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Oxidative stress-induced DNA damage and cell cycle regulation in B65 dopaminergic cell line

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Abstract
Reactive oxygen species and oxidative stress are associated with neuronal cell death in many neurodegenerative conditions. However, the exact molecular mechanisms triggered by oxidative stress in neurodegeneration are still unclear. This study used the B65 rat neuroblastoma cell line as a model to study the molecular events that occur after H_2O_2 treatment. Treatment of B65 cells with H_2O_2 rapidly up-regulated the DNA damage pathway involved in double-strand breakage. Subsequently, proteins involved in p53 regulation, such as sirtuin 1 and STAT1, were modified. In addition, H_2O_2 treatment altered the pattern of cell cycle protein expression. Specifically, a decrease was found in the expression of cyclin D1, cdk4 and surprisingly the levels of cyclin A and the retinoblastoma protein phosphorylated at ser780 were increased. Furthermore, this study shows that pre-treatment of B65 cells with 50 μM trolox confers almost total protection against apoptotic cell death and restores the cell cycle. Likewise, the increase in retinoblastoma phosphorylation was attenuated by KU-55993, a selective ATM inhibitor, and also by trolox. These observations indicate that DNA damage and oxidative stress are responsible for cell cycle regulation. In summary, this study describes the molecular mechanisms involved in cell cycle alterations induced by oxidative stress in B65 cells. These findings highlight the relevance of ATM in the regulation of cell cycle after oxidative stress.

Keywords: *ATM, apoptosis, KU-55933, cell cycle, retinoblastoma protein*

Introduction

Increased production of reactive oxygen species (ROS) plays a central role in numerous pathologies, such as cancer and neurological disorders, as well as in ageing [1–4]. It is widely accepted that an excess of ROS is toxic and damages cell components, including nucleic acids, proteins and lipids. In addition, this excess enhances apoptosis or necrosis [5]. Moreover, ROS causes DNA damage, specifically DNA double-strand breaks (DSBs), considered the most significant nuclear lesion [3–5]. Although DNA damage response involves a complex cellular network, it is now known that the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) phosphoinositide 3-kinase-related kinases (PIKKs) are the main enzymes activated after this damage [6]. Both types of enzyme are involved in the transduction of DNA damage signals to checkpoint control proteins.

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In this regard, ATM is essential for mediating checkpoint control in cells exposed to stimuli which produce DSBs, such as ionizing radiation, camptothecin and other specific DNA-damaging agents [7].

A rapid response to DNA DSBs is the phosphorylation of p53 on Ser15 by ATM [8–11]. However, previous studies identified other potential enzymes that contribute to p53 activation, such as STAT1 (signal transducer and activator of transcription 1) cdk5, c-Jun kinase and SIRT1. SIRT1 is a regulator of p53 function, thus acetylation of p53 occurs at the C-terminal lys-382 and stabilizes it and also increases the binding capacity of p53 to DNA [12,13]. Likewise, stabilized p53 favours the expression of p21, which inhibits cell cycle progression [14]. Thus, p53 is a tumour suppressor protein and the regulation of the p53 pathway in response to DNA stress is crucial since it can induce either apoptosis or cell survival [15–21]. Activated p53 protein binds to specific DNA sequences, thereby initiating one of three programmes that result in cell-cycle arrest, cellular senescence or apoptosis [15,17,20]. Accordingly, the regulatory network of p53 is complex, since it interacts with a large number of other signal transduction pathways and positive and negative regulatory feedback also occurs [17]. For example, interaction between p53 and Akt, MDM-2, Cop-1, Pirh-2, p73 delta N, cyclin G, Wip-1 and Siah-1 p14/19 ARF and Rb have been reported [17–25]. In addition, p53 induces apoptosis by activating downstream cell death effectors such as Bax, Puma and Noxa [17]. Interestingly, the integration of all these stress signals changes in p53 are mainly visualized by changes in the cell cycle phases.

Here we report that multiple intracellular pathways are involved in p53 regulation. Our results also show that oxidative stress contributes to the regulation of retinoblastoma (Rb) protein through phosphorylation in ser780. Rb phosphorylation was an early event and was inhibited by a specific ATM inhibitor, KU-55993 [26]. Accordingly, we propose an intracellular pathway where ROS affects cell cycle progression through ATM activation and Rb phosphorylation.

Material and methods

Methods

Neuroblastoma B65 cell line was purchased from the European Collection of Cell Cultures (ECACC, Alisbury, UK). Cells were seeded 200 cells/mm^2 and cultured in DMEM media containing 10% FCS.

Assessment of cell viability

B65 cells were used after 24 h of culture *in vitro*. Trolox was dissolved in culture media and added to the neuronal preparation at a range of concentrations 2 h before addition of H_2O_2 (50 μM to 1 mM). To assess the loss of cell viability, we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] method. MTT was added to the cells at a final concentration of 250 μM and cells were incubated for 1 h, thereby allowing the reduction in MTT to produce a dark blue formazan product. Media were then removed and cells were dissolved in dimethylsulphoxide. Formazan production was measured by the absorbency change at 595 nm using a microplate reader (BioRad Laboratories, CA, USA). Viability results were expressed as percentages. The absorbency measured from untreated cells was taken to be 100%.

