RESEARCH ARTICLES

Neuroprotective effect of ATM/ATR inhibitor CGK733 on acrylamide-induced inhibition of cytotoxic effects and neurite outgrowth in PC12 cells

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Abstract

Acrylamide is a neurological and reproductive toxicant for both humans and laboratory animals. It inhibits cellular differentiations in variant cells in vitro. We investigated whether the DNA damage response (DDR) pathway is involved in the inhibition of neurite outgrowth and the induction of cytotoxicity by acrylamide in PC12 cells. In this study, CGK733 (ataxia telangiectasia mutated kinase (ATM) and ATM-related kinase (ATR) inhibitor) was used in inhibition of the DDR pathway, which was induced by acrylamide exposure. The results showed that NGF triggered neurite outgrowth and proliferation of PC12 cells. Both neurite outgrowth and cell proliferation induced by NGF were attenuated after acrylamide exposure. The acrylamide-decreased neurite outgrowth and cell proliferation were increased in the administrations of CGK733. In the NGF-stimulated PC12 cells, CGK733 attenuated acrylamide-induced cell cycle alterations and cellular apoptosis. Moreover, the DDR-related molecules were estimated by Western blotting. The results showed that CGK733 can reverse not only acrylamide-induced expressions of ATM, ATR, and p21 but also acrylamide-decreased expression of cyclin D_1 in the NGF-stimulated PC12 cells. These results demonstrated that acrylamide exposure induced the DDR pathway in the NGF-stimulated neurite outgrowth of PC12 cells. Acrylamide-induced inhibition of neurite outgrowth and cytotoxicity in NGF-stimulated PC12 cells can be attenuated by CGK733.

Keywords: neurite outgrowth, acrylamide, NGF, CGK733, PC12 cells.

1. Introduction

Acrylamide, a water-soluble vinyl monomer, has been recognized as a neurological and reproductive toxicant in humans and laboratory animals. It is frequently used in many applications in industry and laboratories. Acrylamide has been identified in breast milk and can cross the human placenta, resulting in deficits in development and motor coordination before weaning.[1] Acrylamide can be found in food processed at high temperatures and in tobacco smoke.[2-4] Children are exposed to two to three times more acrylamide than adults (approximately 0.5 μg/kg body weight).[5-7]

In neurological toxicity, acrylamide causes central-peripheral neuropathy; and axons are the primary site at which acrylamide causes axonopathy after impairment of the neurotransmitter by increasing the number of vesicles in the synapses, swelling the distal nerve terminal axon, and filling it with neurofilaments.[8,9] The disturbance of kinesin-related motor protein has also been reported in acrylamide-induced axonal toxicity.[10] The toxic effects of acrylamide on neurons have been investigated intensively, including the reduction of cell proliferation, the induction of apoptosis, the phosphorylation of p53 and ERK, the formation of perikaryal inclusion bodies, and the transduction of neurodegeneration-related signals.[6, 11-13] In our previous studies, acrylamide at high concentrations could inhibit cell proliferation and cyclin D_1 expression of glioblastoma cells.[14,15] Acrylamide reduced PC12 cells neurite outgrowth through the inhibition of PI3K-AKT-CREB signaling.[16] Retinoic acid-induced neurogenesis of SH-SY5Y and butyric acid-induced astrogliogenesis of U-1240 MG cells were attenuated by acrylamide, which is associated with down-regulation of MAPs expression and JAK-STAT signaling.[17] Furthermore, we developed a stem cell-based model for predictive toxicology of development in neurological systems after acrylamide exposure. The results reveal that acrylamide inhibits neurosphere formation through the disruption of the neurosphere (is frequently used as a standard for evaluating self-renewal ability of neural stem cells) architecture in neural stem cells.[4] However, the developmental toxicity of acrylamide remains unclear.

The CGK733, a thiourea-containing compound, was originally identified as an inhibitor of ataxia telangiectasia mutated kinase (ATM) and ATM-related kinase (ATR).[18] ATM and ATR mediate cellular responses to DNA damage following exposure to diverse genotoxic agents. These responses include induction of cell cycle arrest, DNA repair, maintenance of genomic stability, induction of premature senescence, and cell death. The coordinated activation of these processes has been defined as the DNA damage response (DDR).[19] Investigation of the DDR pathway involved in inhibition of neurite outgrowth by acrylamide. Here we show that acrylamide-inhibited neuronal differentiation and acrylamide-induced cytotoxic effects were attenuated through the inhibition of ATM/ATR signaling in NGF-stimulated PC12 cells.

2. Materials and methods

2.1 Reagents and antibodies

Acrylamide was purchased from Sigma-Aldrich (St. Louis, MO, USA); CGK733 was purchased from Calbiochem (Darmstadt, Germany); human NGF was from PeproTech Inc. (Rocky Hill, NJ, USA); Tris (base) was from J.T. Baker (Phillipsburg, NJ, USA); skim milk powder was from Anchor (Auckland, NZ). All cell culture reagents and saline buffers were from Gibco (Rockville, MD, USA); rat tail collagen I was from BD Biosciences (Franklin Lakes, NJ, USA); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was from Promega (Madison, WI, USA); an antibody against cyclin D_1 was from Cell Signaling Technology (Danvers, MA, USA); and ATM, ATR, p21, and β-actin were from Abcam (Cambridgeshire, UK).

