.5uM STS at 8 hours (Figure 2A). At 5uM,

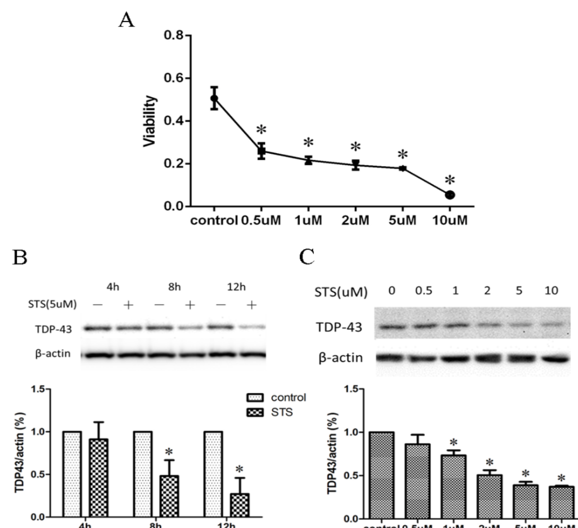

Figure 1. Expression Level of TDP-43 in U87 Decreased After Subjecting to STS. A) Cell viability was measured by MTT assay, the result showed 24 hours of STS treatment can significantly reduce viability of U87 cells. **B)** U87 cells were subjecting to STS(5uM) for different time. Immunoblotting was performed to exam the amount of TDP-43 in U87 after 8 hours STS treatment. **C)** U87 cells were subjecting to treatment with various concentrations of STS (0-10uM) for 8 hours, the result indicated that TDP-43 protein was decreased with increased concentration of STS in U87 cells. Asterisk (*) indicates $P<0.05$ (compared with control group)

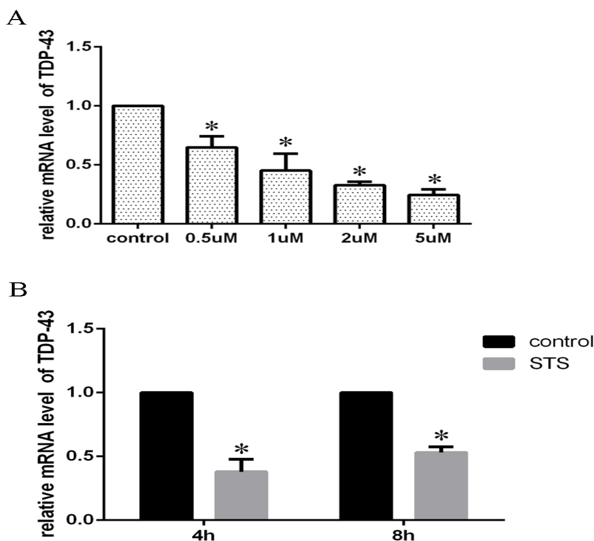

Figure 2. STS Affected mRNA Level of TDP-43 in U87 Cells. (A) The qPCR results showed that transcription level of TDP-43 decreased when treated with various concentrations of STS after 8 hours. (B) When treated with 5uM STS for 4 hours, transcription of TDP-43 was already downregulated significantly. Asterisk (*) indicates $p<0.05$ (compared with control group)

the amount of TDP-43 mRNA was reduced at 4 hours (Figure 2B).

Reduction of TDP-43 protein is Caspase3-dependent in U87

To ask whether transcriptional down-regulation by STS is the only mechanism for reduced levels of TDP-43, we further looked at involvement of caspase-3 in TDP-43 proteolysis as it has been shown that TDP-43