Measurements of ROS

Levels of intracellular ROS were measured using the fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Briefly, cells were incubated for 1 h at 37°C in the presence of 10 μM of H_2DCFDA (added from a 20 mM stock solution in dimethyl sulphoxide). H_2DCFDA diffuses across neuronal membranes, where acetates migrate via intracellular esterases. Oxidation of H_2DCFDA occurs almost exclusively in the cytosol, thereby generating a fluorescent response proportional to ROS generation. After loading the dye, neurons were washed in Locke's buffer and fluorescence was measured at a 488 nm excitation wavelength and an emission wavelength of 510 nm, using a Perkin-Elmer Victor 3 fluorometer.

Western-blot analysis

Aliquots of cell homogenate containing 25 μg of protein per sample were analysed by Western blot. Briefly, samples were placed in sample buffer (0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95–100°C for 5 min. They were then separated by electrophoresis on 10% acrylamide gels and proteins were subsequently transferred to polyvinylidene fluoride sheets (ImmobilonTM-P, Millipore Corp., Bedford, MA) using a transblot apparatus (BioRad). The membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (Tris 50 mM; NaCl 1.5%; Tween 20, 0.05%, pH 7.5). They were then incubated with primary monoclonal antibodies against ser1981 p-ATM (from R&D Systems), ser139 γH2AX, Sirt1 (from Millipore), Cyclin A (from Abcam), lys379 acetyl-p53, Cyclin D1, ser15 p-p53, tyr701 p-STAT1, ser473 p-AKT, tyr15 p-cdc2, ser780 p-Rb (from Cell Signalling), total ATM, Cyclin B1, Cyclin E, CDK2, total cdc2, CDK4, total p53 and actin (from Santa Cruz Biotechnology). After 4 h or incubation overnight, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody (Amersham Corp., Arlington Heights,

IL). Immunoreactive protein was visualized using a chemiluminescence-based detection kit and following the manufacturer's instructions (ECL kit; Amersham Corp.). Digital images were taken with a Chemidoc XRS (Biorad), which allows semi-quantitation of band intensity. The protein load was periodically monitored via immunodetection of actin.

Statistical analysis

Data are given as the mean ± SEM of at least four experiments involving four-to-six independent cultures. In all experiments, data were analysed by ANOVA followed by post-hoc Tukey–Kramer multiple comparisons tests. *P*-values lower that 0.05 were considered significant.

Results

Trolox protects B65 neuroblastoma cells against H_2O_2-induced cytotoxicity

The viability of B65 cells, measured by the MTT method, decreased significantly ($p < 0.05$) to 35% of the control values after cells were exposed to 500 μM H_2O_2 for 24 h (Figure 1A). Pre-treatment with trolox (50 nM to 50 μM, 6 h), an antioxidant, significantly ($p < 0.05$) increased cell viability (Figure 1B). Consistent with these results, phase-contrast images showed an increase in the number of B65 cells after pre-treatment with 50 μM trolox compared with cells treated with 500 μM H_2O_2 (Figure 1C). With respect to ROS production, exposure of cells to a range of concentrations of H_2O_2 for 1 h (250–1000 μM)