2.2 Cell culture

PC12 cell, a rat adrenal pheochromocytoma cell line that can be differentiated into sympathetic-like neurons, is a widely used model system for studies of promoting neurogenesis, neural development, neuronal survival, and functional maintenance of neurons by neurotrophic factors such as nerve growth factor (NGF).[20-24] PC12 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained on collagen-coated plates (0.1 mg/ml rat-tail collagen in 0.02 N acetic acid) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (HS), 5% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. All cells were cultured at 37 °C with 5% CO_2.

2.3 Cell treatments

Acrylamide stock solution (1M) was prepared with water and filter-sterilized. Cell media containing different concentrations of acrylamide (0, 0.5, and 1 mM) were prepared and added to PC12 cells. The treated cells were cultured at 37 °C with 5% CO_2 for different time periods (48 and 72 h). To determine the involvement of ATM and ATR in regulating acrylamide-affected NGF-induced signaling, cells were pretreated with 5 μM CGK733 (ATM/ATR inhibitor) for 1 h and were then treated with 50 ng/ml NGF with or without 0.5 or 1 mM acrylamide for another 72 h at 37 °C with 5% CO_2.

2.4 Neurite outgrowth analysis

In PC12 cells neurite outgrowth, morphological analysis, and quantification of neurite-bearing cells were carried out as in previous studies.[16] PC12 cells were plated on collagen I-coated dishes in normal serum medium for 24 h. For neuronal differentiation, NGF was treated with 50 ng/ml in low-serum medium (DMEM containing 2% HS, 1% FBS, 1% L-Gln, and 1% AA), to co-operate with 0, 0.5, and 1 mM acrylamide, respectively. The NGF-treated cells were cultured for 48 h at 37 °C with 5% CO_2. The definition of PC12 cell morphological differentiation is that the length of neuritis should be at least twice the diameter of the cell body. Images were taken using a microscope (Olympus IX71, Tokyo, Japan).

2.5 Cell proliferation assay

Cell proliferation was evaluated by MTS assay. 1×10^3 cells per well were plated in a 96-well microtiter plate with 100 μl culture medium containing 50 ng/ml NGF. These cells were treated with different acrylamide concentrations (0, 0.5, and 1 mM) for 72 h at 37 °C with 5% CO_2. After treatment, 20 μl MTS reagent was added in each well for 2 h. The enzymatic reduction of MTS to formazan was quantified by Spectra MAX190 photometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. To determine the involvement of ATM and ATR in regulating acrylamide-affected NGF-induced signaling, MTS assay was performed after cells were co-treated with CGK733 for 72 h at 37 °C with 5% CO_2.

2.6 Cell cycle analysis

Cell-cycle phase distribution was analyzed using the hypotonic lysis method by flow cytometry [4]. Single cells were obtained from acrylamide-treated PC12 cells by HyQtase dissociation, and were used in cell-cycle analyses. To determine the cell-cycle profiles, 1×10^6 cells were harvested and washed once with cold phosphate buffered saline (PBS). The cell pellet was harvested by centrifugation (300 × g, 5 min), and the supernatant removed. The cell pellet was treated with 500 μl hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100, 100 μg/ml RNase A, and 50 μg/ml propidium iodide) at 4 °C for 20 min in the dark. Finally, cells were analyzed for DNA content with a BD FACS/Calibur flow cytometer (BD Bioscience, FL, USA). The data were analyzed with BD CellQuest Pro software.

2.7 Detection of apoptosis

The Sub-G_1 assay was performed as described in cell cycle analysis. Annexin V/PI assay was performed according to the manufacturer's protocol (BD Pfarmingen, Heidlberg, Germany). The number of apoptotic cells was calculated using a BD FACS/Calibur flow cytometer (BD Bioscience, FL, USA). The data were analyzed with BD CellQuest Pro software.

2.8 Western blot analysis

Antibodies against ATM, ATR, cyclin D_1, p21, and β-actin were used to detect the corresponding protein levels by the immunoblotting method in PC12 cells. Protein samples were harvested by RIPA Lysis Buffer (Millipore, Billerica, MA, USA) supplemented with 100 μl/ml of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined by a Bio-Rad Laboratories protein assay reagent kit (Hercules, CA, USA). Protein (40 μg) was resolved by a discontinuous 5% stacking and 8% running sodium dodecyl sulfate-polyacylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). Thereafter, membranes were blocked in 5% skimmed milk or 5% bovine serum albumin (Bio Basic, Markham Ontario, Canada) blocking buffer for 30 min at room temperature. The membranes were then incubated with specific primary antibodies in either 5% skimmed milk or 5% bovine serum albumin blocking buffer, followed by incubation with corresponding HRP-conjugated secondary antibodies. Protein levels were detected with a Western lightning kit (PerkinElmer, Boston, MA, USA) with β-actin as an internal control.