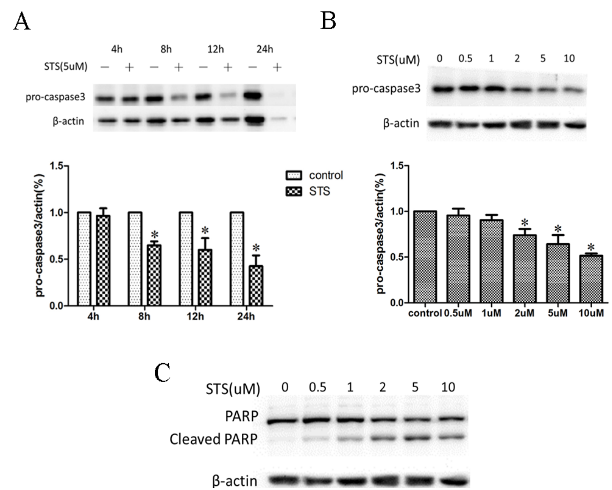

Figure 3. Caspase3 was Activated in STS Treated U87 Cells. A) The amount of pro-caspase 3 in U87 reduced after STS (5uM) treatment for 8 hours or longer. **B)** After 8h STS treatment, pro-caspase 3 decreased with increased concentrations of STS in U87 cells. **C)** PARP was cleaved after 8 hours STS treatment in U87 cells, indicating that caspase 3 was activated in U87. Asterisk (*) indicates $p<0.05$ (compared with control group)

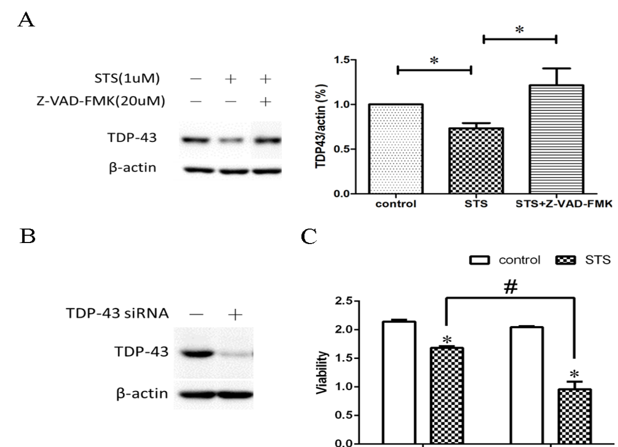

Figure 4. Blocking Activity of Caspases Can Significantly Abolish STS Induced TDP-43 Downregulation in U87, and TDP-43 Knockdown Can Enhance STS Cytotoxicity. A) U87 cells were treated with STS (1uM) in the presence or absent of Z-VAD-FMK (20uM) for 8 hours, asterisks (*) indicate significant difference between two groups. **B)** Western blots of TDP-43 in scrambled siRNA (-) or TDP-43 specific siRNA (+) treated U87 cells. **C)** Viability of U87 cells treated with scrambled siRNA (siRNA-) or TDP-43 specific siRNA (siRNA+) with or without STS. Asterisks (*) indicate significant difference between control and STS-treated group. Number sign (#) indicates significant difference between both STS-treated siRNA- and siRNA+ group ($p<0.05$)

is cleaved by caspase-3 (Dormann et al., 2009; Zhang et al., 2009; Nishimoto et al., 2010). As expected, 5uM STS induced activation of caspase-3 in U87 cells at 8 hours after treatment (Figure 3A), in addition, increased level of caspase-3 was activated when concentration of STS increased after 8 hours treatment (Figure 3B). The activation of caspase-3 was demonstrated in reduction of pro-caspase-3 as well as cleavage of PARP (Figure 3C), one of the caspase-3 substrates. Further, we used pan-caspase inhibitor (general inhibitor of caspase family) Z-VAD-FMK (Slee et al., 1996) to prevent activation of

caspases in the presence of STS in U87. Z-VAD-FMK (20uM) could completely block STS (1uM) -induced reduction of TDP-43 (Figure 4A). This result indicated that caspase family (especially caspase 3) may contribute to the STS induced reduction of TDP-43.