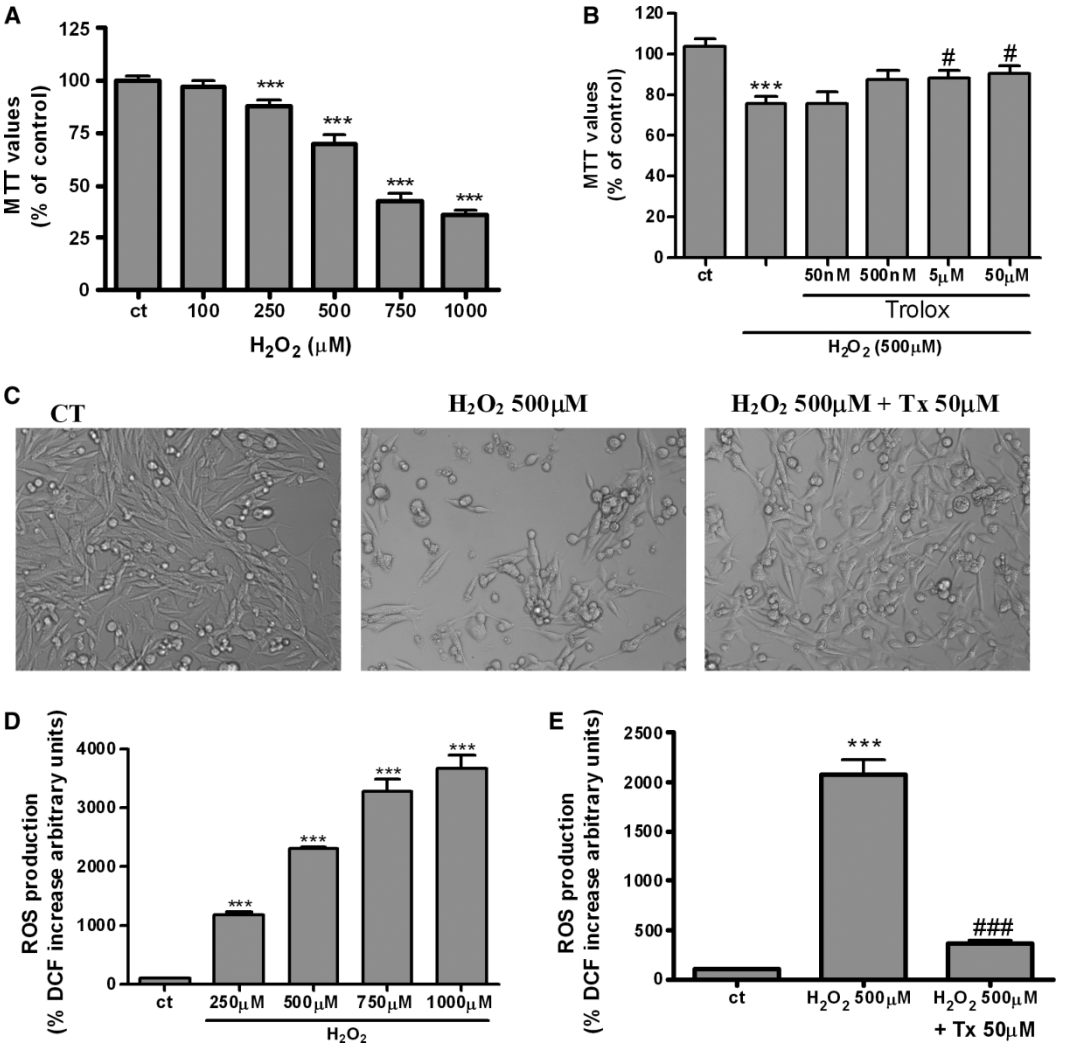

Figure 1. (A) B65 cells were incubated with H_2O_2 (100–1000 μM) and cell viability was evaluated by the MTT method after 24 h (% respect to the control cells). (B) Trolox (0.5–50 μM) attenuated B65 cell loss mediated by 500 μM H_2O_2. Data are expressed as the mean ± SD of four independent experiments; $^{\star}p < 0.05$ compared with H_2O_2. (C) Morphological changes of B65 cells exposed to H_2O_2. Phase contrast microscopic analysis of B65 cells after 24 h of treatment with 500 μM H_2O_2 in the presence of trolox. Calibration bar, 10 μm. (D) Reactive oxygen species (ROS) production in H_2O_2-treated B65 cells. ROS accumulation was detected after B65 cells were treated with a range of H_2O_2 concentrations (250–1000 μM) for 1 h. ROS production was expressed in arbitrary units. (E) Trolox (500 μM) attenuated B65 ROS production mediated by 500 μM H_2O_2. Data are expressed as the means ± SEM of four independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $\#p < 0.05$; $\#\#\#p < 0.001$ compared with H_2O_2-treated cells. $^{***}p < 0.001$ compared with control cells.

caused a significant increase in intracellular oxidative stress generation. Pre-treatment of cells with 50 μM trolox reversed this increase significantly ($p < 0.001$) (Figures 1D and E). H_2O_2 increased the number of apoptotic cells and induced changes in the cell cycle phases (Figures 2A and B). In addition, treatment with 50 μM trolox inhibited the effect of H_2O_2 on cell cycle phases and apoptosis (Figure 2C). Our results show that trolox suppresses H_2O_2-indued cytotoxicity in B65 cells and provides evidence that ROS are responsible for cell cycle changes and cytotoxicity.

H_2O_2-induced changes in the regulation of proteins involved in DNA damage

DNA is a target of oxidative stress [25,27]. ATM is a specific enzyme that is activated after double strand breaks [8]. Thus, first we evaluated ATM activation by phosphorylation at ser1981. We detected the immunoreactive band after 30 min of treatment with H_2O_2 (Figure 2). Next we evaluated the involvement of ATM activation in H_2O_2-induced cell damage. Phosphorylated histone H2AX (γ-H2AX) is involved in the recruitment of DNA damage response proteins to DNA DSBs and facilitates DSB repair. H_2O_2 increased γH2AX expression by 312% and by 450% after 30 min and 1 h of treatment, respectively (Figure 3). This increase was also detected up to 6 h of treatment. p53 activation was also measured by ser15-p53 phosphorylation. We calculated the ser15-p53/p53 ratio on the basis of the results of the Western-blot analyses. There was a 330% increase following a 15 min exposure to 500 μM of H_2O_2. At 3 h the increase was ~800%.