2.9 Statistical analysis

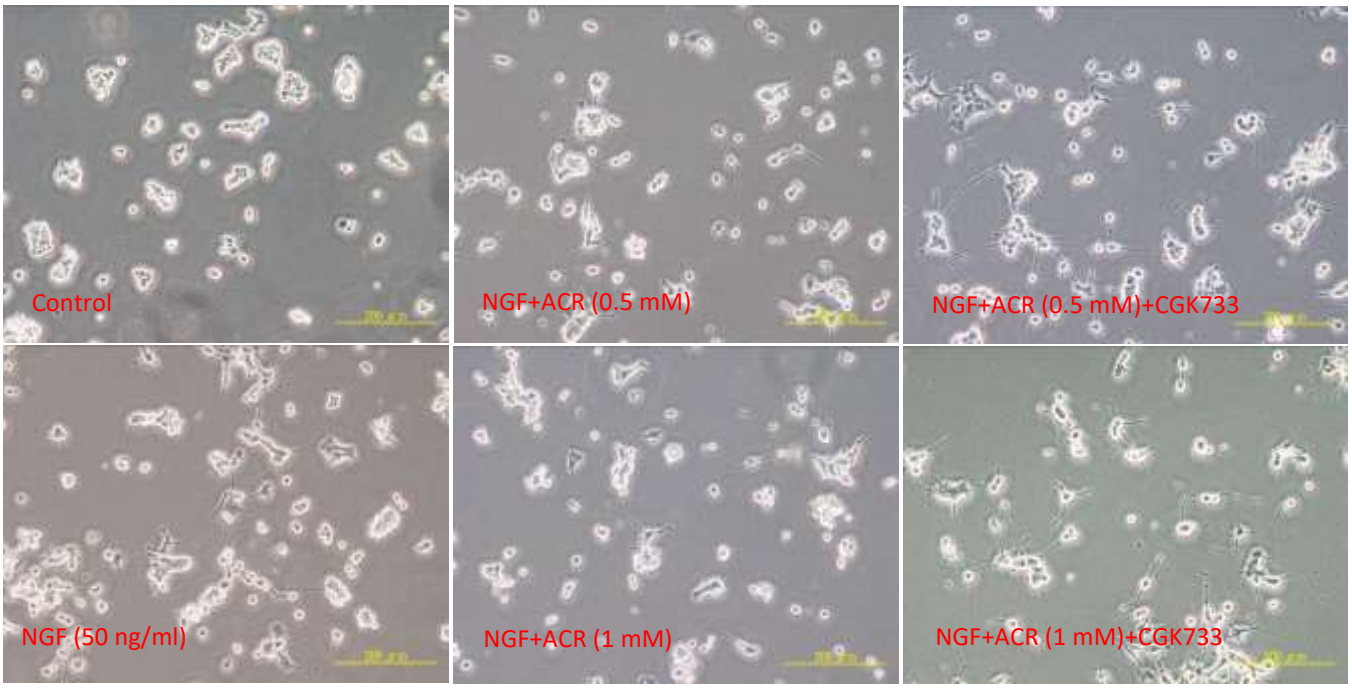
The data are expressed as mean ± SD. The statistical significance was determined by the one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison tests by a statistical package for the social science 13.0 software (SPSS, Chicago, IL, USA). The differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 CGK733 attenuates acrylamide-suppressed neurite outgrowth of PC12 cells

To investigate whether DDR was involved in the acrylamide-inhibited neurite outgrowth in PC12 cells, the cells in low serum medium containing NGF (50 ng/ml) were co-treated with (0.5 and 1 mM) or without acrylamide for analysis of cell morphology change. Cells co-treated with 5 μM CGK733 and acrylamide were used in the evaluation of DDR. As shown in Fig. 1A, NGF triggered PC12 cell neurite outgrowth. Both 0.5 and 1 mM acrylamide treatments reduced NGF-triggered PC12 cell neurite outgrowth under light microscope observation. Quantitative results showed that NGF significantly evoked increase of the percentage of differentiated cells (27.4 ± 4.0%), whereas no neurite outgrowth was found without NGF stimulation in PC12 cells (Fig. 1B). Acrylamide treatments significantly decreased in the percentage of differentiated cells (from 27.4% to 12.4% in 0.5 mM acrylamide, $p < 0.01$; from 27.4% to 6.6% in 1 mM acrylamide, $p < 0.001$) of NGF-stimulated PC12 cells (Fig. 1B). Furthermore, NGF-stimulated PC12 cells co-treated with acrylamide and CGK733 showed a significant increase in the percentage of differentiated cells (from 12.4% to 43.3% in the CGK733 co-treated with 0.5 mM acrylamide, $p < 0.001$; and from 6.6% to 41.6% in the CGK733 co-treated with 1 mM acrylamide, $p < 0.001$) (Fig. 1B). These results demonstrated that acrylamide attenuated NGF-triggered neurite outgrowth, and the acrylamide-attenuated neurite outgrowth was reversed by CGK733.