TDP-43 knockdown enhanced cytotoxicity of STS in U87

To investigate the role of TDP-43 in STS-induced apoptosis in U87, we used TDP-43 siRNA specifically targeted for 3' UTR region of human TDP-43 mRNA (Fiesel et al., 2009) to knockdown endogenous TDP-43 before subjecting to STS (0.5uM) for 40 hours. Immunoblot was performed to exam the knockdown efficiency of siRNA (Figure 4B). Then MTT staining was performed to measure the viability of U87. Our results demonstrated that knockdown of TDP-43 alone had little effect on cell viability. However, drastically enhanced cytotoxicity was observed when combined STS treatment with the specific TDP-43 siRNA (Figure 4C), indicating that normal levels of TDP-43 may be antagonistic for STS-induced apoptosis in U87.

Discussion

Glioma is a certain kind of tumor which grows in brain or spine, it accounts for ~30% of CNS tumors and 80% of malignant brain tumors (Goodenberger et al., 2012). Thusly its pathogenesis is extensively studied recently. Some researchers found overexpression of Mda-9/ syntenin could promote the migration ability of glioma cells (Zhong et al., 2012), someone found two kinds of circadian clock genes, cry1 and cry2, played crucial roles in the survival of human glioma cells (Luo et al., 2011) . Recently, exosomes, cell-derived vesicles which were reported containing TDP-43 inclusion, was found to be promote glioma cells' growth by inhibiting CD8+ T cells (Liu et al., 2013). Yet many related proteins and genes remain to be explored.

Our results demonstrate for the first time that TDP-43, a widely expressed DNA/RNA binding protein which is extensively studied in neurodegenerative diseases, is associated with apoptosis in glioma cells. STS induced cell death in U87 cells is associated with rapid down-regulation of TDP-43 at both mRNA and protein levels. Apparently, normal endogenous levels of TDP-43 is protective for STS induced apoptosis in U87 cells as knocking down TDP-43 by siRNA can significantly enhance the sensitivity of U87 cells to STS induced cell death (Figure 4C).

Although STS caused initial suppression on transcription of TDP-43 mRNA was detected as early as 4 hours (Figure 2B). This down-regulation in mRNA did not seem to be translated into actual reduction of its protein. There is no significant change in the amount of TDP-43 protein at 4 hours after STS treatment. Interestingly, no activation of caspase-3 was evident at the same early stage measured by reduction of pro-caspase-3 (Figure 3A). Thus, the reduction of TDP-43 in STS treated U87 cells seem temporally in parallel to the activation of caspase-3. Previous studies have also showed that TDP-43 protein can negatively regulate its own transcription (Ayala et al., 2010), further suggesting that TDP-43 protein is a determining factor regulating its own level.

In consistence to other studies, our results showed that caspases are the proteases responsible for down-regulation of full-length TDP-43 in glioma cells. While caspase-3 is thought to be the major caspase for the cleavage of TDP-43, caspase 7 may be also involved as reported previously (Zhang et al., 2007; Cassel et al, 2012). Our experiment did not specify exactly which protease is the executer. In addition, some other protease like calpain can cleave TDP-43 in motor neurons of mice (Yamashita et al., 2012), whether they also play a role in the reduction of TDP-43 in glioma cell remains to be investigated.

It is well known that cleavage of TDP-43 by caspase-3 generates 35kD and 25kD C-terminal fragments that are toxic to the cell and may contribute to cytotoxicity of TDP-43 proteinopathies. However, unlike in neural cells where the 35kD fragment is readily detectable, we did not observe any cleaved bands by western blotting, one possible explanation is that the fragments are immediately degraded through protein degradation system in glioma cells, which may prevent the accumulation of toxic cleavage products of TDP-43 as a defense mechanism of cancer cell against apoptosis inducers. Nevertheless, our results show that normal levels of TDP-43 might be an important protective factor for cells under apoptotic insult. Mechanism of cell death induced by lethal concentrations of STS may involve reduction of full-length TDP-43 by caspase-3 cleavage in glioma cells, which is supported by the fact that siRNA knockdown of endogenous TDP-43 sensitized toxicity of STS in U87 cells. Therefore, our work is the first time to suggest that TDP-43 can be a potential oncological target that merits further investigation on the role of this protein in cancer biology.

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