H_2O_2-induced changes in the regulation of SIRT1, pSTAT1, p53-lys379 and p-AKT

We also examined other intracellular signals involved in p53 regulation. In this regard, a significant increase in pSTAT1-ptyr701 expression of 60% occurred at

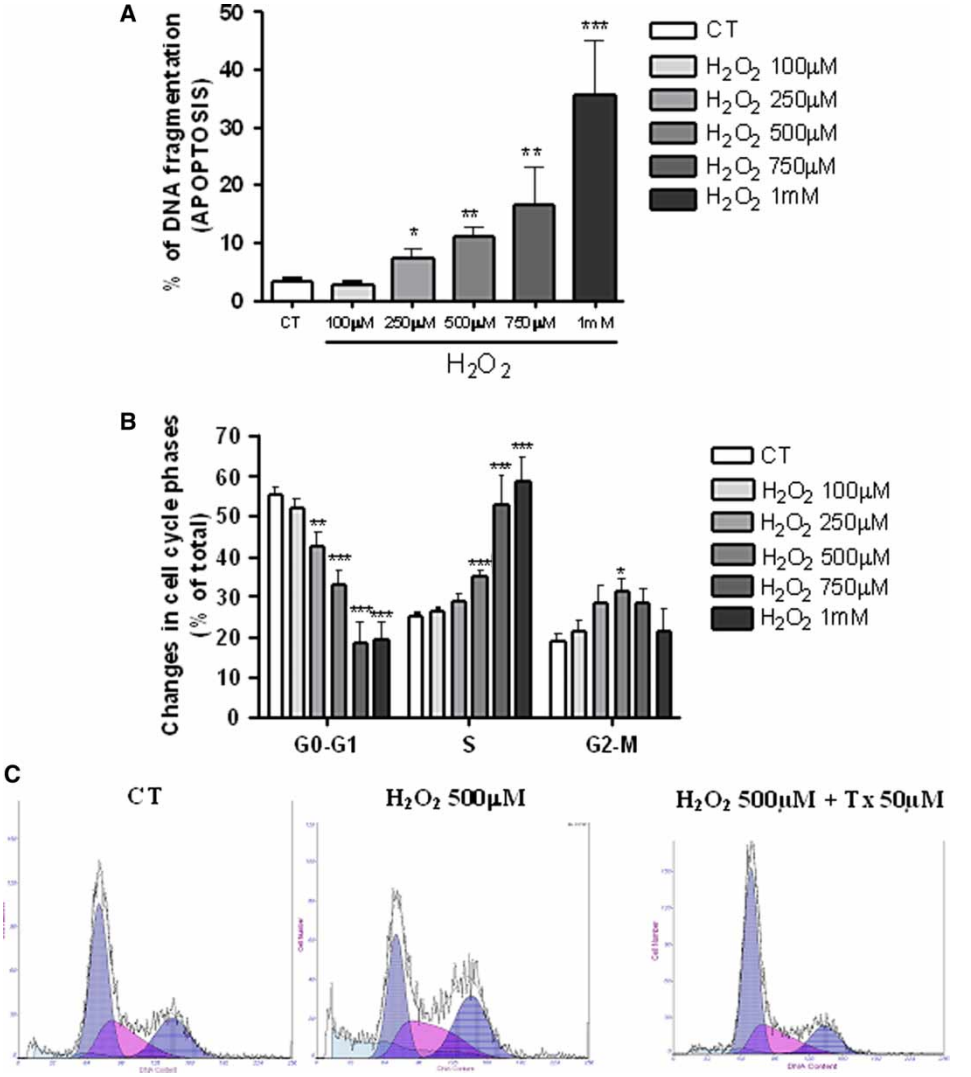

Figure 2. (A) Flow cytometric analysis of cell cycle inhibition by H_2O_2 and after 50 μM trolox treatment in representative B65 cells. (B) Evaluation of DNA fragmentation by flow cytometry in B65 cells treated with H_2O_2 (100 μM to 1 mM). Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control.