A



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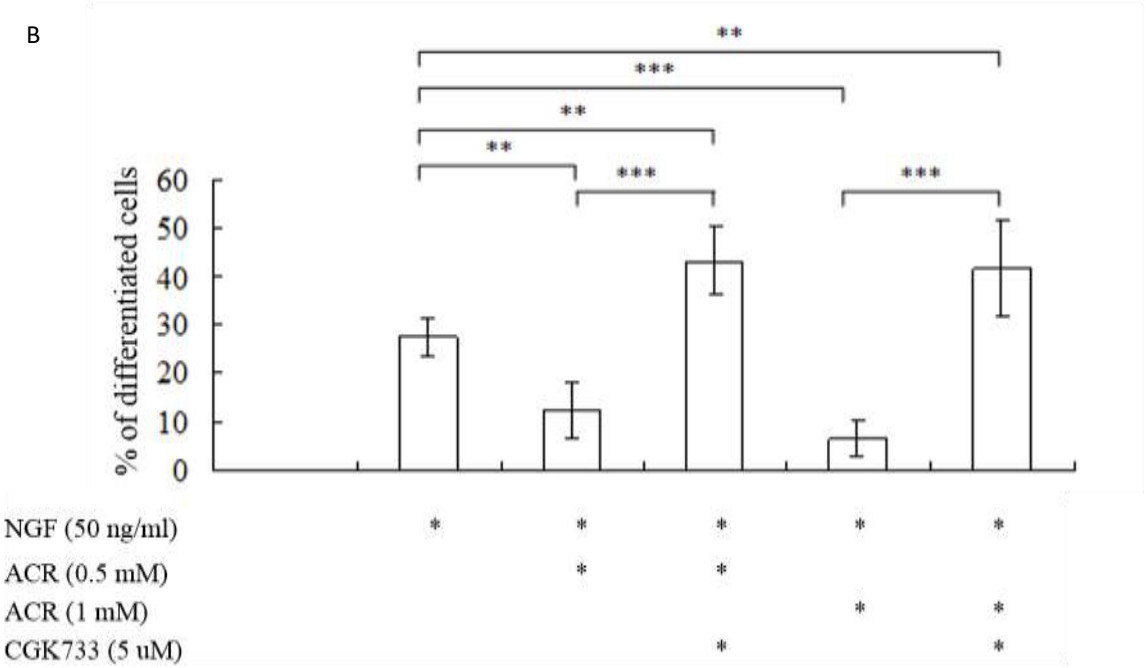

Figure 1. CGK733 attenuated acrylamide-inhibited NGF-induced neurite outgrowth in PC12 cells. 50 ng/ml NGF induced neurite outgrowth in PC12 cells for 48 h. Acrylamide inhibited NGF-induced neurite outgrowth. Representative images of cell morphology by NGF stimulation, NGF and acrylamide co-treatment, or NGF and acrylamide and CGK733 co-treatment are shown in (A). Scale bar, 200 μm. Quantification data of the neurite outgrowth are expressed as percentage of differentiated cells and are presented as the mean ± SD from three independent experiments (B). The definition of differentiation is shown in the Materials and Methods. Statistical differences are shown as *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviation: ACR, acrylamide.

3.2 CGK733 attenuates acrylamide-suppressed PC12 cell proliferation

As shown in Fig. 2, 50 ng/ml NGF treatment for 72 h significantly induced cell proliferation compared with the control (cells without any treatment). PC12 cells co-treated with 0.5 or 1 mM acrylamide for 72 h significantly inhibited NGF-induced proliferation. The results also demonstrated that acrylamide suppresses NGF-induced proliferation of PC12 cells in a dose-dependent manner. CGK733 significantly increased 1 mM acrylamide-decreased NGF-induced cell proliferation. These results demonstrated that acrylamide inhibited NGF-stimulated proliferation in PC12 cells, whereas CGK733 attenuated inhibition of cell proliferation by acrylamide.

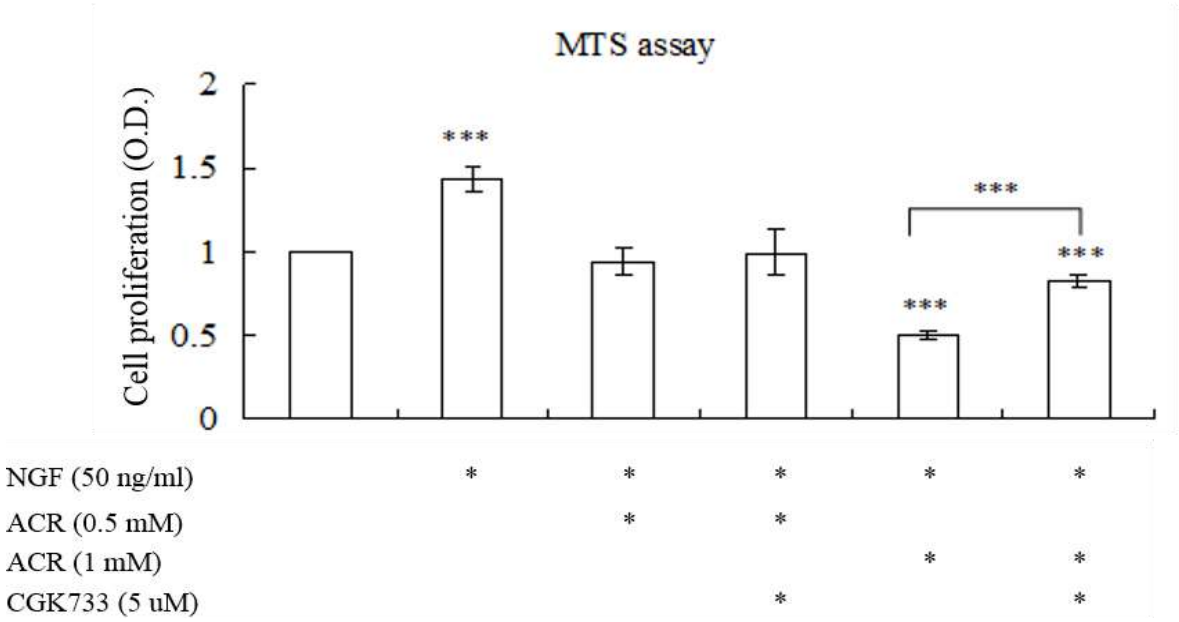

Figure 2. CGK733 increased acrylamide-inhibited NGF-stimulated PC12 cell proliferation. Cell proliferation was analyzed by the MTS assay. 50 ng/ml NGF induced PC12 cell proliferation for 72 h. Acrylamide inhibited NGF-induced PC12 cell proliferation in a concentration (0, 0.5, and 1 mM)-dependent manner. Results are presented as the mean ± SD from three independent experiments. Statistical differences are shown as *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviation: ACR, acrylamide.

3.3 CGK733 attenuates acrylamide-induced cell cycle alterations in PC12 cells

Alterations of each phase of the cell cycle in PC12 cells were analyzed using flow cytometry. Quantitative analyses of cells in each phase of the cell cycle following treatments with different conditions were summarized in Fig. 3. The NGF treatment resulted in significant increases in the G_0/G_1 and G_2/M phase cells and caused a significant decrease in the S phase cells. In the co-treatments of NGF and acrylamide, both 0.5 and 1 mM acrylamide treatments significantly increased in the S phase cells and significantly decreased in the G_0/G_1 and G_2/M phase cells. Further, CGK733 treatment increased in the G_0/G_1 ($p < 0.01$) and G_2/M ($p = 0.08$) phase cells and decreased in the S ($p < 0.05$) phase cells in the PC 12 cells co-treated with 0.5 mM acrylamide and NGF. Similar results also occurred in the cells co-treated with 1 mM acrylamide and NGF. These results demonstrated that CGK733 attenuates acrylamide-triggered cell cycle alterations in NGF-treated PC12 cells.