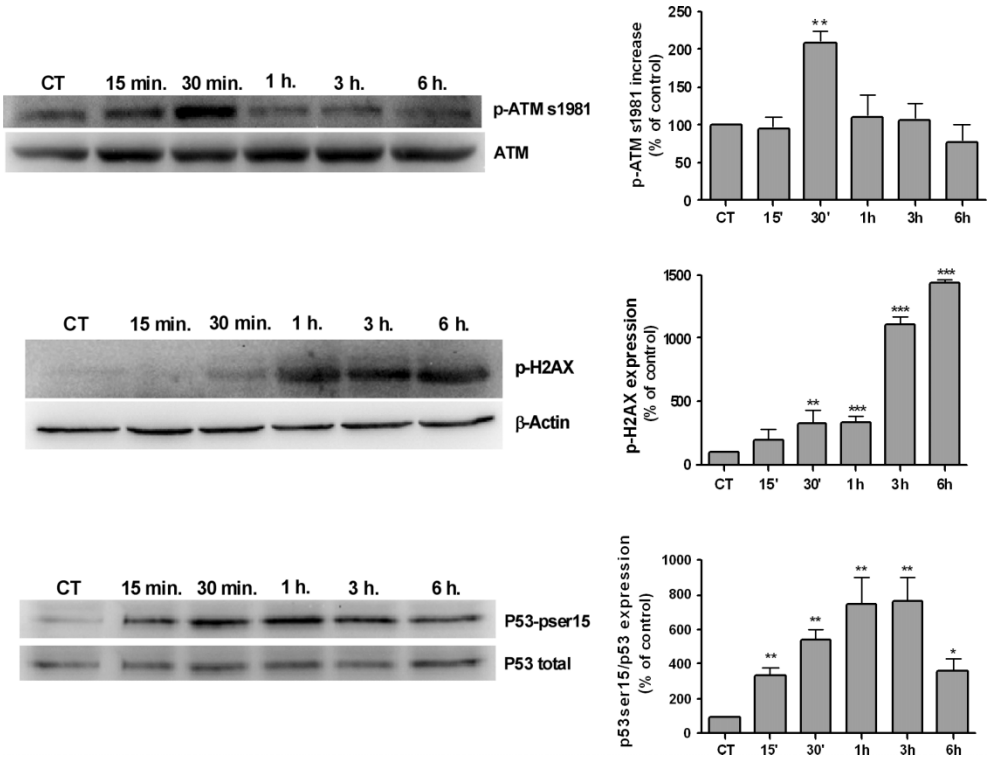

Figure 3. Immunoblots showing the expression of pATM ser1981, p-H2AX ser139 and p53 ser15 in B65 neuroblastoma cells treated with 500 μM H_2O_2 for up to 6 h. Band intensities were calculated as percentages of the control values, which were normalized to β-actin and p53. Bars represent means ± SEM of four or five separate experiments with four or five culture preparations ($n = 4$–5). The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control.

30 min, 100% at 1 hour of 500 μM H_2O_2 exposures and up to 6 h this increase was maintained (Figure 4). Likewise, pSTAT1-ptyr701 expression increased by 60%; at 1 h the increase was 100%, which was maintained for up to 6 h (Figure 4). Sirtuin 1 is also involved in the process of p53 regulation by means of deacetylation. Western-blot data show a 40% and 30% decrease after exposure to 500 μM of H_2O_2. In contrast the decrease in SIRT1 expression was accompanied by an increase in acetylated p53 for up to 6 h (p53 activation). Our immunoblot analysis showed that the p-AKT-ser473 protein content decreased rapidly by 50% 15 min after H_2O_2 exposure and this decrease was maintained for up to 6 h (Figure 4).

H_2O_2-induced changes in the regulation of cell cycle proteins

H_2O_2-induced changes in the cell cycle were analysed by flow cytometry (Figures 2B and C). In agreement with our previous data, H_2O_2 induced DNA damage and an accumulation of B65 cells (cell cycle inhibition) in S phase was detected (Figure 5). This S phase cell cycle arrest was also associated with a 50% decrease in cyclin D protein expression at 12 and 24 h of H_2O_2 treatment (Figure 5). However, a slight decrease in cdk4 expression was detected only at 12 h. Moreover, treatment with 500 μM H_2O_2 increased cyclin E expression, which is involved in G_0–G_1 and S cell cycle phase control and cyclin A, which is also involved in the regulation of S phase. In order to explain the increase in these S phase cell cycle proteins, we studied the effects of oxidative stress on the phosphorylation of the main cell cycle regulator of S phase, namely retinoblastoma protein (pRb), specifically in ser780. Thus, treatment of B65 cells with 500 μM H_2O_2 significantly increased the pRb-ser780 phosphorylation (Figure 5). On the basis of this observation, we hypothesized that DNA damage has a dual effect on B65 cells by arresting cell proliferation in G_0/G_1 and by favouring pRb phosphorylation.

To confirm this hypothesis, we used a specific and selective pharmacological inhibitor of ATM, namely KU-555933. Pre-treatment of B65 cells with 10 μM of this inhibitor caused a significant reduction in H_2O_2 -induced pRb-ser780 phosphorylation at 3 h (Figure 6A). These data indicate that ATM is directly or indirectly involved in the regulation of pRb phosphorylation. Finally, to demonstrate that ROS are responsible for pRb-ser780 phosphorylation, protein measurements were made in the presence of trolox. The antioxidant also caused a significant decrease in H_2O_2-induced pRb-ser780 phosphorylation and also in ATM phosphorylation at 30 min (Figure 6B).