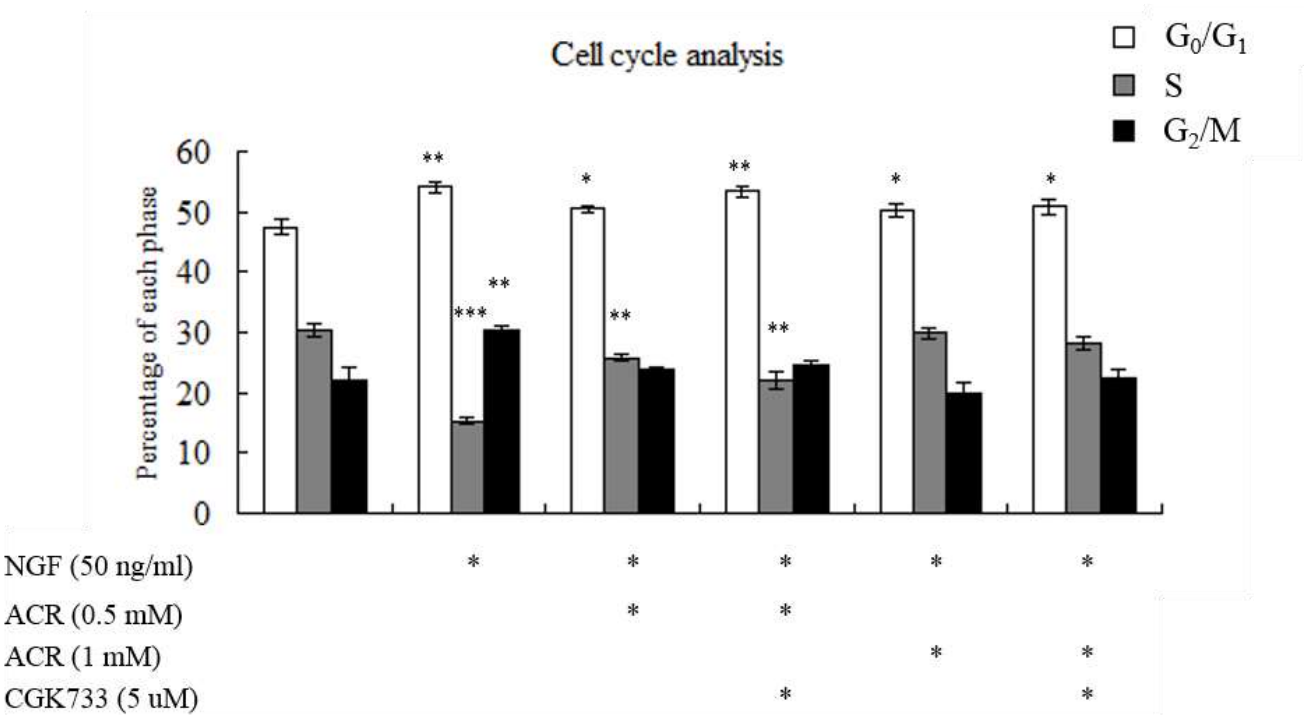

Figure 3. CGK733 attenuated acrylamide-induced cell cycle alterations in differentiating PC12 cells. 50 ng/ml NGF increased in the G_0/G_1 and G_2/M phase cells and decreased in the S phase cells. Both 0.5 and 1 mM acrylamide treatments significantly increased in the S phase cells and decreased in the G_0/G_1 and G_2/M phase cells for 72 h. Quantification data of the cell cycle are expressed as the percentage of each phase and are presented as the mean ± SD from three independent experiments. Statistical differences are shown as $*p < 0.05$, $** p < 0.01$, $*** p < 0.001$ in comparison with control. Abbreviation: ACR, acrylamide.

3.4. CGK733 inhibits acrylamide-induced apoptosis in PC12 cells

In cellular apoptosis, the sub-G_1 and annexin V positive cells were analyzed by flow cytometry.[25] The genomic DNA of cells under apoptosis were degraded into final DNA fragments that were 200 bp long, which were observed on the left side of the G_0/G_1 peak (named the sub-G_1 phase). Annexin V has a strong Ca^{2+}-dependent affinity for phosphatidylserine residues (normally hidden within the plasma membrane) on the surface of the cell, which is an early event in apoptosis and therefore can be used as a probe for detecting apoptosis. As shown in Fig. 4A, PC12 cells co-treated with different concentrations of acrylamide (0.5 or 1 mM) and NGF showed significant reductions of the percentage of the sub-G_1 group by CGK733. PC12 cells co-treated with different concentrations of acrylamide (0.5 or 1 mM) and NGF showed significant reductions of the percentage of the annexin V group by CGK733 (Fig. 4B). These results demonstrated that CGK733 inhibited acrylamide-induced cellular apoptosis.

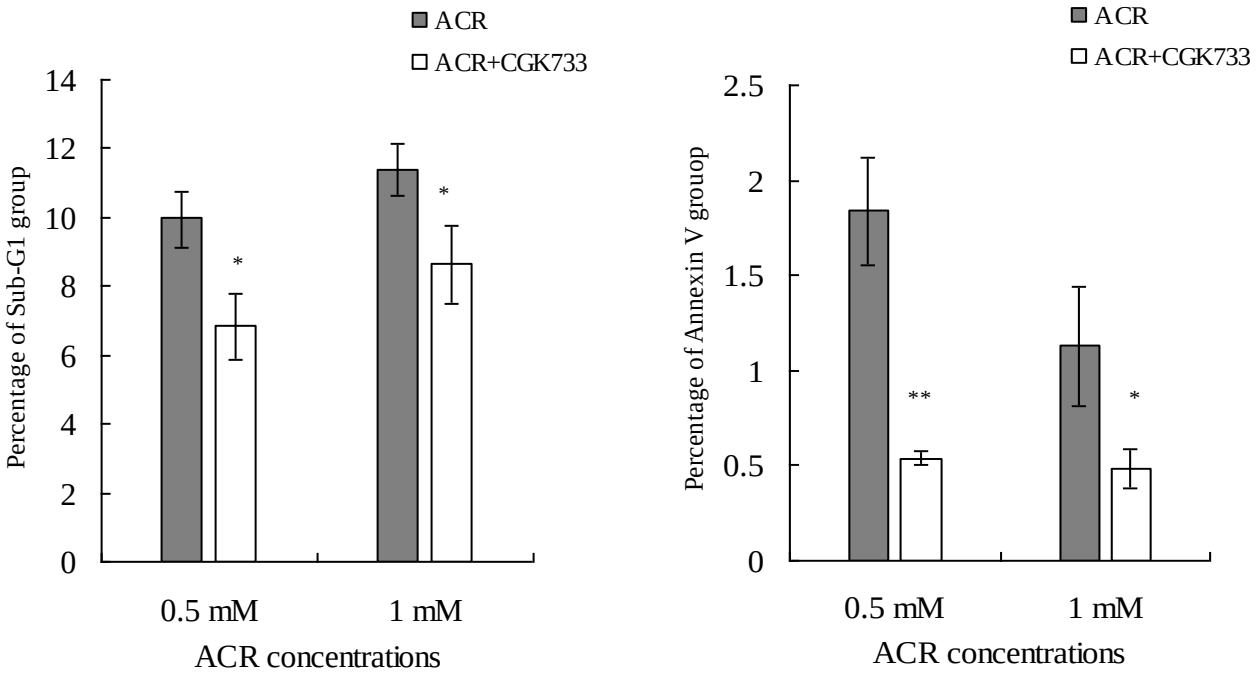

Figure 4. CGK733 reduced acrylamide-induced cellular apoptosis in the differentiating PC12 cells. Acrylamide-induced cellular apoptosis was evaluated by accumulations of the sub-G_1 and annexin V positive cells for 72 h. Quantification data of the sub-G_1 and annexin V positive cells are expressed as the percentage of the sub-G_1 group and the percentage of the annexin V group, respectively. The results are presented as the mean ± SD from three independent experiments. Statistical differences are shown as *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviation: ACR, acrylamide.