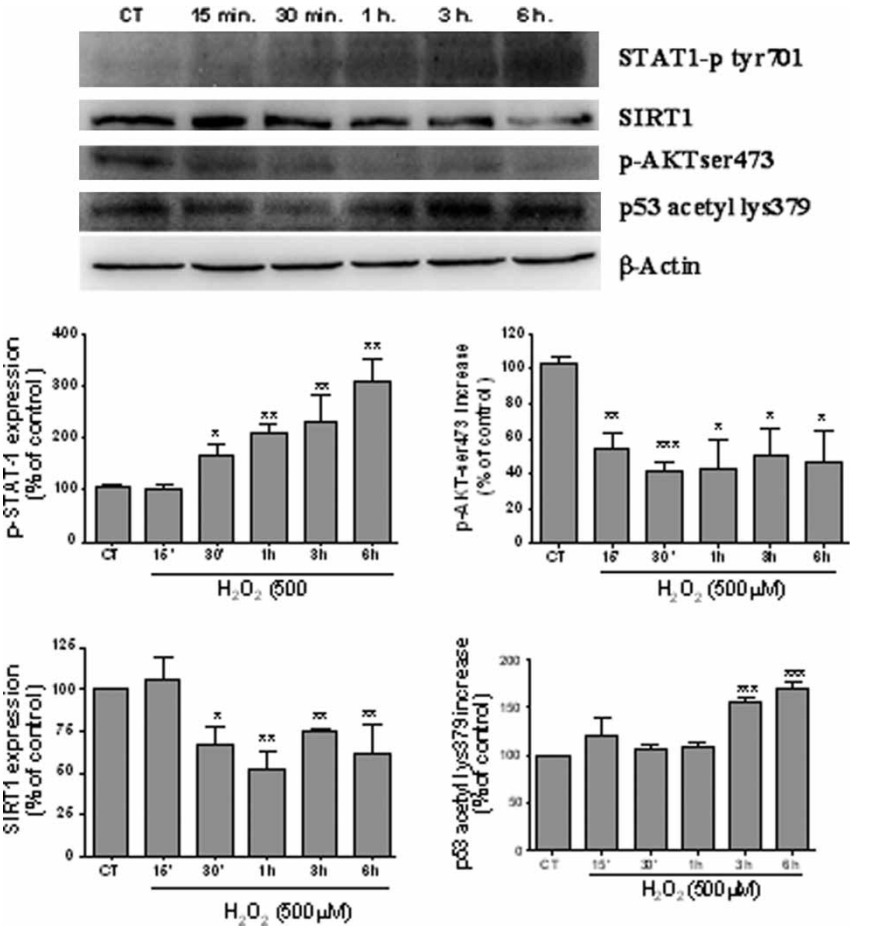

Figure 4. Immunoblots showing the expression of pSTAT1-ptyr701, SIRT1, pAKT ser473, p53acetyl lys379 in B65 neuroblastoma cells treated with 500 μM H_2O_2 for up to 6 h. Band intensities were calculated as percentages of the control values, which were normalized to β-actin. Bars represent means ± SEM of four or five separate experiments with four or five culture preparations ($n = 4$–5). The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control.

Discussion

Oxidative stress is one of the stimuli that contribute to the pathogenesis of neuronal degeneration in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [1–6]. Therefore, a complete understanding of how oxidative stress affects intracellular targets and initiates cell damage is crucial for the design of future therapeutic strategies aimed to prevent or delay these neurodegenerative diseases. Here we focused on the process of DNA damage as first target involved in p53 regulation and addressed how this damage affects cell cycle regulation. The mechanisms by which oxidative stress activate the intracellular DNA damage cascade are not fully understood. Here we first addressed the effect of trolox on cellular apoptosis and cell viability. Treatment of cells with this compound significantly decreased apoptosis and increased the viability of B65 cells challenged with H_2O_2. Once we had demonstrated that oxidative stress is mainly responsible for the loss of cell viability, we next performed experiments to demonstrate the activation of enzymes involved in the DNA damage cascade. Western-blot data showed that H_2O_2 caused rapid DNA DBSs, as measured by ATM activation. ATM activation increased the phosphorylation of typical ATM substrates, such as histone H2AX on ser139, which is a sensitive reporter of DNA damage, and p53 on ser15, which is a marker of p53 activation [11,24,25,27,28]. Interestingly, DNA damage is also involved in the pathogenesis of neurodegenerative diseases such as AD [29]. Furthermore, to address the intracellular signalling pathways that contributed to p53 activation by oxidative stress, we studied additional pathways such as STAT1, SIRT1 and AKT.

STAT1 is required for DNA damage-induced apoptosis through its direct interaction with p53 [30–33]. Thus, STAT1 enhances p53 activity. Our data indicate that cell exposure to oxidative stress caused a rapid increase in STAT1 expression, thereby suggesting that this pathway also contributes to the regulation of p53. Regarding SIRT1 expression, it has been suggested that this protein has a protective effect against DNA damage by enhancing DNA repair capacity and preventing tumourigenesis [12,13]. SIRT1 is also involved in the regulation of p53, since

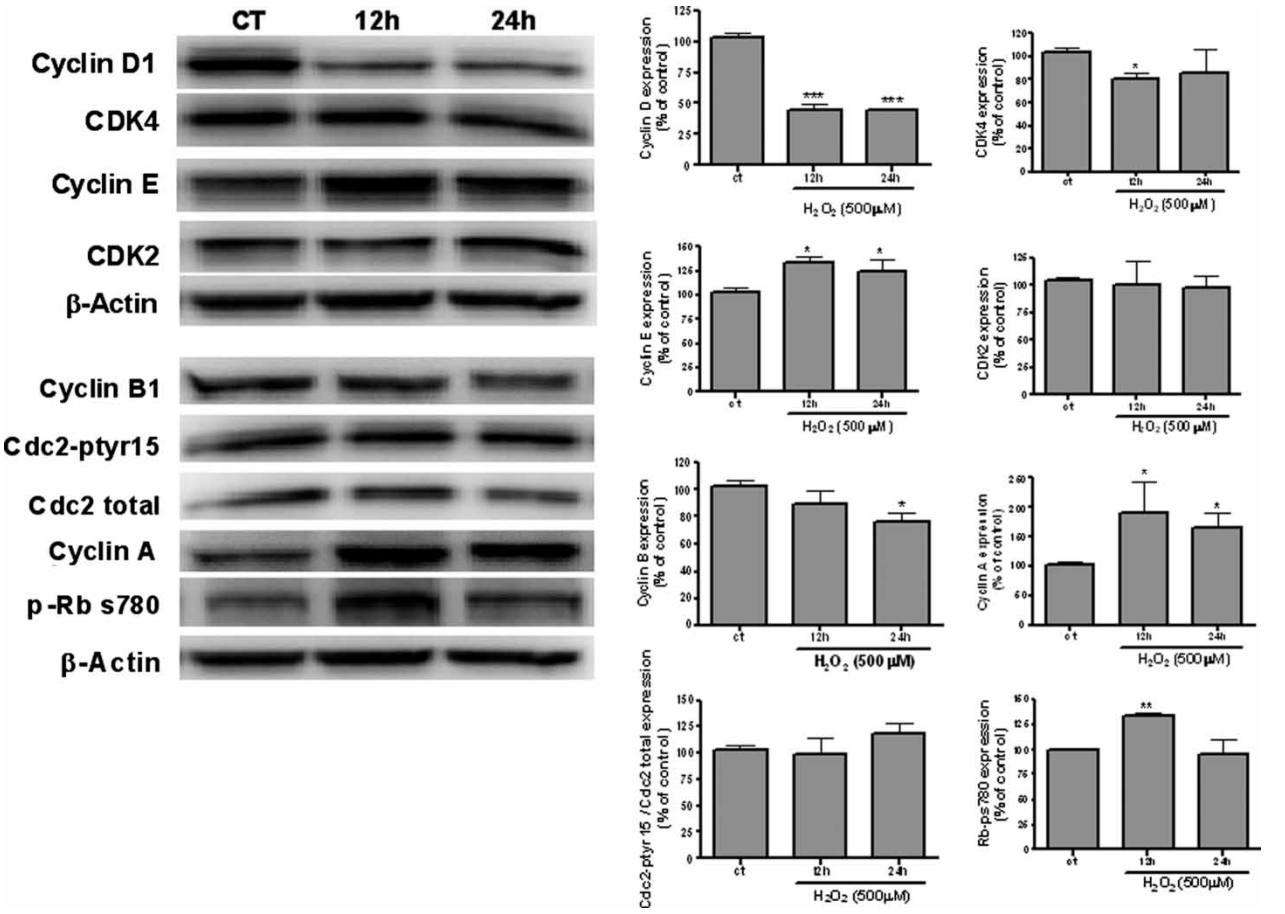

Figure 5. Immunoblots showing the expression of cell cycle proteins in B65 cells treated with 500 µM H_2O_2 for 12–24 h: Cdk4, Cdk2, Cyclin D1, Cyclin E, Cyclin A, Cdc2 tyr15, Cdc2, Cyclin B1and pRb ser780. Band intensities were calculated as percentages of the control values, which were normalized to β-actin. Bars represent means ± SEM of four or five separate experiments with four or five culture preparations ($n = 4$–5). Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control.

it binds to and deacetylates p53 on lys382, thereby negatively regulating p53-mediated transcriptional activation. Our data indicate a rapid (30 min) decrease in SIRT1 expression after oxidative stress. In parallel to the decrease in SIRT1 expression, the levels of p53 acetylated on lys382 were significantly increased. Collectively, all these results indicate that SIRT1 is also involved in p53 regulation after cell exposure to oxidative stress. Finally, the AKT pathway has a pro-survival effect, which is achieved through the inhibition of pro-death signals such as GSK-3β activation, nuclear FOXO localization and inhibition of BAD [34,35]. Moreover, AKT is involved in the regulation of p53-mediated apoptosis, specifically through Mdm2 (murine double minute 2). Therefore, 15–30 min of oxidative stress treatment induced a rapid loss of AKT phosphorylation. On the basis of this observation, we conclude that this pro-survival pathway is also down-regulated and also contributes to p53 regulation in B65 cells. Finally, concerning oxidative stress-induced regulation of p53, given that multiple proteins are rapidly activated or inhibited, it was not possible to identify the main signal involved in p53 activation. Likewise, cross-talk occurs between pathways and in this context we have demonstrated that STAT-1 modulates the phosphorylation of ATM and that SIRT1 is activated by DNA damage. Since, trolox restored the cellular cell cycle phases after H_2O_2 treatment and apoptosis, this finding supports the notion that all changes in cell cycle protein signals are mediated by oxidative stress. Another relevant finding of the present study is that H_2O_2 exerts a dual effect on the cell cycle, by arresting the cells in G_0/G_1 and also by favouring DNA synthesis, as determined mainly by a significant increase in cyclin A expression [36–38]. In addition, we addressed the potential mechanism involved in the increase in S phase proteins. For this purpose, we measured the phosphorylation status of pRb. This protein is a crucial component in the regulation of cell growth and proliferation. Under most cellular conditions, pRb controls cell cycle entry by sequestering the E2F family of transcription factors. Cyclin D/cdk4/6 and cyclin E/cdk2 are responsible for