3.5. CGK733 down-regulated acrylamide-induced apoptotic responses

We have shown that induction of DNA damage, up-regulation of p21, and down-regulation of cyclin D_1 expression occurred after acrylamide exposure. The results suggested that cells were arrested in the G_0/G_1 phase of cell cycle by inhibiting cyclin D_1/CDK4/6 kinase activity.[14,26] Therefore, acrylamide affected factors —including ATM, ATR, cyclin D_1, and p21 of the DNA damage response pathway — were estimated in NGF-triggered neurite outgrowth of PC12 cells. As shown in Fig. 5, 1 mM acrylamide treatment increased in expressions of ATM, ATR, and p21, and decreased in expression of cyclin D_1. CGK733 co-treatments reversed acrylamide-increased ATM, ATR, and p21 expressions, and acrylamide-decreased cyclin D_1 expression. These results indicated that acrylamide causes DNA damage and ATM/ATR kinase activation within neuronal differentiation of PC12 cells.

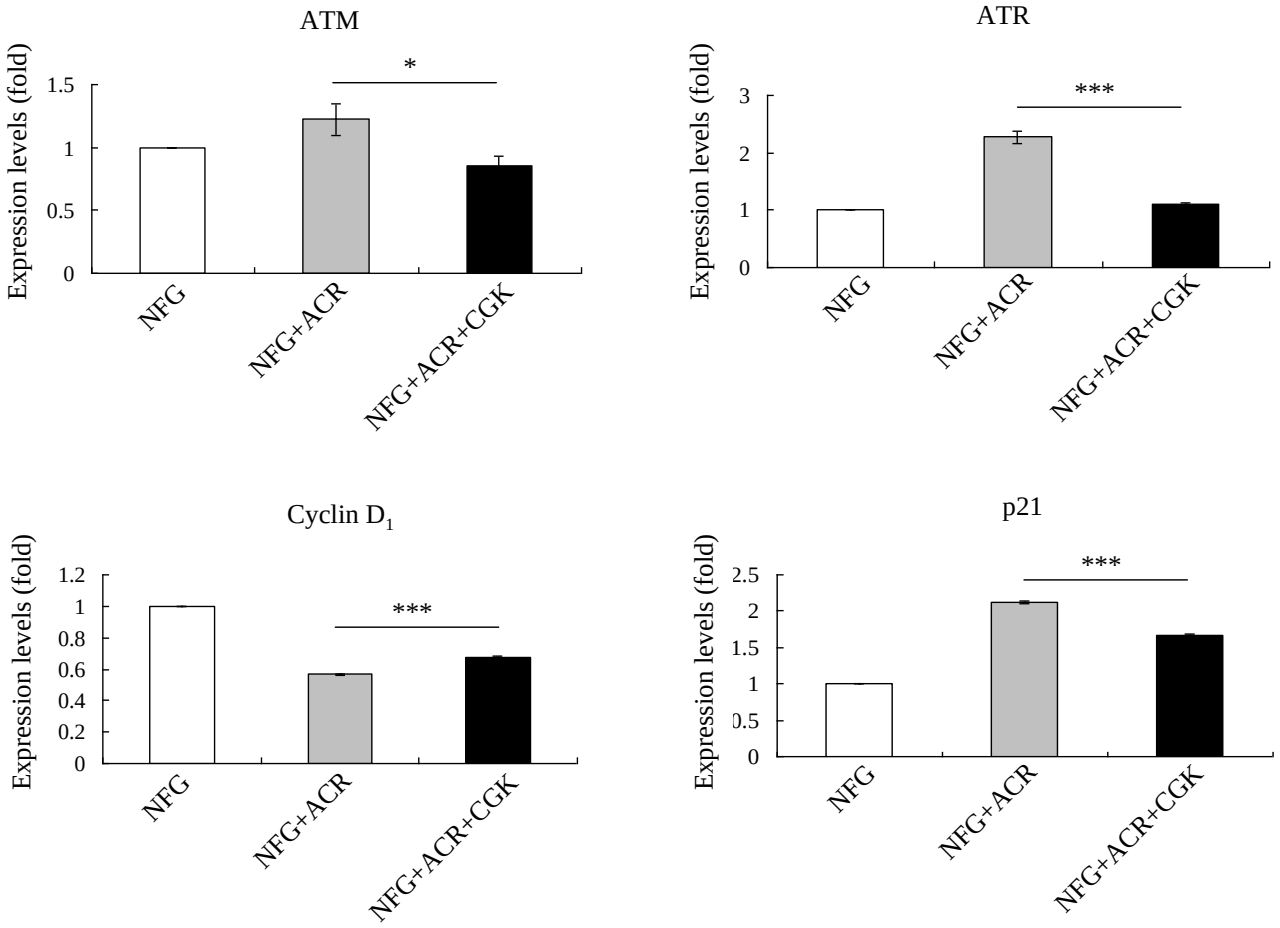

Figure 5. CGK733 attenuated acrylamide-induced activations of DNA damage response-related molecules in the differentiating PC12 cells. Expression of DNA damage response-related molecules (ATM, ATR, cyclin D_1 and p21) in the differentiating PC12 cells was measured by immunoblot. 1 mM acrylamide treatment for 72 h induced DNA damage response-related molecules in the differentiating PC12 cells. The expression levels were normalized by actin protein levels, and then the relative levels were quantified corresponding to control (cells without any treatment). The results are presented as the mean ± SD from three independent experiments. Statistical differences are shown as *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviation: ACR, acrylamide.

Discussion

In this study, ATM/ATR inhibitor CGK733 showed neuroprotective activity on acrylamide-induced inhibition of neurite outgrowth and cytotoxic effects of PC12 cells. CGK733 co-treatment showed increase in acrylamide-inhibited neurite outgrowth (Fig. 1) and cell proliferation (Fig. 2), and decrease in cellular apoptosis (Fig. 4). Acrylamide has been shown to induce DNA damage in various cells by comet assay and the micronucleus test. Our previous findings showed that acrylamide exposure resulted in DNA damage, cell cycle arrest, inhibition of proliferation, generation of reactive oxygen species (ROS), mitochondria collapse, and cellular apoptosis in human astrocytoma cells.[14,26] Acrylamide is chemically reactive toward nucleophiles, including amino