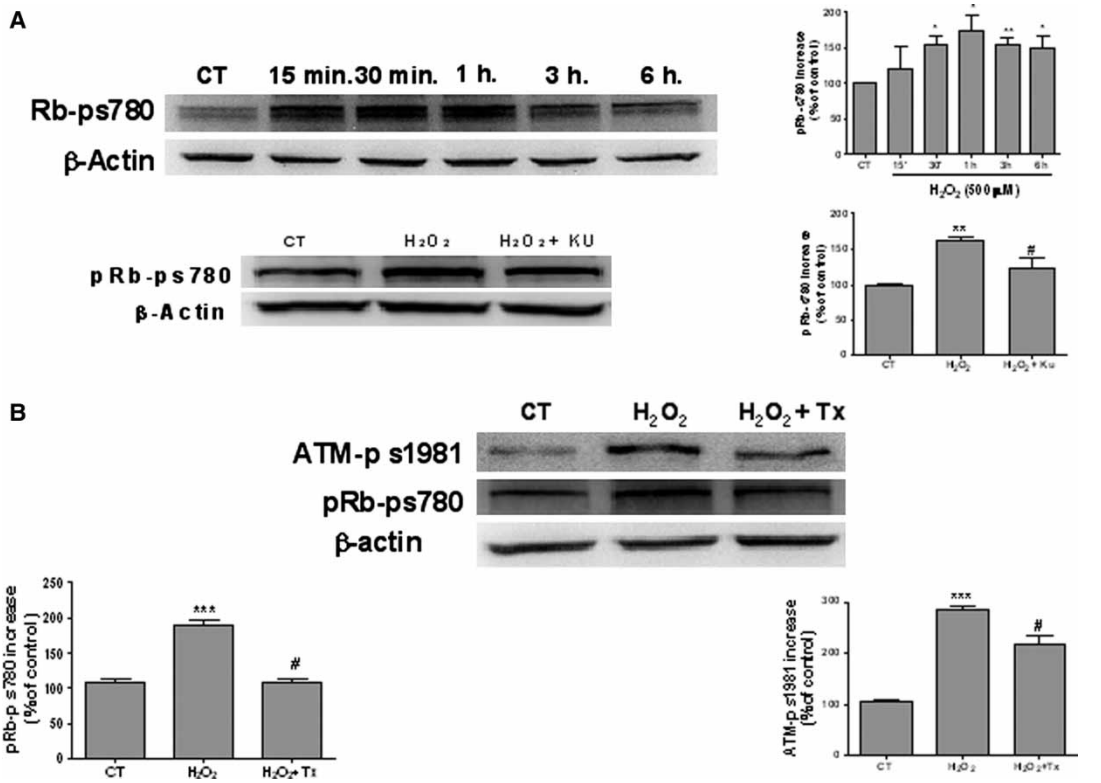

Figure 6. (A) Immunoblots showing the time course expression of pRbser780 in B65 cells treated with 500 μM H_2O_2 for 3 h. Expression of pRb ser780 was attenuated by treatment with KU55993 (10 μM). (B) Immunoblots showing the expression of pATM ser1981 and pRb ser780 in B65 neuroblastoma cells treated with 500 μM H_2O_2 and in the presence of 50 μM trolox at 30 min and 3 h, respectively. Bars represent means ± SEM of four or five separate experiments with four or five culture preparations ($n = 4$–5). Band intensities were calculated as percentages of the control values, which were normalized to β-actin. Bars represent means ± SEM of four or five separate experiments with four or five culture preparations ($n = 4$–5). Statistical significance was determined by one-way ANOVA followed by Tukey's tests: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control. $\#p < 0.05$ vs H_2O_2.

phosphorylating pRb ser780. However, our results indicate that pRb ser780 is not phosphorylated for these proteins since the expression of cyclin D and cdk4 decreased significantly after treatment with H_2O_2. Recently, Inoue et al. [39] demonstrated that, after DNA damage, ATM participates in pRb ser780 phosphorylation. This finding thus supports the hypothesis that other kinases that are not related to typical CDK are involved in pRb phosphorylation [39–41]. Moreover, pRb phosphorylation plays a crucial role in AD and PD, giving support to the concept of neuronal cell cycle re-entry in these neurodegenerative disorders [42]. Thus, we hypothesized that ATM is involved in pRb ser780 phosphorylation. To demonstrate this, a pharmacological approach was used with KU-55993, a selective ATM inhibitor. This treatment led to a significant attenuation in pRb phosphorylation. Accordingly, we propose that ATM participates in the regulation of pRb phosphorylation after H_2O_2 treatment in B65 cells.

We conclude that DNA damage caused by oxidative stress in B65 neuroblastoma cells induces the activation of ATM, thus initiating a DNA response. Moreover, in addition to activating p53, ATM regulates the cell cycle through pRb phosphorylation. Given that trolox inhibits pRb phosphorylation and ATM activation, we propose, in agreement with previous studies, that oxidative stress acts as a switch by mediating the connection between DNA damage cell cycle and apoptosis [43–49].

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