and thiol groups in amino acids and proteins, by Michael additions, but is weakly reactive with the ring nitrogen atoms and the extra-nuclear amino groups of adenine and guanine in DNA.[3,27] However, acrylamide is easily biotransformed to its epoxide, glycidamide, through cytochrome p450 2E1; and glycidamide appears 100–1000 times more reactive with protein and DNA than acrylamide.[28] Because both acrylamide and glycidamide cause depurinating DNA lesions, the repair of the lesions involves the base excision repair pathway, which leads to the formation of DNA breaks.[14,29] Upon DNA damage, ATM and ATR kinases participate in the activation of cell cycle phosphorylation of p53 at Ser-15, which promotes the accumulation and functional activation of p53 and thus leads to cell cycle arrest and cellular apoptosis.[15,30] Therefore, the activation of ATM/ATR kinase in DNA damage responses by acrylamide is involved in the mechanisms of direct activation through interaction with damaged DNA, indirect activation through interaction with DNA repair or maintenance proteins, or a combination of the above. Additionally, acrylamide-induced DNA damage associated with the ROS and 8-hydroxydeoxyguanosine DNA adduct formation in HepG2 cells.[11] Hydroxytyrosol, an efficient scavenger of ROS, could protect HepG2 cells from acrylamide-induced cytotoxicity and DNA damage.[31]

Further, the cytotoxic effects of acrylamide in NGF-induced neurite outgrowth of PC12 cells has been reported before. The results reveal that NGF-stimulation of the neuronal differentiation of PC12 cells is attenuated by acrylamide through the inhibition of PI3K-AKT-CREB signaling, along with the production of ROS.[16] In NGF-induced neurite outgrowth, NGF-mediated ERK activation induces phosphorylation of CREB, which promotes dendritic spine growth, morphology change, and synaptic plasticity.[32,33] The PI3K-AKT signaling cascade also converges signal to CREB by NGF stimulation of PC12 cells.[24,34,35] Activation of TrkA by NGF triggered STAT3 phosphorylation at Ser727, which enhanced the DNA binding and transcriptional activities of STAT3.[36] Oxidative stress results from excess ROS, and it can lead to the disruption of cellular macromolecules, including lipids, proteins, and DNA, and ultimately to cell death via apoptosis.[37-39] Acrylamide-induced ROS production and apoptosis are presented in many cell types, including rat PC12, human astrocytoma, and Caco-2 cells.[26,40] Moreover, acrylamide-induced ROS activities are detected in NGF-treated PC12 cells. The increased ROS activities and cell death were reversed by co-treatment with Trolox (ROS scavenger). The results suggested that ROS production and apoptosis-related signal transduction contributed to acrylamide-inhibited NGF-induced PC12 cell proliferation.[16] Taken together, acrylamide exposure induced DNA damage responses, which resulted in inhibition of neurite outgrowth and induction of cytotoxicity in the differentiating PC12 cells.

In conclusion, the major finding of how acrylamide attenuates NGF-induced neurite outgrowth showed that acrylamide induced DDR pathway which induced cellular apoptosis and inhibition of proliferation of PC12 cells.

Acknowledgments

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